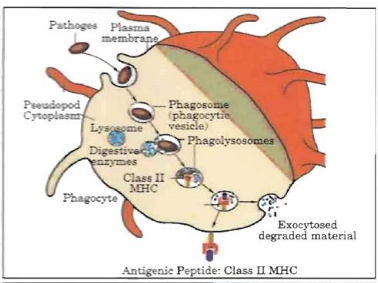
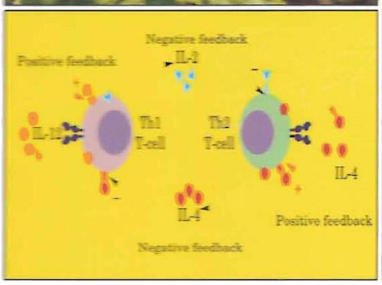
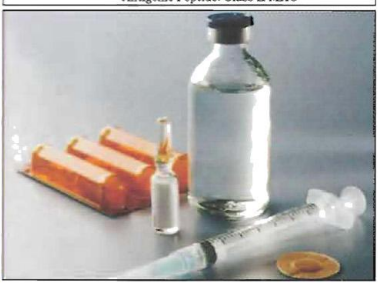
Comprehensive
Bioactive Natural Products

Vol 5 Immune-modulation & Vaccine Adjuvants







V K Gupta

Studium Press

Comprehensive
Bioactive Natural Products

Volume 5
Immune-modulation & Vaccine Adjuvants

V.K. GUPTA

Indian Institute of Integrative Medicine
(Council of Scientific & Industrial Research)
Canal Road, Jammu (J&K State)- 180 001, India

2010

Studium Press LLC, U.S.A.

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Comprehensive
Bioactive Natural Products

Vol. 5: Immune-modulation & Vaccine Adjuvants

ISBN : 1-933699-55-8
SERIES ISBN : 1–933699–50–7

Published by:

STUDIUM PRESS, LLC
P.O. Box-722200, Houston, Texas-77072, USA
Tel. 713-541-9400; Fax:713-541-9401
E-mail: studiumpress@studiumpress.com

Printed at:

Thomson Press (India) Ltd.

"This page is Intentionally Left Blank"

Comprehensive
Bioactive Natural Products:
(*Multi-Volume Set*)

Series Editor* : *V.K. Gupta
E-mail: vgupta_rrl@yahoo.com; vguptaiiim@gmail.com

Volumes Published (2010)

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Vol. 2*: *Efficacy, Safety & Clinical Evaluation I
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Vol. 3*: *Efficacy, Safety & Clinical Evaluation II
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MEMBERS

Dr. A. Panossian: Swedish Herbal Institute Research and Development, Kovlingevagen 21, Vallberga, SE-312 50, Sweden;
E-mail: alexander.panossian@shi.se

Prof. Yu Zhao: Department of TCM & Natural Drug Research, College of Pharmaceutical Sciences, Room 513, Zhejiang University, Zijingang Campus, 388 Yuhangtang Rd., Hangzhou 310058, China;
E-mail: dryuzhao@zju.edu.cn; dryuzhao@126.com

Prof. A. Evidente: Department of Organic Chemistry, Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università di Napoli Federico II, Portici, Italy;
E-mail: evidente@unina.it

Prof. Mirian Salvador: Instituto de Biotecnologia, Universidade de Caxias do Sul, Rua Francisco Getúlio Vargas, 1130, CEP 95070-560, Caxias do Sul, Rio Grande do Sul, Brazil;
E-mail: msalvado@ucs.br

SERIES ISBN : 1–933699–50–7

About the Series

Nature abounds with a rich potential heritage of bioactive natural products as that have been exploited for effective and beneficial use either as a therapeutic resource against a human disease or as a chemopreventive, antioxidant, nutraceutical or diet supplement. A natural product is a substance or a chemical compound produced by a living organism found in nature that usually has a pharmacological or biological activity. Some of these agents are derived from terrestrial plants, whereas, others are obtained from microorganisms, marine or freshwater organisms and even the higher animals.

Past few decades have witnessed an unprecedented worldwide growth in the interest of bioactive natural products based medicines, both in developed and developing countries, and offer a resource-pool for lead identification in drug discovery owing to the reason that isolation of a novel molecule is easier than *de novo* synthesis, especially when the molecular structure of the compound is very complex. On account of such advantages, pharmaceutical companies are opting for bioactive natural products based drugs. More than 20 new drugs, launched world over between 2000 and 2005, originate from natural products. Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases (infectious and non-infectious). The development of new bioassay techniques, biotechnology methods, screening and high performance analytical methods have introduced new concepts and possibilities of rational drug design and drug discovery.

Interest in bioactive natural products research is growing more strongly and can be attributed to several factors, including remarkable diversity of natural products, unmet therapeutic needs, the development of novel and sensitive techniques to detect, isolate, purify and structurally characterize these active constituents. This has opened opportunities for multidisciplinary research that joins the forces of natural product chemistry, molecular and cellular biology, synthetic and analytical chemistry, bio-chemistry, bio-technology, general biology, and pharmacology to exploit the vast diversity of bioactive natural products.

Taking cognizance of these facts, we have initiated efforts in editing the present series "**Comprehensive Bioactive Natural Products**" and brought eight volumes (1 to 8) providing edited information from over 139 original research and review articles by eminent scientists and researchers

from India and abroad, representing 40 countries of the world on wide range of topics in the area of natural products categorized under the themes *viz.*,

1. *Potential & Challenges*
2. *Efficacy, Safety & Clinical Evaluation I*
3. *Efficacy, Safety & Clinical Evaluation II*
4. *Antioxidants & Nutraceuticals*
5. *Immune-modulation & Vaccine Adjuvants*
6. *Extraction, Isolation & Characterization*
7. *Structural Modifications & Drug Development*
8. *Quality Control & Standardization*

These volumes critically evaluate the present state-of-art, current status and future prospects in a well-illustrated manner. It is hoped that this series, which reflects the contributor's research results as well as world wide reviews will be widely read and used by all interested in these fields and shall open new vistas of research and academic pursuits for those engaged in the fields of bioactive natural products.

I would like to take this opportunity to thank each of the authors who have contributed in the preparation of this book series.

Jammu, India

V.K. Gupta
Series Editor

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AND BIOTECH PRODUCTS DEVELOPMENT

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Prof. (Dr.) P. PUSHPANGADAN,
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FNSE, FNESA, FNAASc, FNASc.,
(UN Equator Initiative Laureate)
Director General & Senior Vice President, RBEF
(Former Director, NBRI, Lucknow)

08-06-2009

Foreword to the Series

Medicine among the ancient civilizations was a blend of Religion and Science. Natural products played an important role throughout the world in the prevention and treatment of diseases. These natural products originated from varied sources such as plants, animals, micro-organisms, marine organisms, terrestrial vertebrates and invertebrates. In man's quest for discovering new and potent remedies for diseases, he keenly observed his immediate neighbourhood and experimented not only with organic substances but also with in-organic materials such as metals, minerals, ores etc. Over the years, the accumulated medical wisdom was compiled into excellent treatises and these have later developed into the various classical systems of medicine associated with the ancient civilizations and cultures of the world. Apart from the classical systems of medicines, which by its nature and practice had universal applications, there existed another stream of medicine in the countryside, among the less privileged class of people, among the forest dwellers and among the nomadic tribes which were location specific, confined to small communities, tribes and families. These medical systems were mostly perpetuated by oral traditions. Nevertheless, the value of the oral traditions cannot be overlooked as these localized knowledge systems are veritable treasures of invaluable information on the properties and uses of hundreds of plant, animal and mineral products.

Ethno medical information is now increasingly being utilized in the search for novel bioactive molecules from natural products. Several well known plants such as Licorice (***Glycyrrhiza glabra***) myrrh (***Commiphora sps***) and poppy capsule latex (***Papaver somniferum***) were mentioned in the clay tablets dating back to 2600 B.C, obtained from Mesopotamia. These plants and their derivatives are still used in various forms as herbal drugs as well as pure isolates in different systems of medicine. For instance,

codeine, papaverine, morphine etc isolated from ***P. somniferum*** are still used in modern medicine. Similarly hemisuccinate carbenoxolone sodium, a semi synthetic derivative of glycyrrhetic acid isolated from licorice is used for the treatment of gastric and duodenal ulcers. Plants, especially those with ethno medical history have been the major source of bio active molecules. According to a study by Fabricant and Famsworth (2001), out of 122 plant derived drugs, 80% had their origin from plants with ethno medical use. Since secondary metabolites from plants are products of biosynthetic reactions occurring in living systems, bioactive molecules from natural sources are excellent drug candidates as they are perceived to show more biological friendliness than synthetic drugs.

The ever increasing popularity and acceptance of natural products have evinced an extra ordinary interest and scope in the subject in recent years. Compendium of Bioactive Natural Products- a series of eight books edited by Dr. V.K. Gupta (Volumes 1,2,3 and 5) and Dr. V.K. Gupta, Dr. S.C. Taneja, Dr. B.D. Gupta and Dr. A.K.Verma (Volumes 4,6,7 and 8), published by M/S. Studium press LLC, U.S.A are an excellent compilation, giving comprehensive and reliable data on the current development in the field of bioactive natural products. This series contain 139 original research and review articles contributed by a galaxy of eminent scientists and academicians from 40 countries around the world. The editors have taken care to invite eminent scholars in the relevant areas to contribute each chapter in the series. The series deals with wide ranging topics related to natural products such as extraction, isolation and characterization, structural modifications and drug development, quality control and standardization and, immune modulation and vaccine adjuvants, antioxidants and nutraceuticals, efficacy, safety and clinical evaluation (Parts-1 and 2) and potentials and challenges. The editors of this series Dr. V.K. Gupta, Dr. S.C. Taneja, Dr. B.D. Gupta and Dr. Anil K. Verma are known to me for more than three decades and they are all stalwarts in natural products and related areas.

It is gratifying to know that this series is dedicated to the memory of Col. Sir Ram Nath Chopra, the Founder Director of Regional Research Laboratory, now known as Indian Institute of Integrative Medicine (IIIM), Jammu.

Col. Sir R.N. Chopra (1882-1973) was a pioneer of systematic studies of indigenous drugs, promoter of Indian Systems of Medicine and Patron of Pharmacy in India. Most of the medicinal plants, he studied were in use, in Ayurveda for thousands of years. The major fields of Cot. Chopra's research were general pharmacology, chemotherapy, indigenous drugs, drug addiction and drug assays. At the Calcutta school of Tropical Medicine, he developed a pattern of research in studying the action of medicinal plants which influenced an entire generation of pharmacologists. Pharmacists and physiologists in India. In the words of Dr. G.V. Satyawati, Former Director General of Indian Council of Medical Research, the credit of kindling the interest of Indian Chemists and Pharmacologists in Medicinal Plants should rightfully go to

Sir Ram Nath Chopra who, has been claimed as the Father of Indian Pharmacology'.

I had the privilege and good fortune of meeting Col, Sir Ram Nath Chopra along with Prof. Dr. E.K. Janaky Ammal at his residence in 1969 immediately after my joining Regional Research Laboratory as a Scientific Assistant. My meeting with Col. Sir R.N. Chopra had left a lasting impression of a great scientist in my memory.

It is a fitting tribute to Col. Sir R.N. Chopra that the editors have decided to dedicate these eight volumes to his memory. I congratulate the editors for bringing out this series which is timely, relevant and important to all those who are engaged in the study of natural products and development of therapeutically useful bioactive molecules. I am sanguine that these volumes will receive wider acceptance among the scientific community, especially among those engaged in Natural Product research.

(P. Pushpangadan)

About the Editor

Dr. Vijay Kumar Gupta, Ph.D., FLS, London (born 1953-) Deputy Director & Head, Animal House, Indian Institute of Integrative Medicine (CSIR), Jammu, India. He did his M.Sc. (1975) and Ph.D. (1979) in Zoology both from University of Jammu, Jammu-India. His research capabilities are substantiated by his excellent work on histopathology, ecology and reproductive biology of fishes, turtles, birds and mammals, which has already got recognition in India and abroad. Dr. Gupta has to his credit more than 75 scientific publications and review articles which have appeared in internationally recognized Indian and foreign journals. Founder fellow, life member and office bearer of many national societies, academies and associations. He has successfully completed a number of research/consultancy projects funded by various governments, private and multinational agencies. His current areas of interest are histopathology, toxicology, pre-clinical safety pharmacology and reproductive efficacy studies of laboratory animals. He is also Editor-in-chief of the books 1) Utilisation and Management of Medicinal Plants 2) Medicinal Plants: Phytochemistry, Pharmacology and Therapeutics 3) Perspectives in Animal Ecology and Reproduction (Vols.1-6). The Editor-in-chief of the American Biographical Institute, USA, has appointed him as *Consulting Editor* of *The Contemporary Who's Who*. Dr. Gupta also appointed as Nominee for the *Committee for the Purpose of Control and Supervision of Experiments on Animals* (CPCSEA, Govt. of India). Recently the *Linnaean Society of London, U.K.* has awarded fellowship to him in November 2009 in recognition of his contribution towards the cultivation of knowledge in Science of Natural History.

Preface

Recently the understanding of research on immunomodulators has come up as a new field of immuno-pharmacology. Immunomodulation is a strategy for overcoming incurable autoimmune disease involving cancer, AIDS, arthritis, diabetes and allergies. An in-depth study of the immune system is supposed to provide both the theoretical and therapeutic background of much chronic disorder.

Immunomodulation is basically a process which can alter the immune system of an organism by interfering with its functions. The inference results in either immunostimulation, an enhancement of immune reactions or immunesuppression *i.e.* to reduce resistance against infection, stress which may be because of environmental or chemotherapeutic factors. Advancement in the field of surgery, which has made organ transplantation possible, is endowed with a serious problem of organ rejection, which in fact comes under the preview of immunology associated with immune system, proper handing of this situation through modification of immune response can give new lease of life to patients with organ transplant. In the modern day life, extensive exposure to industry based pollutants and other xenobiotics has resulted into emergence of a variety of immune deficiencies or hypersensitivity, situations which call for changed strategy for handling the patients where immunology plays an important role.

Use of bioactive natural products as a source of immunomodulators is still in its infancy in modern medicine. It is now being recognized that the modulation of immune response could provide an alternate to conventional chemotherapy for vaccine adjuvants.

Adjuvants are foreign substances to the body and may evoke side affects in addition to the desired immune stimulation. The need for appropriate safety testing of any new adjuvant is self-evident. Vaccination remains the most cost-effective biomedical approach to the control of infectious diseases. Vaccines based on killed pathogens or subunit antigens are safer but are often ineffective and require co-administration with adjuvant to achieve greater efficacy. Unfortunately, most conventional adjuvants are poorly defined, complex substances that fail to meet the stringent criteria for safety and efficacy desired in new generation vaccines. Search for plant based adjuvant for human vaccines has become an expanding field of research in the last thirty years for generating stronger vaccines, capable of inducing protective and long lasting immunity in humans.

It hardly needs to emphasize to discover safer and effective limitations like adjuvant toxicity which remains a major concern against virulent diseases. That is why the investigation of bioactive natural products has in recent years assumed a greater sense of urgency in response to the

expanding human population and its subsequent demands for food and good health. The extinction of plant and animals species as mankind encroaches on natural habitat, represents last and irreplaceable resources, the full potential of which is unpredictable. Similarly, the loss of endemic cultures as other cultures become influential in an almost cancerous manner will results in the loss of a fount of empirical ethnobotanical knowledge that has been acquired over the course of thousands of years.

It is owing to a world wide and sustained effort of scientist's that an enormous information is being generated and there has been a series of publications on medicinal plant researches. Based on this rational, the present volume ***"Immune-modulation & Vaccine Adjuvants"*** of the book series entitled, **"Comprehensive Bioactive Natural Products"** presents edited information on 16 research and review communications received from eminent scientists from India and abroad, providing recent and present state of the art data on therapeutic properties, action and uses of bioactive natural products in combating a number of diseases and conditions for which there is lesser satisfactory treatment in modern medicine. Data topics include: Herbal therapies as immune modulators for anti-cancer therapeutics; Neem leaf glycoprotein as a new vaccine adjuvant for cancer immunotherapy; Honey bee products: immunomodulation and antitumor activity; Mitogenic and anti-anticoagulant activity of fucoidan isolated from brown and red seaweed; Development of plant based adjuvants for vaccine antigens; Immunomodulation and vaccine adjuvants; Recent advances in the use of medicinal plants as immunostimulants; Balance of pro-/anti-inflammatory cytokines release in spleen cells from mice treared with *Crotalus durissus* venom; Bioactive agents from herbal sources with immunopharmacological properties abating inflammation and malignancy; Differential effect of subchronic and chronic oral treatments with *Orbignya phalerata* Mart. mesocarp on the inflammatory response; A mistletoe lectin –containing preparation for oral use provokes an immune response induces an increase in the population of activated natural killer cells; Flavonoids from complementary and alternative medicine: mechanism of immunomodulation of macrophages; Herbal immunomodulators; Immunomodulatory activity of botanicals; The potential of immunomodulatory substances of natural origin in contemporary medical practice; Study of immunomodulatory activity of *Sphaeranthus indicus.*

It is hoped the present volume will attract wide acceptance of pharmacologists, phytochemists, medical personals in particular and a host of other scientists as well as the biologists to facilitate further research on bioactive natural products and to contribute material in the search for immune-modulation and vaccine adjuvants.

Jammu, India

V.K. Gupta

ISBN: 1-933699-55-8

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1

Herbal Therapies as Immune Modulators for Anti-Cancer Therapeutics

STEPHEN M. SAGAR[1,*], DANIEL M-Y. SZE[2] AND RAIMOND K. WONG[1]

ABSTRACT

Laboratory studies suggest that some herbs increase the effectiveness of conventional chemotherapy without increasing toxicity. A healthy immune system is necessary for control of malignant disease, and the immune suppression associated with cancer contributes to its progression. Many Chinese herbs contain glycoproteins and polysaccharides (among them, constituents of Coriolus versicolor, Ganoderma lucidum, Grifola frondosa, Astragalus membranaceus, Panax ginseng, and various other medicinal mushrooms) that can modulate metastatic potential and the innate immune system. Phytochemicals such as specific polysaccharides have been shown to boost the innate immune system, especially through interaction with Toll-like receptors in mucosa-associated lymphoid tissue. This intervention can potentially improve the effectiveness of new anticancer vaccines. An increase in virus-associated cancers presents a major public health problem that requires novel therapeutic strategies. A number of herbal therapies have both antiviral activity and the ability to promote immunity, possibly inhibiting the initiation, and promotion of virus-associated cancers.

Key words : Herbal therapies, immune modulators, cancer, Chinese medicine, vaccines, immunity

1. Associate Professor, Departments of Oncology and Medicine, McMaster University, Canada.
2. Associate Professor, Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hong Kong & Director (Cancer Research), Herbal Medicines Research and Education Centre, Faculty of Pharmacy, University of Sydney, Australia.

* *Corresponding author* : E-mail : stephen.sagar@jcc.hhsc.ca

INTRODUCTION

Traditional Chinese Medicine (TCM) may be integrated with conventional Western medicine to enhance the therapy of patients with cancer through biomodulation. Chinese medicine views all cancers as a systemic disorder. The concept of biomodulation is the reactive or associative adjustment of the biochemical or cellular status of an organism. Most modulation events describe an interaction in which a molecule (modulating entity) alters the ability of an enzyme to catalyze a specific reaction, or modifies the ability of a cell to communicate with another cell. Biomodulation includes the use of a substance to augment the host's antitumor response, including immunotherapy. Western science is now exploring the possibility that both hematological and solid cancers may sometimes be a systemic disease from the outset and therefore require systemic biomodulation in addition to the classical therapies, such as surgery, radiotherapy, and cytotoxic chemotherapy (Rafii & Lyden, 2003; Houghton *et al.*, 2004; Kerbal & Kamen, 2004).

THE ROLE OF IMMUNITY IN CANCER PROGRESSION

Recently, the concept of immune enhancement has gained new ground with the discovery that both the cancer itself, as well as cytotoxic therapies, suppresses immunity. The low immune levels may increase the probability of relapse. In addition, an intact innate immune system is necessary for the activity of new cancer vaccines. The interaction of host immunity with the natural history of cancer is suggested by Burnet's immune surveillance theory; the fact that immunodeficiency diseases are associated with an increased risk of cancer; and the fact that immune-enhancing therapies in malignant melanoma and renal cell carcinoma have produced anti-tumor responses. There is evidence that the healthy immune system is necessary for the control of malignant disease and that immune suppression associated with cancer contributes to its progression. Natural immune mediators are implicated in resistance against tumor development (Whiteside, 2006). Innate immunity is often suppressed in tumor-bearing hosts, and specially designed agents are required to boost this defense (Houghton *et al.*, 2004). These agents strengthen the innate immune defenses of the host, and thereby enhance adaptive immunity. We know that specific cytokines and hormones boost natural defense mechanisms (for example, during febrile reactions), termed the acute phase response. Many Chinese herbs contain glycoproteins and polysaccharides that can modulate innate immunity through the biomodulation of immune cells and their associated cytokines.

In addition, the metastasis of malignant tumors may be a specific receptor-mediated process in which organ-specific lectins play a role in the adhesion of disseminated tumor cells. Glycoprotein-mediated membrane identity is part of the HLA antigen histocompatibility system. The abnormal

carbohydrate group on the tumor cell can develop during malignant transformation. The metastatic tumor cell, with its membrane-associated glycoprotein (often identical with the tumor marker) is recognized by the organ specific lectins as belonging to the organ, and is thereby captured. *In vitro* experiments show that galactoglycoconjugates can inhibit the adhesion of tumor cells to hepatocytes (Beuth *et al.*, 1988). Immune suppression in cancer contributes to the metastatic progression and relapse (Kebudie *et al.*, 1995; Maier *et al.*, 1995; Miyazaki *et al.*, 1995; Sasada *et al.*, 2003; Koukourakis *et al.*, 2003; Wichmann *et al.*, 2003; Bang *et al.*, 2006; Baniyash, 2006; Whiteside, 2006).

There are currently multiple strategies to identify candidate tumor antigens, and we now understand more about activation and regulation of immunity against cancer. Vaccines can target tumor-specific antigens, but adjuvants are required to boost the innate immune response, especially in patients who already have depressed immunity from tumor-derived signaling molecules and the effects of cytotoxic therapies (Minev, 2002; Hoffmann *et al.*, 2004; Stevenson, 2005).

MULTI-DIRECTIONAL AND MULTI-TARGETING MECHANISMS TO OPTIMIZE ANTI-CANCER THERAPY

Thalidomide was originally prescribed for alleviating symptoms of morning sickness in pregnant women, but was withdrawn from the market in 1961 after its teratogenicity property was appreciated. However, the teratogenicity was partly due to anti-angiogenesis, and this activity was subsequently recognized as an important anti-cancer therapy (Goh *et al.*, 2008). The first clinical trial of thalidomide was instituted as a single agent to treat multiple myeloma. This is a malignant disease characterized by the infiltration and accumulation of a clone of malignant plasma cells that produce immunoglobulin. Mean survival period is still only 3 years despite the introduction of intensive chemotherapy and transplant regimens. The overall survival of myeloma patients has not significantly improved over three decades. The clinical response to thalidomide is very encouraging (Sze *et al.*, 2006). Why are thalidomide and its analogues that are immunomodulatory drugs so useful? Thalidomide has been shown to exhibit multi-directional and multi-targeting mechanisms as an effective anti-cancer agent in myeloma. Thalidomide exerts effective killing of tumor cells by direct and indirect inhibition of the proliferation and growth of the myeloma cells. Strikingly, thalidomide increases host tumor-specific immunosurveillance, including stimulatory effects on both T-cells and natural killer cells. It is argued that this multi-directional basis of its anti-cancer mechanism is instrumental for its effectiveness. It is important to reiterate the significance of the immunologically important cells in cancer patients (Sze *et al.*, 2001; Sze *et al.*, 2003; Sze *et al.*, 2005). Thalidomide exhibits the desirable differential killing of cancer cells, whilst sparing

or even promoting the immunologically important cells. This treatment paradigm is also present in many anti-cancer traditional Chinese botanicals.

CLINICAL USE OF CHINESE HERBAL THERAPIES AS ADJUVANTS TO BOOST IMMUNITY

There are at least two important cell types that Traditional Chinese Medicine (TCM) could have significant clinical effects on. They are the dendritic cells (DCs) and the cancer stem cells (CSC).

DCs are immune cells that act to present foreign antigens to the immune system for recognition and appropriate response. This response may either be an immunological state of tolerance or active immunity against the foreign antigen concerned, *i.e.* tumor antigens. DCs are widely distributed in various tissues and organs in the body. There are two specific locations: namely, the gut and blood that warrant most intense attention for anti-cancer immunological research (Bebawy & Sze, 2008).

The human gastrointestinal system is the largest immunological organ in the body and can be divided into at least 5 distinct subsets:

(i) DCs in the lamina propria ($CD11b^+$, and $CD172a^+$ phenotype);
(ii) DCs in the subepithelial dome of Peyer's patches ($CD11b^-$, and $CD172a^+$ phenotype);
(iii) DCs in the interfollicular region of Peyer's patches ($CD11b^-$, and $CD172a^-$ phenotype);
(iv) DCs in the mesenteric lymph node ($CD11b^+$, and $CD172a^-$ phenotype);
(v) Plasmacytoid type DCs of the CD11c intermediate ($CD4^+$ phenotype).

Similar to the presence of diverse DC subsets in gut, there are also at least 5 mainly non-overlapping DC subpopulations that can be categorized as $CD16^+$; $CD1b/c^+$; $BDCA\text{-}3^+$; $CD123^+$ and $CD34^+$ subsets. Our laboratory has reported, that blood DCs in cancer patients are defective or tolerogenic and that this presence of defective blood DCs is correlated with the high levels of serum IL-10 and TGF-β (Brown *et al.*, 2001). In turn this defectiveness is found to be alleviated following the addition of antibodies targeting these two cytokines or by addition of gamma IFN or IL-12 to the DCs (Brown *et al.*, 2004). It will be important to determine whether some of the commonly used TCM herbs have beneficial effects to reverse the defectiveness of DCs in cancer patients.

The second potential TCM immunological anti-cancer target is cancer stem cells (CSC). These cells are demonstrated in a number of solid and non-solid tumors including multiple myeloma (Loh *et al.*, 2008). They are capable of self-renewal, proliferation, and differentiation, as are normal adult and embryonic stem cells. As these cancer cells are capable of such high and indefinite proliferative rates, only a small number are required to maintain a population of cancer cells and to metastasize. As the functional unit behind cancer growth and persistency, these cancer cells have become known as the CSC. Based on the paradigm that CSC is

the only cell capable of initiating true cancer growth, as long as some CSCs remain, the cancer will inevitably relapse. On the other hand, once the CSC are destroyed, the cancer mass will gradually disappear as the CSC daughter cells eventually succumb due to their own mortality and external factors. It is therefore important to investigate selective anti-cancer TCM herbal medicines that would have this desirable anti-CSC property.

Immunotherapy is mediated by the specific group of TCM herbs called *Fu Zheng* herbs. (Ning *et al.*, 1988; Chen, 1990; Yu *et al.*, 1990; Hou *et al.*, 1991; Rao *et al.*, 1991; Li, 1992; Yu *et al.*, 1993; Cao *et al.*, 1994; Cheng, 1994; Horie *et al.*, 1994; Lin *et al.*, 1995). There is some limited evidence that improvement of the immunological function of cancer patients is associated with an improvement in their survival. In China, *Fu Zheng* herbs have been reported to increase survival when combined with radiotherapy for patients with nasopharyngeal cancer (Pan *et al.*, 1985) and when combined with chemotherapy for patients with stomach and liver cancer (Wang, 1990) but the clinical evidence is weak due to the lack of randomized controlled trials. Fu Zheng herbs, including *Rx Ginseng, Ganoderma, Rx Astragali membranaceus, Rx Angelicae sinensis, Cordyceps sinensis* and *Fructus lycii*, have been shown to enhance the body's defense mechanisms. Clinical studies, including two randomized trials, have found that the NK-cell and OKT4 (immune-enhancing lymphocyte) cell counts were increased with the use of *Fu Zheng* herbs. These immunocytes are known to attack cancer cells. In a study of gastric cancer patients, increased survival was found in the combined treatment group receiving both *Fu Zheng* herbs and chemotherapy, versus the chemotherapy alone group. Many of these herbs are associated with an increase in cytokines, such as interferon and interleukin (Kawakita *et al.*, 1990). Most evidence for successful herbal treatment of cancers is from case reports or case series that may be biased by selection or spontaneous remission. A meta-analysis of existing randomized controlled trials provided promising evidence that combining Chinese herbal medicine with chemotherapy may benefit patients with hepatocellular carcinoma (HCC) (Jin *et al.*, 1994; Feng *et al.*, 1995; Shu *et al.*, 2005). A major weakness of the data is that high-quality, rigorously controlled trials were lacking. Chinese studies also suggest that healing of normal tissues may be enhanced. Anti-inflammatory constituents may diminish radiation-induced ulcers and chemotherapy-induced stomatitis (Zhu & Zhang, 1993; Zhu, 1994). These studies still need to be verified in the West, using acceptable standards and quality assurance.

THE POTENTIAL ROLE OF TOLL-LIKE RECEPTORS AND DENDRITIC CELLS IN MUCOSA ASSOCIATED LYMPHOID TISSUE OF THE GASTROINTESTINAL TRACT

Phytochemicals, such as specific polysaccharides, have been shown to boost the innate immune system, especially through interaction with Toll-like

receptors (TLRs) in Mucosa Associated Lymphoid Tissue (MALT) (Sen *et al.*, 2005; Rezaei, 2006; Tsan *et al.*, 2006). TLRs evolved to interact with polysaccharides found in the walls of bacteria and are an essential part of developing and maintaining a competent immune system (Heine, 2005). Polysaccharide extracts and complexes from Chinese medicinal herbs and mushrooms may have a potential role for enhancing innate immunity. There is some evidence from clinical trials that they can improve survival (Chang, 2002). The polysaccharide complexes and extracts include constituents of *Coriolus versicolor* (extract is Krestin, PSK or PSP) (Mitomi *et al.*, 1994; Nakazato *et al.*, 1994; Ogoshi *et al.*, 1995; Hayakawa *et al.*, 1997; Munemoto *et al.*, 2002; Koda *et al.*, 2003; Tsang *et al.*, 2003; Ito *et al.*, 2004; Kanazawa *et al.*, 2004; Ohwasa *et al.*, 2004; Wong *et al.*, 2004; Wong *et al.*, 2005; Zeng *et al.*, 2005), *Ganoderma lucidum* (Shao *et al.*, 2004; Lin, 2005; Kuo *et al.*, 2006; Gao *et al.*, 2003), *Grifola frondosa* (maitake MD-fraction) (Atsuyuki *et al.*, 2002; Kodama *et al.*, 2002; Kodama *et al.*, 2003; Kodama *et al.*, 2005), *Astragalus membranaceus* (Shao *et al.*, 2004), *Panax ginseng* (Shin *et al.*, 2002; Lim *et al.*, 2004), and various other medicinal mushrooms (Ooi *et al.*, 2000; Lindequist *et al.*, 2005; Zaidman *et al.*, 2005). Molecular mechanisms for the immuno-biological functions may be through various receptors on macrophages, monocytes and NK-cells, which activate NF-κB and anti-tumor cytokine secretion. Interactions may include complement receptor type 3, CD14, mannose, and β-glucan receptors. There is evidence of interaction with TLRs, especially TLR4, with polysaccharides derived from *Astragalus membranaceus*, *Acanthopanax senticosus/koreanum*, *Ganoderma lucidum* and *Platyloden grandiflorum* (Han *et al.*, 2003; Han *et al.*, 2005; Schepetkin & Quinn, 2006).

Regulatory T-cells (Treg) and myeloid suppressor cells (MSC) inhibit the anti-cancer activity of NK and T-helper cells and are partly responsible for tumor progression, resistance to chemotherapy and ineffective anti-tumor vaccines. Enhancement of innate immunity seems to improve anti-cancer therapies. Treg are characterized by CD25 and FoxP3 expression. Their normal role is to control the adaptive immune response through cell contact-dependent mechanisms. The interplay between Treg and antigen responsive T-cells is modulated by dendritic cells (DC): whereas immature myeloid precursors of DC suppress T-cell activation and induce Treg development, mature monocytes (macrophages) override T-reg mediated suppression. Mature DC macrophages can be activated through the TLR pattern recognition receptors found on monocytes in the GI tract. They then secrete IL-6, which renders T helper and NK-cells refractory to the suppressive effect of Tregs (Kabelitz *et al.*, 2006). Other studies have shown that elimination of Tregs can significantly improve the outcome of cancer immunotherapy in preclinical models. For example, Sutmuller *et al.* (2001) showed that therapeutic whole-cell vaccination against melanoma was significantly more effective upon depletion of $CD4^+$ $CD25^+$ Treg with an anti-CD25 monoclonal antibody. Unfortunately, they also showed that Treg

depletion with anti-CD25 antibody carries an inherent risk of depleting tumor-specific effector $CD4^+$ and possibly $CD8^+$ T-cells, thus negatively affecting treatment efficacy. MSC may have additional properties that can compromise anti-cancer therapies, such as promotion of angiogenesis (Yang *et al.*, 2004). Specific cytokines also play a role in immune suppression. IL-13 and IL-4 are cytokines that suppress NKT-cell immunosurveillance (Terabe *et al.*, 2004).

Treg cells that suppress immune responses may limit the efficiency of cancer immunotherapy. Recent findings indicate that TLRs directly regulate the suppressive activity of Treg cells. Linking TLR signaling to the functional control of Treg cells may offer new opportunities to improve the outcome of cancer immunotherapy by coadminstration of certain TLR ligands (Wang, 2006). TLR stimulation blocks generation of DCs from progenitor cells, and diverts them to mature macrophage monocytes. This is achieved by inhibition of GM-CSF signaling through the induction of SOCS-1. Microbial ligands are able to skew the dichotomy of macrophage versus DC differentiation from common progenitors. In uninflamed tissues, GM-CSF induces the generation of immature DCs, preparing the host for the sensing of infectious danger. However, during infectious inflammation, TLR stimulation will drive incoming monocytes not to differentiate to DCs but to behave more like macrophages. This could be of help for the direct antimicrobial defense, which is more effectively mediated by macrophage-like cells with a high capacity to phagocytose. Pre-existing resident DCs are sufficient to perform the task of antigen sampling and transduction of information to the adaptive immune system. Thus, TLR stimulation would guide the innate immune system to assure a sufficient supply of phagocytic cells in inflamed tissues (Holger *et al.*, 2006). Garay reviewed the potential benefits of TLR agonists when added to chemotherapy TLR2/4 agonists to induce a well-controlled tumor necrosis factor-alpha (TNF-α) secretion, at plasma levels known to permeabilize neoangiogenic tumor vessels to the passage of cytotoxic drugs (Garay *et al.*, 2007). Moreover, TLR2/4 agonists induce inducible nitric oxide synthase (iNOS) expression, and nitric oxide is able to induce apoptosis of chemotherapy-resistant tumor cell clones. Finally, TLR2/4-stimulation activates dendritic cell traffic, macrophage production and cytotoxic T-cell responses.

POTENTIAL ROLE OF HERBAL DERIVATIVES AS ADJUVANTS FOR ANTI-CANCER VACCINES AND ENHANCEMENT OF CYTOTOXIC CHEMOTHERAPY

Breast cancer patients have increased levels of Treg cells (Lyanage *et al.*, 2002). Administered vaccine peptides need to be combined with strong adjuvants, such as TLR agonists. A peptide vaccination strategy that incorporates a TLR agonist could prevent the occurrence of spontaneous

breast tumors. Transgenic mice that carry the activated rat HER-2/neu oncogene were vaccinated with a synthetic peptide from the rat HER-2/neu gene product, in combination with a TLR agonist adjuvant. The results show that to obtain tumor antigen-specific T lymphocyte responses and antitumor effects, the function of CD4/CD25 T regulatory cells had to be blocked with anti-CD25 antibody therapy. Mice that were vaccinated with this approach remained tumor-free or were able to control spontaneous tumor growth and exhibited long-lasting T lymphocyte responses. The results suggest that similar strategies should be followed for conducting clinical studies in patients (Nava-Paradan *et al.*, 2007).

The polysaccharide β-glucans stimulate leukocyte anti-infective activity and enhance (murine) hematopoietic activity. In a study of human bone marrow mononuclear cells, PGG-glucan acts on committed myeloid progenitors to enhance activity by direct action independent of IL-3 (Turnbull *et al.*, 1999). β-glucans and polysaccharides are potent stimulators of TLR. Some specific polysaccharides have already been shown to boost the innate immune system through interaction with TLRs in mucosa associated lymphoid tissue (MALT) (Sen *et al.*, 2005; Rezaei, 2006; Tsan, 2006). Polysaccharide extracts and complexes from Chinese medicinal herbs and mushrooms seem to have a potential role for enhancing innate immunity. There is some evidence from clinical trials that they can improve survival (McCulloch *et al.*, 2006). Polysaccharide extracts from *Panax ginseng* can increase immunity and enhance chemotherapy (Lim *et al.*, 2004; Shin *et al.*, 2004; Han *et al.*, 2005). There is evidence of interaction with TLRs, especially TLR4 (Han *et al.*, 2003; Ahn *et al.*, 2006; Schepetkin *et al.*, 2006).

Maitake D-Fraction, a polysaccharide extracted from maitake mushrooms (*Grifola frondosa*), has been reported to exhibit an antitumor effect through activation of immunocompetent cells, including macrophages and T-cells, with modulation of the balance between T-helper 1- and 2-cells. It can decrease the effective dosage of mitomycin chemotherapy in tumor bearing mice by increasing the proliferation, differentiation, and activation of immunocompetent cells (Kodama *et al.*, 2005). Further evidence of the potential usefulness of polysaccharides in stimulating an enhanced immune response comes from a study of orally administered betaglucans (from maitake mushroom) that demonstrates an enhancement of the anti-tumor effects of monoclonal antibody targeted therapies (Cheung *et al.*, 2002). A meta-analysis of another immune-enhancing botanical, *astragalus*, reported an enhancement of the efficacy of platinum-based chemotherapy for lung cancer (McCulloch *et al.*, 2006) and PSK (*Coriolus versicolor*) for the enhancement of Tegafur for colorectal cancer (Ohwada *et al.*, 2004).

Immuno-suppression in cancer patients can reduce the efficacy of anti-cancer vaccines and increase complications from opportunistic infections.

Polysaccharides (mainly β-D-glucans alone or linked to proteins) from the cell walls of various traditional Chinese medicinal mushrooms and plants show anti-tumor and anti-infection activities through activation of monocytes, macrophages and NK-cells. A future research strategy should authenticate the source of these polysaccharide extracts and screen them for interaction with TLRs in the gastrointestinal tract of animals. Oral agents that boost cell-mediated immunity through the MALT may be subsequently evaluated in human phase I studies for dose-response (cytokine and immune cell assays) and safety. Optimized, authenticated polysaccharides may play a role in enhancing the potency of anti-cancer vaccines and other therapeutic modalities. These non-cytokine molecules appear to signal primarily through the TLRs, which are expressed by dendritic cells. In the MALT, these agonists can induce a host of proinflammatory cytokines such as tumor necrosis factor-α, IL-12, and IL-6, as well as $CD4^+$ and $CD8^+$ T-cells.

POTENTIAL ROLE OF HERBAL DERIVATIVES AS ADJUVANTS FOR RADIATION THERAPY AND TARGETED ANTIBODY THERAPIES

Combining radiation therapy and TLR agonists may reduce the amount of radiation therapy required to eradicate tumors, thus acting as an "immunosensitizer" (Koski & Czierniecki, 2005; Demaria *et al.*, 2005). Evidence of the potential usefulness of polysaccharides in stimulating an enhanced immune response is strengthened by the study of orally administered betaglucans (from maitake mushroom) that enhance the anti-tumor effects of targeted monoclonal antibodies (Cheung *et al.*, 2002). Ganopoly (a *Ganoderma lucidum* polysaccharide extract) modulated immune function in advanced-stage cancer patients. Treatment for 12 weeks resulted in a significant increase in the mean plasma concentrations of IL-2, IL-6, and IFN-γ, whereas IL-1 and TNF-α were decreased. NK activity was increased, but there was no significant change in the levels of $CD4^+$, $CD8^+$ or the $CD4^+/CD8^+$ ratio (Gao *et al.*, 2003). Lymphoproliferative neoplasms, such as lymphomas and leukemias, may be particularly sensitive to changes in cytokine balance. The Memorial Sloan-Kettering Cancer Center (New York, NY, USA) has commenced an NCI-sponsored phase I study of β-glucan and rituximab in pediatric patients with relapsed or progressive CD-20 positive lymphoma or leukemia (Clinical Trials gov, 2006).

PREVENTION OF CANCER PROGRESSION THROUGH IMMUNO-MODULATION WITH AND WITHOUT SPECIFIC VACCINES

In China there is a high incidence of chronic viral infections resulting in cancer. The sites of cancer include, liver, stomach, esophagus and nasopharynx. In addition, an increase in cervix cancer has made this the

second most common cancer in women. The cause of the relatively higher incidence of virus-associated cancers, compared with the West, is unclear. Factors include spread of infection, genetic predisposition, poor diet, and smoking. An inadequate response of the immune system to eradicate chronic viral infections and cancer cells is a common determinant. The total number of new cases of cancer is expected to increase by almost 15% by 2005. The increase in virus-associated cancers presents a major public health problem that requires more data to develop novel therapeutic strategies based upon local evidence-based remedies.

Hepatocellular carcinoma (HCC) is ranked second in cancer mortality in China and is now also increasing in frequency in males in many other countries. Hepatitis B (HBV) and C viruses remain major etiological factors, and the hepatitis G virus and other transfusion-transmitted viruses cannot be excluded (Tang, 2000). HBV infection is virtually ubiquitous in HCC patients in China. The tight association of HBV with HCC strongly suggests the dominant role of HBV infection in causing the cancer, Almost 12% of HCC patients have co-infection with hepatitis B and C (Gao *et al.*, 2005). A meta-analysis concludes that HBV and HCV infections are important independent risk factors for HCC, and that dual infection by HBV and HCV is associated with a higher risk of causing HCC than each infection alone, suggesting a synergism between HBV and HCV (Shi *et al.*, 2005). Nasopharyngeal cancer is associated with Epstein-Barr virus infection. (Zong *et al.*, 2001; Cheng *et al.*, 2002). It may also be associated with some cases of Hodgkins lymphoma (Jarett, 2003) and gastric cancer (Lo *et al.*, 2001). Its highest incidence is in the Southern Chinese population, with familial aggregation. The annual detection rate is 433 per hundred thousand for males and 499 for females in high-risk families, whereas the average overall annual incidence per one hundred thousand in Hong Kong is 24 males and 10 females respectively (Ng *et al.*, 2005). The incidence of cervix cancer is increasing. The combination of human papilloma virus (HPV) infection and smoking cigarettes is synergistic for the induction of cervix cancer and anal cancer. In the West, the sub-type HPV16 is the most common cause, whereas in China, other types of HPV viruses (*e.g.* HPV18 and 59) are more commonly associated with cervix cancer. The HPV16 E7 variant protein may induce a host humoral immune response, but not a special cellular immune response against it (Li *et al.*, 2004). This is made worse by smoking. Inducing an appropriate cell mediated immune response may be a key to eradicating the virus and its potential to induce and promote cancer. HPV16 virus is also a major factor in the development of esophageal cancer in China, but not yet in the West (Chang *et al.*, 2000; Li *et al.*, 2001). In addition, both the West and China are experiencing an increase in HPV associated head and neck cancers (Mellin *et al.*, 2000; Almadori *et al.*, 2002). Another major concern is the rise in breast cancer in women. Although a high fat diet, less exercise, and reduced parity are

contributing factors, infection with the HPV33 virus appears to play a role in China (Yu *et al.*, 2001; Cheng *et al.*, 2004).

There is reasonable epidemiological evidence that *Panax ginseng* is a non-organ specific cancer preventive, having a dose response relationship (Yun *et al.*, 2001). Ginseng extracts and its synthetic derivatives should be examined for their preventive effect on various types of human cancers. Some of the herbs modulate the immune system. *Astragalus, Ligusticum* and *Schizandrae* have a long history of medicinal use within the traditional Chinese system. Recently Western Science has begun to understand their pharmacological possibilities and clinical applications. *Astragalus* has demonstrated a wide range of immuno-potentiating effects and has proven efficacious as an adjunct cancer therapy. *Ligusticum*, and its active components, have been investigated for enhancement of the immune system, treatment of ischemic disorders, and as an anti-inflammatory. Clinically, the hepato-protective and antioxidant actions of *Schizandrae* have proven beneficial in the treatment of chronic viral hepatitis (Sinclair, 1998). More data are required to determine the clinical effects of Chinese herbs on immunity and to prevent cancer progression (Block & Mead, 2003). Research into the effectiveness of Chinese herbs on immunity may also help people with Acquired Immune Deficiency Syndrome (AIDS). The prevalence of AIDS is increasing rapidly (Qian *et al.*, 2005). The acquired immunodeficiency syndrome (AIDS) is a result of human immunodeficiency virus (HIV) infection, which subsequently leads to significant suppression of immune functions. The search for effective therapies to treat AIDS is of paramount importance. Several chemical anti-HIV agents have been developed. However, besides the high cost, there are adverse effects and limitations associated with using chemotherapy for the treatment of HIV infection. Thus, herbal medicines have frequently been used as an alternative medical therapy by HIV positive individuals and AIDS patients in China. For example, *Scutellaria baicalensis* Georgi and its identified components (*i.e.* baicalein and baicalin) have been shown to inhibit infectivity and replication of HIV (Wu *et al.*, 2001). Some preliminary evidence of efficacy has recently been published (Tani *et al.*, 2002).

Data from controlled clinical trials suggest that medicinal mushrooms may be beneficial as adjunctive anti-cancer therapies (Nakazato *et al.*, 1994; Matsui *et al.*, 2002). A randomized controlled trial of colorectal cancer patients receiving curative resection compared adjuvant chemotherapy alone to chemotherapy plus an extract from the fungus *Coriolus versicolor* (PSK). Both disease-free and overall survivals are significantly higher in the PSK group (Mitami *et al.*, 1992). Medicinal mushrooms contain the class of polysaccharides known as β-glucans that promote anti-tumor immunity. They may act synergistically with some of the new therapeutic antibodies and chemotherapy agents and protect normal marrow (Fullerton *et al.*, 2000; Cheung *et al.*, 2002; Lin *et al.*, 2004). *Maitake* mushroom, and

Ganoderma lucidum are both Chinese medicinal mushrooms showing preliminary evidence that they can suppress viral infections and inhibit cancer progression through modulation of the immune system (Kodama *et al.*, 2002; Sliva, 2003; Slikova *et al.*, 2004).

Future goals for cancer prevention in China will include public education to reduce the risk of infection, mass immunization, antiviral drugs, and chemotherapy. These are extremely expensive programs and more education and research are required prior to implementing the programs. Vaccination programs will not be effective without the use of appropriate adjuvants to enhance innate immunity. Many Chinese currently have access to traditional herbal remedies within the culture of Traditional Chinese Medicine (TCM). Some of these herbs and their derivatives are effective immuno-modulators. An opportunity exists to develop, refine and evaluate the effectiveness of Chinese herbal medicine to prevent the development of cancers secondary to virus infections. The idea of using Chinese herbs to prevent cancer progression is already being tested in the West. A TCM herb combination may reduce the risk of lung cancer in ex-smokers. An NCI (USA) sponsored study through the British Columbia Cancer Agency, led by Dr. Stephen Lam, is recruiting participants age 45–74, who are ex-smokers, to evaluate the efficacy of a herbal combination called Anti-Cancer Preventive Health Agent (ACAPHA) (Editorial, 2006). This contains *Sophora tonkinensis; Polygonum bistorta; Prunella vulgaris; Sonchus brachyotus; Dictamnus dasycarpus* and *Dioscorea bulbifera.* This herbal mixture modulates multiple physiological pathways that include the immune system. In Chinese studies, ACAPHA reduced the risk of esophageal cancer by 50%, through reversing severe esophageal dysplasia. In addition, their pilot study of 20 former heavy smokers with bronchial dysplasia treated with ACAPHA showed that, after 6 months, 50% had complete regression of dysplasia, compared to only 13% in the placebo group. Further evidence includes *Panax quinquefolium* (American ginseng), which appears to reduce death and increases quality of life in survivors of breast cancer, suggesting that it may prevent recurrence (Cui *et al.*, 2005).

CONCLUSIONS

The evidence indicates that the healthy immune system is necessary for the control of malignant disease and that immune suppression associated with cancer contributes to its progression. Tumors have developed strategies to successfully evade the host immune system, and various molecular and cellular mechanisms responsible for tumor evasion have been identified. Some of these mechanisms target immune antitumor effector cells. Dysfunction and apoptosis of these cells in the tumor-bearing host creates an immune imbalance that cannot be corrected by targeted immunotherapies alone. Reversal of existing immune dysfunction and normalization of lymphocyte homeostasis in patients with cancer needs to

be a part of future cancer immunotherapy (Whiteside, 2006). Therapeutic strategies are being designed to correct the immune imbalance, deliver adequate *in vivo* stimulation, transfer effector T-cells capable of *in vivo* expansion and provide protection for the immune effector cells re-populating the host. Survival of these cells and long-term memory development in patients with malignancy are necessary for improving clinical benefits of cancer immunotherapies. Polysaccharides derived from Chinese herbs and mushrooms are emerging agents that seem to enhance cytotoxic drugs, radiotherapy, surgery, and the newer targeted therapies and vaccines (Chang, 2002; McCulloch *et al.*, 2006). Rigorous authentication and quality control of these phytoceuticals are necessary prior to clinical studies (Sagar, 2007).

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Neem Leaf Glycoprotein as a New Vaccine Adjuvant for Cancer Immunotherapy

BARAL RATHINDRANATH[1,*], SARKAR KOUSTAV[1], MANDAL-GHOSH ISHITA[1] AND BOSE ANAMIKA[1]

ABSTRACT

An aqueous preparation of neem (Azadirachta indica) leaf (NLP) was tested for its adjuvant function to augment the immunogenicity of three tumor associated antigens, namely, B16 melanoma cell surface antigen (B16MelSAg), breast tumor associated antigen (BTAA) and carcinoembryonic antigen (CEA). NLP enhances the antibody response against these antigens to participate in antibody dependent cellular cytotoxicity. NLP is also efficient to augment the tumor antigen specific cytotoxic T lymphocyte reactions. This NLP generated anti-tumor immunity was translated in restriction of the tumor growth in vivo. NLP contains a glycoprotein participates in the observed immune functions of NLP and termed as NLGP. For further betterment of adjuvant like action, NLGP was tested in next set of experiments with an objective to generate effective CEA specific anti-tumor immune response in Swiss mice. CEA was presented using macrophages with adjuvant help from NLGP. Such vaccination generates significantly higher antibody (IgG2a) and T-cell response than immunization protocol without NLGP. NLGP controls the function of both B-cells and macrophages by altering expressions of various regulatory molecules, like, CD19, CD11b etc. This NLGP generated anti-CEA immune response would be effective as a vaccine to lyse CEA$^+$ tumors in vitro and in vivo. Overall results are encouraging to establish NLGP as an immunoadjuvant in connection to the development and application of several tumor antigens or its derivatives as cancer vaccines.

1. Department of Immunoregulation and Immunodiagnostics, Chittaranjan National Cancer Institute (CNCI), 37, S.P. Mukherjee Road, Kolkata-700 026, India.

* *Corresponding author* : E-mail : rbaral2@hotmail.com

Key words : Adjuvant, immunity, neem leaf glycoprotein, tumor antigen, vaccine

ADJUVANTS IN IMMUNE RESPONSE

Adjuvants have been used for decades to improve the immune responses against antigens (Audibert & Lise, 1993). Adjuvants work by stimulating innate, humoral, cellular or all wings of the immune system (Kensil *et al.*, 1991) by activating NK-cells, macrophages, B-cells, T-cells etc. (Lipford *et al.*, 1997; Kawarada *et al.*, 2001). It can potentiate the immune response either antigen specific or non-specific manner (Baral, 2005). In antigen specific vaccines, incorporation of adjuvants into vaccine formulations is aimed to enhance, accelerate and prolong the specific immune response of vaccine antigens (Gary & Gary, 2007). Advantages of adjuvants include the enhancement of the immunogenicity of antigens, modification of the nature of immune response, reduction of the antigen amount and frequency of booster immunizations needed (Cooper *et al.*, 1991; Koike *et al.*, 1998). In non-specific vaccines, adjuvants can stimulate various wings of the immune system, thereby, boost up the immune functions to fight against a disease (Marciani, 2003).

TUMOR ASSOCIATED ANTIGENS ARE KEY FOR VACCINE DEVELOPMENT

Cancer cells are well identified by expression of one or more antigens, known as tumor associated antigens (TAAs) (Stevanovic *et al.*, 2002; Renkvist *et al.*, 2001). TAAs received considerable interest as a vaccine candidate for active specific or passive immunotherapy (Slovin *et al.*, 2005; Schuster *et al.*, 2006). In active specific immunotherapy, TAA or a peptide representing part of the TAA, were used as vaccines (Conry *et al.*, 1995; Disis *et al.*, 1999). TAA or generated peptide alone was not detected as immunogenic in patients due to several reasons including self tolerance and needed the help of immunostimulating adjuvants (Lipford *et al.*, 1997). Additionally, cancer patients are severely immunosuppressed (Shibuya *et al.*, 2003), thus, generation of antigen specific immune response depends on the activation of several immune compartments to work in a co-ordinated manner (Bocchia *et al.*, 2000).

DIFFERENT ADJUVANTS

Several adjuvants are identified, characterized and tested in clinical settings as described in the Table 2.1. Among many, a discussion on adjuvant should mention the discovery of Jules T. Freund (1890–1960), Hungarian-born American Immunologist. Adjuvants described by him, known as Freund's complete and incomplete adjuvants, are very useful to boost up the immune system. Unfortunately, these adjuvants are not suitable for human use

Table 2.1. Comparison of NLGP with other available adjuvants

S. No.	Adjuvant name	CTL response	Humoral response	Th1/Th2	Non-TLR or TLR patways	Delivery	Toxicity	Antigen if any	Clinical trials	Ref.
1.	**NLGP** (Neem Leaf Glyco Protein)	Ag-specific CTL responses	Antibody response & ADCC reaction	Induces Th1 response in mice and humans	Yet to be determined	Non-oral route	100% Non-toxic	Tested with CEA, BTAA & B16MelSag	After successful preclinical animal and human (*in vitro*) studies, clinical trial is going to be initiated	(1–9)
2.	**QS21** (a saponin purified from the bark of the *Quillaja saponaria* Molina tree)	Ag-specific CTL responses	Antibody response & ADCC reaction	Induces Th1 response from Th2 in humans		Active in injectable form	Non-Toxic, Local skin reaction & mild fever	Tested with Ovalbumin & several other antigens	Tested in several clinical trials on several diseases including cancer	(10–15)
3.	**ISCOM AND ISCOMATRIX** (a saponin preparation, a sterol and a phospholipid)	Optimal induction of CTL responses	Generate a humoral responses	Promote Th1-biased immunity.			No reportable toxicity		Showed promise in late-phase human clinical trials for viral vaccines	(16–20)
4.	**Alum** (Aluminium hydroxide)	No CTL response	Strong humoral response	Generates strongly Th2-polarized responses	Non-TLR pathway	Injectable route	No significant toxicity	Tested with diff. antigens and anti-idiotype vaccines	It is one of the most common adjuvants used in clinical trials for cancer vaccines	(21–24)

Table 2.1. *Contd.*

S. No.	Adjuvant name	CTL response	Humoral response	Th1/Th2	Non-TLR or TLR patways	Delivery	Toxicity	Antigen if any	Clinical trials	Ref.
5.	**CFA** (complete Freund's adjuvant water in oil emulsion with *M. tuberculosis*)	Ag-specific CTL response	Humoral immune responses	Th1 type	Increases TLR2 mRNA but TLR4 in mice livers	Sub-cutaneous injection	Toxic for human use	Tested with diff. antigens and anti-idiotype vaccines	It is one of the most common adjuvants used in experimental studies	(25–28)
6.	**CpGODN** [Oligodeoxy-nucleotides containing unmethylated CpG motifs]	Ag-specific CTL responses	Humoral immune response	Th1 type immune response	Works through TLR9	Injectable route	Non-toxic	Tested with diff. antigens and anti-idiotype vaccines	Clinical trials are ongoing	(29–34)
7.	**IC31** (a mixture of a synthetic ODN and an 11-mer cationic peptide)	Ag-specific CTL responses	Not known	Th1 Type	TLR9 agonist			Tested with influenza vaccine	Phase I clinical trials are ongoing	(35–36)
8.	**Iomai Patch** (patch-based adjuvant)	T cell responses	Elicits systemic antibody response	Mixed Th1/Th2 phenotype but with a Th2 bias		Through patch	Yet to be determined		Tested in clinical trials for influenza vaccine	(37–40)
9.	**Vaxfectin** (A noval cationic and neutral lipid formulation)	CTL response	Enhances antigen specific antibody titers	Maintains Th1 type immune responses		Intrader-mal and intra-muscular routes are effective	Non-toxic	Tested for complexing with an antigen encoding plasmid	Tested in clinical trials for plasmid vaccines	(41–43)

Table 2.1. *Contd.*

S. No.	Adjuvant name	CTL response	Humoral response	Th1/Th2	Non-TLR or TLR patways	Delivery	Toxicity	Antigen if any	Clinical trials	Ref.
10.	**Resiquimod** (low mol. wt. compounds, the imidazoquinolinamines)	Augments cell mediated immune responses	Augments humoral responses	Th1 biased	Binds to TLR7		Non-toxic			(44–46)
11.	**Rantes** (A chemokine)	Induces cell-mediated immunity	Augments humoral responses	Th1 biased	Not known		No toxicity was observed		Clinical studies initiated	(47–50)

References included in Table 2.1

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due to its toxic nature (Audibert & Lise, 1993; Steiner *et al.*, 1960). Aluminium hydroxide (Alum) is another popular adjuvant used in human clinical trials and this adjuvant is potent to stimulate humoral immune system (Maruyama *et al.*, 2000). A saponin extract of the tree *Quillaja saponaria* (QS-21) (Evans *et al.*, 2001) and oligodeoxynucleotide containing unmethylated CpG motif (Klinmann *et al.*, 2004) currently received much attention in adjuvant biology. These are good immunostimulators, can be used along with some antigens in antigen specific immunotherapy (Baral *et al.*, 2003). Moreover, these can also be used in non-specific immunotherapy. With description of several adjuvants till date, demand to search a non toxic, immunostimulatory and economical adjuvant is not fulfilled completely.

NEEM-TRADITIONAL VIEW

The world has been learned on natural management of several diseases from Indian Ayurveda (Diasio & LoBuglio, 1996; Atal *et al.*, 1986). Among several natural resources, neem (*Azadirachta indica*) received the key interest in traditional medicine as SARBOROGANIBARANI (can cure all forms of diseases) and eight parts of this plant have wonderful biological actions (Biswas *et al.*, 2002). Most of these bioactivities are proved in daily life, not in laboratories. These daily experiences of people from all communities made a stable platform for research in various directions. In 1992, US National Academy of Science designated this tree, as 'A tree solving global problem' (National Research Council, 1992). In our studies on active specific immunotherapy in mice tumor models, three tumor antigens have been used along with an aqueous preparation from neem leaf as an adjuvant (Baral *et al.*, 2005; Mandal-Ghosh *et al.*, 2007). This preparation is termed as neem leaf preparation (NLP) (Baral & Chattopadhyay, 2004).

IMMUNOMODULATION BY NEEM LEAF PREPARATION

In course of our work, we found the reflection of earlier hypothesis that neem derivatives have immunomodulatory functions (Ray *et al.*, 1996; Upadhyay *et al.*, 1992). The hypothesis was elaborated by a series of investigations from our group (Baral *et al.*, 2005; Bose *et al.*, 2005; Haque & Baral, 2006; Bose *et al.*, 2007). Although, all parts of neem tree have enormous biological significance, we are concentrated on leaf and a preparation made from neem leaf, designated as NLP is being used. First reported observation of our work is: NLP causes prophylactic growth inhibition of murine carcinoma and melanoma by significant immunostimulation (Baral & Chattopadhyay, 2004). This NLP is non-toxic, hematostimulatory and immunostimulatory (Haque *et al.*, 2006). In mouse system, activation of NK-cells and NK-T-cells by NLP was detected (Haque & Baral, 2006), which is also true for macrophages, NK-cells, NK-T-cells, T-cells of human origin (Bose & Baral, 2007). This activation is chiefly reflected on cytokine signaling and we observed that NLP stimulates

macrophages to release IL-12. This IL-12 further activates NK and T-cells to secrete the signature Th1 cytokine, IFNγ. IL-12 dependence of the NLP mediated release of IFNγ was confirmed by neutralization of IL-12 in the system (Bose & Baral, 2007; Bose *et al.*, 2009a). On the other hand, NLP downregulates the release of Th2 cytokines IL-10 (Mandal-Ghosh *et al.*, 2007) and IL-4 (Bose *et al.*, 2009b). NLP cannot induce the tumor cell apoptosis, however, NLP stimulates the release of cytotoxic cytokines from immune cells that can induce tumor cell apoptosis (Bose *et al.*, 2007).

NEEM LEAF PREPARATION AS AN IMMUNOADJUVANT

An ideal adjuvant should be a good immunostimulant (Kensil *et al.*, 1991; Kawarda *et al.*, 2001; Lipford *et al.*, 1997). Immunostimulatory activities of NLP described above encouraged us to use it as an immune adjuvant. Testing of the adjuvanicity of NLP was done in association with antigens expressed on three different malignant tumors, namely, melanoma, breast carcinoma and colon carcinoma. Immunogenecity of the poorly immunogenic B16 melanoma cell surface antigen (B16MelSAg), breast tumor associated antigen (BTAA) and carcinoembryonic antigen (CEA) was enhanced by combining these antigens with NLP in mice (C57BL/6, Swiss, Balb/c) and rats (Sprague dawley) as evidenced by ELISA (Fig 2.1a), flow cytometry (Fig 2.1b) and immunocytochemistry (Fig 2.1c) (Baral *et al.*, 2005; Mandal-

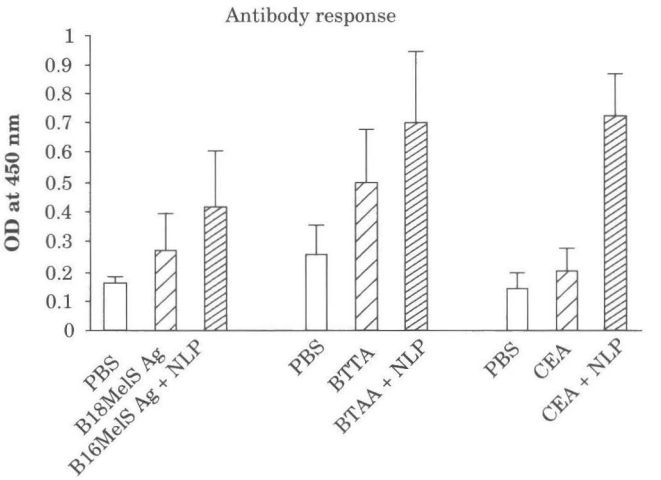

Fig 2.1a. NLP augments antibody response against B16MelSAg, BTAA and CEA. Three groups of mice (n = 6 in each group/set) were immunized weekly for four weeks with PBS, Antigens (B16MelSAg/BTAA/CEA) and Antigens + NLP respectively. Sera were tested for the presence of antigen specific antibodies by ELISA

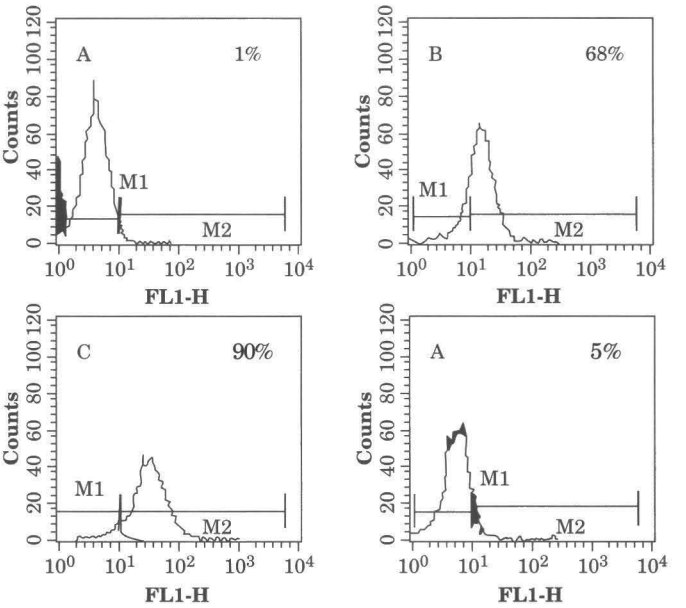

Fig 2.1b. Reactivity of NLP generated B16Mel antibody to B16MelSAg present on cell surface by Flow Cytometry. Viable B16Mel cells were incubated with control and immune sera (1: 50 dilution in 1% BSA), collected from mice immunized with either PBS (A) or B16MelSAg (B) or B16MelSAg + NLP (C) weekly for 4 weeks. In a control experiment, cells were incubated with goat anti-mouse IgG labeled with FITC only (D). Cells present in the zone M2 are considered reactive with B16Mel antibody

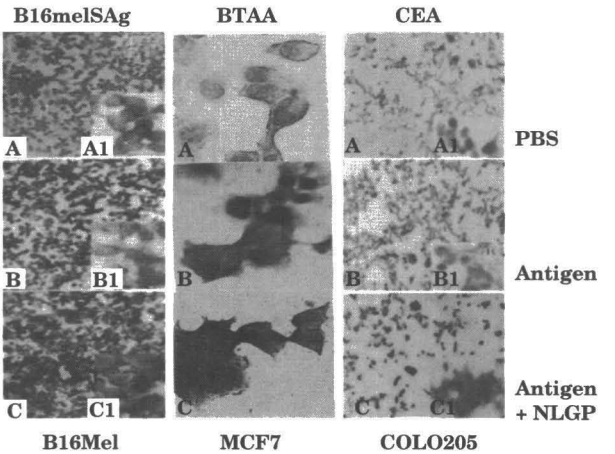

Fig 2.1c. Reactivity of the NLP generated antibody to B16Melanoma, MCF7 and COLO205 cells. Cells were grown on tissue culture slides and incubated with sera (at 1: 100 dilution) from PBS, Antigens (B16MelSAg/BTAA/CEA) and Antigens + NLP immunized mice. Antigen specific antibody binding to cells was determined by immunoperoxidase-staining. Panel 1 & 3, 100X; Panel 2 and inset, 400X

Ghosh *et al.*, 2007). NLP is as effective as Freund's complete and incomplete adjuvant to generate antibody recognizing the B16MelSAg. The NLP generated antibody was a gamma globulin with a subtype of IgG1 during immunization with B16MelSAg (Baral *et al.*, 2005), whereas, it is IgG2a after vaccination with BTAA (Mandal-Ghosh *et al.*, 2007) and CEA (Sarkar *et al.*, 2008) (Fig 2.2a). Generation of IgG2a type immune response by adjuvant effect of NLP directs the creation of Th1 type microenvironment required for optimum anti-tumor immune response. In addition, vaccination with BTAA generates antibody response with IgM predominance.

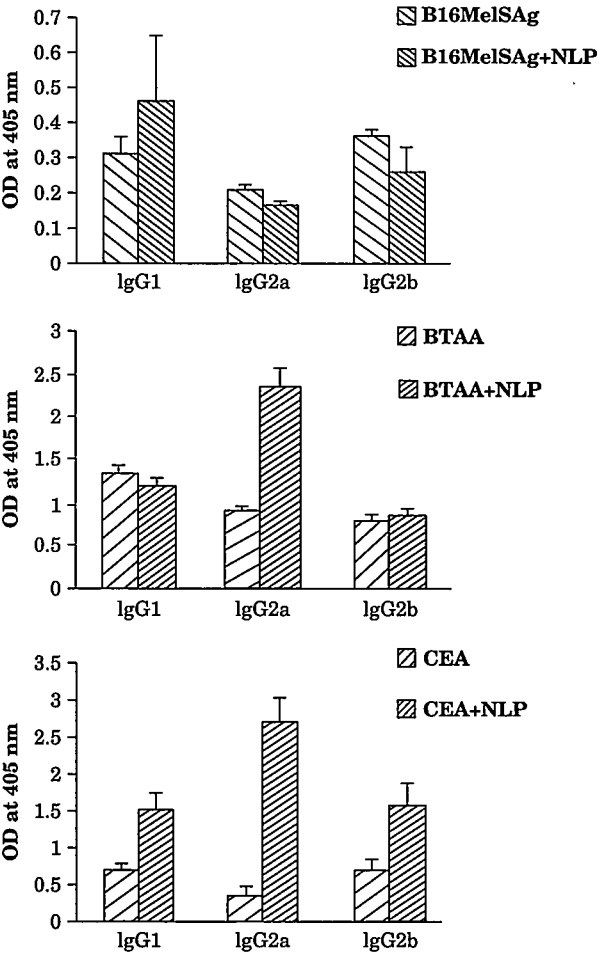

Fig 2.2a. Analysis of the IgG subclasses present in the sera of immunized mice. Three groups (n = 4 in each group) of mice were immunized with PBS, Antigens (B16MelSAg/BTAA/CEA) and Antigens + NLP weekly for 4 weeks in total. Seven days after last injection, sera were assessed for IgG1, IgG2a and IgG2b antigen specific-antibodies by ELISA. Each bar represents the group mean of each response

Combination of BTAA with NLP reduces IgM with production of more IgG (Fig 2.2b). Generation of the antigen specific immune response with IgG predominance, rather than IgM is a goal of immunotherapy (Sen *et al.*, 1998; Baral, 2005). Adjuvant property of NLP to enhance the antigen specific immune response is not only restricted in single species, as we observed the antibody response in various mice strains (C57BL/6, Swiss, Balb/c) and rats (Sprague dawley). Maximum adjuvant effect of NLP was observed

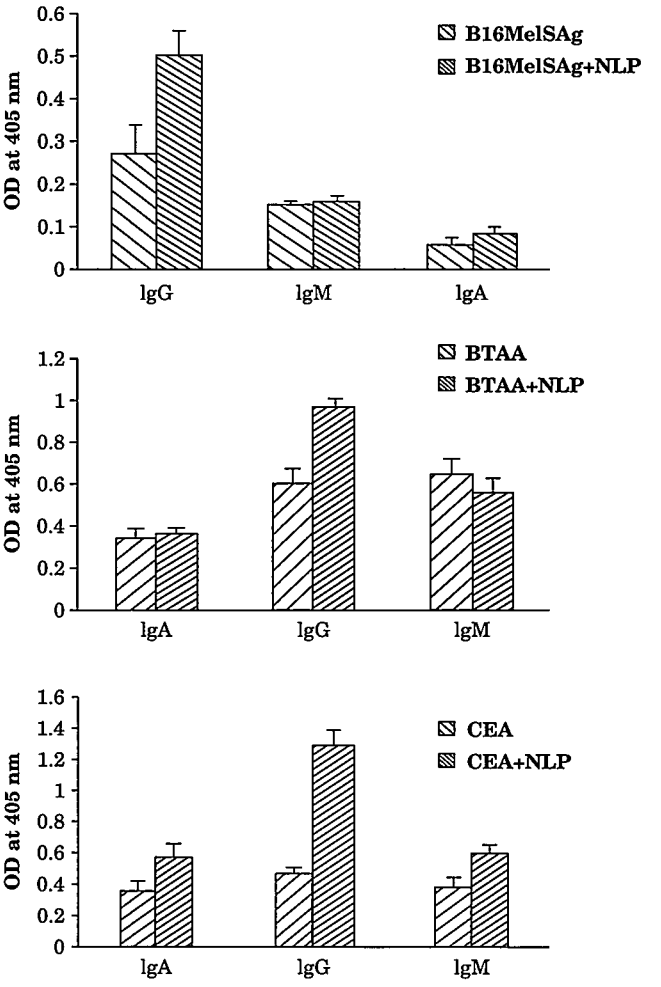

Fig 2.2b. Analysis of the immunoglobulin isotypes in the sera of immunized mice. Three groups (n = 4 in each group) of mice were immunized with PBS, Antigens (B16MelSAg/BTAA/CEA) and Antigens + NLP weekly for 4 weeks in total. Seven days after last injection, sera were assessed for IgA, IgG and IgM content by ELISA. Each bar represents the group mean of each response

when immunogen was administered intravenously. However, effect was also prominent when immunization was given in peritoneal route (Fig 2.3) (Mandal-Ghosh *et al.*, 2007). Exposure of immunogens to the antigen presenting cells may play the vital role in production of immune response and mechanism involved in the NLP mediated antigen presentation is a crucial area of research.

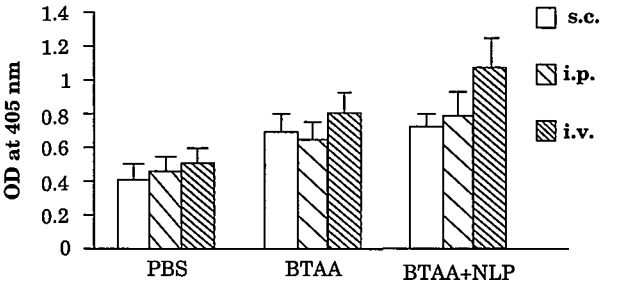

Fig 2.3. Route of immunization influences generation of BTAA reactive antibodies. Balb/c mice were immunized weekly for four weeks with PBS, BTAA and BTAA + NLP by *s.c.*, *i.p.* and *i.v.* routes. After seven days of last immunization the mice were bled and anti-BTAA antibodies in the sera were measured by ELISA. Data represent mean ± SD of six observations

NLP GENERATED ANTIBODY RESPONSE AND ANTITUMOR IMMUNITY

NLP assisted generation of IgG type antibody response is closely associated with the development of anti-tumor immune response. The immune sera generated by either B16MelSAg or BTAA or CEA in presence of NLP in mice and rats caused enhancement of the antibody dependent cellular cytotoxicity (ADCC) (Fig 2.4a) *in vitro* specifically towards antigen positive cells, *e.g.* B16Melanoma, MCF7 and COLO-205 respectively (Baral *et al.*, 2005; Mandal-Ghosh *et al.*, 2007). NLP mediated augmentation of the ADCC was further proved by insignificant killing of antigen negative cells. Besides *in vitro* studies, it was also observed that vaccination of mice with B16MelSAg + NLP, more efficiently prevented the growth of B16 melanoma tumor than mice immunized with B16MelSAg or NLP alone (Fig 2.4b). In another experiment, the immune sera (B16MelSAg + NLP) was mixed with B16Mel tumors and injected subcutaneously into syngenic C57BL/6 mice. Tumor burden was less in mice receiving tumor along with B16MelSAg + NLP generated immune sera than other groups (Fig 2.4c). These experiments proved that NLP generated antibodies play the significant role in the induction of anti-tumor immunity (Baral *et al.*, 2005).

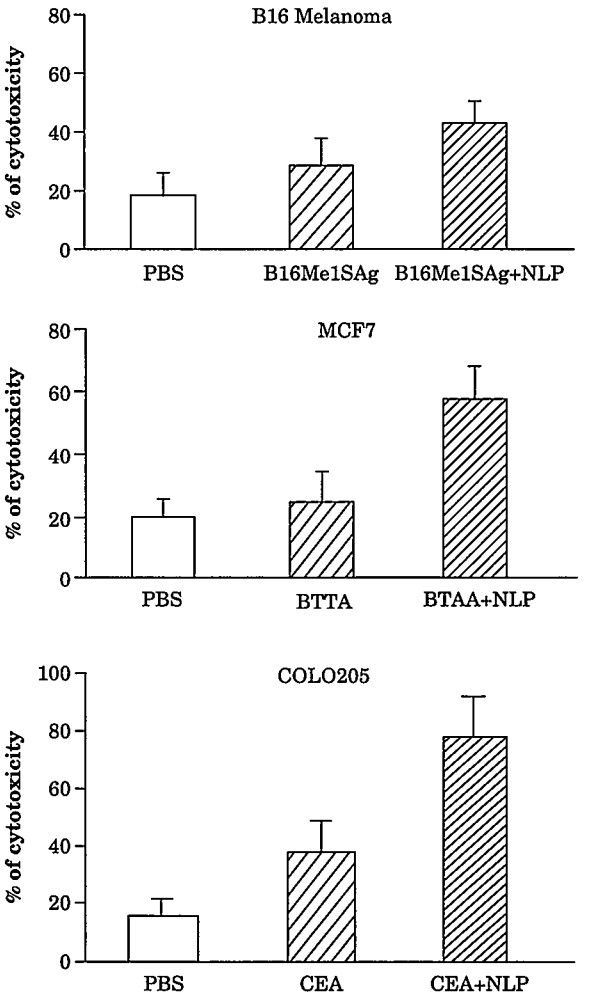

Fig 2.4a. Assessment on the induction of antibody dependent cellular cytotoxicity by immune sera. Tumor cells (Target) (B16Melanoma, MCF7 and COLO205) were incubated with normal mice spleen cells (Effector) in presence of 1 : 100 dilution of either PBS or immune sera (from B16MelSAg/BTAA/CEA immunized mice in presence or absence of NLP) for 4 h using an effector-target ratio, 1: 10. Tumor cell lysis was determined by LDH release assay. Data obtained by the incubation of Effector and Target in absence of sera were always substracted from the data from Effector + Target + Sera

NLP GENERATED CELLULAR IMMUNE RESPONSE

With demonstration of the role of NLP generated antibody in the restriction of tumor growth, involvement of the cellular immune system was also investigated. Splenic lymphocytes from Antigen + NLP treated mice

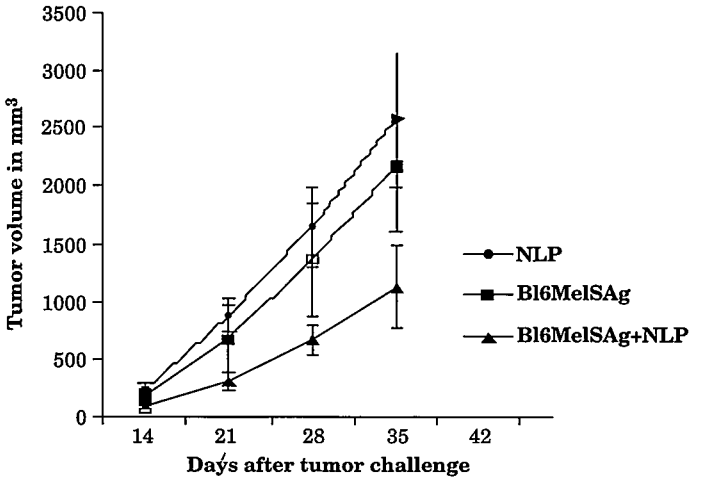

Fig 2.4b. *In vivo* growth of B16Mel tumors in mice. Three groups of mice (n = 6 in each group) were immunized with by either NLP or B16MelSAg or B16MelSAg + NLP weekly for 4 weeks. Seven days after the last injection mice were subcutaneously inoculated with B16Mel tumor cells (1×10^6) in each mice. Tumor growth was monitored weekly by caliper measurement

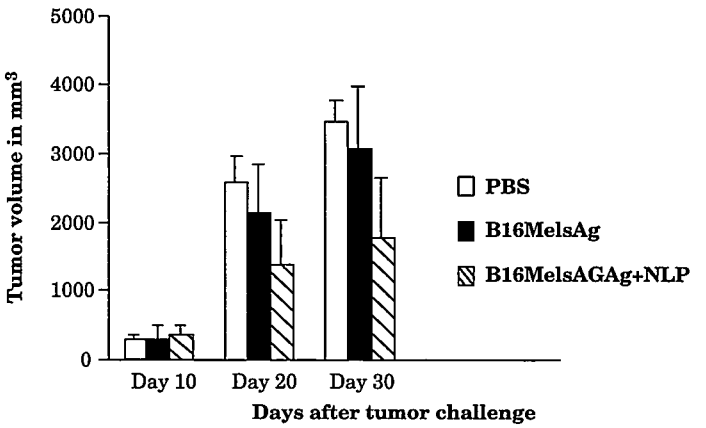

Fig 2.4c. *In vivo* growth of B16Mel tumors treated *in vitro* with immune sera. B16Mel tumor cells (6×10^6) were co-cultured with immune sera (300 mL) for 2 h. Immune sera were generated by immunizing mice with either PBS or B16MelSAg or B16MelSAg+ NLP. Treated tumor cells (1×10^6) were then injected subcutaneously to three groups of C57BL/6 mice (n = 6 in each group). This figure is the representative of two identical experiments

proliferated more rapidly *in vitro* when stimulated by specific (B16MelSAg/BTAA/CEA) and nonspecific (ConA) stimulators, in comparison to the proliferation detected in Antigen and NLP treated groups (Baral *et al.*, 2005; Mandal-Ghosh *et al.*, 2007). Splenic cells isolated from these mice

undergone treatment with three different sets of antigens along with NLP and cultured *in vitro* in presence of specific antigen for 5 days. These cells were further co-cultured with three different antigen positive tumor cells. It was observed that spleen cells obtained from Antigen + NLP immunized mice were cytotoxic maximally to the antigen positive tumor cells, in comparison to those spleen cells obtained from mice immunized with antigen only (Fig 2.5). These results suggest that NLP helps in the generation of cytotoxic tumor cells that may ultimately participate in the *in vivo* restriction of tumor growth.

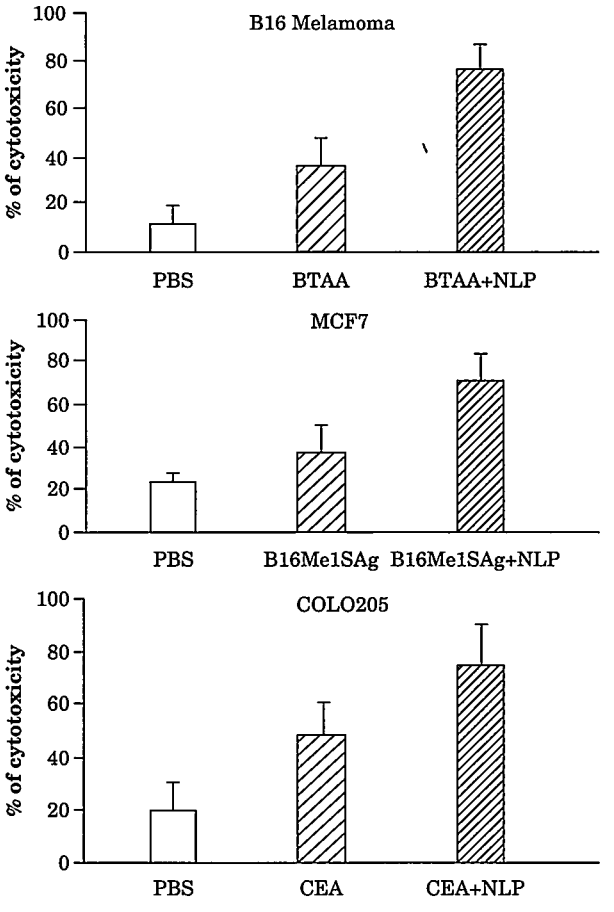

Fig 2.5. CTL activity of the splenic cells of immunized mice. Three groups (n = 4 in each group) of mice were immunized with PBS, Antigens (B16MelSAg/ BTAA/CEA) and Antigens + NLP weekly for 4 weeks in total. Seven days after the last injection, splenic cells from individual mice were isolated, cultured in presence of antigens for 3–5 days and CTL assays were conducted using these cells as effectors and B16melanoma/MCF7/COLO205 cells as target at an E: T ratio 10: 1

ACTIVE PRINCIPLE OF NLP IS A GLYCOPROTEIN, TERMED NLGP

With progression of the work to this point, it was felt necessary to upgrade the adjuvant preparation with identification and characterization of it and to use the partially purified form as a vaccine adjuvant. In our work to fulfill this objective, we have demonstrated the presence of a glycoprotein in NLP, which is designated as neem leaf glycoprotein (NLGP) (Chakraborty *et al.*, 2008). Purified NLGP appeared in nondenatured PAGE as a single band, whereas, three bands in SDS-PAGE (Fig 2.6a). This glycoprotein constitutes the carbohydrate moiety of about 33%, consisting of arabinose, galactose and glucose. Protein moiety of the glycoprotein consists of sixteen amino acids, except arginin. Scanning electron microscopy reveals the protein like structure of the NLP constituents. Immunogenecity of this protein was defined by strong reaction of the anti-NLP sera with NLP by ELISA and immunoblot analysis. NLGP is the active constituent of NLP in relation to the immunomodulation and tumor growth restriction. This hypothesis was proved by exposing NLGP in an array of temperature, pH and enzymes (Fig 2.6b). Exposure to the adverse temperature (100°C), pH (5.7) and proteolytic enzymes (papain) resulted complete disappearance of the tumor growth restricting activity of the NLP/NLGP.

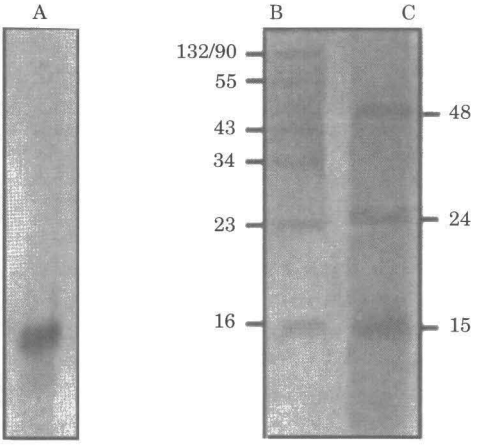

Fig 2.6a. Analysis of NLGP by PAGE. NLGP was analyzed by non-denatured (A) and denatured (B) PAGE. Gel was stained with silver nitrate method. Molecular weight markers were electrophorsed in each run

NLGP IN THE IMPROVEMENT OF ANTIGEN PRESENTATION

In continuation with the effort to demonstrate the adjuvant function of NLP to enhance the immunogenicity of the poorly immunogenic B16MelSAg (Baral *et al.*, 2005), BTAA (Mandal-Ghosh *et al.*, 2007), CEA (Sarkar *et al.*, 2008), immunization protocol was modified with NLGP instead of NLP. To improve the antigen presentation, a macrophage-based system

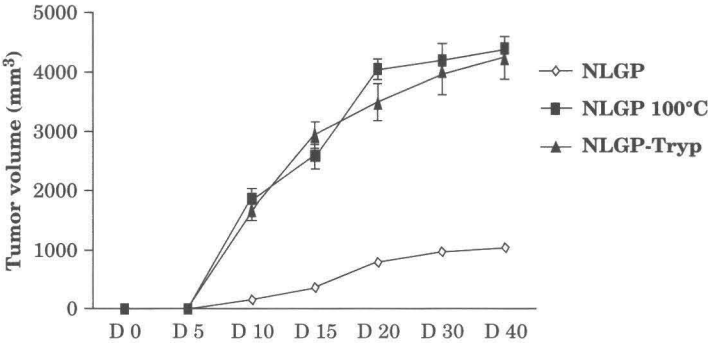

Fig 2.6b. *In vivo* tumor growth restriction by NLGP and NLGP exposed to 100°C and trypsin. Three groups of mice were injected with either NLGP or NLGP exposed to 100°C and trypsin weekly for four weeks. Seven days after the last injection mice were inoculated with Ehrlich's carcinoma cells subcutaneously (1×10^6) (n = 6 in each group). Tumor growth was monitored regularly

was used besides direct immunization of animals with antigen with or without NLP. For this purpose, macrophages were pulsed with CEA and assessment was performed on the morphological alterations of such macrophages during antigen pulsation in presence of NLGP. Significant difference in macrophage adherence and extension of pseudopodes were noticed due to exposure of NLGP (Sarkar *et al.*, 2008). These findings suggest that NLGP promotes the adsorption of CEA by macrophages to present to MHC molecules for necessary B-cell and T-cell functions. Next, we have analyzed the B-cell functions by monitoring anti-CEA antibody against immunization with CEAMϕNLGP (CEA pulsed macrophages in presence of NLGP) and CEAMϕ (CEA pulsed macrophages). Significant greater anti-CEA antibody response was observed when immunization was made by CEA pulsed Mϕ along with NLGP (Fig 2.7). One fact should be mentioned here that NLGP is also able to increase the anti-CEA antibody response, when CEA was administrated without its pulsation with macrophages (unpublished observation). However, pulsation of macrophages with CEA always developed better antibody response than CEA administration alone as tested in mice system. This approach may be explored as a vaccine protocol to break the tolerance that is generated occasionally in human system during CEA vaccination due to self antigenic nature of CEA (Bhattacharya-Chatterjee *et al.*, 2000; Saha *et al.*, 2004). Additionally, NLGP as an adjuvant would be effective to enhance the generation of anti-CEA antibody response more effectively than total preparation, NLP.

NLGP IN THE ALTERATION OF B-CELL PHENOTYPIC MARKERS

To explore the alteration in B-cell phenotypes during generation of the better antibody response by CEA pulsation of macrophages in presence of

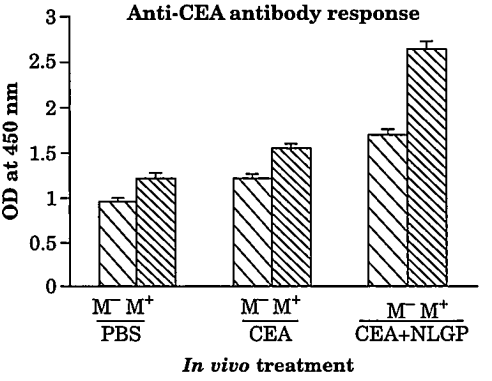

Fig 2.7. NLGP augments antibody response against CEA. Three groups of mice (n = 6 in each group) were immunized weekly for four weeks with PBS, CEA and CEA + NLGP respectively. In another set of experiment, three groups of mice (n = 6 in each group) were immunized weekly for four weeks with macrophages, pulsed with either PBS, CEA and CEA + NLGP. Sera from these six groups of mice were tested for the presence of CEA specific-antibodies by ELISA

NLGP, we have determined the expression of three different B-cell markers, *e.g.* CD19, CD23 and CD5. *In vitro* treatment of normal splenocytes with CEA + NLGP resulted upregulation of CD19 on cell surface. CD19 has immense role in generation of humoral response as ablation of the B-cell surface glycoprotein CD19 severely impairs the humoral immune response (Buhl *et al.*, 1997). CD19 is responsible for B-cell antigen receptor stimulation and enhancement of CD19 by NLGP resulted better production of anti-CEA antibody response. Downregulation of CD23 was associated with the B-cell activation (Dinkel *et al.*, 1997). However, our observation on no significant change in the expression of CD23 marker on B-cells was detected after CEA presentation with NLGP, may indicate the steady maintenance of the B-cell activity. On the otherhand, a slight down-regulation of CD5 may reflect a decrease in IgM secreting B1-cells after CEAMϕNLGP vaccination (Sarkar *et al.*, 2008). This incidence may denote the transformation from early to late stage of lymphopoiesis (Barker & Verfaillie, 2000) with generation of a burst of IgG2a response, which is specific for CEA, present on tumor cell surface.

NLGP IN THE ALTERATION OF PHENOTYPIC MARKERS ON MACROPHAGES AND ENHANCEMENT OF ADCC

NLGP enhances the $CD14^+$ cells either in CEA pulsed or unpulsed conditions. As CD14 is a marker of macrophages, it indicates the NLGP mediated upregulation of the number of macrophages, those may participate both in antigen presentation and ADCC reaction. In our study to observe the alteration in Fc receptor (CD16/32), we found the upregulation of it

during CEA pulsation in presence of NLGP. Fc receptors are generally present on macrophages and NK-cell surfaces and these cells participate in ADCC reaction as effector cells (Sánchez-Mejorada & Rosales, 1998). In support of this observation greater ADCC was demonstrated due to vaccination with CEAMϕNLGP (Fig 2.8). NLGP may facilitate the antigen delivery to B-cells to generate anti-CEA IgG2a first, then this CEA specific antibody might react with CEA^+ tumor cells and make a bridge with CD16/32 expressing macrophages and NK-cells through their Fc region for ADCC reaction. In CEA pulsed macrophages with NLGP influence, expression of CD11b was also increased. CD11b non-covalently associates with CD18 to form Mac-1 ($\alpha_m \beta_2$ integrin) and participate in adhesive cell interaction. We have also observed the increase in adhesiveness of CEA pulsed macrophages in presence of NLGP and may have some role in presentation of CEA to B-cells (Sarkar *et al.*, 2008).

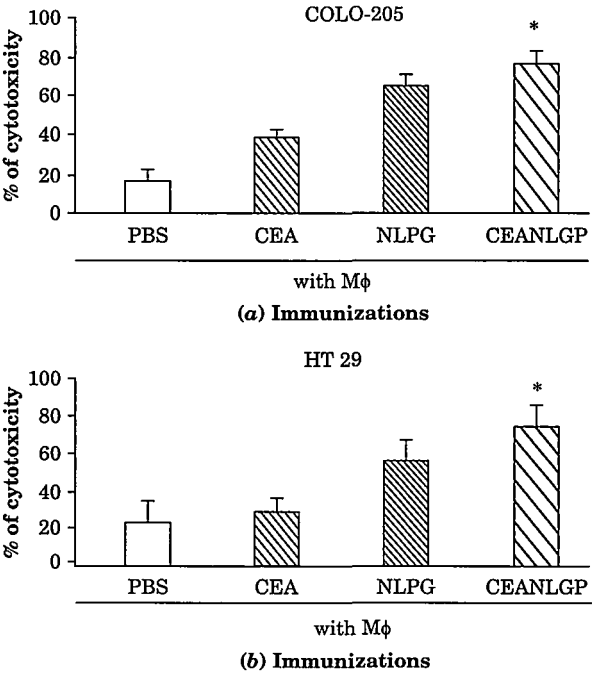

Fig 2.8. Sera from CEAMϕNLGP immunized mice exhibits enhancement in ADCC to CEA^+ cells. Four groups of mice (n = 6 in each group) were injected with Mϕ, CEAMϕ, NLGPMϕ and CEAMϕNLGP weekly for 3 weeks. Seven days after the last injection, sera from individual mice were collected and tested in ADCC. Splenocytes isolated from a syngenic mice were used as effector and CEA^+ colon tumor cells, COLO 205 (a) and HT 29 (b) were used as target. Data obtained by using an E : T ratio of 10 : 1 and immune sera at a dilution of 1: 100 is presented. Data represent the mean value ± SD. *$p<0.0001$, in comparison to CEAMϕ group

NLGP ENHANCES TUMOR GROWTH RESTRICTION DURING CEA VACCINATION

In continuation of our demonstration of cellular cytotoxicity of CEA^+ COLO-205 and HT-29 cells in antibody dependent manner, we have examined tumor growth restriction *in vivo* after immunization of mice with either CEAMϕ or CEAMϕNLGP. In accordance with *in vitro* demonstration, significant tumor growth restriction was noted in mice immunized with CEAMϕNLGP (Fig 2.9), where significantly higher antibody response was

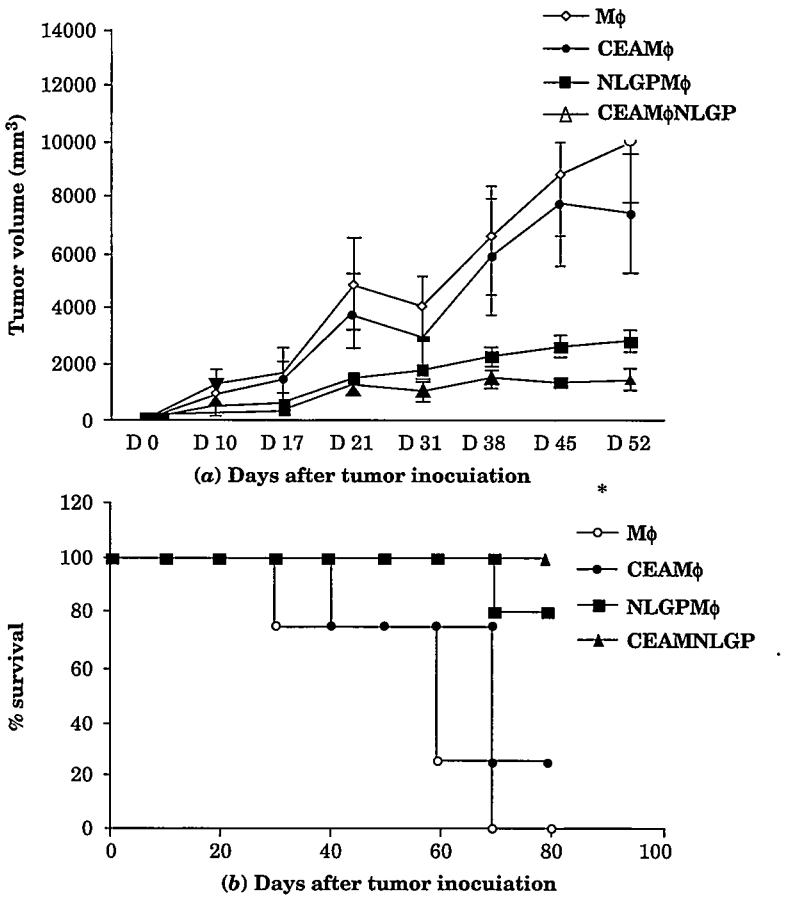

Fig 2.9. CEAMϕNLGP immunization prevents *in vivo* growth of CEA^+ tumors in mice. Four groups of mice (n = 6 in each group) were injected with Mϕ, CEAMϕ, NLGPMϕ and CEAMϕNLGP weekly for 3 weeks. Seven days after the last injection mice were subcutaneously inoculated with CEA^+ colorectal tumor cells. Tumor growth was monitored regularly by caliper measurement. The fig is the representative of two identical experiments. *$p<0.0001$, in comparison to CEAMϕ group (a). Survivability of mice was recorded by regular observation till day 80 after tumor inoculation (b). Data presented are a representative of two identical experiments

detected in comparison to CEAMϕ treated group. For further confirmation of the role of NLGP generated anti-CEA antibody in tumor restriction, CEA^+ cells were incubated with immune sera before inoculation into mice and complete restriction of tumor growth was observed. This antibody mediated tumor growth restriction may have some positive co-operation by T-cells after CEAMϕNLGP vaccination. Demonstration of IgG2a type anti-CEA antibody response indicates the chance of generation of Th1 type immune skewness in vaccinated mice (Collins & Dunnick, 1993). In this perspective, we have assessed the CEA specific proliferation of splenocytes and cytokine secretion. Splenic lymphocytes containing both B-cells and T-cells, isolated from CEAMϕNLGP immunized mice were proliferated more efficiently after *in vitro* stimulation with CEA, CEA + NLGP and ConA compared to the lymphocytes obtained from mice immunized with CEAMϕ (Fig 2.10). This result suggests that in addition to B-cell response, NLGP may help to create a CEA specific memory, which may participate in anti-tumor response following introduction of CEA as a vaccine.

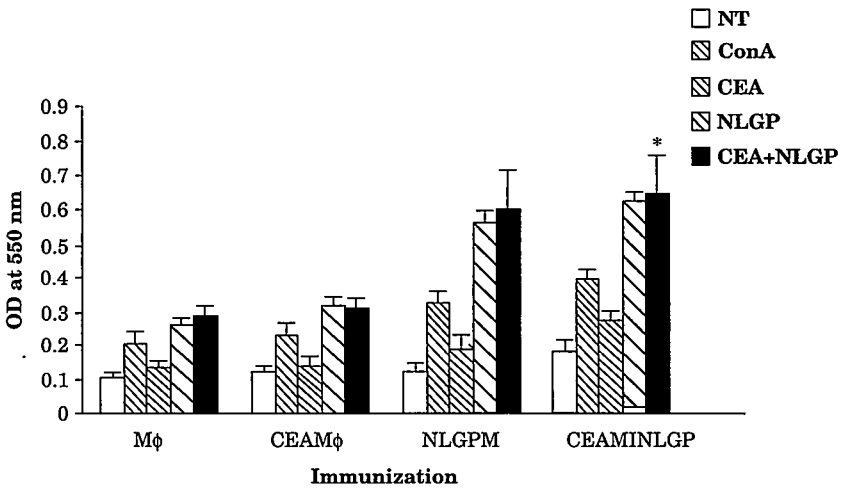

Fig 2.10. NLGP stimulates CEA specific splenocytic proliferation. Four groups of mice (n = 6) were immunized with Mϕ, CEAMϕ, NLGPMϕ and CEAMϕNLGP weekly for 3 weeks. RBC depleted spleen cells were cultured *in vitro* with PBS, ConA, CEA and CEA + NLGP to check their proliferation by MTT assay. Data represent the mean ± SD. *$p<0.0001$, in comparison to spleen cells from CEAMϕ immunized mice after *in vitro* stimulation with CEA + NLGP

NLGP AND INDUCTION OF TH1 TYPE IMMUNE RESPONSE

CEA specific Th1 cytokine response was also demonstrated which may participate in anti-tumor immune response. We have previously reported

that NLGP induced secretion of IFNγ (Mandal-Ghosh *et al.*, 2007) and IL-12 (Bose & Baral, 2007) has significant role in anti-tumor immune functions. NLGP assisted creation of Th1 microenvironment during CEAMϕNLGP vaccination have great help to mediate B-cell or T-cell dependent anti-tumor immune responses. At the same time, downregulation of the Th2 cytokine response, especially IL-10, facilitate to reduce immunosuppression and favors the desired immune conditioning. Lastly, we have checked the generation of cytotoxic T lymphocytes (CTLs) after CEAMϕNLGP vaccination in mice to kill the CEA^+ tumor cells. NLGP stimulation increases to increase the number of $CD8^+$ T-cells in the spleen of mice (data not shown) and may participate in the observed CTL reaction. In addition, role of NLGP dependent released IFNγ in the CTL generation is important. Although, role of these CTLs in *in vivo* tumor growth restriction in CEAMϕNLGP vaccinated mice was not explored yet, we are definite for their involvement in the observed tumor growth restriction in association with the role of anti-CEA antibodies.

FUTURE DIRECTIONS IN VACCINATION PROTOCOL WITH NLGP ADJUVANT

Primary goal to use an adjuvant in cancer vaccine is to enhance the antigen specific immunity and optimization of the other immune conditions. As immune functions in various compartments work in a co-ordinated manner, attention should be given in associated immune functions for maximum utility of adjuvant in a vaccine setting. It is discussed earlier that antigen presentation plays a crucial role in success of a vaccine. Accordingly, our interest in current research is to know the role of NLGP in antigen presentation through dendritic cells. It is also important to optimize the interaction between dendritic cells and T-cells for tumoricidal activity. In this interaction, expression of co-stimulatory molecules, like, B7.1, B7.2 on antigen presenting cells and CD28 on T-cells are important. Interestingly, NLGP participates in the upregulation of these immune molecules (unpublished observation). Normal immune functions in cancer are generally suppressed by upregulated activity of T-Reg cells. Success in adjuvant function is also dependent on the downregulation of the suppressive activity of T-Reg cells. Role of NLGP on T-Reg cell functions is another field of research in relation to adjuvant function under study. Signal less trafficking of effector T-cells and T-Reg cells at tumor site is a hurdle of cancer immunotherpy using adjuvant. An ideal adjuvant may control the signaling process by releasing an array of chemokines and cytokines. Participation of NLGP in these particular aspects is also partially elucidated (Chakraborty *et al.*, 2009). Overall results are encouraging to establish NLGP as an immunoadjuvant in connection to the development and application of several tumor antigens or its derivatives as cancer vaccines.

ACKNOWLEDGEMENTS

This work was supported in part by ICMR grant No. 61/3/BMS-2001. Authors acknowledge the Director, CNCI for providing research facilities and Dr. Subrata Baskar, Burdwan University, India, to help in characterization of NLGP.

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Honey Bee Products: Immunomodulation and Antitumor Activity

NADA ORŠOLIĆ[1,*], DAMIR SIROVINA[2], IVAN KOSALEC[3] AND IVAN BAŠIĆ[1]

ABSTRACT

There is growing recognition that many polyphenolic compounds present in most plants and bee products such as propolis, honey or pollen may have beneficial effect on human health. Epidemiological studies have revealed the important role that foodstuffs of vegetable origin have to play in the prevention of numerous illnesses including cancer. The natural antioxidants present in such foodstuffs, among which the flavonoids are widely present, may be responsible for such an activity. Chemoprevention of tumor with natural components, including honey bee products, especially propolis and propolis related polyphenolic/flavonoid compounds has recently drawn attention as a strong antitumor approach to be used. Flavonoids are known to affect proliferation, differentiation and apoptosis in cancer cells and may play an important role in cancer chemoprevention. Chemoprevention includes: inhibition, delay or reverse the process of carcinogenesis and/or inhibition of proliferation of already established tumor cell. Chemoprevention via nontoxic agents could be one approach for decreasing the incidence of cancer and its growth. Many naturally occuring agents have shown chemopreventive potential in a variety of bioassay systems and animal models. In the present study, results clearly demonstrated that combination of chemotherapeutic agents with polyphenolic/flavonoid components found

1. Department of Animal Physiology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, HR - 100 00 Zagreb, Croatia.
2. Department of Zoology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, HR - 100 00 Zagreb, Croatia.
3. Department of Microbiology, Faculty of Pharmacy and Biochemistry University of Zagreb, Schrottova 39, HR - 100 00 Zagreb, Republic of Croatia.

* *Corresponding author* : E-mail : norsolic@yahoo.com

in propolis, vegetables, fruits, and plant-derived beverages, having no side effects in animals, could reduce the dose of chemotherapeutic agents and improve anticancer activities in different type of murine tumors such as mammary carcinoma, and Echrlich ascites tumor. Their chemopreventive activity in animal models and cell cultures are likely to be the result of their ability to inhibit DNA synthesis in tumor cells, their capability to induce apoptosis of tumor cells, and their property to activate macrophage to produce factors capable of regulating the function of B-, T- and NK-cells, respectively. These findings suggest that propolis preparation and related flavonoids may enhance anticancer efficacy of chemotherapeutics and reduce cytotoxicity to immunocompetent cells. Pretreatment with flavonoids both in vitro and in vivo sensitizes cancer cell to growth inhibition and apoptosis induced by chemotherapeutic agents. It is likely that flavonoids by inhibition of P-gp activity increase the accumulation of chemotherapeutic in P-gp expressing cancer cells render accumulation of chemotherapeutic in cancer cell which results in altered absorption / bioavailability of these drugs after coadministration of flavonoids with cytostatic drug. These findings suggest that bee products and their polyphenolic flavonoid components may serve as a potent adjunct to chemotherapy in the treatment of cancers. However, further in-depth studies including clinical trials are needed to fully evaluate the value of flavonoids in combination with chemotherapeutic agents for the treatment of human cancers.

Key words : Honey, bee venom, propolis, royal jelly, pollen, flavonoids, tumour, metastasis, immunomodulation, radioprotection

INTRODUCTION

In recent years, the physiological functionality of natural foods has received much attention, due to increasing interest in human health. Among natural products, honey bee-derived apicultural products such as propolis, honey royal jelly, bee venom and pollen have been applied for centuries in traditional medicine as well as in food diets and supplementary nutrition. Much work has been conducted on the chemistry and properties of honey bee products. Hundreds of chemical compounds isolated from them are studied in the biomedical area and rerepresent an unlimited source for antitumor activity because of their immunostimulating activity (Šver *et al.*, 1996; Kimoto *et al.*, 1998; Oršolić & Bašić, 2003a, b, c, 2005a, b, 2007a; Oršolić *et al.*, 2003b, c, 2005a, b, e, 2008c; Sforcin, 2007). The enhancement of host immune response has been recognised as a possible means of inhibiting tumor growth without harming the host.

The immune system plays an important role in maintenancing of the body homeostasis by eliminating endogenously formed mutated cells such as virus-infected or tumour cells as well as exogenous by invading microbial organisms. Thus, so far many immunological modulators capable of

improving immune responses have been developed; some being widely used in clinics such as lentinan, cyclosporine and ascorbate derivatives. One might expect the advent of novel immunostimulators, because safe and effective drugs are of critical importance in control of infectious diseases, tumour development, and various types of allergic diseases. Anti-infectious drugs such as antibiotics or antiviral have begun to exhaust their therapeutic capabilities. The occurrence of dysfunction of the immune system requires new approaches. Immunomodulation through natural or synthetic substances may be considered an alternative for the prevention and cure of infectious diseases (Azuma & Jolles, 1987) and of neoplastic diseases (Oršolić & Bašić, 2000, 2003a, b, c, 2005a, b, 2006c, 2007a; Oršolić *et al.*, 2001a, b, 2003a, 2004b, 2005a, c, d, e), respectively.

The target of much research has been on the discovery of natural and synthetic compounds that can be used in prevention and/or in treatment of cancer. Many plants have shown to possess various biological activities like immunopotentiating and antitumor efficiency. Honey bee products and their flavonoid components are of the most promising as antitumor (Oršolić & Bašić, 2007a; Oršolić *et al.*, 2003a, b, c, 2005, 2007, 2008), immunomodulatory (Šver *et al.*, 1996; Kimoto *et al.*, 1998; Oršolić & Bašić, 2003b, 2007a; Oršolić *et al.*, 2003, 2004, 2005, 2007; Sforcin, 2007) and radioprotective (Oršolić *et al.*, 2004c, 2006a, 2007a, b, 2008a; Benković *et al.*, 2008a, b) agents.

There has been a revival of interest in medical properties of honey bee products because they are thought to exhibit a broad spectrum of activities including antibacterial, antifungal, cytostatic, wound healing, antitumour effects and antiinflammatory proprties (Oršolić & Bašić, 2008a, b, c; Bašić *et al.*, 2008; Oršolić *et al.*, 2008b, c; Kosalec *et al.*, 2008). Bees themselves were used for health (apitherapy) and their products serve as nutritious food and as health products.

PROPOLIS

Propolis (bee glue) is the generic name for the resinous substance collected by honey bees from the buds of various plant sources and used by bees to seal holes in their honeycombs, smooth out the internal walls, and protect the entrance of bee hive against intruders. It is rich in biochemical constituents, including mostly a mixture of polyphenols, flavonoid aglycones, phenolic acid and their esters, and phenolic aldehydes and ketones, terpenes, sterols, vitamins, amino acids, etc. (Marcucci, 1995). Healing properties of propolis are known in folk medicine from antiquity. Recently, the interest for propolis as a harmless medicine is increasing. There have been many attempts to validate biological effects of propolis and elucidate its composition (Marcucci, 1995). It was shown that propolis and its constituents have strong antimicrobial effect, acting on viruses, bacteria, and fungi

(Sforcin, 2007). It was also demonstrated that propolis and some of its active substances have a pronounced cytostatic (Oršolić *et al.,* 2004a, 2006; Bašić *et al.,* 2008), anticarcinogenic and antitumor effect both in *in vitro* and *in vivo* tumor models (Kimoto, 1998; Oršolić & Bašić, 2007a; Sforcin, 2007).

MACROPHAGE ACTIVATION BY PROPOLIS AND RELATED POLYPHENOLIC COMPOUNDS IS IMPORTANT EFFECTOR MECHANISM OF ANTITUMOR ACTIVITY

We demonstrated that macrophage activation by propolis and related polyphenolic compounds was likely to be the most important effector mechanism of antitumor activity of test compounds *in vivo* (Oršolić & Bašić, 2003b, c; Oršolić *et al.,* 2005a, b, e, 2006b). These findings suggested that propolis and some of its components stimulated macrophages and reduced the number of mammary carcinoma (MCa) metastases in CBA mouse (Oršolić *et al.,* 2003a, b; Oršolić & Bašić, 2005a). Results also demonstrated a reduction of tumor volume and increase in life span (ILS) for 14.89 to 40.76% when test compounds were given before tumor cell inoculation (Oršolić *et al.,* 2005d).

Preventive treatment with water-soluble derivative of propolis (WSDP) given perorally (*po)* or with caffeic acid (CA) was very effective in inhibition of tumor growth; antitumor activity was mediated by stimulated macrophages acting directly on tumor cells. Indirect antitumor activity is likely to be mediated by the products from stimulated macrophages such as NO (Oršolić *et al.,* 2006b) as was also described by others (Elgert *et al.,* 1998; Orsi *et al.,* 2000).

To test both direct and indirect effects on tumor growth we injected WSDP or CA or caffeic acid phenethyl ester (CAPE), subcutaneously (*sc*) and immediately thereafter at the spot of their introduction tumor cells were inoculated. Results (Oršolić *et al.,* 2005c) indicated that the presence of CA or CAPE in the tissue of tumor cells inoculation inhibited tumor growth and increased life span (ILS) of treated animals (29.30% to 51.74%), while WSDP was less effective (Oršolić *et al.*, 2005d). These findings suggested that CA or CAPE suppressed mammary carcinoma growth via other mechanism(s) different from those mediated by WSDP. These include the ability of CA and CAPE to inhibit DNA synthesis in tumor cell cultures (Oršolić *et al.,* 2004b), and their capability to induce apoptosis of tumor cells (Oršolić *et al.,* 2003b, 2004a). Furthermore, the ability of CA and CAPE to induce apoptosis suggested their potential use in preclinical and clinical trials as anticancer therapeutic agents.

Moreover, we showed that WSDP and/or its related polyphenolic compounds were effective at reducing tumour volume as measured by the total number of cells in peritoneal cavity in mice-bearing EAT (Oršolić *et al.,* 2005a), the analysis of cells present in peritoneal cavity of mice revealed

that all experimental groups inoculated with tumor cells in the presence of WSDP or related polyphenolic components, exhibited a significant reduction of tumour cells. The survival rates of EAT-bearing mice were increased after treatment with test components (Oršolić & Bašić, 2005a). These findings suggested that test components might have interfered with the growth of Ehrlich ascites tumour cells directly during early phase of treatment leading to a considerable elimination of these cells as showed with studies *in vitro* (Oršolić *et al.*, 2005a).

Other possible antitumor mechanism of WSDP and related polyphenolic compounds may involve macrophage activation conjoined with production of many different soluble factors by them which may exert direct or indirect effect to tumor cells (Kimoto *et al.*, 1998; Oršolić *et al.*, 2006b, c; Bašić *et al.*, 2008).

Spreading ability of macrophages is an important marker of their activation, and it was shown that its capacity was associated with an increase in adherence and phagocytic activities of these cells. Results of our studies on macrophage spreading revealed that the treatment with WSDP or its polyphenolic components significantly increased spreading ability of cells compared to untreated control (Oršolić & Bašić, 2005b). Findings implied an enhanced ability of macrophages to phagocyte; thus, the increase of macrophage spreading might have been responsible for the slower growth of tumour cells. It is well known that mononuclear cells (MN), mainly macrophages, are the major cells involved in tumour destruction (Kimoto, 1998; Oršolić & Bašić, 2003b, c, 2005b; Sforcin, 2007). Since as compared with other tratments the strongest antitumor effect was achieved by WSDP treatment it is likely that the antitumor activity of WSDP was the consequence of synergistic activities of polyphenolic compounds present in WSDP. Stimulation of macrophages might induce production and release of several cytokines such as IL-1, IL-6, IL-8, TNF-α (Dimov *et al.*, 1992; Oršolić & Bašić, 2003b) and NO (Oršolić & Bašić, 2003b, c; Oršolić *et al.*, 2006b). Some of these cytokines may express direct cytotoxic effect on tumour cells while others act on NK-cells and cytotoxic T lymphocytes stimulating their activities (Oršolić *et al.*, 2005e). In addition, these cytokines might stimulate production of antibody, C-reactive protein and complement factor C3 that could act as opsonin to tumor cells (Oršolić & Bašić, 2005b, 2007a; Oršolić *et al.*, 2005e) and as such activate antybody-dependent cellular toxicity (ADCC). Combination of these effects might impede tumour growth and lead to elimination of tumour cells. It is likely that WSDP and its polyphenolic compounds can trigger various host defence mechanisms. Destruction of tumor cells observed with test components may be the results of one or more mechanisms described above.

It has been suggested that the therapeutic activities of propolis depend mainly on the presence of flavonoids (Oršolić & Bašić, 2007a; Oršolić *et al.*, 2005a, c, 2007c). Flavonoids have also been reported to induce the immune

system (Oršolić & Bašić, 2003b; Oršolić *et al.,* 2005b, e), and to act as strong oxygen radical scavengers (Oršolić & Bašić, 2005a; Oršolić *et al.,* 2007a, b, 2008a, b; Benković *et al.,* 2007; Kosalec *et al.,* 2008). Dietary intake of antioxidant has been associated with a diminished risk of cancer at various anatomical sites (Oršolić *et al.,* 2005a, c, d, 2006b; Oršolić & Bašić, 2007a).

PROPOLIS AND RELATED POLYPHENOLIC COMPONENOTS AS IMMUNOMODULATING AGENTS

Immunomodulatory effects of propolis were recorded (Dimov *et al.,* 1992; Kimoto *et al.,* 1998; Oršolić & Bašić, 2003b, c; Oršolić *et al.,* 2005a, b, e). Our studies (Oršolić & Bašić, 2003b, c; Oršolić *et al.,* 2005a, b, e) and those by others (Dimov *et al.,* 1992; Kimoto *et al.,* 1998; Sforcin, 2007) suggested that WSDP stimulated macrophages and influenced specific and non-specific immune defense mechanism(s), through the release of migration inhibitory factor, macrophage phagocytosis, elevation of number of rosette-forming and antibody producing cells, respectively. Activated macrophages were shown to be a major component of host defense against neoplastic growth in experimental tumor systems (Oršolić & Bašić, 2000; Oršolić & Bašić, 2003b, c). It was demonstrated that increased levels of IL-1 and TNF (Dimov *et al.,* 1992) produced by activated macrophages correlated directly with two other criteria for macrophage activation: enhanced *in vitro* responsiveness to chemotactic stimuli and macrophage-induced tumor cytotoxicity (Oršolić & Bašić, 2003b, 2007a; Oršolić *et al.,* 2006b). Among the best-characterized lytic factors produced by activated macrophages are hydrogen peroxide (H_2O_2) and other reactive oxygen intermediates, TNF-α, and NO and reactive nitrogen intermediates (Oršolić & Bašić, 2003b, c, 2006b, 2007a). Macrophages were capable of destroying microorganisms and tumor cells by their products such as, H_2O_2 including oxygen radicals and NO.

Since immunomodultaion is known to be of importance to control tumour growth and its spread, we studied the effect of WSDP and its polyphenolic components on hematological and immunological parameters in mice. Findings demonstrated that the mitogenic effect of supernatant of macrophages from mice treated with WSDP exerted strong activity on production of lymphocyte activation factor (LAF) that influenced incorporation of ^{3}H-thymidine in primary culture of syngeneic mouse thymocytes (Oršolić & Bašić, 2003b). Increased level of LAF activity produced by WSDP activated macrophages correlated directly with the reduction of metastases in the lung of treated mice and with tumour cytotoxicity *in vitro* (Oršolić & Bašić, 2003b). These findings suggested that WSDP possesses the property to activate macrophage to produce factors capable to regulate the function of B- and T-cells, respectively. The elevation of both $CD4^+$ and $CD8^+$ T-cell subsets in tumour-bearing mice after treatment with WSDP showed a dose-dependent effect of WSDP that leads

to progressive reduction of the $CD4^+/CD8^+$ ratio in favour of $CD8^+$ cells (Oršolić *et al.*, 2005b, e). It thus appears that, the antimetastatic activity of WSDP was, at least in part, the consequence of immunomodulation of the host's immune system. These results are consistent with the observations by Kimoto *et al.* (1998) who reported that the artepilin C from Brazilian propolis suppressed tumor growth after intratumor injection of 500 mg; in contrast results of these studies showed that increased ratio of CD4/CD8 was in the favour of CD4 cells.

Since interferon gamma (INF-γ), TNF-α and IL-2, produced by Th-1 lymphocytes as promoters of host defence, induce the synthesis of NO by macrophages (Elgert *et al.*, 1998; Orsi *et al.*, 2000), it was of our interest to check whether WSDP and related polyphenolic compounds (CAPE, CA) influence the synthesis of NO. Studies revealed that peritoneal macrophages from mice treated with 50 mg/kg of WSDP or CAPE when cocultured with HeLa cells produced significantly higher amount of NO ($p<0.05$) than control cells (Oršolić *et al.*, 2006b). At the same time the percentage of ^{3}H-TdR incorporation into tumour cells was lower than in control. In contrast, peritoneal macrophages from mice treated with CA expressed very strong cytotoxicity to HeLa cells as compared to control, suggesting that (an) other mechanism(s) different from that of WSDP and CAPE should be considered. It was shown by Orsi *et al.* (2000) that treatment with CA elevated the production of H_2O_2 by macrophages of treated mice. Chan *et al.* (1995) demonstrated that CA can act as a prooxidant and an effective irreversible inhibitor of glutathione S-transferases that causes a decrease in the generation of NO by activated macrophages. Activated macrophages produce increased level of reactive oxygen species, including H_2O_2, which are known to modulate cellular functions including those of lymphocytes (Oršolić *et al.*, 2005b). Our results supported these findings since the response of spleen cells to polyclonal mitogens was supressed in mice treated with CA (Oršolić *et al.*, 2005b) while WSDP exerted an oposit effect. These pro-proliferative effects may predominate since WSDP increased LAF (Oršolić & Bašić, 2003b) activity that might be associated with enhanced T- and B-cell proliferation.

Our studies also confirmed the different modulating effect of test compounds on the haematological parameters. Treatment of mice with WSDP or related polyphenolic compounds caused profound changes in the spleen weight and cellularity of treated mice, which could be explained by increased production of macrophages (Oršolić *et al.*, 2005b). Findings from these experiments also confirmed that WSDP was a strong activator of the processes included in production of antibodies; whether the activation of antibody production is connected with macrophage activation (Orsi *et al.*, 2000) caused with WSDP or whether other fenomena of immunological reactivity are involved remains to be revealed. These findings, however, confirmed that the dose of WSDP was an important factor for activation of

the mechanisms involved in antibody production as well as the time intervals between antigen introduction and treatment with WSDP. Thus, findings suggested that a continuous presence of WSDP is necessery to assure activation of mechanisms involved in antibody production, such as macrophage activation and production of factors regulating the functions of B- and T-lymphocytes (Oršolić & Bašić, 2003b). Scheler *et al.* (1988) also described that ethanolic extract of propolis (EEP) was capable of increasing the number of plaque-forming cells in spleen cell population of immunized BALB/c mice, demonstrating the ability to produce antibodies. The single EEP dose exerting the maximal (a three-fold increase over control) is 500 μg/mouse. When this dose of EEP was repeated within 24 h the plaque forming effect was even stronger, but further increases of the dose or in the number of administration, had an inhibitory effect on the plaque formation. This suggested that time interval between administration of the EEP and the immunization process should not exceed 48 h.

Concerning the plaque formation, the number of IgM PFC of spleen in our studies was significantly increased in WSDP treated mice when SRBC were given to mice at the time of injection of WSDP and 24 or 48 h before WSDP respectively; most pronounced effect was achieved in group of mice receiving SRBC 24 h before WSDP treatment (Oršolić *et al.*, 2005b). This finding suggested an adjuvant effect of the WSDP. Although PFC is an endpoint to evaluate the humoral immune response, the response to SRBC requires the cooperation of a number of cell populations, including B-cells, T helper cells, and macrophages. Findings from these experiments confirmed that WSDP could strongly activate the processes included in production of antibodies. These findings, however, confirmed that the dose of WSDP was an important factor for activation of the mechanisms involved in antibody production (Oršolić *et al.*, 2005b) as well as the time intervals between antigen introduction and treatment with WSDP. WSDP also increased, in a time-depnedent manner, the levels of γ-globulins in treated mice when mice were given WSDP before immunization with antigen; γ-globulin levels were higher when time between WSDP and antigen injection was longer (Oršolić *et al.*, 2005b). Treatment with WSDP at the time of antigen injection resulted in the lowest level of γ-globulins.

Our findings also suggested that WSDP possesses the property to activate macrophage to produce factors regulating the function of B- and T-cells, respectively (Oršolić & Bašić, 2003b). The main roles of macrophages are to phagocytize antigens, present them to helper T-cells, and generate various cytokines. Moreover, the increase of macrophage activity by WSDP and its polyphenolic compounds might have been responsible for the slower growth of tumour cells. The mechanisms for the macrophage mediated cytotoxicity were proposed: a soluble cytotoxic factor released by macrophages, which might alter the integrity of the tumor cell membrane and/or pericellular environment of these cells would be expected to be acid,

depleted of oxygen, and perhaps to contain lysosomal enzymes (Oršolić & Bašić 2003b; Oršolić *et al.*, 2005e). Macrophage spreading revealed that the treatment with test components in our studies affected the functional state of macrophages. Results showed that macrophage spreading involved an increase in size and content of large cytoplasmic vacuoles (Oršolić *et al.*, 2005a, 2008c). The highist macrophage spreading was achieved with preparations of WSDP (Croatian & Brazilian) as compared by single flavonoids (Oršolić *et al.*, 2006b). It is likely that the antitumor activity of WSDP was the result of synergistic activities of its polyphenolic compounds. It should also be pointed out that for the tumor–bearing mice treated with test components as compared to control, there was an increase in the median values of percentage spreading accompanied with an increase in cytotoxicity on EAT-cells and in their effect on induction of apoptosis in tumor cells. Treatment with WSDP and polyphenolic components also yielded an increase in the percentage of PMN cells when compared with control group (Oršolić *et al.*, 2005a). According to Elgert (1998) PMN cells could lead to the elimination of cells that are strange to the host by oxidative and non-oxidative mechanisms. However, in spite of a quantitative increase of PMN cells in test component treated mice a marked decrease in the percentage of tumour cells were not seen in our studies. This suggests that PMN cells alone were not capable of inhibiting tumour growth. It is well known that MN cells, mainly macrophages, are the major cells involved in tumour rejection. Namely, activated macrophages have higher capacity to survive in acid environment, since the oxidative burst is a sequential step after the processes, such as phagocytosis, enzyme liberation, free radical generation as well as production of mediators of inflammatory processes. Our findings and those by others (Kimoto *et al.*, 1998; Oršolić & Bašić, 2005; Oršolić *et al.*, 2005a, 2008c) suggested that the immunostimulatory and antitumor activity of propolis may be associated with macrophage activation and enhancement of macrophage phagocytic capacity (Oršolić & Bašić, 2005; Oršolić *et al.*, 2005a, 2008c).

PROPOLIS AND CHEMOTHERAPEUTIC DRUGS IN COMBINED THERAPHY OF MURINE TUMORS

We showed that WSDP preparation from natural propolis was capable to prevent proliferation of tumor cells and metastasis formation in the lung (Oršolić & Bašić, 2005a). This investigation clearly demonstrated the inhibitory effects of WSDP on metastasis formation of a mammary carcinoma in CBA mouse. WSDP expressed strong antimetastatic effect in mice treated either preventively or curatively (Oršolić & Bašić, 2005a). The combined treatment with WSDP and Epirubicin profoundly inhibited metastasis formation as shown in (Oršolić & Bašić, 2005a), this synergistic effect is maximal when Epirubicin and WSDP were administrated after tumor cell inoculation. In preventive treatment the combination of

chemotherapy and WSDP was ineffective; it is possible that the antioxidative capability of propolis through scavenging of oxiradical induced by Epirubicin reduced their bindings to DNA of tumor cells thus making them less sensitive to Epirubicin as suggested by Sheller *et al.* (1989). Furthermore in curative treatment it is likely that more expressed antitumor effect of Epirubicin is elevated by the flavonoids present in WSDP which through their property to inhibit different kinases and topoisomerase II activities reduced the growth of tumor cells (Galati *et al.*, 2000). Similar inhibitory effect of flavonoides and cisplatin on melanoma cell growth *in vivo* was reported by Caltagirone *et al.* (2000), Suzuki *et al.* (2002) demonstrated that oral administration of crude water-soluble propolis (CWSP) concurrently with 5-fluorouracil (5-FU) or Mitomicin (MMC), significantly increased tumor regression as compared with the respective chemotherapy alone, illustrating the adjuvant effect of orally administered CWSP for tumor regression when combined with chemotherapeutic agents. In addition, same authors showed that orally administered CWSP significantly ameliorated the cytopenia induced by 5-FU or MMC, resulting in recovery of white as well as red blood cell counts. Our results are in consistence with the data mentioned above; WSDP prevent the Epirubicin induced hematological toxicity in mice bearing metastases of the mammary carcinoma. In mice treated with WSDP and Epirubicin number of leucocytes and erythrocytes were elevated as compared to untreated and mice treated with Epirubicin alone.

Apart from immunopotentiation (Dimov *et al.*, 2002; Kimoto *et al.*, 1998; Oršolić & Bašić, 2003b, c; Oršolić *et al.*, 2005a, b, e; Sforcin, 2007) and proliferating (Oršolić & Bašić, 2005a) activities as well as inhibition of DNA synthesis in tumor cells (Oršolić *et al.*, 2004a, b; Bašić *et al.*, 2008), and induction of the apoptosis of tumor cells (Oršolić *et al.*, 2003b, 2005b), WSDP antitumor activity in conjuntion with Epirubicin is likely to be mediated by other mechanisms which include inhibition of CYP1A2 enzyme activity and perheps inhibition of multidrug resistance protein 1 (MRP1) responsible for the eflux of chemotherapeutics from tumor cells. Our results (Oršolić & Bašić, 2007a) provide the first demonstration for the possible mechanism based on inactivation of mice CYP1A2 by WSDP; CYP1A2 is expressed principally in the liver, where it is responsible for metabolism and biotransformation of carcinogens and chemotherapeutics. Results showed that WSDP inhibited CYP1A2 responsible for transformation of 3-methylcholanthrene (MC) in hepatocytes of mice.

In addition to inhibition of MRP1 as shown by Leslie *et al.* (2001) who demonstrated that most flavonoids inhibited MRP1 responsible for the efflux of antracyclines from tumor cells and that this inhibition was enhanced by GSH. Our findings suggest that flavonoids might prolong the retain of cytostatic drugs in tumor tissue. Thus, inhibition of MRP1 as well as CYP by flavonoids from WSDP may be important strategy to improve

antimetastatic effect of chemotherapeutics since the metabolism of drug substrate by CYP enzymes typically leads to drug inactivation and facilitates drug elimination. Adequate biovariability and period of drug exposure are also a major determinant of anticancer therapy; our results demonstrated that WSDP was potential inhibitor of CYP activity prolonging chemotherapeutic action in tumor cells thus increasing their antimetastatic effects. Collectively, these results suggested that the antimetastatic effect of WSDP reflects its multiple mechanisms of action that include antioxidant potential, immunomodulation and effects on xenobiotic metabolizing system.

Present results on antimetastatic activity of propolis are in concordance with the findings by Caltagirone *et al.* (2000), who demonstrated similar effect of flavonoids on melanoma metastatic potential in mice.

The combination of WSDP with chemotherapeutic epirubicin influenced the proliferation of leukocyte populations in peripheral blood (Oršolić & Bašić, 2005a) that was inhibited by chemotherapeutic agents. These results are in line with previous results from this laboratory and others (Oršolić & Bašić, 2005a, 2007a; Oršolić *et al.,* 2008c; Suzuki *et al.,* 2002), suggesting that flavonoids from WSDP possess hemostimulative, antioxidative, protective and regenerative properties.

Flavonoids have biochemical and pharmacological activities beneficial for human health, including antioxidant, anticarcinogenic, anti-inflammatory, antiproliferative, antiangiogenic, and antiestrogenic, estrogenic effects (see review Oršolić & Bašić, 2007a; Sforcin *et al.,* 2007), and that their ingestion produces no or very little toxicity (Oršolić & Bašić, 2007a, 2008a, b; Oršolić *et al.,* 2008a, b, c). A few *in vivo* studies support the concept that antioxidants selectively enhance the effect of standard therapy on tumor cells by increasing tumor response. The purpose of study (Oršolić *et al.,* 2008a, b, c) was to analyze the possible use of propolis and related flavonoids as an adjunct to standard cancer therapy. Another part of our proposed hypothesis is that propolis and its flavonoids compounds in combination with standard tumor therapeutic agents may reduce the toxicity of chemotherapeutic agent (irinotecan) on normal cells. Several studies using animal models also support this part of the hypothesis (Scheller *et al.,* 1989; Dimov *et al.,* 1992; Kimoto *et al.,* 1998; Caltagirone *et al.,* 2002; Suzuki *et al.,* 2002; Oršolić & Bašić, 2007a).

Our previous work (Benković *et al.,* 2007) has shown that WSDP, EEP, naringin and quercetin given in combination with chemotherapeutic agent irinotecan delayed Ehrlich ascites tumor (EAT) growth and increased life span of EAT-bearing mice. EEP and WSDP in combined treatment with irinotecan increased median survival time (59.00 ± 9.87; 70.00 ± 9.22) comparing to control group or the group treated with irinotecan alone (39.00 ± 2.67). In this study, the analysis of the total number of cells present

in the peritoneal cavity of mice revealed that all the experimental groups inoculated with tumor cells in the presence of WSDP or polyphenolic compounds of propolis exhibited significantly lower number of cells in peritoneal cavity as compared to control. Combined treatment of test components with irinotecan showed strong antitumor activity; total number of cells in peritoneal cavity of mice treated with irinotecan in combination with quercetin or naringin was reduced for 87.89% or 81.12% as compared to control and for 28.21% or 21.44% as compared with irinotecan alone, respectively (Benković *et al.*, 2007; Oršolić *et al.*, b, c). The exact mechanism(s) of action by which test components interacted with irinotecan including the suggestions given in reference (Suzuki *et al.*, 2002) still left to be elucidated. Some of possibilities include: (a) maintaining high circulating levels of irinotecan by WSDP or related flavonoids due to P-glycoprotein pump efflux activity, (b) test component may act as an efficient vehicle for selective delivery of chemotherapeutic agents to tumor cells, rather than acting to tumor cells, (c) test components may act on tumor cells through enhanced immunity and direct DNA damage induced by apoptotic processing, (d) test components may have a potential to alter metabolic activation of therapeutically administered drug, (e) synergistic action of flavonoids and chemotherapeutic on topoisomerase I and II. We determined that efficacy profile of propolis and related polyphenolic compounds treatment alone, or in combination with chemotherapeutic irinotecan, enhanced the activity of immunological effector cells and hematopoiesis in mice bearing tumor. In addition this study showed that propolis and its polyphenolic compounds may reduce the toxicity of irinotecan to normal cells (liver, kidney and blood) (see review Oršolić *et al.*, 2008b, c). The major new findings are that the pre-treatment of mice-bearing tumor with propolis and propolis related compound such as naringin or quercetin in combination with irinotecan resulted in (i) decreased number of total cells in peritoneal cavity, (ii) increased number of WBC, (iii) enhanced macrophage and PMN activity, (iv) protection of liver and kidney cells against irinotecan-induced toxicity, (v) decrease of the number of micronucleated cells in peripheral blood. According to this observation, it is likely that activation of the immune system is involved in *in vivo* tumor regression. Thus, in treated mice with propolis preparation or flavonoids the number of polymorphonuclear (PMN) cells in the peritoneal cavity was significantly ($p<0.05$) increased (Table 3.1) as well as the number of macrophages in combined treatments. WSDP combined with irinotecan in preventive and EEP combined with irinotecan in therapeutic treatment increased the macrophage spreading activity (Fig 3.1).

It is likely that macrophage activation and their effect on other immunological effectors are responsible for the destruction of tumor cells. Activated macrophages were shown to be a major component of host defense

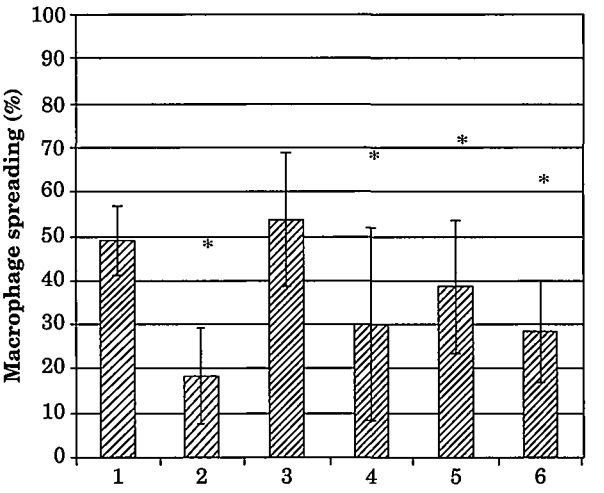

Fig 3.1. The percentage of macrophage spreading in the peritoneal cavity of the animals on the 5th day of Ehrlich ascites tumor growth after intraperitoneal treatment with irinotecan and combination of irinotecan with WSDP, EEP, naringin or quercetin [(1) control, (2) Irinotecan, (3) Irinotecan + WSDP, (4) Irinotecan + EEP, (5) Irinotecan + naringin, (6) Irinotecan + QU)]. Test components were given *i.p.* daily for 3 days prior tumor inoculation (2×10^6/cells/mouse) or 3 days after tumor cell inoculation starting on day 3 after inoculation; the daily dose contained 100 mg kg^{-1} body weight. Irinotecan was injected *i.p.* at dose of 50 mg kg^{-1} on days 3, 4, and 5 after tumor cell inoculation starting 1 h after injection of test components. Mice (n = 7) of each group were sacrificed on the 5th day after *i.p.* tumor cell inoculation

* The value was significantly different ($p<0.05$) from the corresponding value of untreated animals analyzed by Student's *t* test.

against neoplastic growth in experimental tumor systems (Oršolić *et al.*, 2006b; Oršolić & Bašić, 2007a). Consistent with these results are our findings of restored lymphocyte/polymorphonuclear leukocyte ratio (L/P) activity indicating that the overall immunological activity may be significantly elevated in animals receiving propolis preparations (Table 3.2). These results suggest that test components used in this study might interfere with the growth of EAT-cells by activation of macrophages and neutrophils. The functional activity of neutrophils and/or macrophages are related to the amount of reactive oxygen species (ROS) produced during the respiratory burst after their activation (Elgert *et al.*, 1998; Watson, 2002; Oršolić & Bašić, 2003b, c; Oršolić *et al.*, 2006b). The ROS production in activated neutrophils seems to influence their lifespan which in turn can be modulated by antioxidants (Watson, 2002). Therefore, manipulation of these processes is likely to be a key strategy in the acceleration or delay of apoptotic events in the entire population of circulating neutrophils and/or macrophages before they are replaced by mature cells released

from the bone marrow. However, little is known about the effects of a combined treatment on neutrophils with antioxidants and drugs, on their lifespan and functions. Many authors suggest that antioxidants used as an adjunct in chemotherapy enhance efficacy of antineoplastic drugs and/or lower their adverse effects on surrounding normal tissue (Caltagirone *et al.,* 2000; Suzuk *et al.,* 2002; Watson, 2002; Conklin *et al.,* 2004).

Table 3.1. The total number and percentage of cells present in the peritoneal cavity of mice–bearing Ehrlich ascites tumor after intraperitoneally treatment with WSDP, EEP, naringin, quercetin[a] and irinotecan[b] on the 5[th] day of Ehrlich ascites tumor growth

Experimental group	Total number of Cells ($N \times 10^6$)	%			
		Tumor Cells	Macrophages X ± SD	Lymphocytes X ± SD	Neutrophils X ± SD
Control	220.50 ± 26.73	82.00 ± 4.00	14.38 ± 4.31	1.88 ± 1.46	1.75 ± 1.58
WSDP	192.19 ± 26.20	77.36 ± 9.65	16.35 ± 2.03	2.03 ± 0.78	3.98 ± 0.98*
EEP	186.52 ± 43.58	76.35 ± 11.89	18.78 ± 3.65	1.76 ± 1.01	3.89 ± 1.45*
Naringin	204.30 ± 28.91	81.48 ± 14.30	15.23 ± 1.98	1.91 ± 1.87	1.98 ± 0.65
Quercetin	98.83 ± 31.13*	76.98 ± 11.65	18.08 ± 3.36	2.90 ± 2.65	2.04 ± 2.13
Irinotechan	88.91 ± 07.61*	73.45 ± 13.47	23.69 ± 10.05	1.63 ± 1.78	4.25 ± 4.68*
WSDP + Irino	88.53 ± 18.60*	54.86 ± 20.53	42.88 ± 18.35	2.38 ± 3.63	2.94 ± 2.86
EEP + Irino	77.16 ± 12.62*	69.40 ± 16.31	22.56 ± 19.37*	8.13 ± 7.54*	2.94 ± 2.52
NAR + Irino	41.65 ± 19.18*	80.26 ± 10.44	11.06 ± 8.83	6.38 ± 3.36*	2.19 ± 5.08
QU + Irino	26.72 ± 11.20*	70.33 ± 12.92	13.19 ± 6.56	6.19 ± 3.02*	12.81 ± 12.42*

(a) Swiss albino mice were treated preventively with WSDP, EEP, quercetin and naringin daily for 3 three consecutive days (100 mg kg^{-1} body weight) before the *i.p.* injection of EAT-cells (2×10^6).

(b) Irinotecan was injected *i.p.* at dose of 50 mg kg^{-1} on days 3, 4, and 5 after tumor cell inoculation.

* The value was significantly different ($p<0.05$) from the corresponding value of untreated animals analyzed by Student's *t* test.

It is known that standard therapy with cytostatics produces acute toxicity during treatment and that toxicity can be severe enough to cause discontinuation of therapeutic agents. Agents, which could reduce the toxicity of standard cytostatic therapy on normal cells and/or which could increase the response of tumor cells to standard therapy, may markedly improve the current management of human cancer. Usually, in cancer chemotherapy, the major problems that are being encountered are of myelosuppression and anemia (Oršolić & Bašić, 2005a; Suzuki *et al.,* 2002). So, it is known that the major toxicities of irinotecan in clinical use are myelosuppression and diarrhoea. Our results have clearly shown that propolis and related flavonoids (100 mg kg^{-1}) in combined treatment with

Table 3.2. The effect of WSDP, EEP, naringenin or quercetin[a] alone and/or in combination with irinotecan[b] on hematological parameters in mice-bearing tumor[c]

Experimental group	Hematological parameters								
	Leucocytes 10^9 L^{-1}	L/P Ratio	Erythrocytes (× 10^{12} L^{-1})	Hemoglobin (gL^{-1})	Hematokrit (LL^{-1})	MCV (fL)	MCH (pg)	MCHC (gL^{-1})	Platelets (× 10^{12} L^{-1})
Control	3.84 ± 2.80*	2.6	9.30 ± 1.90	138.45 ± 26.87	0.44 ± 0.09	47.05 ± 0.28	14.90 ± 0.35	317.50 ± 6.70	788.00 ± 328.01
Control (EAT)	9.36 ± 2.14	1.83	9.24 ± 1.44	138.5 ± 18.05	0.44 ± 0.06	47.40 ± 0.31	14.97 ± 0.03	315.67 ± 2.67	1101.53 ± 270.90
WSDP	7.17 ± 0.61	4.59	8.16 ± 0.54	123.67 ± 13.02	0.40 ± 0.04	47.23 ± 0.28	14.46 ± 0.13*	307.00 ± 2.52*	1029.00 ± 279.28
EEP	7.27 ± 5.65	2.37	9.14 ± 1.08	132.55 ± 11.87	0.43 ± 0.04	46.73 ± 0.28	14.33 ± 0.12*	306.66 ± 3.18*	831.70 ± 622.73
Naringin	11.72 ± 6.16	1.69	8.28 ± 0.89	127.55 ± 10.27	0.44 ± 0.03	47.47 ± 0.55	15.00 ± 0.35	316.00 ± 4.16	929.00 ± 323.24
Quercetin	10.13 ± 3.71	0.96	8.43 ± 0.27	123.55 ± 4.30	0.40 ± 0.01	47.77 ± 0.62	14.83 ± 0.44	310.00 ± 5.29	882.00 ± 121.60
Irinotecan	2.30 ± 1.46*	1.56	8.16 ± 0.47	121.35 ± 6.58	0.39 ± 0.02	47.60 ± 0.53	14.77 ± 0.07	310.00 ± 1.73	1133.00 ± 234.15
WSDP+ Irino	3.01 ± 2.31*	1.71	8.34 ± 0.25	122.80 ± 6.68	0.40 ± 0.02	47.37 ± 0.41	14.87 ± 0.23	314.00 ± 1.73	992.00 ± 189.36
EEP + Irino	4.46 ± 2.21*	1.25	8.22 ± 0.96	122.65 ± 13.15	0.40 ± 0.04	47.43 ± 0.55	14.73 ± 0.54	310.33 ± 2.33	1100.00 ± 213.26
NAR + Irino	3.63 ± 1.56*	1.19	8.11 ± 0.81	120.50 ± 8.91	0.38 ± 0.03	46.97 ± 0.52	14.60 ± 0.36	310.67 ± 4.67	1033.00 ± 168.01
QU + Irino	5.49 ± 1.98*‡	0.76	8.28 ± 0.41	124.35 ± 4.37	0.40 ± 0.09	47.57 ± 0.52	14.77 ± 0.28	310.67 ± 3.48	1102.00 ± 173.61

a Preparation of propolis and their flavonoids were given *i.p.* for three consecutive days prior tumor inoculation, and the daily dose contained 100 mg kg^{-1} body weight.

b Irinotecan was injected *i.p.* at dose of 50 mg kg^{-1} on days 3, 4, and 5 after tumor cell inoculation.

c Tumor cells (2 × 10^6) were injected to mice intraperitoneally; group comprises 7 mice each.

L/P, lymphocyte/polymorphonuclear leukocyte ratio activity.

* The value was significantly different ($p<0.05$) from the corresponding value of untreated animals analyzed by Student's *t* test.

‡ The value was significantly different ($p<0.05$) from the corresponding value of irinotecan treated animals analyzed by Student's *t* test.

cytostatic may protect WBC, but have not effect to the hemoglobin content and red blood cells (RBC) (Table 3.2). Moreover, propolis and related flavonoids may guard RBC in the peripheral blood from irinotecan—induced toxicity as shown in Tables 3.2, 3.3 and 3.4 by micronucleus assay. In this experiment and our studies performed previously (Benković *et al.*, 2007) we did not observed side effect such as diarrhoea and a loss of body weight in combined treatment.

Table 3.3. Number of reticulocytes in peripheral blood with micronucleus after treatment of mice with WSDP, EEP, naringenin or quercetin[a] alone and/or in combination with irinotecan[b]

Experimental groups	Number of cells			
	Without MN ± SD	With MN ± SD	1 MN ± SD	2 MN ± SD
Control	1996.0 ± 3.0	4.0 ± 3.0	4.0 ± 3.0	—
Control DMSO	1995.5 ± 2.5	4.5 ± 2.5	4.5 ± 2.5	—
Control (Ett-OH)	1994.5 ± 1.5	5.5 ± 1.5	5.5 ± 1.5	—
WSDP	1996.5 ± 2.5	3.5 ± 3.5	3.0 ± 2.0	0.5 ± 0.5
EEP	1993.0 ± 2.0	7.0 ± 2.8*	6.5 ± 1.5	0.5 ± 0.5
Naringin	1995.0 ± 3.0	5.0 ± 4.2	4.5 ± 2.5	0.5 ± 0.5
Quercetin	1993.0 ± 3.0	7.0 ± 4.2*	6.5 ± 2.5	0.5 ± 0.5
Irinotekan	1953.0 ± 2.0	47.0 ± 2.8*	46.5 ± 1.5	0.5 ± 0.5
WSDP + Irino	1977.0 ± 1.0	23.0 ± 1.4*‡	22.0 ± 1.0	1.0 ± 0.0
EEP + Irino	1965.0 ± 1.0	34.5 ± 2.1*‡	34.5 ± 2.1	—
NAR + Irino	1970.0 ± 0.5	29.5 ± 0.7*‡	29.0 ± 1.0	0.5 ± 0.5
QU + Irino	1984.5 ± 0.5	15.5 ± 0.7*‡	15.0 ± 0.0	0.5 ± 0.5

a The test components (WSDP, EEP, naringin or quercetin) were given *i.p.* daily for 3 days; the daily dose contained 100 mg kg^{-1} body weight.

b Irinotecan was injected *i.p.* at dose of 50 mg kg^{-1} on days 3, 4 and 5 starting 1 h after injection of test components.

* The value was significantly different ($p<0.01$) from the corresponding value of untreated animals analyzed by χ^2 test.

‡ The value was significantly different ($p<0.01$) from the corresponding value of irinotecan treated animals analyzed by χ^2 test.

This observations are in concordance with Fitzpatrick *et al.* (2001) and Ballester *et al.* (2006), observations suggest that for biological modulation of undesirable effects such as diarrhoea, flavonoids may be of great utility in states of acute or chronic diarrhoea through the inhibition of intestinal secretion and motility, and may also be beneficial in the reduction of chronic inflammatory damage in the intestine, by affording protection against oxidative stress and by preserving mucosal function. Mechanism involved in intestinal anti-inflammatory activity of the flavonoids have been proposed: (i) antioxidant and/or antiradical properties; (ii) the effect of NO

on the metabolism; (iii) the inhibition of lipoxygenase and the reduction of leukotriene B4 (LTB4) production; (iv) inhibition of proinflammatory cytokine production (v) preservation of colonic absorptive function; (vi) some flavonoid compounds have even been demonstrated to have the capacity to increase intestinal glutathione content, when orally administered to normal rats (Fitzpatrick *et al.*, 2001; Ballester *et al.*, 2006).

Table 3.4. Number of reticulocytes in peripheral blood with micronucleus after treatment of mice-bearing tumor with WSDP, EEP, naringenin or quercetin[a] alone and/or in combination with irinotecan[b]

Experimental groups	Number of cells			
	Without MN ± SD	With MN ± SD	1 MN ± SD	2 MN ± SD
Control DMSO	1996.5 ± 0.5	4.5 ± 2.5	4.5 ± 2.5	—
Control (ET-OH)	1994.5 ± 1.5	5.5 ± 1.5	5.5 ± 1.5	—
WSDP	1993.0 ± 3.0	7.0 ± 4.2*	6.5 ± 3.5	0.5 ± 0.5
EEP	1996.5 ± 0.5	3.5 ± 0.5	3.5 ± 0.5	—
Naringin	1995.5 ± 0.5	4.5 ± 0.5	4.5 ± 0.5	—
Quercetin	1998.0 ± 1.0	2.0 ± 1.0	2.0 ± 1.0	—
Irinotecan	1952.0 ± 2.0	47.5 ± 4.9*	47.0 ± 4.0	0.5 ± 0.5
WSDP + IRINO	1964.0 ± 5.0	36.5 ± 7.8*	35.0 ± 6.0	1.5 ± 0.5
EEP + IRINO	1948.5 ± 2.5	51.5 ± 3.5*‡	49.5 ± 1.5	2.0 ± 1.0
NAR + IRINO	1973.5 ± 1.5	26.5 ± 1.5*‡	26.5 ± 1.5	—
QU + IRINO	1839.5 ± 12.5	165.5 ± 24.7*‡	158 ± 17.0	7.5 ± 0.7

a The test components (WSDP, EEP, naringin or quercetin) were given *i.p.* daily for 3 days prior tumor inoculation (2×10^6); the daily dose contained 100 mg kg^{-1} body weight.

b Irinotecan was injected *i.p.* at dose of 50 mg kg^{-1} on days 3, 4, and 5 after tumor cell inoculation (2×10^6).

* The value was significantly different ($p<0.01$) from the corresponding value of untreated animals analyzed by χ^2 test

‡ The value was significantly different ($p<0.01$) from the corresponding value of irinotecan treated animals analyzed by χ^2 test.

Moreover, propolis and related flavonoids reduced irinotecan-induced DNA damage of kidney, liver, and leucocytes (Brozović *et al.*, 2005; Oršolić *et al.*, 2008c) as well as chromosomal breakage (Tables 3.2 & 3.3) in groups of mice treated with test compounds without tumor and in all groups with tumor except group of mice treated with quercetin combined with irinotecan. In micronucleus assay quercetin in combination with irinotecan increased number of micronucleated cells indicating again prooxidative effect of quercetin. It is possible that autooxidative activity of quercetin may be result of high concentration of quercetin and its inhibition of mitochondrial respiration with concomitant production of the superoxide anion and hydrogen peroxide. Hydrogen peroxide causes DNA strand breakage by

generation of hydroxyl radicals close to the DNA molecule, by the Fenton reaction. When the superoxide anion accepts a proton, it forms the hydroperoxyl radical ($HO_2^{\bullet}$) which crosses a membrane and then conceivably create damage. In addition, the superoxide anions is converted to H_2O_2 and O_2 by superoxide dismutase and then, in the presence of transition metal ions, H_2O_2 forms $OH^{\bullet}$. It is well known that superoxide anions produced in a significant quantity can directly or indirectly damage biomacromolecules by forming hydrogen peroxide, hydroxyl radicals, and peroxynitrite or singlet oxygen (Oršolić *et al.*, 2008b, c). According to our data (Table 3.4), it is likely that quercetin promoted generation of superoxide anion in reticulocytes.

Chemotherapeutic drugs are often associated with some degree of toxicities, which are caused by reactive metabolites generated by the biotransformation of anticancer drugs in the liver. The activities of liver marker enzymes and their variation reflect the overall change in metabolism that occurs during malignancy (Oršolić & Bašić, 2005a; Padmavathi *et al.*, 2006) or organ dysfunction. These enzymes have clinical importance. Since the levels of these marker enzymes are proportional to the extent of malignancy, the activity of these enzymes can be used for diagnosis as indicators of the prognosis of disease as well as a potential molecular biomarker for assessing exposure to any toxic agent (Oršolić *et al.*, 2008b; Sforcin, 2007). Tissue damage is the sensitive feature in the cancerous conditions so any deterioration or destruction of the membrane can lead to the leakage of these enzymes from the tissues. These enzymes are widely distributed in tissues: AST is predominantly found in the heart, liver, skeletal muscle, kidney and pancreas; ALT in the liver, kidney and heart. LDH isoenzymes are used to diagnosis of specific organ injury, mainly in cardiatic damage and they are important as index cell viability.

Combination of flavonoids and chemotherapy with irinotecan produced a clear reduction of drug toxicities (Table 3.5) while irinotecan alone increased activities of ALT and AST indicating organ dysfunction and cellular injury (Sforcin, 2007). Administration of propolis and its flavonoids with cytostatic caused the activities of these enzymes to return to normal levels; protective effect against organ dysfunction and cellular injury of liver or kidney was more expressed in preventive than in curative treatment with test components. This may be due to antineolpastic property of the test components and drug indicating the protective role on tissue damage. Propolis and related flavonoid administered alone to mice showed no alterations in the specific activities of AST and ALT or LDH, suggesting that it did not affect the pancreas and liver, respectively what is in accordance with other findings (Oršolić *et al.*, 2008b; Sforcin, 2007). In this work, the evidence that propolis did not induce kidney damage (Table 3.5) came from the findings indicating urea and creatinin levels. Urea concentration in blood is a consequence of its production rate during amino

Table 3.5. The effect of WSDP, EEP, naringenin or quercetin[a] alone and/or in combination with irinotecan[b] on biocemical parameters in mice-bearing tumor[c]

Experimental Group	Biochemical Parameters							
	AST (UL^{-1})	ALT (UL^{-1})	LD (UL^{-1})	Bilirubin (mmol L^{-1})	Total protein (gL^{-1})	Creatinine (mmol L^{-1})	Glucosa (mmol L^{-1})	Urea (mmol L^{-1})
Control	369.43 ± 107.07‡	100.29 ± 14.98	2361.43 ± 921.30	22.97 ± 2.31	49.77 ± 21.43	46.84 ± 22.38	9.10 ± 4.07	8.99 ± 3.66‡
Control (EAT)	377.33 ± 161.12	154.00 ± 67.85	2622.75 ± 307.84	22.35 ± 4.32	39.60 ± 5.29	42.75 ± 3.75	9.09 ± 0.23	5.93 ± 0.97
WSDP	303.00 ± 161.67	243.00 ± 126.21	3143.25 ± 452.09‡	29.63 ± 6.02	42.98 ± 3.37	33.75 ± 3.33*‡	6.86 ± 0.30‡	8.33 ± 0.64‡
EEP	354.75 ± 60.86	104.25 ± 17.81	3079.50 ± 150.77‡	34.43 ± 5.77	41.85 ± 1.92	33.00 ± 3.46*‡	7.64 ± 0.13‡	7.57 ± 0.35‡
Naringin	270.00 ± 64.11	124.50 ± 8.26	2747.25 ± 126.38‡	47.85 ± 15.73	41.85 ± 5.50	36.75 ± 5.25	7.14 ± 0.36‡	6.90 ± 0.50
Quercetin	412.50 ± 296.07	200.25 ± 29.04	4191.75 ± 294.02	29.775 ± 8.41	45.07 ± 2.86	47.25 ± 4.31	7.77 ± 0.73	7.95 ± 0.51‡
Irinotecan	738.75 ± 403.12*	156.75 ± 19.03	5718.75 ± 903.69	23.77 ± 1.79	48.82 ± 3.13	49.50 ± 1.94	9.85 ± 0.70	6.07 ± 0.43
WSDP + Irino	581.25 ± 366.72	197.25 ± 51.43	4695.00 ± 525.16	30.67 ± 2.31	45.52 ± 1.08	42.00 ± 3.24	9.10 ± 0.28	6.75 ± 0.31
EEP + Irino	498.00 ± 189.63	160.5 ± 32.99	4629.00 ± 417.26	30.67 ± 5.50	43.65 ± 2.41	46.50 ± 3.12	8.86 ± 0.29	8.40 ± 0.44‡
NAR + Irino	369.00 ± 61.04	210.00 ± 32.79‡	4675.50 ± 499.01	18.08 ± 3.39	38.02 ± 1.96‡	44.25 ± 4.96	8.94 ± 0.48	7.95 ± 0.46‡
QU + Irino	534.75 ± 200.42	169.50 ± 15.32	4332.75 ± 167.08	20.47 ± 3.09	42.37 ± 0.72	49.50 ± 0.87	8.14 ± 0.45	6.90 ± 0.37

a Preparation of propolis and its related flavonoids were given *i.p.* for three consecutive days prior tumor inoculation, and the daily dose contained 100 mg kg^{-1} body weight.

b Irinotecan was injected *i.p.* at dose of 50 mg kg^{-1} on days 3, 4, and 5 after tumor cell inoculation.

c Tumor cells (2×10^6) were injected to mice intraperitoneally; group comprises 7 mice each.

* The value was significantly different ($p<0.05$) from the corresponding value of untreated animals analyzed by Student's *t* test.

‡ The value was significantly different ($p<0.05$) from the corresponding value of irinotecan treated animals analyzed by Student's *t* test.

acid catabolism and its excretion by the kidney. Creatinine concentration in blood is a result of the balance between creatinine production by muscle and excretion by the kidney. So, urea and creatinine are important parameter of kidney damage.

Sforcin (2007) and Padmavathi *et al.* (2006) reported that natural flavanoids have the ability to decrease serum transferases activity in intoxicated animals. More than 38 flavanoids have been found in propolis (Padmavathi *et al.*, 2006) and these flavonoids might be responsible for the beneficial effect of propolis on these enzymes; findings indicate reduction in ALT and AST activities up on propolis treatment.

Our results confirm that pre-treatment with natural antioxidant can reduce the adverse effects of same chemotherapeutic agents on normal cells with equal or increased efficacy to tumor cells. In addition, the results of the present study in mice have given us the hope that propolis and flavonoids can confer in the prevention or the reduction of the side effects due to chemotherapeutic agents, and may be used in humans in the future as an adjunct to standard cancer therapy. The haematological, liver and kidney toxicity due to common drugs such as irinotecan may be avoided by the use of the propolis and related flavonoids.

PROPOLIS AND RELATED FLAVONOIDS AS RADIOPROTECTORS

Ionizing radiation causes damage to living tissues through a series of molecular events, such as photoelectric, Compton and Auger effects, depending on the radiation energy. Because human tissues contain 80% water, the major radiation damage is due to the aqueous free radicals, generated by the action of radiation on water. The major free radicals resulting from aqueous radiolysis are OH, H, eaq^-, HO_2, H_3O^+, etc. (34–39, 65). These free radicals react with cellular macromolecules, such as DNA, RNA, proteins, membrane, etc, and cause cell dysfunction and mortality. These molecules cause radiation injury to living cells, to large extent, due to oxidative stress (Fang, 1991; Oršolić *et al.*, 2007a, b; Benković *et al.*, 2008a, b). Exposure to ionizing radiation leads to undesirable damage, both on molecular, cellular and on the organism level. Studies in experimental animals have included protection against radiation-induced lethality due to haematopoietic or gastrointestinal (GI) injury, other specific tissue damage, apoptosis, mutagenesis, and carcinogenesis. Ionising radiation can induce a wide range of DNA lesions, including damage to bases, DNA–DNA and DNA–protein cross-links, DNA single (SSB) and double strand breaks (DSBs). It is generally agreed, that the formation of DSBs is the critical radiation-induced damage that leads to cromosomal aberrations such as dicentrics, reciprocal translocations and rings, which involve interaction of DSBs with each other. The majority of studies use lymphocytes, which besides their availability are known to be very sensitive to ionising radiation. The alkaline comet assay, as employed in our study

(Oršolić *et al.*, 2006a, 2007a, 2008a; Benković, 2008a, b), is capable for the detection of single strand breaks and alkali labile sites present in DNA. Therefore, chemical alterations of nucleic acids, such as breaks of hydrogen bounds, breaks of base-sugar binding, sugar oxidation, break of nucleotide strand and release of terminal phosphates caused by reaction of free radicals can be sensitively detected by means of the technique used (Oršolić *et al.*, 2007b).

Our data suggest that propolis preparation (water or ethanolic extract of propolis; WSDP or EEP) and propolis polyphenolic compounds (caffeic acid, naringin, chrysin, or quercetin) posses promising radioprotective effects, comparable to well-established chemical radioprotector aminoethyl isothiourea (AET). Synthetic protector AET at dose of 1 mm kg^{-1} was used as a positive control in our studies. Propolis and its flavonoids given to mice before or after whole body γ-irradiation (WBI) (9 Gy) may protect mice on molecular (Oršolić *et al.*, 2006a, 2007b; Benković, 2008a, b), and on the organism level (Oršolić *et al.*, 2006a, 2007b; Oršolić *et al.*, 2008a). It is observed that propolis and its polyphenolic compounds cause a decrease in the tail lentgh, tail intensity and tail moment showing radioprotective effects on DNA. Results showed that test components possess radioprotective effect similar or even in some instances better than AET. WSDP and EEP were the most effective against reduction of DNA damage of lymphocytes indicating synergistic antioxidative effect of different polyphenolic flavonoid components present in EEP or WSDP. Pretreatment of mice with WSDP or EEP or flavonoids produced the reduction in oxidative DNA damage of lymphocytes as compared with control and they were ranked in decreasing order of potency as follows: naringin (2.98%); chrysin (16.84%); quercetin (33.67%); AET (48.52%); caffeic acid (49.51%); EEP (53.47%); and WSDP (54.46%), respectively. Treatment with propolis or its polyphenolic/flavonoids compounds after irradiation also resulted in a significant reduction of DNA damage as follows: caffeic acid (32.31%); AET (75.39%); naringin (78.46%); EEP (80%); chrysin (83.08%); quercetin (84.62%); and WSDP (89.24%), respectively. Based on these results, natural compounds WSDP, EEP as well as their flavonoids showed to be effective in cytoprotection. According to our observations, therapeutic treatment of mice with WSDP and flavonoids, might lead to misinterpretation of real DNA damage levels present at the moment of blood sampling. Despite of treatment with test compounds, on third day after lethal whole body irradiation (WBI) severe disturbances in the leukocyte counts in mice were present; haematopoietic syndrome occurs at dose 2.5–8 Gy and is manifested by haematopoietic stem cell depletion, and ultimately by depletion of mature hemopoietic and immune cells (Fang, 1991; Singh & Yadav, 2005). Therefore, the majority of white blood cells measured by the alkaline comet assay in our study could be considered as new produced young and undamaged cells, since highly damaged cells after irradiation died by means of apoptosis/necrosis and were obviously destroyed.

Test components did not induce significant genotoxicity to the white blood cells of non-irradiated mice, but offer a quite measurable protection against DNA damage caused by ionizing radiation (Singh & Yadav, 2005; Oršolić *et al.,* 2006a, 2007b; Benković, 2008a, b).

Moreover our studies indicated statistically significant differences in the survival times of WBI mice pretreated with test compounds as compared to control (solvent: H_2O or ethanol). The most effective compound regarding survival of mice was QU, showing protection similar to that achieved by the AET; such a huge protective effect of QU could result from its chemical structure, which consists of the most suitable structural form for scavenging free radicals (Oršolić *et al.,* 2006a, 2007b; Benković, 2008a, b; Kosalec *et al.,* 2008). Treatment with test components after irradiation was ineffective. All other polyphenolic compounds used were also effective in protection against radiation induced damage as well as propolis preparations (EEP and WSDP); the effectivity in radiation protection between WSDP and EEP could be explained by higher contents of polypehonls present in both WSDP and EEP preparations, as presented in (Oršolić *et al.,* 2007b). The Kaplan-Meier method and the log-rank test have revealed that the surviving time of mice was as follows: quercetin, WSDP ($p<0.0001$), naringin ($p = 0.0001$), caffeic acid, chrysin ($p = 0.0003$) and EEP ($p = 0.0081$).

A single whole-body exposure to ionizing radiation results in a complex set of symptoms which onset, nature, and severity are a function of both total radiation dose and radiation quality. The haematopoietic syndrome occurs at a dose 2.5–8 Gy; it is manifested by haematopoietic stem cell depletion, and ultimately by depletion of mature haemopoietic and immune cells (Oršolić *et al.,* 2007b; Oršolić *et al.,* 2008a). In this study, the pathological cellular emptiness of bone marrow and spleen in mice which died on day-5 after lethal WBI indicated that their deaths could have been prescribed to the haemopoietic syndrome. Administration of several immunomodulators including propolis and propolis derived compounds has been shown to stimulate hemopoietic recovery and enhance the survival of irradiated animals (Singh & Yadav, 2005; Oršolić & Bašić, 2005a; Oršolić *et al.,* 2006a, 2007b; Benković, 2008a, b). Dimov *et al.* (1992) observed that oral and parenteral administration of WSDP enhanced the survival rate and the mean survival time in experimental bacterial and fungal infections in normal and immunodepressed mice. The authors suggested that the broad therapeutic spectrum of propolis includes a pronounced immuno-modulatory activity directed mainly toward augmenting of non-specific anti-infectious resistance *via* macrophage activation. Our recent findings imply that the antitumor activity of WSDP and polyphenolic compounds of propolis enhanced host resistance in the Ehrlich ascites tumor model, increasing the activities of macrophages, cytotoxic T-cells, B-cells and NK-cells as well (Oršolić *et al.,* 2005e). Moreover, WSDP stimulated peritoneal macrophages to produce IL-1 (Oršolić & Bašić, 2003b, 2005a), serving as a differentiation-

and maturation-inducing agent for a variety of cells. There are also indications that IL-1 could serve as a signal that initiates radioprotective events *in vivo* (Oršolić & Bašić, 2005a, 2008a).

Our recent observations (Oršolić & Bašić, 2005a; Oršolić *et al.*, 2008a, c) and those by others (Caltagirone *et al.*, 2000; Suzuki *et al.*, 2002) proved the protective effect of propolis on bone marrow and lymphoid tissues of mice to cytotoxic drugs and radiation. Augmented immunological activity as seen in increased activity of macrophages, cytotoxic T-cells, B-cells and NK-cells by propolis and related compounds (Oršolić *et al.*, 2005b, e; Oršolić *et al.*, 2008c) seems to play a central role in preventing secondary infections associated with irradiation, contributing to further acceleration of haemopoietic regeneration and increasing survival following radiation-induced lympho- and myelo-suppression.

The exact mechanism of action in protecting mice from the lethal effects of acute whole-body irradiation by propolis and related flavonoids is not known. In addition to modulation of immunohaematopoiesis, scavenging of radiation-induced free radicals should be the important mechanisms of radiation protection by test components, considering those confirmed by Chen *et al.* (2004) who showed that propolis and related flavonoids exercise their activity through the scavenging of hydroxyl, superoxide free radicals, and lipid peroxides. The antioxidant activities of propolis and its polyphenolic/flavonoid components are related to their ability to chelate metal ions and to scavenge singlet oxygen, superoxide anions, peroxyl radicals, hydroxyl radicals and peroxynitrite (Oršolić *et al.*, 2008a; Kosalec *et al.*, 2008). Our review papers (Oršolić *et al.*, 2008a) described that flavonoids from propolis elevate catalase, superoxide dismutase and glutathione peroxidase mRNA synthesis; the elevation of these enzymes by flavonoids was considered to be responsible for the observed protection against radiation-induced damage. By increasing the activities of antioxidant enzymes, flavonoids from propolis reduce the number of free radicals and ROS and increase the production of molecules capable of protecting against oxidative stress. It is possible that propolis and its polyphenolic/flavonoid components may influence the survival of the WBI mice *via* increased activities of SOD, CAT, GPx, GR, and GSH, (Chen *et al.*, 2004; Oršolić *et al.*, 2007b) through the free radical scavenging ability, tissue regeneration properties, and immunostimulatory effects. It is likely that more different co-operative and synergistic mechanisms of propolis and its polyphenolic compounds are included in the protection of the whole organism against radiation.

Other radioprotectors also can elicit their action by various mechanisms, such as: (i) suppression of formation of reactive species, (ii) detoxification of radiation induced species, (iii) target stabilization and (iv) enhancing the repair and recovery processes. Several amino thiol radioprotectors, such as cysteamine, guanidoethyldisulfide and glutathione disulfide, bind to DNA

and their DNA binding paralleled their radioprotective potency. Moreover it was shown that thiols, such as GSH, may be involved in the repair of DNA single-strand breaks. Cells genetically deficient in GSH synthesis or cells in which GSH deficiency is produced by dl-Buthionine-sulfoxime or by hypoxia or misonidazole show a lack of DNA single-strand break repair (Fang, 1991). Our results indicate that preventive treatment of irradiated mice with WSDP and flavonoids significantly decreased the levels of primary DNA damage in their white blood cells, as compared to untreated animals. Other studies have confirmed the role of flavonoids in the deactivation of the free radicals, (Fang, 1991; Suzuki *et al.,* 2002; Conklin, 2004). Flavonoids possess antioxidant activity based on ability of direct free radicals scavenging, or stabilizing the ROS by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxyl substituents of the flavonoids, radicals are made inactive (Chen *et al.,* 2004). Flavonoids can also increase the function of the endogenous antioxidant enzyme systems (Oršolić & Bašić, 2005a; Chen *et al.,* 2004). Furthermore, antioxidant effects may be a result of a combination of radical scavenging and an interaction with enzyme functions. Our results strongly support these observations. Therefore, stable doses of WSDP or flavonoids present in an organism prior to the irradiation could possibly diminish the frequency of DNA-strand breakage or stimulate the repair processes with consequent decrease of the frequency of double-strand breaks (considered to be the primary lesion involved in cellular death). It is also possible that both WSDP and flavonoid components stimulate other intracellular enzyme systems capable to protect cells against free radicals and DNA damage (Oršolić *et al.,* 2007b; Oršolić *et al.,* 2008a; Benković *et al.,* 2008a, b).

Similar radioprotective potentials of other flavonoids on radiation-induced chromosomal damage in human lymphocytes (*e.g.* genistein, luteolin, naringin, quercetin, apigenin, curcumin, orientin and vicenin), have been reported. However, the genotoxicity of these flavonoids has also been reported (see review Oršolić *et al.,* 2007a, 2008a). Several studies provided the evidence of an increase in MN frequency, at the highest concentrations of flavonoids used in the study (Oršolić *et al.,* 2008a).

We conducted experiments to investigate potential genotoxic activity of propolis preparations and its polyphenolic compounds (caffeic acid, quercetin, chrysin, naringin) in human lymphocytes and compared the effect of test components with that of AET. In these the well established *in vitro* cytokinesis-block micronucleus (CBMN) assay to determine whether test components are capable of inducing chromosomal damage, measured as the presence of MN in binucleated cells, in treated human lymphocytes was used. Results indicated that WSDP, quercetin and caffeic acid exerted the decrease in the frequency of MN as compared to that in the group with no test components. However, EEP, chrysin, and naringin slightly incresed the frequency of MN, as compared with conrol. AET was very toxic to human

lymphocytes and induced the highest increaase in the frequency of MN (see review Oršolić *et al.,* 2008).

Flavonoid compounds could behave as both prooxidants and antioxidants depending on concentration and free radical source. Flavonoids autooxidize in aqueous medium and may form highly reactive OHS radicals in the presence of transition metals. Moreover, polyphenols may act as substrate for peroxidases and other metalloenzymes, yielding quinone or quinomethide type prooxidant (Galati *et al.,* 2000). Recent reports have linked polyphenols to ROS production, especially hydrogen peroxide and subsequent apoptosis. It is likely that high dose of γ source of radiation may induce prooxidative effect of flavonoids in *in vitro* studies on human lymphocytes pretreated with flavonoids and irradiated with 4 Gy. Following treatment with EEP, caffeic acid, chrysin and naringin, a significant increase of MN frequency was found, while after treatment with WSDP and quercetin a decrease of MN frequency in irradiated samples *in vitro* was noticed (see review Oršolić *et al.,* 2008). Our results are in concordance with other studies in which described prooxidative mechanism of flavonoids and flavonoid-induced impairment of the antioxidant defence system consisted of GSH and GST (Fanf *et al.,* 1991; Galati *et al.,* 2000; Chen *et al.,* 2004). The hydroxyl radical generated by the autooxidation and redox cycling of the polyphenolic compounds may initiate peroxidation in the nuclear membrane; the level of lipid peroxides and intermediate radicals may then be amplified by chain reaction. Their pro-oxidant activity may deplete the nuclear antioxidant defence and lead to oxidative DNA damage, which may be responsible for their mutagenicity. Besides of radiation-induced nuclear effects, the experiments indicate that ROS formed in the cytoplasm, resulted in minimal cell killing but significant mutagenic potential. A number of antioxidant nutrients and phytochemicals exhibit antimutagenic properties when administered after radiation exposure but it is unclear if this results in an anticarcinogenic effect.

THE POSSIBLE MECHANISM OF RADIOPROTECTIVE AND HAEMATOSTIMULATIVE ACTION OF PROPOLIS PREPARATIONS AND OF RELATED FLAVONOIDS

In general, immunotherapeutic agents, cytokines, or growth factors provide a large window of protection, although protection against lethality is lower than that afforded by phosphorothioates. Administration of cytokines and growth factors after radiation exposure combined with preirradiation administration of phosphorothioates appears synergistically to reduce radiation damage (see review Oršolić *et al.,* 2008a). Propolis and its flavonoids have a pronounced immunomodulatory and hemostimulative activity directed mainly toward augmenting of non-specific activity *via* macrophage activation. One possible mechanism of haematopoietic action of WSDP is its influence on macrophages activty and production of

interleukin-1 (IL-1) by these cells (Oršolić & Bašić, 2003b; Oršolić *et al.*, 2005b). It is known that IL-1 stimulates stem cells and haematopoiesis. Increased level of IL-1 activity produced by WSDP activated macrophages correlated directly with haematopoietic activity of treated mice as well as macrophages cytotoxicity to tumor cells (Oršolić & Bašić, 2005b; Oršolić *et al.* 2005a, e). It was shown that IL-1 might protect mice from the lethal effect of irradiation (Oršolić & Bašić, 2005a; Oršolić *et al.*, 2008b). It has been suggested that radioprotective activity conferred by immunomodulators can be attributed to their capacity to enhance haematopoietic and immune functions (Oršolić & Bašić, 2005a, 2006d). Results described here suggest the protective role of the WSDP against radiation-induced destruction of the lymphoid and haematopoietic system (Oršolić & Bašić, 2005a, 2006d) as well as against the toxicity of the chemotherapeutic agents which induce bone marrow aplasia and other haematological failures in experimental animals and in human.

Numerous microbial components such as bacterial lipopolysaccharide (LPS), muramyl dipeptide, *Mycobacterium bovis* strain BCG, and glucan showed to have radioprotective effect when administered before irradiation (described in review Oršolić *et al.*, 2008a). There is a similarity between our studies and those described in the literature (Oršolić *et al.*, 2008a; Borek, 2004). WSDP given orally throughout the period of 20 consecutive days induced the extensive proliferation of nucleated cells, mainly macrophages (Oršolić & Bašić, 2005a, 2006a) as well as haematopoietic cells in the spleen and bone marrow of treated mice. Stimulated haematopoietic activity in WSDP—treated animals, as evidenced by the increased number of cells capable of producing haematopoietic colonies in the spleen of lethally irradiated recipients (Oršolić & Bašić, 2005a; review Oršolić *et al.*, 2007a) has also been evidenced in animals treated with other biological response modifier *C. parvum*. WSDP caused a significant elevation of leucocytes in peripheral blood of treated mice (Oršolić & Bašić, 2005a). WBI of mice with either 5 Gy or 7 Gy decreased the number of leucocytes in same proportion in normal and WSDP—treated mice respectively. However, the recovery of leucopenia was three times faster in WSDP—treated mice, as compared to control, suggesting thus that proliferation of leukocyte precursors from pluripotent stem cells is increased in mice after treatment with WSDP. In these studies no changes in erythrocyte cell count in peripheral blood of WSDP—treated mice were noticed during the single or combined treatment with WSDP plus WBI. Concerning the specific type of CFUs (Oršolić & Bašić, 2005a), the only difference between untreated and WSDP—treated mice was noticed in mice receiving treatment for 40 consecutive days in exogeneous spleen CFU assay; their spleen cells injected to WBI recipients gave rise to more myeloid and megakaryocytic colonies. These findings may explain faster recovery of leukocyte count in peripheral blood after WBI irradiation. Data on the effect of WSDP showing increased resistance of mice to metastasis formation in the lung (Oršolić *et al.*, 2004b, 2005b, d)

suggest that antitumor effect of WSDP might have been mediated by the effects of WSDP on macrophages and production of IL-1 (Oršolić & Bašić, 2003b; Oršolić *et al.,* 2005b). In addition, the anti-IL-1 antibody reduced the survival of untreated and irradiated mice, suggesting that natural levels of IL-1 contributed to radioresistance to mice. Forthermore, IL-1 was also shown to have a role in stimulating bone marrow to overcome the myelosuppressive effect of irradiation (Oršolić & Bašić, 2005a; Singh & Yadav, 2005) and was able to protect bone marrow progenitor cells from irradiation injury *in vitro* (Singh & Yadav, 2005).

Preclinical and clinical studies also demonstrated that a large number of cytokines could serve to accelerate bone marrow restoration after treatment with cytotoxic drugs that ablate the bone marrow or after exposure to radiation (Singh & Yadav, 2005) It is possible that cytokines such as IL-1, IL-4 or IFN-γ, also have restorative effects on bone marrow. Moreover, it was shown that IL-1 plays an important role in the regulation of normal haematopoiesis directly, by stimulating the most primitive stem cells and indirectly, by raising the production of other haematopoietic factors such as G-CSF, M-CSF, GM-CSF, and IL-6 (Singh & Yadav, 2005). IL-1 is the most extensively investigated cytokine for radioprotection in animal models (Singh & Yadav, 2005; Oršolić & Bašić, 2005a; Oršolić *et al.,* 2007a, 2008a) and single dose of IL-1 given to mice prior to irradiation protected several strains of mice (DBA/1, C3H/HeN, B6D2F1, CDF1, & Balb/c) from radiation damage (Singh & Yadav, 2005; Oršolić & Bašić, 2005a; Oršolić *et al.,* 2007a, 2008a).

Studies on radioprotection by immunomodulators date from reports indicating that endotoxin provides protection when administered 20 to 24 h before irradiation and/or shortly before or after exposure. The possible mechanisms of protection by cytokines have been reviewed by Singh and Yadav (2005) and Oršolić *et al.* (2008a). The potential utility of cytokines and growth factors as therapeutic agents and/or protective agents is great, and future studies should lead to a specific agent to treat specific tissue damage (Singh & Yadav, 2005).

It has been shown that tumor cell glutathione cycle is rate-limiting factor of their survival after treatment with different agents possessing antitumor cytotoxic property (Oršolić *et al.,* 2004a; Oršolić & Bašić, 2005a). Inhibition of the pathways of glutathione synthesis makes tumor cells more susceptible to the action of different antitumor agents; glutathione deficiency sensitizes cells to the disadvantageous effect of radiation (Oršolić & Bašić, 2005a; Bhosle *et al.,* 2005). It was demonstrated that the tumor cell resistance to various antitumor agents was partially associated with an overproduction of glutathione synthesis in those cells, and that its production could be reversed by treatment with selective inhibitors of glutathione synthesis (Oršolić *et al.,* 2004a; Oršolić & Bašić, 2005a; Oršolić *et al.,* 2008b, c). Our findings (Oršolić *et al.,* 2004a) showing that the

glutathione content of tumorigenic cell line was higher than in nontransformed cell line are in accordance with the above mentioned findings. The increase in glutathione in bone marrow cells after treatment with WSDP suggest that the elevation of glutathione level may spare normal haematopoietic cells from the deleterious effect of irradiation or chemotherapy. Our results (Oršolić & Bašić, 2005a) show that WSDP protected more of those cells possibly due to elevation of glutathione synthesis in their bone marrow which was not compromised by either 3 Gy or 6 Gy WBI. It is likely that treatment with WSDP increases the ability of haematopoietic tissue to synthesize glutathione in bone marrow compartment, making treated mice more resistant to the harmful damages of irradiation.

These data are in agreement with other (Conklin, 2004; Chen *et al.*, 2004; Singh *et al.*, 2005), suggesting that intracellular glutathione level may be implicated in the control of cell proliferation. More CFUs in haematopoietic tissues of mice as shown by exogenous spleen CFU assay indicate that WSDP used *po* for long period of time exercised stimulative effect on haematopoiesis. The stimulative effect of WSDP on haematopoiesis may be due to IL-1 production and its action to stem cells (Oršolić & Bašić, 2003b, 2005a; Oršolić *et al.*, 2006d) or through its influence on glutathione level. Moreover, some data suggest that IL-1α and IL-β may increase mRNA of MnSOD (Oršolić *et al.*, 2008a). It is likely that the antioxidative effect of flavonoids in biological system is related to a great deal of events including: (i) their ability to scavange ROS including $^1O_2$, $OH^\bullet$, H_2O_2, $O_2^{\bullet-}$, $HO_2^\bullet$, lipid radical ($LO^\bullet$) and lipid peroxy radical ($LOO^\bullet$; (ii) ability to scavange nitric reactive radical (HOONO, NO, NO3 i dr.); (iii) inhibition of oxidative enzymes; (iv) metal ion chelation (Cu^{2+}, Fe^{2+}, Zn^{2+} i Mg^{2+}); (v) increase the activity of antioxidant enzymes and their protection (Chen *et al.*, 2004; Oršolić *et al.*, 2008a; Kosalec *et al.*, 2008).

Our studies demonstrate that propolis and flavonoids present in propolis have radioprotective, stimulative and regenerative properties on haematopoiesis and suggest a clinically potential use of propolis and related flavonoids in the treatment of various cytopenias induced by radiation and/or chemotherapy. In addition, these studies provided the first evidence that propolis and its flavonoids acts directly on haematopoietic bone marrow and spleen cells and enhances their growth and differentiation into colony forming cells.

RADIOPROTECTION OF NORMAL TISSUES AND RADIOSENSITIZATION OF MALIGNANT CELLS

Exposure to ionizing radiation leads to undesirable damage, both on cellular and on the whole organism level. To diminish the hazards of the radiation exposure, many protective strategies have been developed, or are still under investigation. However, most of the compounds used failed in their

transition from laboratory to clinic. Acute toxicity and their inability to differentiate between tumor and normal cells are the main reasons for their failure in clinical applications. An effective radioprotector may be directed towards compounds which can protect normoxic cells and afford no protection under a hypoxic environment, which is a common feature in all solid tumors. Also relevant are compounds which become enzymatically converted into toxic derivatives in hypoxic tumor cells while remaining unchanged and protecting normal cells.

Ionizing radiation, alone or in combination with other therapies, is one of the most used anticancer treatments. It is well know that haematopoietic toxicity and immune suppression are one of the major problems after irradiation of patients.The radioprotective agents appear related to free radicals in competition with oxygen or with increased repair of radiation injury. On the contrary, radiosensitizers are chemical agents that have the capacity to increase the lethal effects of radiation.

The development of drugs that radiosensitize the malignant cell and radioprotect the normal tissues, is yet a challenge for oncologists and radiobiologists.

Whole body exposure of tumor-bearing animals to γ-radiation (4 Gy) resulted in an increase in the comet parameters (such as tail lenght, % DNA in tail, tail moment) of blood lymphocytes as well as in tumor cells as a results of damage to cellular DNA.Using the comet assay, we clearly demonstratet that propolis and related flavonoids applied to mice-bearing tumor before or after irradiation have different effect on normal and tumor cells; a significant decrease was in comet parameters of blood lymphocytes but not in the tumor cells of irradiated animals (Oršolić *et al.*, 2008a). The comet assay results in irradiated tumor-bearing animals show that the administration of WSDP, caffeic acid or quercetin for three consecutive days prior to whole-body gamma radiation significantly decreased the tail moment in blood cells when comet parameters were measured 1 h after irradiation. The minimal or maximal tail migration (tail length) of blood cells was 9.62–51.92 mm in control group while tail migration in propolis and/or polyphenolic treated group was lower (8.97 to 32, 69 μm) (Oršolić *et al.*, 2008). At the same time minimal or maximal tail migration of tumor cells of animals treated with propolis and related polyphenolic compounds was higher (10.26 to 52.56) as compared to tail length of control group where tail length was from 10.90 to 45.51 mm (Oršolić *et al.*, 2008a). Moreover, number of tumor cells in peritoneal cavity of mice treated preventively with test component was significantly decreased ($p<0.05$) as compared to control (review Oršolić *et al.*, 2008a). It is possible that synergism beetween radiotherapy and polyphenolic compounds due to selectively induced apoptosis in cancer cells was the base for these findings. When the administration of propolis or related polyphenolic compounds was performed after irradiation the significant decrease was noticed in

comet parameters of blood cells in all treated groups as compared to the control group (Oršolić *et al.*, 2008a), unlikely there was no decrease in comet parameters (tail moment) of tumor cells (see review Oršolić *et al.*, 2008a) when tumor-bearing mice were exposed to radiation and test components were administered. A significant decrease of tumor cells in peritoneal cavity was found as compared to conrol (Oršolić *et al.*, 2008a).

Our results obtained with propolis and related flavonoids are in concordance with result by Bhosle *et al.* (2005) and Maurya *et al.* (2004). We proposed that variability in antioxidant defense or DNA repair capability, induction of apoptosis as well as above mentioned difference between cell types and growth state, could be important in determining the susceptibility of the cells to genetic destabilization, cell death or mutation. It is possible that catalase (CAT) activity was lower in tumor cells than in the blood lymphocytes. It was shown that CAT activity and H_2O_2 sensitivity have been inversely related where human lymphocytes had the lowest CAT activity and were the most H_2O_2 sensitive (Oršolić *et al.*, 2008a).

Antioxidants may interfere with the initial mediation of apoptosis by ROS (Maurya *et al.*, 2004; Bhosle, 2005), as well as later membrane lipid peroxidation, which is characteristic of radiation-induced apoptosis (Oršolić *et al.*, 2008a). Central issues involve whether radiation-induced apoptosis can be promoted by some antioxidants in tumors but not in normal tissue, and when is it useful to protect against radiation-induced apoptosis in normal cells. Thus, experimental studies showed that antioxidant vitamins and some phytochemicals selectively induce apoptosis in cancer cells but not in normal cells and prevent angiogenesis and metastasis spread, suggesting a potential role for antioxidant as adjuvants in cancer therapy (Borek, 2004).

To conclude, the DNA damage caused by test components and radiation in the our study (Oršolić *et al.*, 2008a) can be based on the experimental evidence of radiation and different mode of action of the test component on tumor as compared to normal cells. The mode of action may include: (i) inhibition of various enzymes involved in DNA repair; (ii) induction of reactive oxygen species (ROS) capable of inflicting DNA damage, (iii) the unableness of tumor cells to use extra antioxidants in a repair capacity, (iv) the difference in cellular biochemistry or a lack of sufficient concentration of the propolis and polyphenolic compounds in tumor tissue to elicit radioprotection (v) the biodistribution of this compound in tumor and normal tissues, the hypoxic environment of the tumor and the poor vasculature in the tumor, (vi) the variations in the physiological and biochemical status of the cells of the tumor compared to normal cells at the time of irradiation and (vii) a selective protection of normal tissues from damage induced by irradiation and cytotoxicity to tumor cells.

Thereby, it is possible to conclude that the combination of propolis and related polyphenolic compounds with chemotherapeutics could increase the antimetastatic potential of chemotherapeutic agents and suggests the benefits of potential clinical trials using propolis and its flavonoids combined with chemotherapeutic agents in order to maximize enhanced immunity while potentially minimizing postchemotherapeutic or radiotherapeutic deteriorated reactions. Since the test components showed effectiveness in radioprotective studies, it appears that propolis and its related compounds should be considered for protection against radiation in humans. Presented results suggest that propolis and its polyphenolic compounds may be a promising adjunct treatment for patients exposed to radiation as well as to a hazardous radiation environment. Further studies are needed to implement the use of such natural compounds in human protection against ionizing radiation, especially in treatment of cancer patients exposed to radiotherapy.

HONEY

Honey is the foodstuff made by honey bees from the nectar of flowers or secretions from other parts of the plants, which they gather, transform together with their own specific materials, and store in honeycomb. It is a supersaturated solution of sugars, mainly fructose, glucose, and maltose-like sugar, with traces of sucrose- and glucose-oxidase, hydrogen peroxide, phenolic acid, flavonoids, terpenes, etc. Some studies indicated that honey possessed moderate antitumour and pronounced antimetastatic effects (Gribel & Pashinskii, 1990; Swelam *et al.,* 2003; Al-Waili, 2003) Furthermore, honey potentiated the antitumour activity of chemotherapeutic drugs such as 5-fluorouracil and cyclophosphamide (Gribel & Pashinskii, 1990).

It is shown (Oršolić *et al.,* 2003a, 2005d) that honey significantly affected the formation of lung metastasis when applied before tumour cell inoculation; however given to animals after tumour cell inoculation honey enhanced metastasis formation in the lung. The latter suggests that the antitumour effect of honey mostly depedend on the time of application; it is likely that polyphenolic components present in honey stimulate host antitumour defence, while in the presence of tumour nutritive constituents of honey prevail the effect of the former. It is possible that honey in presence of tumour alleviates tumour growth since it contains a mixture of vitamins, minerals and amino acids as those present in plasma, as well as large amounts of glucose. In addition, its high osmolarity induces an outflow of lymph which enhances nutrification and oxigenation, and its acidity favours release of oxigen from haemoglobin in the capillaries of adjacent tissues. Hydrogen peroxide in honey is responsible for angiogenesis which also improves oxigenation of tumour cells and their food supply (Oršolić *et al.,* 2003a; Al-Waili, 2003).

The combination of honey with chemotherapeutics in studies (Oršolić & Bašić, 2004a, b) influenced the proliferation of leukocyte populations in peripheral blood (Oršolić & Bašić, 2004a, b) that was inhibited by chemotherapeutic agents. These results are in line with previous results from this laboratory and others (Caltagirone *et al.*, 2000; Suzuki *et al.*, 2002; Oršolić *et al.*, 2003a; Oršolić & Bašić, 2004a, b; Conclin, 2004; Oršolić *et al.*, 2005d) suggesting that flavonoids from honey possess hemo-stimulative, antioxidative, protective and regenerative properties. Flavonoids possess the ability to capture and deactivate the free radicals (Caltagirone *et al.*, 2000; Suzuki *et al.*, 2002; Chen *et al.*, 2004), they act by inhibiting the binding of the free radicals to the DNA, by the activation of the detoxication system and by the protection of the capillary partitions (Caltagirone *et al.*, 2000; Suzuki *et al.*, 2002; Chen *et al.*, 2004). The family of flavonoids expresses this ability due to its unique structure, which allows the trap of the free radicals and their neutralization by two atoms of hydrogen, provided by two thiols (Chen *et al.*, 2004). Al-Waili (2003) showed that honey increased the amount and activity of antioxidant agents such as blood vitamin C concentration by 47%, b-carotene by 3%, uric acid by 12%, and glutathione reductase by 7%. These compounds reduce the activity of lipid peroxidation which is the consequence of toxic metabolites generated by the biotransformation of chemotherapeutic agents and their affect on the lipids of the normal cells (Al-Waili, 2003; Oršolić *et al.*, 2005d). It is suggested that the antioxidant capacity of honey appeared to be a result of the combined activity of a wide range of compounds including phenolics, peptides, organic acids, enzymes, Maillard reaction products, and possibly other minor components and that the phenolic compounds contributed significantly to the antioxidant capacity of honey but were not solely responsible for it.

In summary, preventive administration honey resulted in marked reduction of number of metastases in the lung. Application of honey with chemotherapeutic agents decreased metastasis formation and ameliorated the cytopenia induced by the chemotherapeutic agents alone. These results suggest the benefits of potential clinical trials using honey combined with chemotherapeutic agents in order to increase antimetastatic effect of some chemotherapeutics to maximise protective effect on leukopoiesis.

ROYAL JELLY

Royal jelly, which is secreted from the salivary glands of worker bees, serves as food for all young larvae and as the only food for larvae that are developing into queen bees.The physical and chemical properties of the royal jelly and its constituents, such as free amino acids, proteins, sugar, fatty acids (mainly 10-hydroxy-2-decenoic acid), minerals (mainly iron and calcium) and vitamins (thiamin, niacin, riboflavin) have been described in detail elsewhere (Šver *et al.*, 1996) Antitumour effects of royal jelly were

investigated employing transplantable tumours of mouse, advance leukemia L1210 and P388 strains and Ehrlich ascites tumour, Sarcoma-180 ascites and solid tumours (Swelam *et al.,* 2003). Our data demonstrated that royal jelly given *i.p.* or *sc* before or after tumour cell inoculation had no effect on metastasis formation (Oršolić *et al.,* 2003a, 2005d). However, royal jelly administered to mice *iv* synchronously with tumour cells exerted significant effect on metastases formation by these cells in the lungs ($p<0.001$). It is likely that direct effect of 10-hydroxy-2-decenoic acid and suturated fatty acid present in royal jelly directly affect metastatic ability of injected tumour cells (Oršolić *et al.,* 2003a, 2005d). In addition, the royal jelly enhanced the cell mediated immune response in the irradiated mice, and the synthetic 10-hydroxy-2-decenoic acid stimulated phagocytosis by mononuclear and phagocytic cells in the immunosupressed mice (Kimura *et al.,* 2003; Okamoto *et al.,* 2003; Kohno *et al.,* 2004; Salazar-Olivo *et al.,* 2005; Bincoletto *et al.,* 2005). Our data indicated that royal jelly exhibited immunomodula-tory properties by stimulating antibody production and immunocompetent cell proliferation in mice or depressing humoral immune function in rats (Šver *et al.,* 1996). Both phenomena, though species-related in this model, cysteamine could probably be reversed by changing the dose or the route of royal jelly application.

BEE VENOM

Bee venom aparatus has a prime role of defence to the bee colony. Bee venom from the venom gland located in abdominal cavity contains major components that are histamine, catecholamines, polyamines, melittin, and phospholipase A_2 (Oršolić *et al.,* 2003a, c, 2005d; Raghuraman & Chattopadhyay, 2007). Melitin, present in bee venom about 50–70%, is antimicrobial peptide of bee venom. Some of antimicrobial peptides display a wide range of biological activities and at least some of antimicrobial peptides isolated from insects, including melittin cecropin related peptides and the magainins have been shown to exhibit antitumor activity for cells derived from mammalian tumors (Oršolić *et al.,* 2003a, c, 2005d). One such peptide, melittin has been shown to revert the tranformed fenotipe of H-ras transformed cells (Sharma, 1993). It was demonstrated that melittin is one of the most potent inhibitors of calmodulin activity, and as such also is a potent inhibitor of cell growth and clonogenicity (Oršolić *et al.,* 2003). There is also some evidence suggesting that calmodulin inhibitors are cytotoxic to malignant cells both *in vitro* (Oršolić *et al.,* 2003c) and *in vivo* (Oršolić *et al.,* 2003a, c, 2005d; Raghuraman & Chattopadhyay, 2007). Drugs that inhibit the activity of calmodulin have been shown to inhibit DNA synthesis in a glioblastoma cell line, to block the movement of chromosomes during metaphase to inhibit the growth of Chinese hamster ovary cells and to enhance the cytotoxicity of vincristine, deoxorubicin and bleomycin (Tsuro *et al.,* 1982; Chafouleas *et al.,* 1984). Mechanisms of antitumour action were described as apoptosis, necrosis and lysis of tumour cells (Liu *et al.,* 2002;

Oršolić *et al.,* 2003c; Oršolić & Bašić, 2003a). In this report, we demonstrate direct antitumor effect of bee venom given intratumourally at different times after tumour cell inoculation in mice and cytotoxic effect of bee venom on HeLa and on primary culture of MCa cells. In our study (Oršolić *et al.,* 2001b, 2003c) we demonstrated that antitumour and antimetastatic effects of bee venom could be highly dependent on the route of injection and on close contact between bee venom and tumour cells. Here we were particularly interested in antitumour activities of bee venom given intratumourally at different times after tumour cell inoculation and in clarification of the mechanisms by which bee venom produced these effects. When bee venom was injected intratumourally tumours decreased in size; some sort of shrinkage of tumours occurred, and the delay of tumour growth was evident. Survival of bee venom treated mice was prolonged compared to control mice. Bee venom significantly inhibited tumour growth; tumour inhibition effect of bee venom was dose- and time depedent (Oršolić *et al.,* 2005d). We have recently shown that inhibited tumour growth by bee venom was the consequence of apoptosis and/or necrosis of tumour cells or it was the result of activation of immune system by bee venom (Ćurić *et al.,* 1993; Oršolić *et al.,* 2003c). It was also shown that less differentiated cells (leukaemic cells, tumour cells) were 2–4 fold more sensitive to the lytic effects of bee venom than normal splenocytes or bone marrow cells (Killion *et al.,* 1986); probably the reason for this is the loss of amino/carbohydrate binding structures which led to destruction of tumour cells that were in close contact with bee venom. It is likely that higher doses of bee venom exhibited toxic effect on tumor tissues as was shown for blood, muscle and heart tissues in human and animals treated with bee venom or other components of it such as melittin and phospholipase A_2 (Killion *et al.,* 1986; Oršolić *et al.,* 2003c, 2007c).

Requirements for close contact between MCa and bee venom for *in vivo* effect was also shown in *in vitro* studies (Oršolić *et al.,* 2003c). The degree of growth inhibition of MCa cells in the presence of bee venom was dose dependent up to 24 h. It is likely that bee venom has a short half-life for the effect on thymidine incorporation since its active ingredients could be unstable in tissue culture medium. This may be explained by inhibition of calmodulin or by the effect of bee venom on induction of apoptosis and necrosis of tumor cells described in our (Oršolić & Bašić, 2003a; Oršolić *et al.,* 2003c, 2007c) and other studies (Raghuraman *et al.,* 2007; Liu *et al.,* 2002). The mechanism by which agents exerting anticalmodulin activity inhibit the growth of cells is unknown. One possible mechanism might be the inhibition of a calmodulin sensitive enzyme such as cyclic nucleotide phosphodiesterase. The mechanism of inhibition appears to be mediated through the formation of a calcium dependent high affinity complex between calmodulin and melittin (Sharma, 1993; Liu *et al.,* 2002; Raghuraman *et al.,* 2007). Although phosphodiesterase was the first enzyme demonstrated

to be activated by calmodulin, it is now known that numerous enzymes and structural proteins are dependent on this pervasive calcium binding protein (Raghuraman *et al.*, 2007). Calmodulin is essential for many processes that are necessary for normal cellular function, including the assembly and disassembly of microtubules, calcium extrusion from cells by a calcium-magnesium ATPase, and the activation of numerous intracellular enzymes, such as protein kinases, phosphatases, and cyclic nucleotide phosphodiesterase (Sharma, 1993; Liu *et al.*, 2002; Raghuraman *et al.*, 2007). Interfering with any of these known functions, calmodulin would be potentially toxic to cells. Other cellular functions inhibited by calmodulin inhibitors may or may not be mediated exclusively through the inhibition of calmodulin. These effects include the depletion of intracellular ATP, the destabilisation of membranes and inhibition of protein-kinase C (Sharma, 1993; Liu *et al.*, 2002; Oršolić *et al.*, 2003; Raghuraman *et al.*, 2007). Futhermore it has also been reported that direct contact of bee venom components with different tissues (cells) caused instability of cell membrane receptors and damaged of enzyme systems bound to cell membrane such as cation activated adenosine triphosphatases, damaged acetylcholine esterase's, or inhibited thromboplastic potency of tissue (Sharma, 1993; Liu *et al.*, 2002; Raghuraman *et al.*, 2007).

It is likely that incubation of MCa for 48 h is important for the appearance of resistant tumor cells to bee venom (dose 1.43). Acquisition of resistance to bee venom could be accompanied by a decrease in the number of copies of *ras* genes, decrease in expression of the *ras* oncoprotein, and concomitant reversion of transformed cells to a normal morphology as suggested by Sharma (1993). Also, our results on other cells (HeLa and V79) showed that glutathione levels might be involved in their resistance to bee venom.

The responses of regional lymph node cells were increased in animals treated with bee venom (Oršolić *et al.*, 2003c), as was similarly shown by Schneider and Urbanek (1984). The results related to the lytic activity of popliteal lymph node cells on MCa cells (Oršolić *et al.*, 2003c) indicate that bee venom is a strong activator of antitumor lytic activity of lymphoid cells deriving from the regional lymph node. Inactivity of spleen cells in this respect indicates that concentration of bee venom is an important factor for activation of antitumor lytic activity in mice. The possible mechanism(s) of antitumor lytic activity may include factors related to activation of cytotoxic T lymphocytes. Local treatment with bee venom increased the $CD8^+$-T-cell subset and led to a progressive reduction of the immune index ($CD4^+/CD8^+$ ratio) in favour of $CD8^+$ cells. A low $CD4^+/CD8^+$ ratio in lymph node, of 0.75, is observed in the treated group compared with the control group (1.43). There was a significant difference between the two groups ($p<0.01$). The $CD4^+/CD8^+$ ratio in spleen between control and treated group was 1.45 and 1.35, respectively. This implies that the phenotype of $CD8^+$

cells increased by local stimulation of regional lymph nodes by bee venom may have an important role in tumor cytotoxicity. Moreover, according to Magnan *et al.* (2001) and Ribardo *et al.* (2002), the release of precursors of pro-inflammatory mediators caused by bee venom components such as phospholipase A2 and melittin might increase synthesis of IL-1 and TNF-α in monocytes and the cellular response of T lymphocytes. These findings contrast to those indicating that bee venom has an inhibitory effect on immunocompetent cells (Rekka *et al.,* 1990). Moreover, treatment with bee venom increased the response of spleen cells to polyclonal mitogens (Oršolić *et al.,* 2003c). Consistent with this observation is a report of Schneider and Urbanek (Schneider & Urbanek, 1984), who also found an increased stimulation index among lymphocytes from lymph nodes when cultured with bee venom *in vitro.* Thus, bee venom might have direct and indirect action on tumor cells by stimulating the host cells, mainly macrophages and cytotoxic T lymphocytes (Rekka *et al.,* 1990; Magnan *et al.*, 2001; Ribardo *et al.,* 2002).

Taken together the data presented here support and extend our findings and the findings of other that bee venom has direct antitumor effect *in vivo* and *in vitro.* This study together with previous one demonstrate that apoptosis, necrosis and lysis of tumor cells could be the possible mechanisms by which bee venom inhibited tumor growth. Concomitant to these is the development of local cellular immune response in lymph node draining the region of bee venom introduction. Furthermore it is likely that more attention should be paid to natural inhibitor of tumor growth such as bee venom and other products of bee hive in testing their antitumor activity for future possible use in clinical practice.

POLLEN

»Bee pollen« is actually pollen from flowers that is collected by bees. Pollen granules stick to the bees' legs and other body parts as they help themselves to nectar inside the flowers. Pollen are the male reproductive cells of flowers. Flower pollens are bees' primary food source, and they contain concentrations of phytochemicals and nutritients and are rich in carotenoids, flavonoids and phytosterols (Orzaez *et al.,* 2002; Nagai *et al.,* 2005; Aliyazicioglu *et al.,* 2005). Findings (90) indicated that quallity of bee pollen may be at the top of the list of food exhibiting antioxidant activity; total polyphenol content in pollen is found to be 15.05 mg/g. In addition to most vitamins and minerals, bee pollen also provides amino acids, enzymes and coenzymes, fatty acids, carbohydrates and 25% protein by weight. Bee pollen possesses antimicrobial effects, but more common claim is that it increases energy levels (Orzaez *et al.,* 2002; Nagai *et al.,* 2005; Aliyazicioglu *et al.,* 2005).

Studies concerning antimetastatic capacity of pollen reveal that some pollens such as *Castanea* and *Raphanus* given to mice orally before tumor

cell inculation manifested significant ($p<0.05$) antimetastatic effect while the treatment of mice with pollen from *Cistus, Salix,* and *Papaver* was ineffective (Oršolić *et al.,* 2007c). These results suggest that diferent antimetastatic effects of pollens from differrent plant species may be prescribed to their constituents and probable polyphenolic compounds of pollens (Orzaez *et al.,* 2002; Aliyazicioglu *et al.,* 2005). According to the literature antitumor effecacy of pollen *in vitro* (Aliyazicioglu *et al.,* 2005) and *in vivo* (Roberts *et al.,* 1998) was described but presented data are the first ones describing antimetastatic effect of pollen in animal model of metastases formation.

CONCLUSIONS

In conclusion, results presented here indicate that honey bee products might be a potential useful tool in the control of tumour growth in experimental tumour models. The chemopreventive activity of honey bee product in animal models and cell cultures are likely to be the result of their ability to inhibit DNA synthesis in tumor cells, their capability to induce apoptosis of tumor cells, and their property to activate macrophage to produce factors capable to regulate the function of B-, T- and NK-cells, respectively. Moreover, these results suggest the protective role of the flavanoids from propolis or honey against the toxicity of the chemotherapeutic agents in mice giving a hope that they may have similar protective action in humans. The result from the present study indicate that propolis and its flavonoids are able to alter the activities of liver marker enzymes by its protective effect against tissue damages and can alter both phase I and II metabolism of chemotherapeutics thus meeting the criteria for an ideal chemotherapeutic agent and adjuvant along with chemotherapeutic. In addition, these results suggest the benefits of potential clinical trials using propolis preparation, honey or their flavonoids combined with chemotherapeutics in order to maximize antitumor activity and potentially minimize postchemotherapeutic deteriorating reaction of chemotherapeutic drug on haematological toxicity. Taken together, this information provides a basis for attempts to use the honey bee products and related polyphenolic compounds in preclinical and clinical practices for many diseases, since these components have wide pharmacological and biological activities.

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Mitogenic and Anticoagulant Activity of a Fucoidan Isolated from Brown and Red Seaweed

LYDICE C.M. CARVALHO[1], HUGO A.O. ROCHA, FERNANDA W. OLIVEIRA, SUELY F. CHAVANTE, SELMA M.B. JERÔNIMO AND EDDA L. LEITE

ABSTRACT

Brown algae synthesize several polysaccharides, some of which, such as cellulose, closely resemble those of the higher plants, whereas laminaran, fucans and alginic acid are unique in brown seaweed. Fucans are sulfated polysaccharides found in Phaeophyceae and the main sugar of these polymers is α-L-fucose. These compounds are located in the intracellular tissues or in the mucilaginous matrix and have been found along with alginic acid in drops that exude from the leaves of several species of brown algae. Sulfated fucans are among the most widely studied of all the sulfated polysaccharides that exhibit biological activities in mammalian systems. Algal fucans may have some regular repeating structure but are clearly more heterogeneous than the echinoderm fucans. The structures of the sulfated fucans from brown algae also vary from species to species. The results indicate that different structural features determine not only anticoagulant and antiinflammatory activity, but also induce platelet activation. In this work we studied the mitogenic action of sulfated polysaccharides from algae and their anticoagulant effect.

Key words : Fucan, galactan, fucoidans, mitogenic activity, anticoagulant action, *Fucus vesiculosus*

1. Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Salgado Filho, s/n, Lagoa Nova, Natal, RN, Brazil.

* *Corresponding author* : E-mail : eddaleite@cb.ufrn.br

INTRODUCTION

Fucan is the term used to define a polysaccharide composed mainly of sulfated L-fucose (Fig 4.1) and containing other monosaccharides (Percival & Mc Dowell, 1967). Fucoidans or fucans are a family of sulfated homo and heteropolysaccharides composed mainly of α-(1 → 2) and α-(1 → 3) linked by other sugar residues. They are isolated in brown seaweeds and echinoderms (Berteau & Mulloy, 2003). Another source of sulfated polysaccharides from invertebrates have specific patterns of sulfatation and glycosidic linkages α-(1 → 3) and are ordered structures with different positions of the sulfate groups in positions 2 and 4 in the α-L-fucoses (Patankar *et al.*, 1993; Berteau & Mulloy, 2003). Fucan structures vary among species and sometimes among different parts of the algae (Mourão & Pereira, 1999; Berteau & Mulloy, 2003). In contrast to animal polysaccharides, the algae fucans contain, in addition to neutral sugars, xylose, uronic acid and sulfated fucose (Dietrich *et al.*, 1995; Berteau & Mulloy, 2003; Leite *et al.*, 1998). These sulfated polysaccharides are involved in several biological activities, including anticoagulant, antiinflammatory, antitumoral and mitogenic (Patel *et al.*, 2002; Koyanagi *et al.*, 2003; Mourão, 2004; Queiroz *et al.*, 2006).

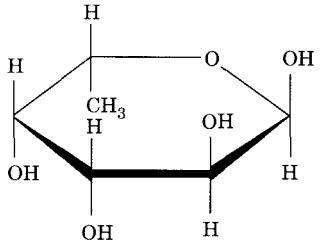

Fig 4.1. Structure of α-L-fucose

Red marine algae contain galactans, sulfated polysaccharides composed mainly of sulfated galactose. Carrageenan is a generic name for a family of linear, sulfated galactans, obtained from certain species of marine red algae. They are classified according to the presence of 3, 6-anhydro-D-galactose (AG) in the 1→4-linked residue and the position and number of sulfate groups. They could potentially be used as texturizing, viscosity-building and gel-forming ingredients in the food and pharmaceutical industries (Falshaw *et al.*, 1996; Navarro & Storz, 2005; Zhou *et al.*, 2006; Chattopadhyay *et al.*, 2007). We assessed the action of these polysaccharides as part of our ongoing research program, which involves the search for bioactive polysaccharides from brown seaweed. We determined the structure of a sulfated polysaccharide a fucan from *S. scroederii* (Leite *et al.*, 1998) (Fig 4.2). These saccharides have both anticoagulant and

antiinflammatory properties. Our aim is to study the biological activity of these compounds, which may become future substitutes for synthetic drugs.

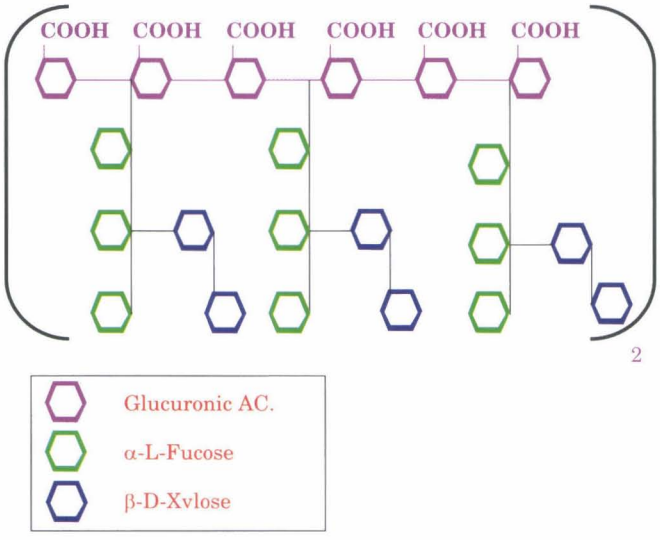

Fig 4.2. Structure proposed by Leite *et al.* (1998) for fucans obtained from the algae *Spatoglossum schröederi*

Biological Activities

Fucan and fucoidan are terms to define a family of L-fucose-containing sulfated polysaccharides extracted from brown algae and sea urchins (Farias *et al.*, 2000; Rocha *et al.*, 2005). Patel *et al.* (2002), showed that the structurally different polysaccharides, heparin, fucoidan (and fucans), have distinguishable effects on mitogenesis and ERK1/ERK2 activation, suggesting that different mechanism(s) mediate these actions. The potent antimitogenic action of fucoidan and its efficacy in heparin resistant vascular smooth muscle cells (VSMC) emphasize the need to further investigate its action mechanism in human VSMC and suggest this agent could have therapeutic properties. Mitogens are substances that encourage a cell to commence cell division, triggering mitosis. They trigger signal transduction pathways in which mitogen-activated protein kinase is involved, leading to mitosis. Mitogen as concanavalin A has been found to induce killer cells in human peripheral blood mononuclear cells. The incorporation of tritiated thymidine into cultures of phytohemagglutinin-stimulated lymphocytes is routinely used as an indication of the immunocompetence of the cells and of their proliferation (Rocha *et al.*, 2005). The activity on RT (reverse transcriptase) was also available.

Anti-HIV Activity

Extracts with anti-HIV activity are also active against other retroviruses such as herpes simplex virus, but the amount of antiviral activity varies with the compound and the virus. Most of the research has focused on sulfated homopolysaccharides and heteropolysaccharides (Witvorouw & De Clercq, 1997). Sulfated homopolysaccharides are more potent than sulfated heteropolysaccharides. The presence of the sulfate group is necessary for anti-HIV activity, and potency increases with the degree of sulfation (Baba *et al.,* 1988; Witvorouw & De Clercq, 1997). We also assessed the activity of several fucans, fucoidans and modified fucans, such as decarboxylation and desulfatation, in the inhibitor HIV from reverse transcriptase (RT). The activated DNA was used as well as template-primer poly(rA)-oligo(dT). We found that fucans and the fucoidan *F. vesiculosus* had a pronounced inhibitory effect *in vitro* on reverse transcriptase from HIV, with the exception of desulfated fucans and 4 xylogalactofucan isolated from *Spatoglossum schroederi,* which have no inhibitory activity on this enzyme (Baba *et al.,* 1988; Witvorouw & De Clercq, 1997; Queiroz *et al.,* 2008). These modified conditions reduced the inhibitory activities of these polysaccharides for RT approximately four fold. However, the activity of these polysaccharides is not narrowly related to the presence of polyanionic charges (Fig 4.3). The length of the sugar backbone and its structure can also act on reverse transcriptase activity. Some studies have suggested that other characteristics of these molecules, such as the polymeric backbone, carbohydrate moieties, and the degree of polymerization, could play a role in influencing their antiviral properties (Baba *et al.,* 1988; Queiroz *et al.,* 2008).

Anticoagulant Activity

Anticoagulant activity was measured in red and brown algae. Several sulfated green algae and polysaccharides from red algae have exhibited anticoagulant activity and have been extracted from different seaweeds (Haroun-Boundeja *et al.,* 2000; Shanmugam & Mody, 2000; Rocha *et al.,* 2005). One way to determine the relationship between structure and biological activity of sulfated polysaccharides is to compare their activity in various assays where the contributions of the polysaccharide backbone, and the extent and position of sulfation have been fully characterized. In this line of work, new sulfated galactans and the sulfated fucan from invertebrates constitute a valuable tool (Shanmugam & Mody, 2000). Anticoagulant and antithrombotic activities are among the most widely studied properties of sulfated polysaccharides. However, there are few reports of their action mechanism (Colliec *et al.*, 1991; Shanmugam & Mody, 2000). In general, the proposed mechanism is predominantly mediated by antithrombin and/or heparin co-factor II. Anti-factor Xa and fibrinolytic

activities are also proposed (Mourão, 2004). The prothrombine time (PT) and activated partial thromboplastin time (APTT) tests are used to distinguish the effects on extrinsic and intrinsic coagulation pathways, respectively. Fucan preparations have been proposed as alternatives to the anticoagulant heparin (Farias *et al.*, 2000; Rocha *et al.*, 2005) which is prepared from mammalian mucosa. Fucans are less likely to contain infectious agents, such as viruses or prions, since they are extracted from organisms of marine origin. The literature on the action mechanism of fucoidan or fucans is complex and somewhat contradictory (Mourão, 2004; Silva *et al.*, 2005). For example, some reports have suggested that fucans act on thrombin inhibition by antithrombin or heparin cofactor II, whereas other investigators have reported that this enhanced thrombin inhibition is caused by direct thrombin inactivation (Boisson-Vidal *et al.*, 2000).

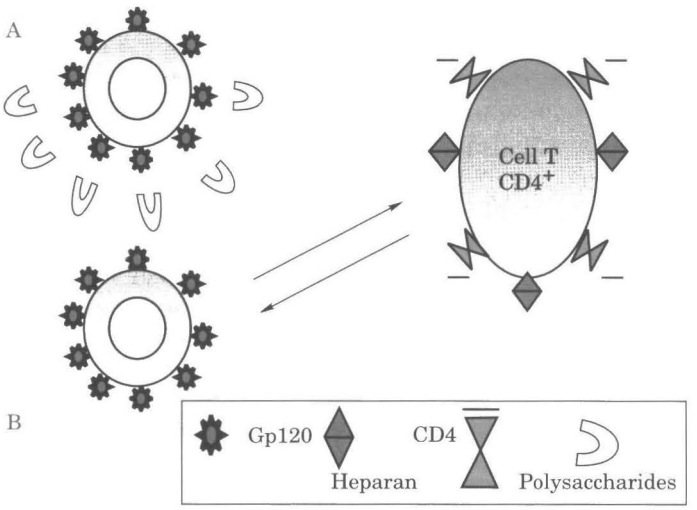

Fig 4.3. Interaction of HIV with surface T $CD4^+$ cell. (A) HIV-virus and sulfated polysaccharides fucans. (B) HIV-virus without sulfated polysaccharide

Antiinflammatory Activity

Inflammation, a response of the organism to invasion by an infectious agent or to a physical lesion, attracts leukocytes and plasma molecules to the infection site or to the tissue lesion. The main effects are: increased blood supply to the area and increased capillary permeability, which allows protein exudation and leukocyte migration to the tissues. Several inflammatory response phenomena are mediated by three interrelated factors originating in blood plasma: coagulation, kinins and the complement system that acts as a link between immunological events and inflammatory processes (Luster, 1998; Zhou *et al.*, 2008; Shimaoka *et al.*, 1996).

This complement system encompasses a group of more than thirty proteins found in plasma and on the cellular surface. The system functions in the innate and adaptive immunity to defend against microbial agents, culminating in microbial lysis by the membrane attack complex (Farries & Lipowsky, 1989). In addition to cell lysis, this system has the following biological functions: opsonization; inflammation activation; solubilization and phagocyte purification of the immunocomplexes and promotion of humoral immune responses. The activation of the complement components occurs by two pathways: the classic and the alternative. This activation may be beneficial to the host by eliminating the infectious agents, but may also damage the cells and tissues due to deposition in the circulation of the immunocomplexes formed (Farries & Lipowsky, 1989; Berteau & Mulloy, 2003).

Carrageenans, sulfated polysaccharides from red algae, inhibit the hemolytic activity of the complement *in vitro* by interfering in C1, the first component of the complement cascade. The effects on the complement depend on the class of carrageenans, their origin and their molecular weight (Shanmugan & Mody, 2000). According to these authors, low molecular weight fucans (LMWF) are also potent activation inhibitors of the human complement system *in vitro*, inhibiting the initial steps in both pathways. They avoid the formation of the C3 convertase in the classic pathway by interfering in the activation mechanism of C1 and/or the cleavage of C4 by C1s (Kozlov *et al.*, 1986). These fucans also inhibit the formation/function of the C3 convertase found in the alternative pathway by suppressing the link between factor B and cell-bound C3b, and by interfering in the stabilization of the properdin function (Tissot & Daniel, 2003). However, they do not inhibit the terminal components of the complement pathways (Tissot & Daniel, 2003; Landis, 2007). This activity depends on the molecular weight increasing with the content of xylose, galactose and glucuronic in the LMWF polymers (Blodin *et al.*, 1994). This suggests that these residues must be essential in determining the maximum anticomplement activity of these compounds. However, in contrast to its anticoagulant activity, the presence of sulfate groups is not a sufficient condition for the anticomplement activity displayed by the fucans (Colliec *et al.*, 1991; Mourão 2004).

The implantation of organs or artificial biomaterials and the xenotransplantation of vascularized organs induce the activation of complement proteins that damage the patient's tissue (Platt & Bach, 1991). Inflammatory mediators produced during this activation contribute to stimulating the activation of endothelial cells and subsequent adhesion of leukocytes and macrophages to these cells or to the artificial polymer surface. Before leukocytes adhere to the endothelial cells, they marginate and roll along the endothelium (Firrel & Lipowsky, 1989). This rolling and margination requires interaction between the leukocytes and the endothelial

cells through the adhesion molecules, such as selectins, laminins, fibronectins and integrins (Penberthy, 1997).

High molecular weight fucans inhibit leukocyte rolling in the venules (Bodycote *et al.,* 1986; Ley *et al.*, 1991; Nasu *et al.,* 1997) likely through the L-selectin bond and subsequent inhibition of lymphocyte adhesion to the endothelium, which is mediated by this adhesion molecule (Weston & Parish, 1999; Dwir *et al.,* 2001).

Colliec *et al.* (1991) established that fucans can be used as a good anticomplement (Farries & Atkinson, 1987) and antiinflammatory agent and that they are able to inhibit the activation of a small concentration of complement proteins (Blodin *et al.,* 1994). Consequently, if these polysaccharides were used clinically, they would deprive the patient of native complement proteins, controlling their pathologic activation and decreasing the risk of hemorrhage, given that fucans exhibit moderate anticoagulant activity (Platt & Bach, 1991). With respect to anti-inflammatory activity, fucans (Weston & Parish, 1997) inhibiting complement activation impede the triggering of its biological functions, provoking the non-activation of the inflammatory process. However, this action mechanism is not entirely clear.

MATERIALS AND METHODS

The sulfated polysaccharides, fucoidan or fucan, were fractionated with different acetone volumes into three fractions. The molecular weight of 170-kDa polysaccharides from brown algae *F. vesiculosus* contains different levels of sulfate. In heterofucans, proteolysis with maxataze resulted in low levels of protein contamination (Dietrich *et al.*, 1995). This step was important because fucans bind to a large number of proteins through an ion-exchange process. Subsequently, the extract was fractionated with different acetone concentrations. These polysaccharides were characterized by different electrophoretic mobilities in agarose gel electrophoresis (see Fig 4.4) (Dietrich & Dietrich, 1976). Chemical analysis, total sugars (Dubois *et al.,* 1956), sulfate (Dodgson & Price, 1962) uronic acid (Dische, 1962) and proteins (Lowry *et al.,* 1951) showed the compound components. At least three different polysaccharides have been shown in heterofucan preparations from *Sargassum vulgare*, *Dictyota mertensis* and *Spatoglossum schröederi* (Dietrich *et al.,* 1995; Rocha *et al.,* 2005). The NMR and IR were the spectroscopic analyses used in the characterization of these polysaccharides (Leite *et al.*, 1998; Albuquerque *et al.,* 2004; Silva *et al.,* 2005).

The activated partial thromboplastin time (APTT) was determined using citrated normal human plasma according to manufacturer specifications. For the prothrombin time (PT) assay, citrated normal human plasma was mixed with a sulfated polysaccharide solution at different concentrations

and incubated at 37°C. The PT assay reagent, preincubated for 10 min at 37°C, was then added and the clotting time was measured in a coagulometer. The anticoagulant activity of several heterofucans from algae was compared with that of heparin and low molecular weight heparin using sheep serum.

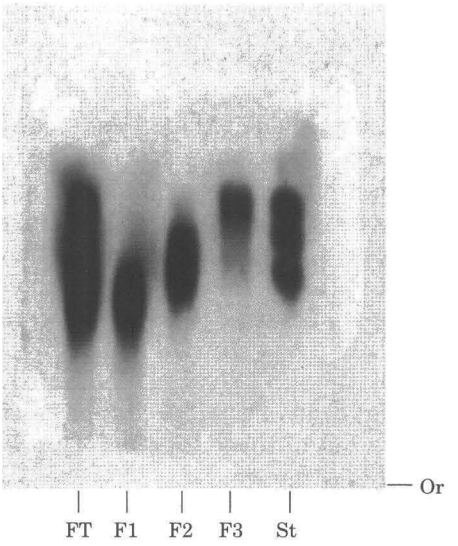

Fig 4.4. Agarose gel electrophoresis in 1, 3 diaminepropane acetate buffer of fucoidan fractions from *F. vesiculosus*. St (standard of sulfated glycosaminglycans). TF-total fucoidan; F1-purified fractions with acetone with 1, 2 and 3 volumes respectively; Or-origin

Mononuclear cells from peripheral blood were separated using a Ficoll-Hypaque gradient. For proliferation assays, mononuclear cells were resuspended in RPMI-1640 supplemented with penicillin/streptomycin, L-glutamine and bovine serum (10%) and cultured in plates with or without fractions from fucans (Ft, F1, F2 and F3) in an atmosphere containing 5% CO_2. The positive control was also performed with concanavalin A. The cells were pulsed with 1 [H^3]-thymidine per well and label incorporation was assessed by liquid scintillation counting.

RESULTS AND DISCUSSION

Structural Aspects of Fucans or Fucoidans

Fucans or fucoidans have recently become attractive for their drug development potential (Berteau & Mulloy, 2003). The effect of fucans isolated from other seaweed species on cell proliferation, cell cycle and adhesiveness of both wild-type Chinese hamster ovary cells and the xylosyltransferase-deficient mutant to several extracellular matrix proteins has been described (Rocha *et al.*, 2005). The results suggest that these polysaccharides from brown seaweed species are different in terms of their

mitogenic activity, probably because their structural features are not identical. In this study, crude commercial fucoidan was purified into three fractions and their action on the mitogenic activity of the fucoidan from *F. vesiculosus* in mononuclear cell peripheral blood was observed. The characterization of their mobilities and molecular weight was performed by polyacrylamide agarose gel electrophoresis in diaminepropane acetate and gel-permeation chromatography. These polysaccharides from brown algae were extracted and purified with 1, 2 and 3 volumes of acetone (Leite *et al.,* 1998; Rocha *et al.*, 2005). A highly active mitogen, isolated from fucan A of the brown algae *Spatoglossum schroderi,* was characterized by agarose get permeation chromatography (Fig 4.4) and chemical analyses, including methylation and NMR spectroscopy. This fucan from Dictyotaceae is a 21 kDa heterofucan containing mainly fucose, galactose, glucose, xylose, and/or uronic acid. An 18 kDa heterofucan precipitated with 1.0 volume of acetone (0.25–3 v) was fractionated with increasing volumes of acetone and fucans from the brown seaweed *Padina gymnospora* (Silva *et al.,* 2004).

Anticoagulant Activity

An alternative pathway of the complement system was evaluated by assay hemolytic using red blood cells from rabbits. The results of APPT for *F. vesiculosus* showed that TF, F1 and F2 at 5 µg/ml have high anticoagulant activity (240.0 s). However, the fraction F3 showed coagulation time at 73.7 s. The results obtained for Ft, F1, F2 and F3 using the PT test were 81.5 s, 120 s, 57.1 s and 32.5 s respectively. The results of the complement system assay showed that F2 and F3 decreased the hemolytic activity of this system by 30% and 40% respectively compared to the control. Ft and F1 did not show any effect. The results obtained suggest that fucoidans F2 and F3, in addition to exhibiting high anticoagulant activity, inhibit the hemolytic profile of the alternative pathway of the complement system.

Fucan-Binding L-Selectin

Cell surface carbohydrate are presents in the regulation of lymphocyte homing and the inflammatory recruitment of leukocytes (Kannagi, 2002). In the routine homing process, lymphocytes home at a slow and steady speed, avoiding excess accumulation of cells in the vascular beds. This is partly mediated by a negative-feedback system employing post-translational modification of the ligand sialyl 6-sulfo Lewis x *via* the sialic acid cyclase pathway. Research performed with hetero and homofucans suggests their antiinflammatory action. In contrast, massive accumulation of lymphocytes at inflammatory lesion sites is mediated mainly by nonsulfated sialyl Lewis x, the expression of which is augmented by transcriptional activation. Medeiros *et al.* (2008), suggest that fucans of *Lobophora variegata* has antinflammatory actionn pathway L-selectin.

Mitogenic Action

Based on the lymphocyte stimulation method described in the Methods section, we can observe that fucana Ft *Fucus vesiculosus*, when incubated with the cell membrane stationary phase (CMSP), had a stimulation index of 51.5 at a concentration of 20 μg/mL, indicating that this fraction has considerable mitogenic activity. Fractions F2 and F3, at a concentration of 50 g/mL had no stimulatory effect on CMSP proliferation. The mitogenic reference used was concanavalin A, which had a high stimulation index at a concentration of 5 μg/mL. The aforementioned results are as shown in Table 4.1. A highly active mitogen was isolated from fucan A of the brown algae *Spatoglossum schroederi*. The stimulation index on peripheral blood mononuclear cells was near that of Concanavalin A.

Table 4.1. Molar relations of the sugars and sulfates and the percentage of proteins present in the polysaccharide acid fractions from *Fucus vesiculosus*

Fraction	Polysac.* %	Protein*** %	Molar Relation			
			Fucose	Xylose**	Uronic Acid	Sulfate
F1	99.7	0.24	1	n.d	0.3	1.0
F2	99.8	0.16	1	n.d	0.2	0.8
F3	87.4	1.25	1	n.d	0.1	0.7

*Calculated according to total sugar content (Dubois *et al.*, 1956).

n.d**–not detected.

***Calculated according to Lowry method 1951.

Data are represented as means of stimulation indices, calculated by dividing the mean cpm of stimulated cultures by the mean cpm of unstimulated cultures. The types of mononuclear cells stimulated by fucan fractions were analyzed by flow cytofluorimetry using previously described procedures. The flow cytometry was performed with monoclonal antibodies labeled with fluorescent substances (fluorescein and phycoerythrin). The proliferative response of human peripheral blood mononuclear cells (PBMC) stimulated by fucans purified from brown algae species was studied and compared to that of lectin Concavalin A. Two fractions stimulated human PBMC to proliferate, with variable mitogenic activity efficiency. The data obtained through the cytometric flow showed that fractions Ft and F1 introduced an immunophenotype percentile for T-CD4, T-CD19 and T-CD22 adult cells, when compared to the control. The Ft fraction also introduced an immunophenotype percentile for T-CD7 adult cells. The results are as shown in Table 4.2 and Table 4.3.

Table 4.2. Mitogenic action of fucan fractions on peripheral mononuclear cells*

Samples	Concentration mg/mL	Stimulation Index	CPM**
Ft	20	49.2	13310
Ft	50	92.1	26675
F1	20	51.5	13798
F2	50	4.5	1207.5
F3	50	0.6	149
Concanavalin A	**5**	**100**	**26782**

* CMPS

* *CPM-Counts per min.

Table 4.3. Percentage of immunophenotyping of fucan fractions on PBMC

Phenotyping	Blank (%)	Ft (%)	F1 (%)	F2 (%)	F3 (%)
CD8	50	46	33	43	40
CD4	35	47	45	30	25
CD19	6	8	8	2	2
CD3	86	76	70	75	77
CD22	6	10	8	5	5
CD5	80	81	62	69	72
CD7	84	88	73	83	80
CD16	3	3	3	3	3

Mitogens are known to stimulate lymphocyte transformation, leading to their proliferation. Because the incorporation of [3H] dThd into the chromosomes of proliferating cells sequesters the radioactive nucleoside from the medium, activity loss from the medium is often used as an indication of cell proliferation.

As a result of the multiplicity of biological activities associated with fucans and their importance as a possible pharmaceutical, substantial research effort has been dedicated to the discovery of heparin-like compounds. Studies have shown that the purification of new fucans and the determination of their structures may lead to the discovery of new drugs.

ACKNOWLEDGEMENTS

This work was supported by the Coordenação de Aperfeiçoamento de Pesquisa e Ensino (CAPES) and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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5

Development of Plant Based Adjuvants for Vaccine Antigens

GUPTA AMIT[1,*], KHAJURIA A.[1], SINGH J.[1], TABASUM SIDIQI[1], SINGH S.[1], GUPTA V.K.[1], SURI K.A.[1], SATTI N.K.[1], SRINIVAS V.K.[2], ELLA KRISHNA[2] AND QAZI G.N.[1]

ABSTRACT

Vaccination remains the most cost-effective biomedical approach to the control of infectious diseases. Vaccines based on killed pathogens or subunit antigens are safer but are often ineffective and require coadministration with adjuvants to achieve efficacy. Unfortunately, most conventional adjuvants are poorly defined, complex substances that fail to meet the stringent criteria for safety and efficacy desired in new generation vaccines. Search for plant based adjuvants for human vaccines has become an expanding field of research in the last thirty years for generating stronger vaccines, capable of inducing protective and long lasting immunity in humans. Inspite of such efforts, it hardly needs to emphasize to discover safer and effective limitations like adjuvant toxicity remains a major concern against virulent diseases, which have endangered the humanity. In this review, it highlights the role of plant based immunomodulators used as an adjuvant for vaccines.

Key words : Adjuvant, infectious disease, vaccine

INTRODUCTION

Different strategies have been exploited for vaccine development. Whole cell vaccines are produced by inactivating the virulent organisms (*e.g.* by chemicals, heat). Alternatively, pathogens can be attenuated, in such a way

1. Department of Pharmacology, Indian Institute of Integrative Medicine (IIIM), Canal road Jammu Tawi-180 001, India.
2. Bharat Biotech International Limited Genome Valley, Turkapally, Shameerpet Mandal, Hyderabad-500 078, A.P., India.

* *Corresponding author* : E-mail : amitrrl@yahoo.com; amitrrl@gmail.com

that they are unable to cause disease in their natural hosts, but are still able to induce protective immunity. Thus, attenuated organisms are not able to induce clinical disease, but they retain the ability for transient colonization and/or self-limiting replication. Finally, purified subcellular components from the pathogens, which are critical targets for the elicitation of protective responses, can also be used to develop subunit vaccines (*e.g.* inactivated toxins, capsular polysaccharides, structural or non-structural proteins).

Subunit vaccines usually exhibit a better safety profile than whole cell vaccines. However, purified antigens are often extremely poor immunogenic. To overcome this bottle-neck and in order to modulate the elicited response, antigens need to be co-administered with adequate adjuvants. Unfortuna-tely, up to now only few adjuvants, such as alum and MF59, have been licensed for human use (Guy & Burdin, 2005; Burdin *et al.*, 2004). However, recent advances in our understanding of the immune system, particularly with respect to early pro-inflammatory signals, have led to the identification of plant based identified immunomodulator used as an adjuvant for vaccines.

Adjuvants

The term 'adjuvant' is derived from the Latin word adjuvare which means 'to help'. In the context of vaccines, this means any material that helps increase the humoral response and/or cellular immune response to an antigen.

Adjuvants are foreign substances to the body and may evoke side effects in addition to the desired immune stimulation. Table 5.1 lists some of the real and theoretical risks. More theoretical risks include the induction of autoimmunity or cancer (Gupta *et al.*, 1993). This has slowed down the development of some otherwise promising adjuvants. The need for

Table 5.1. Real and theoretical risks from adjuvants

Risk
• Local reaction-chronic inflammation, abscess, nodule, ulcer, draining lymphadenitis.
• Anaphylaxis/hypersensitivity reaction.
• Influenza like illness.
• Toxicity.
• Cross reaction with human antigens *e.g.* glomerular basement membrane.
• Immune suppression.
• Carcinogenesis.
• Teratogenesis.
• Abortogenesis.

appropriate safety testing of any new adjuvant is self-evident. Recommendations of the WHO and other experts have been used to develop a composite list of factors that should assure the safety of adjuvanted vaccines.

Substances Used Currently as Adjuvants (Gupta & Siber, 1995)

Actual and candidate adjuvants are a group of highly diverse materials that have in common only their adjuvant properties. Table 5.2 lists some of the principle substances currently used or being tested as adjuvants.

Table 5.2. Some substances currently used as adjuvants

Substance
• Local reactions.
• Aluminium and calcium salts.
• Bacterial and plant products.
• Surface acting agents *e.g.* saponins (*e.g.* Quill A, ISCOMS).
• Polyanions.
• Polyacrylics.
• Vitamins.
• Cytokines.
• Harmones.
• Imidazo-quinolines.
• Other.

Similar to adjuvants in certain properties are "vehicles" *e.g.* mineral or vegetable oil emulsions, squalene etc., "carriers" *e.g.* bacterial toxoids, outer membrane proteins, live vectors and immunomodulators. These three additional classes of immunomodulators affect the immune system in different ways -formation of a depot of antigen at the site of inoculation which is slowly released, the presentation of antigen to immune-competent cells and the production of different lymphokines such as interleukins and tumor necrosis factors.

Examples of Identified Plant Based Immunomodulator Used as Adjuvants that are Currently under Investigation

The evaluation and development of plant based immunomodulators, as the alternative adjuvants for providing maximum and lasting protective immune responses with existing vaccines, is justified because of their being safer in comparison with synthetic variants. One such plant derived QS21 from *Quillaja saponaria*, which possess significant immunomodulatory activity and is being chemically tested (Jacobsen *et al.*, 1996). In view of credibility of plant-based adjuvants with numerous supporting and related bioactivities, it seems worthwhile that the directional approach may help grant potential protection to immune system in combination with vaccines.

Strategy for the development of immune adjuvants of plant origin may thus open a new era of vaccination therapy. The ability of plant-based vaccine adjuvant to deliver sufficient antigen to induce protective immune responses is now well established for a wide range of antigens (Streatfield & Howard, 2003). A plant-based vaccine adjuvant may offer a range of advantages over traditional vaccine production and delivery systems (Webster *et al.*, 2002). Today's development of novel vaccines stresses the need for plant-based adjuvants that are inexpensive, easily administered and capable of being stored and transported without refrigeration. Without these characteristics, developing countries find it difficult to adopt vaccination as the central strategy for preventing their most devastating diseases. A promising approach is the production of vaccines containing plant-based adjuvant. Two major obstacles have been encountered in developing vaccines. First, the expression level of foreign antigens tends to be low and second, co-expression of an adjuvant may be required to facilitate an appropriate immune response. Plant based adjuvants are emerging as suitable candidates and increasing efforts are being made to discover potential suitable adjuvants.

Plant based material may be attractive candidates for use as vaccine adjuvants that stimulate both Th1 and Th2 immune responses. The specific goal of this research was to produce plant based molecule as adjuvant for the vaccines. There are several plant based adjuvants as described below:

1. **Glycoside (RLJ-NE-299A):** RLJ-NE-299A is a standardized mixture of iridoid glycosides Picroside I & Picroside II by HPLC fingerprinting (Chander *et al.*, 1992; Li *et al.*, 1998).

HPLC Standardized Fraction RLJ-NE-299A from Roots of *Picrorhiza kurroa*

Preparation of the material involves extraction with organic solvents and crystallization. No chromatography operation is required. The root powder (500 g) of *Picrorhiza kurroa* is extracted with dichloromethane while refluxing and the extract is rejected. The marc is extracted with EtOAc while refluxing in a Soxhlet for 20 h. The EtOAc extract is centrifuged to remove suspended matter and concentrated under vacuum to $1/4^{th}$ of its volume and allowed to stand at 20 ± 5°C for 36 h. The separated solid is filtered off and recrystallizes from MeOH, yield RLJ-NE-299A (1.23 g). Pure iridoids *i.e.* Picroside I & Picroside II (Chander *et al.*, 1992; Li *et al.*, 1998), which were used for the standardization of RLJ-NE-299A isolated in pure form from fraction by column chromatography.

Male BALB/c mice were immunized intraperitoneally with HBsAg 20 μg dissolved in phosphate buffered solution (PBS) containing alum (1.45 mg/mL). The polarization of the immune response in favour of the Th1 and Th2 subset is dependent on the variable doses of adjuvant; RLJ-

NE-299A (0.312–40 μg/mL) immunized intraperitoneally in comparison with standard adjuvant alum (1.45 mg) with constant dose of hepatitis antigen (20 μg) immunized on days 0 and 15 has been selected for detailed investigation.

There is a very clear surrogate marker for protection after vaccination with HBsAg; an anti-HBsAg titre of 10 milli-international units (mIU/ml) has been shown to be sufficient to protect mice against HBV infection (Centers for disease control, 1987). The most important role of RLJ-NE-299A (2.5 μg) was to improve the seroconversion rate against HBsAg in Balb/c mice, titres greater than 10 milli international units (mIU/mL) are considered to be protective against HBsAg. Those adjuvants that elicit the highest anti-HBsAg titres would be predicted to maintain these titres at protective levels for the longest periods of time. A significant feature of RLJ-NE-299A in comparison with alum, is its unique ability to stimulate anti-HBsAg titre in immunized animals (Fig 5.1). The anti-HBsAg antibody response induced by RLJ-NE-299A adjuvanted HBsAg largely exceeds the antibody levels induced by classical aluminum-based vaccines. Two weeks after the challenging dose all subjects had reached protective antibody levels (≥10 mIU/mL). The anti-HBsAg titre obtained with 2.5 μg/dose of RLJ-NE-299A after the challenging injection reached unprecedented levels (188.2 mIU/mL), which predict a long persistence of protection.

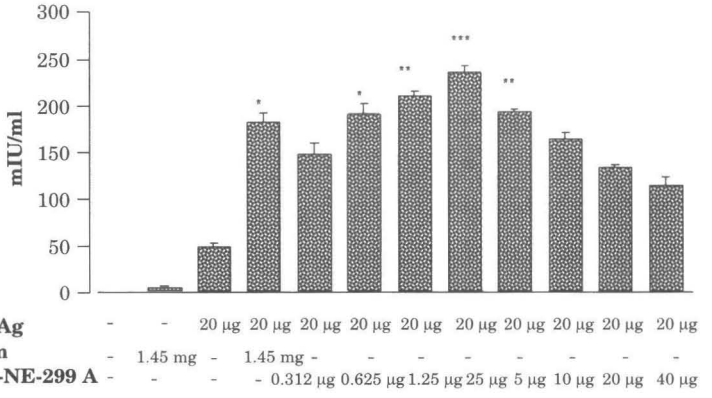

Fig 5.1. Dose-dependent effect of adjuvants in serum anti-HBsAg antibody titre. Groups of Balb/c mice (n = 10) were immunized *i.p.* with 20 μg HBsAg alone or with variable doses of RLJ-NE-299A. Serum anti-HBsAg antibody (IgG) titre in Balb/c mice after two immunizations on days 0 and 15 and bleed out at 2 weeks post-immunization. Anti-HBsAg titre was determined by ELISA kit. The results are presented as Mean ± S.E. *P* values: $*p<0.05$, $**p<0.01$ and $***p<0.001$ when compared to the value of control

The increase in the potency of the humoral immune response, however, was detected when RLJ-NE-299A was associated to HBsAg. The neutralizing antibody titers, in this case, were the highest among all

experimental groups, compared only to the alum associated HBsAg. The addition of 2.5 μg/dose of RLJ-NE-299A to the HBsAg without aluminum hydroxide increased the potency of the humoral immune response when compared to the HBsAg with alum, determined by neutralizing antibody (IgG1 and IgG2a) titers. IgG1 and IgG2a anti-HBsAg antibody titres induced by RLJ-NE-299A (2.5 μg) were greater than those elicited by alum in both primary and secondary responses (Fig 5.2). While RLJ-NE-299A adjuvanted HBsAg induced extremely strong immune responses and alum adjuvanted HBsAg turned out to be poorly immunogenic and low antibody levels were detected and virtually no cellular response was seen. These observations demonstrate RLJ-NE-299A to induce the better immunogenicity with HBsAg compared with alum to induce adequate immune responses.

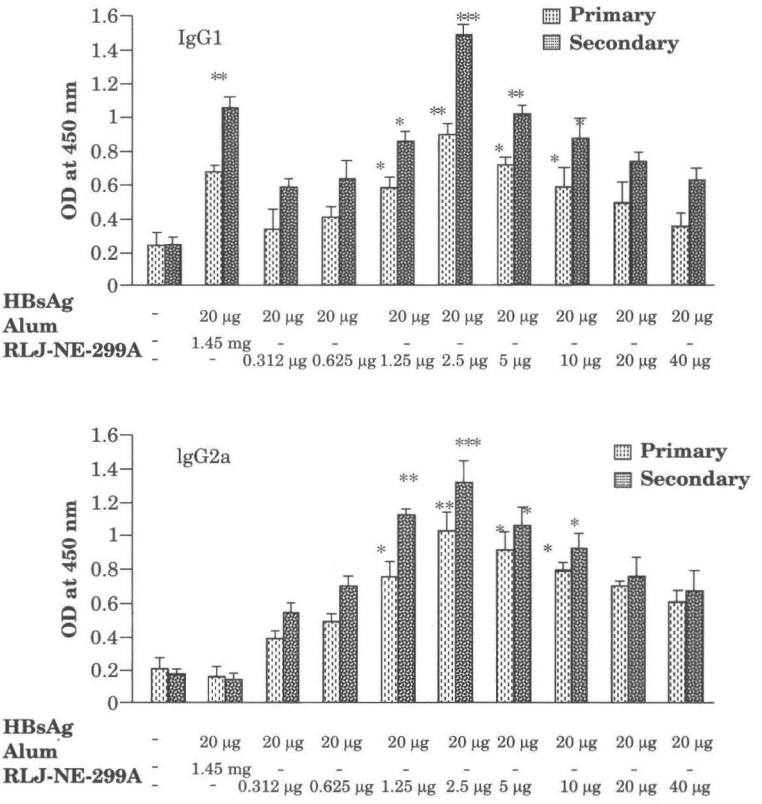

Fig 5.2. Influence of adjuvanted antigen on immunoglobulin subtypes (IgG1 and IgG2a) in sera of mice. IgG (IgG1 + IgG2a) anti-HBsAg antibody levels in sera on day 15 (primary response) and 28 (secondary response) were determined by ELISA assay and measured the optical density at 450 nm. The results are presented as Mean ± S.E. *P* values: $*p<0.05$, $**p<0.01$ and $***p<0.001$ when compared to the value of control

Furthermore, unlike alum, RLJ-NE-299A was able to induce an important anti-HBsAg T helper cell response as shown by the strong antigen specific cell proliferation observed. Cytokine determination at 2.5 µg/mL showed that RLJ-NE-299A adjuvanted with HBsAg induced more Th1 (IL-12, IFN-γ & TNF-α) and Th2 (IL-4 & IL-10) cytokines than either the alum adjuvanted vaccine or the HBsAg alone (Fig 5.3). It can be concluded on the basis of the profile of antibody isotypes (high IgG1 & IgG2a levels) and the cytokines (IL-4, IL-10, IL-12, IFN-γ & TNF-α) elicited that the RLJ-NE-299A induces Th1 as well as Th2 responses. These observations suggest that the use of RLJ-NE-299A as an adjuvant for HBsAg may provide better protection against HBV by eliciting both humoral and cellular immune responses. However, correlation of long term protection with high

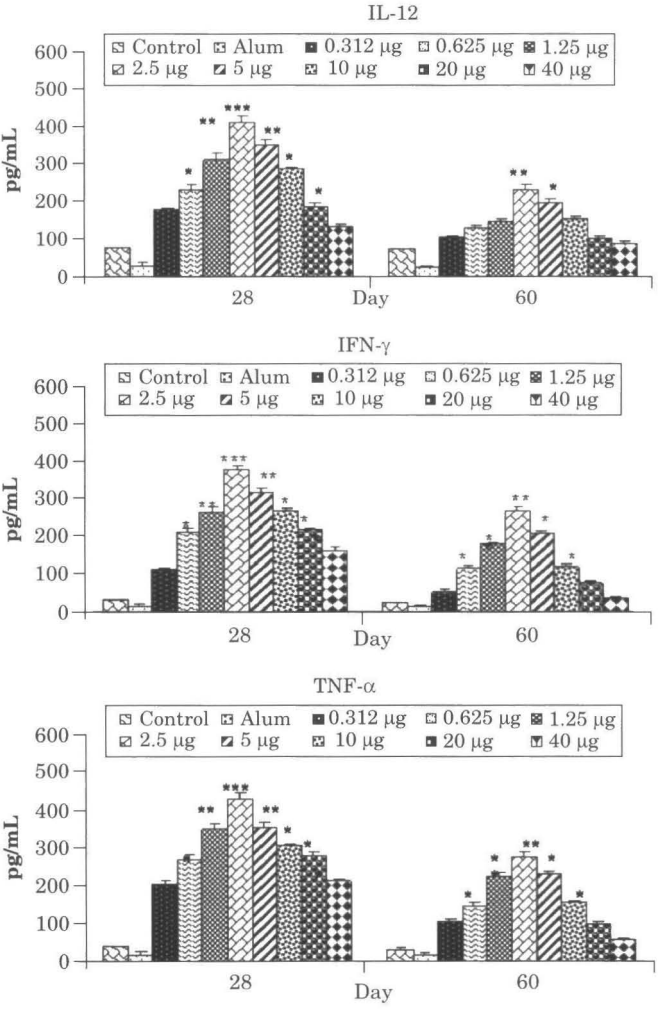

Fig 5.3. *Contd.*

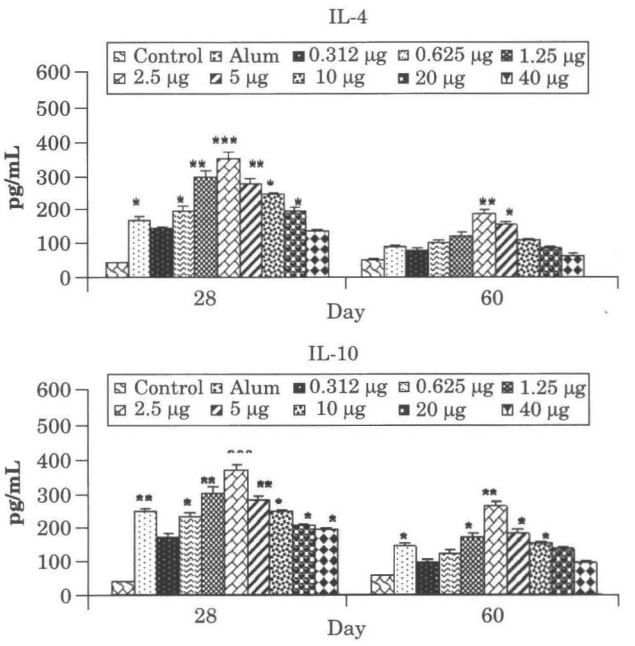

Fig 5.3. ELISA assay. Groups received variable doses of RLJ-NE-299A (0.312-40 µg) on day-0 and-15 along with the antigen. Another five animals were not immunized but served as negative controls (control). Spleen cells were collected on 28 and 60 days after the secondary immunization and the spleen cell supernatant were evaluated for the estimation of Th1 (IFN-γ, TNF-α and IL-12) and Th2 (IL-4, IL-10) cytokines. Each bar represents the group mean (n = 10). Value for the concentration of cytokine expressed in pg/mL. *P* values: $*p<0.05$, $**p<0.01$ and $***p<0.001$ when compared to the value of control

HBV infection may persist even though antibody levels are low or undetectable (Krugman, 1994). Furthermore, cell mediated immunity has been shown to be strongly involved in protection against viruses. (Coutelier *et al.*, 1988; Mahon *et al.*, 1995; Mosmann & Sad, 1996). Thus, HBsAg and adjuvant RLJ-NE-299A function as both a unique system of delivering antigen and also as an adjuvant. Out of the adsorption assay (Fig 5.4), it is suggested that antigen (HBsAg) may, upon adsorption is presented to immunocompetent cells in a particular manner that could facilitate antigen targeting (favour uptake by APC).

The ability of an adjuvant RLJ-NE-299A to reduce the antigen (HBsAg) dose from 20 µg to 15 µg (Fig 5.5) without having a detrimental effect on vaccine efficacy would be an effective way of increasing vaccine-manufacturing capacity, without the need for investment in new plant and equipment. Furthermore, the ensuing reduction in the cost of goods could facilitate a more rapid uptake of vaccines in the developing countries, where pricing is a critical issue. Here we have shown using a recombinant vaccine

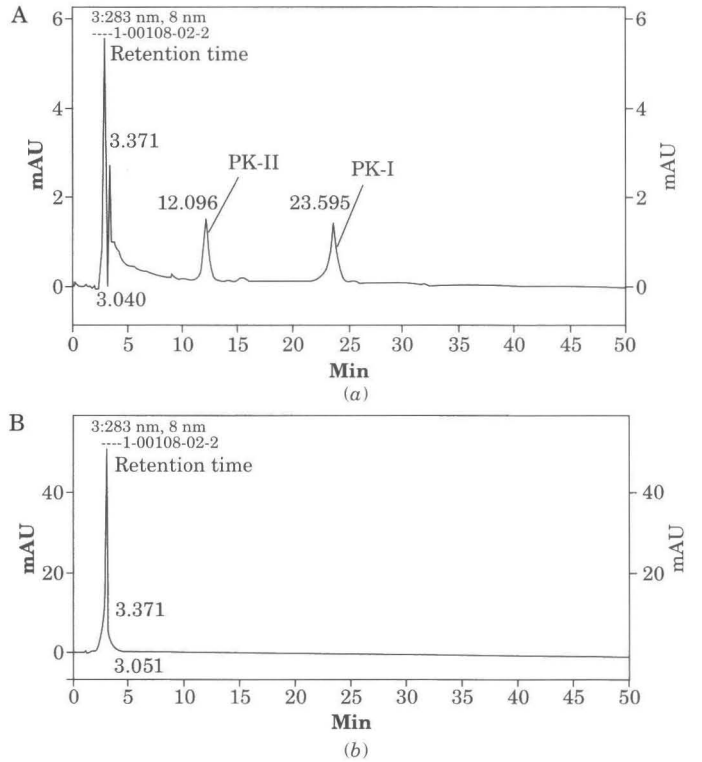

Fig 5.4. Adsorption assay by HPLC: (A) HPLC of RLJ-NE-299A; (B) Bulk protein HBsAg adsorbed on RLJ-NE-299A adjuvant

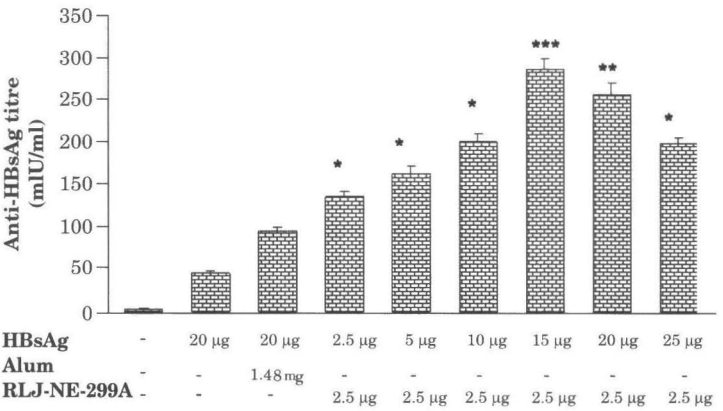

Fig 5.5. Dose-dependent effect of hepatitis antigen (HBsAg) in serum anti-HBsAg antibody titre. Serum anti-HBsAg antibody (IgG) titre in Balb/c mice after two immunizations on days 0 and 15 and bleed out at 2 weeks post-immunization. Anti-HBsAg titre was determined by ELISA kit. Titres were expressed in milli international units per mL (mIU/mL). The results are presented as Mean ± S.E. *P* values: **p*<0.05, ***p*<0.01 and ****p*<0.001 when compared to the value of control.

antigen (HBsAg) from *Pichia pastoris* that reduction of dose of adjuvant RLJ-NE-299A from 20 to 15 μg is superior to alum in terms of the magnitude of responses, resulting in a significant enhancement in neutralizing antibody responses. These findings suggest that both RLJ-NE-299A and alum potentiate the IgG1 response intrinsically induced by HBsAg without adjuvant but that RLJ-NE-299A, in addition upregulates the antigen specific production of IgG2a (Fig 5.6). These observations suggest that adjuvants have varying effects on immunoglobulin isotype switching and can induce antibody responses with different isotype and IgG subclass profiles.

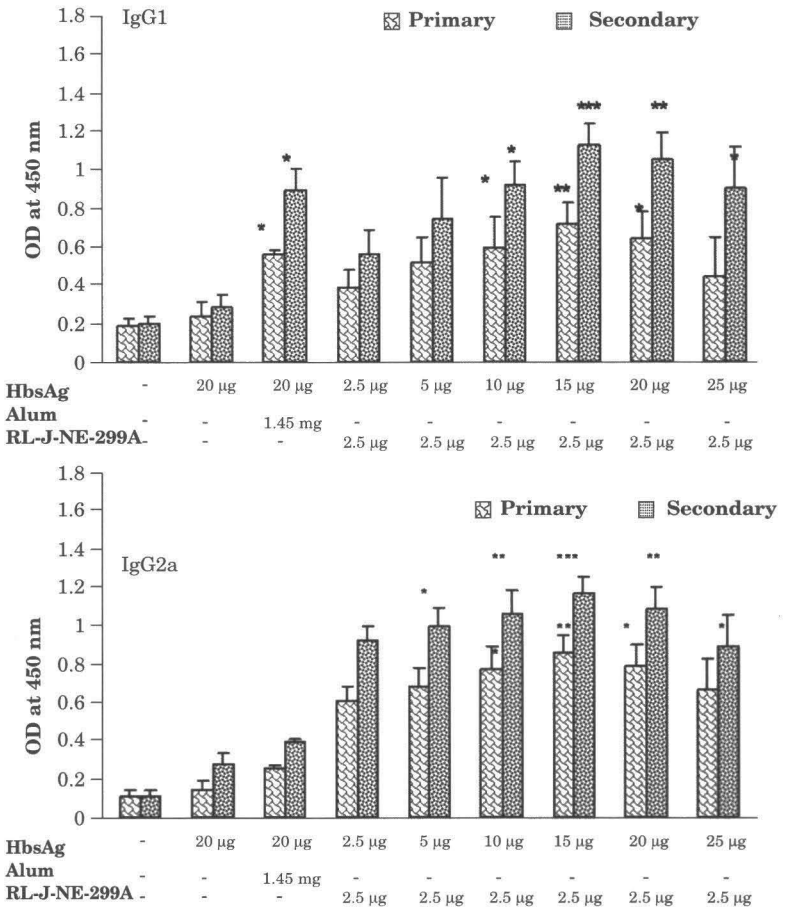

Fig 5.6. Influence of adjuvanted antigen on immunoglobulin subtypes (IgG1 and IgG2a) in sera of mice. IgG (IgG1 + IgG2a) anti-HBsAg antibody levels in sera on day 15 (primary response) and 28 (secondary response) were determined by ELISA assay. Anti-HBsAg (IgG1 and IgG2a) titre was determined by ELISA kit and optical density measured at 450 nm. The results are presented as Mean ± S.E. *P* values: $*p<0.05$, $**p<0.01$ and $***p<0.001$ when compared to the value of control

2. Biopolymeric fraction (BOS 2000)

BOS 2000 isolated from *Boswellia serrata* having anti-arthritic (Banerjee *et al.*, 2000), anti-inflammatory (Singh *et al.*, 1996), immunomodulatory (Sharma *et al.*, 1996), anti-cancer activity (Hostanska *et al.*, 2002) and also showed adjuvant activity in mice.

The organic solvent exhausted material (0.5 kg) of the plant *B. serrata* was thrice extracted with methanol (3 × 1.5 l) at room temperature in a percolator. The marc 175 g was air dried and extracted twice with water using 700 mL of water for the first extraction and 200 mL for the subsequent extraction. The combined aqueous extract was clarified by centrifugation and to the clear solution (600 mL) alcohol 1.2 l was added. The light brown solid, which separated on keeping, was collected by filtration and purified by dissolving in water and precipitating with alcohol. The purification process was repeated once more to get the biopolymeric fraction BOS 2000 as white solid (70 g).

Male BALB/c mice were immunized intraperitoneally with HBsAg 20 μg dissolved in phosphate buffered solution (PBS) containing alum (0.5 mg/mL) in a final volume of 1 mL. All experiments were performed using 20 μg of HBsAg. The dose response experiments were carried out with various doses of BOS 2000 (10, 20, 40 and 80 μg) against HBsAg immunized on days 0 and 15. Phosphate buffered solution-treated animals were included as controls. A challenging injection was given on day 15. Sera and splenocytes were collected 2 weeks after the second injection for HBsAg specific antibody titers were obtained by ELISA and spleen cell proliferation.

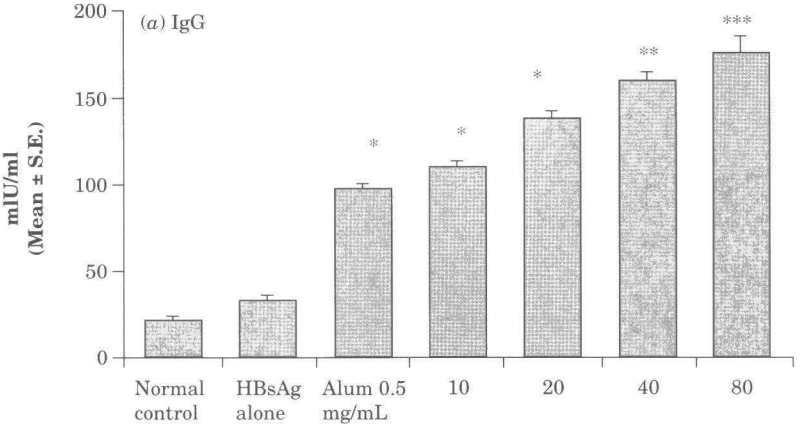

Fig 5.7. Dose-dependent increase of anti-HBsAg antibody titre in serum by adjuvant BOS 2000. Groups of Balb/c mice (n = 10) were immunized *i.p.* with 20 μg HBsAg alone or with variable doses of BOS 2000. Serum anti-HBsAg antibody (IgG) titre in Balb/c mice after two immunizations on days 0 and 15 and bleed out at 2 weeks post-immunization. Anti-HBsAg titre was determined by ELISA kit. The results are presented as Mean ± S.E. *P* values: $*p<0.05$, $**p<0.01$ and $***p<0.001$ when compared to the value of control

The serum anti-HBsAg IgG titer was assayed by ELISA and titers obtained after challenging immunization are presented in Fig 5.7. The dose response curve for BOS 2000 reveals that the production of anti-HBsAg antibodies was strongly enhanced in mice treated with a dose of 80 μg BOS 2000 in comparison with HBsAg alone or associated with alum. Those vaccines containing adjuvant especially BOS 2000 in HBsAg that elicit the highest anti-HBsAg titers would be predicted to maintain these titers at protective levels for the longest periods of time. Moreover, antibody titers induced by BOS 2000 adjuvanted HBsAg after two injections were still higher than those elicited by alum-adjuvanted vaccine.

The HBsAg-specific IgG1 and IgG2a antibody titers in the serum were measured by indirect ELISA as shown in Fig 5.8. The serum IgG (IgG1 and IgG2a) titer in HBsAg immunized mice was significantly enhanced by BOS 2000 (80 μg). Alum (0.5 mg/mL) and BOS 2000 (80 μg) significantly

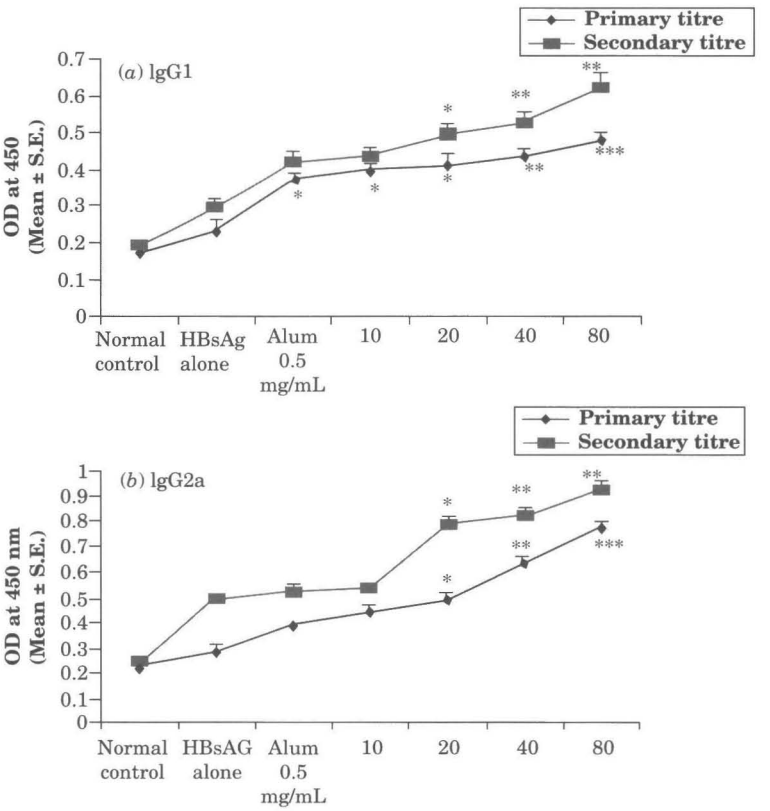

Fig 5.8. Influence of adjuvanted antigen on immunoglobulin subtypes (IgG1 and IgG2a) in sera of mice. IgG (IgG1 + IgG2a) anti-HBsAg antibody levels in sera on day-15 (primary response) and 28 (secondary response) were determined by ELISA assay and measured the optical density at 450 nm. The results are presented as Mean ± S.E. *P* values: *$p<0.05$, **$p<0.01$ and ***$p<0.001$ when compared to the value of control

enhanced the serum IgG1 titres in HBsAg-immunized mice. Significant enhancements in HBsAg-specific serum IgG2a titres were observed in BOS 2000 (80 μg) immunized mice compared with control group. Moreover, IgG2a antibody titre in the mice immunized with BOS 2000 at a dose of 80 mg was higher than that in the alum-treated mice. There were, however, no significant differences in the serum IgG2a levels between mice groups immunized with alum and HBsAg alone. Thus, findings indicated that BOS 2000 significantly enhanced serum HBsAg-specific antibody production in mice immunized with HBsAg.

In order to clearly establish that Th cell-derived cytokines were involved in the adjuvant activity of BOS 2000, we analyzed cytokine secretion patterns by HBsAg specific immunized mice. There was a high frequency of HBsAg specific IFN-γ secreting cells in mice immunized with BOS 2000 + HBsAg while all the four groups had high numbers of IL-4 producing cells. These experiments confirmed that the adjuvant effects of BOS 2000 involved Th1 and Th2 type cytokines (Fig 5.9). The high frequency of IFN-γ secreting cells in HBsAg + BOS 2000 immunized mice was consistent with the high IgG2a titres seen in these mice. All groups of mice secreted high levels of IL-4 and this was consistent with the high levels of IgG1 antibodies seen in all the groups at the end of the experimental period. Together, the HBsAg-specific antibody isotypes and cytokine profiles confirm that BOS 2000 promoted mixed Th1/Th2 type immune responses, while alum was associated with predominantly Th2 type immune responses. In this paper, we have amplified the concept that BOS 2000 provide signals to induce Th1 and Th2 type of immune response. The Th1 (CD4) helper cells, is characterized by the production of cytokines like IFN-γ and enhanced the production of IgG2a in mice. Th2 response is characterized by the production of IL-4. Therefore, BOS 2000 could be a good alternative to the existing adjuvants for use in future vaccines and can be effectively used as an adjuvant of whole cells, peptides and soluble proteins. We demonstrate that potent antigen-specific CD4/CD8 T-cell responses, essential for protective immunity against intracellular pathogens and cancer are generated under the influence of BOS 2000.

Apart from cellular responses, strong protein-specific humoral responses are also induced by BOS 2000, implying its potency as adjuvant in vaccines directed against extra cellular pathogens. Therefore, BOS 2000 may represent a powerful alternative to currently available adjuvants in both prophylactic and/or therapeutic vaccine applications.

3. Saponin

(a) **ISCOMS** are a particulate adjuvant system composed of antigen, cholesterol, phospholipids and saponins from the bark of the tree *Quillaia saponaria* Molina. Since their discovery by Morein and co-workers in 1984. ISCOMS have been formulated with a broad range

of antigens of viral, bacterial or parasite origin. They have been shown to induce antibody and cellular immune responses in a number of animal species including non-human primates. The adjuvant properties of ISCOMS including their *in vivo* distribution, interactions with antigen presenting cells (APC), stimulation of T helper (Th) cell subsets and activation of cytotoxic T lymphocytes have been described in several review articles. ISCOMS have also been shown to be a potent adjuvant in a number of animal species besides mice, including guinea pig, turkey, rat, rabbit, dog, seal, sheep, pig, cow and horse. A licensed ISCOM based vaccine is used to protect horses from equine influenza (Barr *et al.*, 1998).

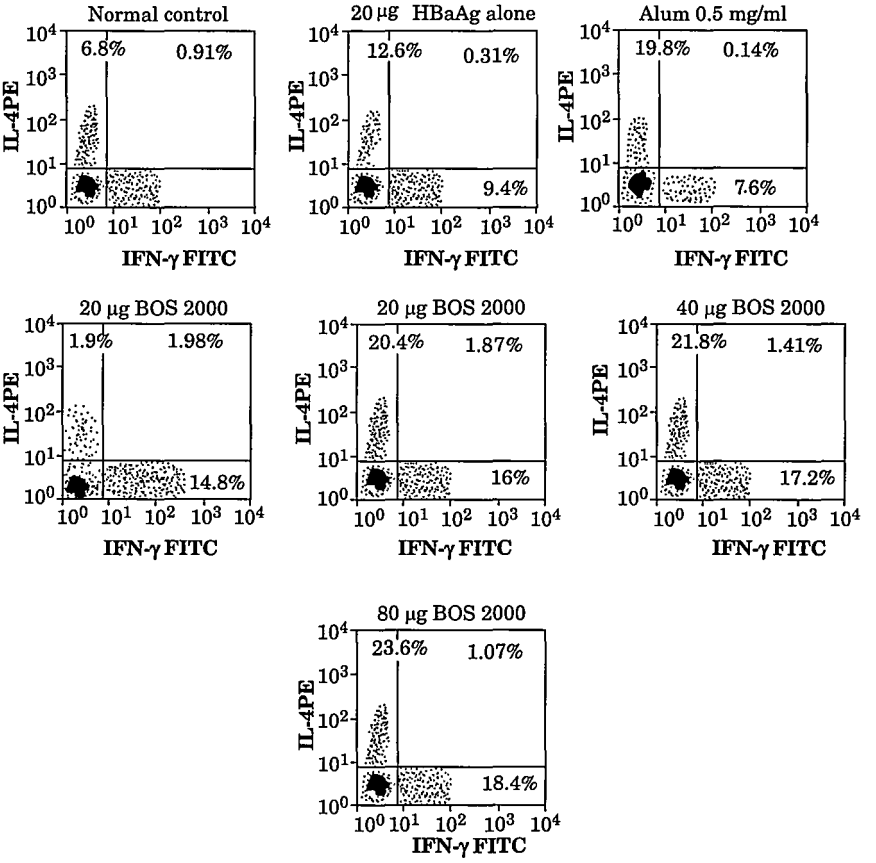

Fig 5.9. Upregulation of intracellular (IFN-γ and IL-4) production by spleen cells from immunized mice. On day-28, splenocytes were harvested and cultured with or without HBsAg antigen for 24 h. These cells were then stimulated with 20 ng/mL PHA plus 500 ng/mL ionomycin to detect surface markers of lymphocyte subsets and stained for intracellular cytokines. A FACScan flowcytometer with cell quest software was used for data acquisition and analysis

(b) **Quill A** is a semi-purified preparation of *Quillaia* saponin that is composed of a heterogeneous mixture of probably more than 100 closely related saponins. Quill A is suitable for veterinary applications but is considered unsatisfactory for human applications (Kensil, 1996).

(c) **QS21** The vaccine adjuvant QS-21 (**Fig 5.10**) is a highly purified saponin derived from the bark of the South American tree, *Quillaja saponaria* Molina (Kensil, 1996). It is a water-soluble quillaic acid-based triterpene with a complex acylated 3, 28-*O*-bisglycoside structure (Jacobsen *et al.*, 1996). QS-21 has been shown to be an effective immunological adjuvant with a wide variety of antigens and to have a relatively low toxicity in preclinical studies in mice. It enhances antibody responses, including IgG2a (Coughlin *et al.*, 1995; Kensil, 1996; Ma *et al.*, 1994) and antigen-specific interferon-gamma and IL-4 production (Sasaki *et al.*, 1998). It also enhances $CD8^+$ cytotoxic T lymphocytes in animal studies (Wu *et al.*, 1992).

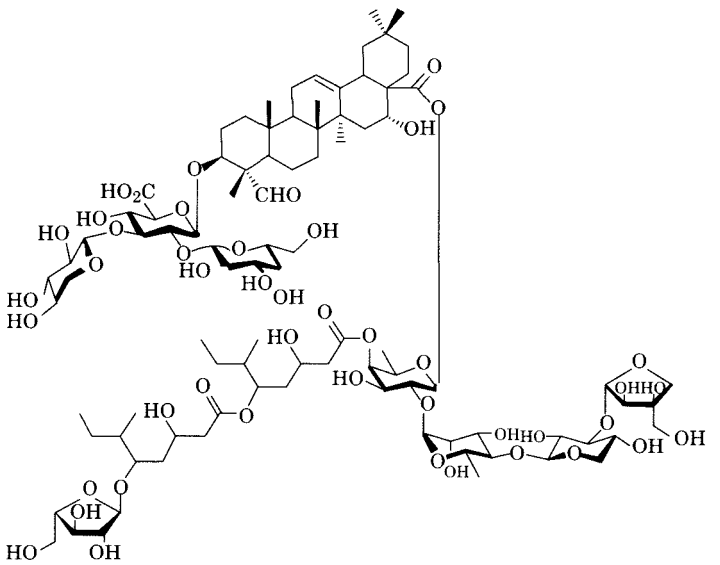

Fig 5.10.

QS-21 has been evaluated in a large number of vaccines in Phase I and Phase II human clinical trials (Kensil & Kammer, 1998). These vaccines include cancer immunotherapeutics (Helling *et al.*, 1995), HIV recombinant envelope (Keefer *et al.*, 1997) and malarial antigens (Stoute *et al.*, 1997). QS-21 has been tested in more than 2600 individuals to date. In most of these studies, QS-21 doses of 50–100 μg were utilized. All vaccines were given either by the intramuscular or subcutaneous route. QS-21 was shown

to strongly enhance the responder rate and antibody titers to the ganglioside portion of the conjugated melanoma antigen GM2-KLH (Helling *et al.*, 1995). This vaccine is now in Phase III clinical trials. QS-21 was also a critical component of the adjuvant formulation of the first malaria recombinant antigen vaccine to protect the majority of vaccinated volunteers against parasite challenge (Stoute *et al.*, 1997). Hence, it appears to be a promising adjuvant for human vaccines. Vaccines containing QS-21 have generally been well tolerated. Systemic side-effects are infrequent. Local reactions, typically consisting of transient mild or moderate pain, tenderness and induration have been seen in most studies. However, a more marked local reaction, consisting of severe immediate injection pain was observed in some individuals in two studies. Immediate injection pain, categorized as severe and a burning sensation was reported by 12% of individuals receiving intramuscularly administered QS-21 in an HIV-1 gp120 vaccine trial (Keefer *et al.*, 1997). QS-21 however, is a surfactant with lytic effect on cells (Kensil *et al.*, 1991), a factor that may be associated with immediate injection site pain. In addition, formulation characteristics such as buffer, pH and excipients are known to affect immediate injection pain to some pharmaceuticals. Various strategies may reduce or eliminate injection-associated pain. These strategies include a change of injection route or site, pH, distraction of recipient during immunization, formulation with an excipient that forms an emulsion, complex or mixed micelle with the pharmaceutical (Brazeau & Fung, 1989) or inclusion of a local anesthetic such as lidocaine or procaine (Brazeau & Fung, 1989).

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6

Immunomodulation and Vaccine Adjuvants

TEENA MOHAN[1], BHAT A. AJAZ[1] AND RAO D.N.[1,*]

ABSTRACT

The problems with pure recombinant or synthetic antigens used in modern day vaccines are that they are generally less immunogenic than older style live / attenuated or killed whole organism vaccines. This has created a major need for improved and more powerful adjuvants for use in these vaccines. With few exceptions, alum remains the sole adjuvant approved for human use in the majority of countries worldwide. Although alum is able to induce a good antibody (Th2) response, it has little capacity to stimulate cellular (Th1) response, which is so important for protection against many pathogens. Consequently there is a major unmet need for safer and more effective adjuvants suitable for human use. We can improve the quality of vaccine production by incorporating immunomodulators or adjuvants with modified delivery vehicles viz. liposomes, ISCOMs, and microspheres apart from alum, being used as gold standard. Adjuvants are used to augment the effect of a vaccine by stimulating the immune system to respond to the vaccine, more vigorously, and thus providing increased immunity to a particular disease. Adjuvants accomplish this task by mimicking specific sets of evolutionary conserved molecules which includes lipopolysacchrides, components of bacterial cell wall, endocytosed nucleic acids such as dsRNA, ssDNA and unmethylated CpG dinucleotide containing DNA. In this review we discuss clinical trial results for various vaccine adjuvants and delivery vehicle being developed that are approximately nanoscale (<1000 nm) in size. Humoral immune responses have been observed for most adjuvants and delivery platforms. While only viral vector ISCOMs and Montanide™ ISA51 and 720 have shown cytotoxic T-cell response in the clinical trails. MF59 and

1. Department of Biochemistry, All India Institute of Medical Sciences, New Delhi - 110 029, India.

* *Corresponding author* : E-mail : dnrao311@rediffmail.com

MPL® have elicited Th1 responses, and virus like particles, non-degradable nanoparticle and liposomes have also generated cellular immunity. Such vaccine components have also been evaluated for alternative routes of administration with clinical success reported for intranasal delivery of viral vectors and proteosome and oral delivery of VLP vaccine.

Key words : Peptide, vaccine, epitope, antigen, antibody, MHC, adjuvant, liposomes, ISCOMs, microsphere

INTRODUCTION

The creation of vaccine is one of medicines most important accomplishment in the modern era. Their influence on humanity is aptly expressed by vaccinologist-Stanley Plotkin when he says: "The impact of vaccination on health of the world's is hard to exaggerate. With the expression of safe water, no other modality not even ambitious has had such a major effect on mortality reduction and population growth".

Diseases such as measles, mumps, rubella, diphtheria, tetanus, pertussis, haemophilus influenza type B (Hib) diseases, polio and yellow fever are now under control because of effective vaccination. Smallpox has been completely eradicated and polio is on the verge of elimination, thanks to aggressive vaccine campaigns. Other diseases, including influenza, hepatitis B virus (HBV), typhoid and pneumococcal infections are being partially controlled by vaccines, but there is still much to be done to eliminate many more fatal diseases (Plotkin, 2004).

Innate and Adaptive Immunity

The historical basis for the concept of immune memory following a primary exposure to an infection dates back to 430BC when Thycydides described the plague of Athens, "It was with those who had recovered from the disease that the sick and the dying found most compassion. It was from experience for the same man was never attacked twice-never at least fatally. Approximately 200 years later, Edward Jenner conclusively shown that vaccination with cowpox virus could protect individuals following an infectious challenge with small pox virus. After Jenner observation, Lewis Pasteur developed first live and inactivated rabies vaccine. Today, various vaccine formulations have been shown to be successful with a wide variety of viral and bacterial infection and showed enormous success in reducing the mortality and morbidity from infectious diseases.

When a microbe enters the body, the immune system responds through a diverse set of mechanisms in order to eliminate the infectious agent. The immune response is of two types: The innate and adaptive. The innate response relies on immediate recognition of antigenic structures common to many micro-organisms by a selected set of immune cells with rapid

effector function. The effects include phagocytic cells, antimicrobial peptides and complement. The innate immune recognization of infectious pathogen is mediated by germline receptors that recognize relatively limited number of highly conserved microbial structures named pathogen associated molecular patterns (PAMPs). These PAMPs interact with pattern recognition receptors called toll-like receptors that are expressed on various cells including APCs leading to T-cell activation. This finally leads to an increase in APC effector function and play a key role in the initiation of adaptive immune response. The adaptive immune response is made up of B&T-cells that have unique receptors specific to various microbial antigens. The specific binding of microbial antigens with these cells leads to activation and clonal expansion that finally protects the host through humoral and cellular immune responses.

$CD4^+$ T-Cell Response

The goal of immunization is to generate and sustain a population of antigen specific immune cells that will mediate effector functions to prevent or control disease. The primary immune response is initiated by the presentation of antigen to naïve $CD4^+$ T-cells. Each $CD4^+$ T-cell express a unique receptor that recognize antigen associated with major histocapatibility complex (MHC) class II molecules expressed on specific APCs. Processing of antigen by MHC class II pathway (exogenous pathway) leading to epitope or peptide antigen within the APC and then associated to class II molecule on the surface of the cell. The peptide—MHC II complex then binds to $CD4^+$ T-cell. For effective $CD4^+$ T-cell proliferation, APC with costimulatory molecules like B7.1 and B 7.2 has to interact with CD28 on T-cells. This finally leads to the activation of naïve T-cell to effector T-cell with the release of cytokines. These cytokines then cause activation of B-cell to stimulate antibody secretion. Additionally, the activated T-cells also cause effector function of $CD8^+$ T-cell through IL-2 production. Thus, $CD4^+$ T-cell activation is a critical component of successful vaccination. Depending upon the type of cytokine the $CD4^+$ T-cells produce, two distinct subsets, such as Th1 & Th2 are recognized in the mouse and human. These two subsets of varied effector functions with prime function is to handle the pathogen.

$CD8^+$ T-cell Response

$CD8^+$ T-cells recognize processed antigen, in context of class I molecule. The antigen processing takes place within APC through endogenous pathway resulting in the generation of peptides that are loaded onto MHC class I molecule in endoplasmic reticulum and then transported to the cell surface. This peptide—MHC class I molecule then binds to $CD8^+$ T-cell leading to effector T-cell. This effector T-cell then produce the function cytokines such

as IFN-γ and TNF-α leading to further activation of MΦ and NK-cells etc. to perform their biological functions. The other mechanism is through direct cytolytic mechanism through the release of granules such as perforin and granzyme from activated $CD8^+$ T-cells. The net result is the formation of pores in the lipid membrane of pathogen infected cells leading to lysis.

With the discoveries of newer technologies and greater understanding of the molecular biology of the pathogens, the conventional empirical approaches to vaccine development have given way to more rational vaccine design. Immunoprophylaxis through vaccination now offers the prospect of substantially reducing the mortality caused by microbial pathogens to human race. A prophylactic vaccine aims to elicit immune effector elements such as circulating antibodies and various antigen specific memory lymphocytes. These host elements are readily available for immediate neutralization of the pathogen upon entry or for production of cytotoxic molecules for destroying the infected cells. Rational vaccine design requires sensible formulated adjuvant/delivery vehicles (Anderer, 1966). In view of the fast literature available on various aspects of the vaccines, the present review limit to peptide based vaccine with immuno-potentiator.

Synthetic Peptide Vaccines

The basis for synthetic peptide vaccines was laid by the pioneering work of Anderer who showed that short fragments of a protein from tobacco mosaic virus could inhibit the precipitation of the virus by antiserum. Also, when a hexapeptide from the fragment is coupled with a carrier, it induced specific virus precipitating and neutralizing antibodies. Further work by Arnon *et al.* extended this concept to show that chemically synthesized peptides could also induce antibodies that specifically recognize the intact virus particle from whose coat-protein the amino acid sequence was derived and generated antibodies in a suitable host. With the advent of gene cloning and nucleic acid sequencing techniques, a large number of amino acid sequences of biologically important proteins are now available. This is undoubtedly responsible for the greatly increased activity in the search for synthetic peptide vaccine in many laboratories. The first step in developing a synthetic peptide vaccine is to identify the relevant antigen(s) and determine its amino acid sequence. This is now mostly achieved by deducing from the nucleic acid sequence of the gene encoding at protein. The next step is to identify the relevant antigenic determinant. This is perhaps the most difficult part and may be achieved using the following parameters:

1. Chemical and enzymatic cleavage of purified proteins and subsequent analysis of their immunological properties.
2. The use of monoclonal antibodies to identify and select the smallest component of the antigen still capable of specific binding activity.

3. Predictions based on regions of hypervariability when amino acid sequences of a number of variants are available.
4. Predictions of secondary and tertiary structures by computer chemistry, which indicate regions of hydrophilicity, accessibility and mobility based on the state of lowest free energy.
5. Random synthesis of overlapping peptides.
6. Regions around disulphide bridges that locks the native conformation.
7. The use of hydrophilicity parameters of individual amino acids derived from the HPLC (high performance liquid chromatography) retention times to predict surface residues on the protein antigens (Deber, 1985).
8. Isolation of the peptide sequence that is released during proteolysis of the antigen monoclonal antibody complex (Jemmerson, 1986).
9. Developing a peptide microarray for the antigen of interest to delineate the helper, cytotoxic and humoral sites.

Concept of Synthetic Vaccines

The concept of synthetic vaccine must not only include the synthesis of immunogenic epitopes eliciting a protective antibody response, but also have the epitopes for sensitizing the specific T-helper/T-cytotoxic cells for long lasting immunity. This way, it can eliminate the intracellular pathogens through various effector mechanisms (Shirai, 1994).

Thus for a peptide vaccine to be effective in a broader population of diverse HLA alleles, the following points should be met:

1. The immune response should be directed against the determinant(s) that are invariant within the population.
2. The immune response should be elicited in almost all the individuals of an outbred population *i.e.* there should be no genetic restriction at the host level against the synthetic vaccine.
3. Both the T- and B-cells should cooperate with each other in the immune response in order to produce long lasting immunity and memory.
4. Cross-reactive antigens/supressive epitopes should be eliminated in order to obviate any undesirable immune response.

Advantages of Synthetic Peptide Vaccines over other Vaccines

The main advantages of using synthetic vaccines may be summarized as follows:

1. They can be produced in large quantity.
2. The peptide vaccines are stable at room temperature.

3. The stability of the peptide vaccine makes it suitable for applications in delayed release vehicles and thus the slow release profile mimics the booster response of vaccine.
4. It is possible to attach several peptides, representing the relevant portions of different pathogens to the same carrier molecule or develop multiple antigen peptide (MAP) approach effectively giving a multivalent vaccine.
5. Their use will eliminate immunization against many irrelevant antigenic determinants of the virus or irrelevant proteins that contaminate the viral preparation.
6. Many vaccines need an adjuvant to enhance the immune response. In synthetic vaccine, it is always desirable to introduce certain groups that augment antigenicity. Thus, the synthetic vaccine should contain built-in adjuvanticity and may prove to be less hazardous and are of better quality for humans.
7. The peptide vaccine is defined in chemical terms and is free from infectious material/any contamination.
8. Unlike the conventional vaccine, synthetic vaccine need not to be propagated in the unnatural host hence, no fear of autoimmunity/ cancer. Antibodies against the synthetic peptide immunogens may provide reagents for passive immunity, antitoxin therapy, targeted immunotherapy of neoplasia, radioimaging of tumors and finally for use in various immunodiagnostics (Manzar, 1991).

Synthetic Vaccines

Synthetic Vaccines can be divided into two Categories:

1. Chemically synthesized or peptide vaccines.
2. Recombinant vaccines.

Peptide Design

The synthesis of peptides corresponding in sequence to the primary structure of antigenic regions of a pathogen represents another way of developing non-infectious surrogate vaccines. The potential effectiveness of a synthetic peptide vaccine is usually reflected in its ability to elicit the formation of neutralizing antibodies and/or immunological memory. Synthetic antigens might be of considerable importance in the future development of vaccines (Manocha, 2002). They may be converted into potent immunogens after coupling with proteins that activate the immune system and invite the participation of T-helper antigenic sites. The development of synthetic molecules along with the B-cell epitopes will allow

the design and development of inexpensive, efficient vaccines. They should avoid possibly deleterious sites, such as suppressor T-cell epitopes or sites involved in tolerance (Frangione-Beebe, 2000). The following guidelines may help in making vaccines better:

1. Identification of precise epitopes within the predicted sequence by synthesizing overlapping peptides with single amino-acid deletions from the N- or C-terminus of the predicted sequence which is finally recognized by T-cells in association with many MHC class II/class I molecules.
2. Construct multiple antigen system.
3. Inclusion of a universal T-helper epitope into the vaccine since the immune response to a given epitope is normally under genetic restriction.
4. Mimicking of the conformation of the native epitope by disulphide bonding to form a circle that enhances the antigenicity of the synthetic oligopeptides.
5. Linking of hydrophilic epitopes between hydrophobic segments to provide correct folding to stimulate immune responses against different epitopes in a single synthetic chain.
6. Introduce some motifs that allow polymerization/cross-linking of each epitope.
7. Palmitoylation of peptides leads to CTL response.

Structural Factors in Peptide Vaccine Design

In order to be effective, a synthetic peptide vaccine must possess a high level of immunogenicity and induce antibodies that cross-react extensively with the pathogen. Developing peptides suitable for vaccination is a far more difficult task than selecting peptides able to induce antibodies that simply cross-react with the cognate protein. At times, certain structural modifications have shown a marked increase in the antigenicity/immunogenicity of synthetic vaccines. Little evidence support the role of carbohydrates as immunogens in protection or prevention of a disease but majority of the studies show that they have a very little role to play in elimination of the pathogen. The majority of the studies involving bacterial pathogens show that anti-carbohydrate antibodies have a negative effect either in enhancing the multiplication of the pathogen or are involved in pathogenesis of a disease. The role of carbohydrates observed in majority of diseases is in receptor recognition and also the site of protein modification or protection of the antigen from degradation by burying some potential tryptic and chymotryptic sites (Wilson, 1983). Addition of carbohydrate residues also helps in the modulation of antigenicity by stabilizing the three-

dimensional structure of the molecule. Masking and unmasking of the protein is another important function of the carbohydrate. This may lead to exposure of the antibody binding sites in the areas previously buried in the native molecule. Thus, efficacy may be greatly enhanced by altering the position and number of carbohydrate residues in a synthetic protein vaccine. Local secondary structures can also be dramatically altered by changes in the sequence. Substitution of a polar residue for a hydrophobic residue may lead to the exposure of buried residues *e.g.* foot and mouth disease peptide VPI (Rowlands, 1983). Further in some cases disulphide bridges have been shown to be critical for the maintenance of the native antigenic and/or immunogenic activity. In the hepatitis B surface antigen peptide, the presence of an intra-chain disulphide bond (between 124 and 137) makes this cyclic peptide exceptionally immunogenic (Dressman, 1982). Acylation of the N-terminal of synthetic peptides by long chain fatty acids has also been shown to increase the immunogenicity. Myristylated, as opposed to unmyristylated pre-S peptide of the hepatitis B envelope protein has been shown to be immunogenic in sub human primates (Neurath, 1989). Unless the protective determinants are identified and isolated, it is easy to make tailor made sequences retaining their immunogenic properties, though it would not be possible to produce a highly specific vaccine free from infectious material.

Mapping of T- and B-Cell Antigenic Determinants

Successful induction of immunity to most antigens requires the recognition by T- and B-cell of every different epitope. T-cells are important regulators of immune responses and it has become increasingly clear that the class of T-cells preferentially activated in an immune response is of pivotal importance for its strength and for the generation of effector mechanism. Hence, the ability to predict regions of protein sequence most likely to elicit T-cell immunity would be of potential use in vaccine development. A computer algorithm designed to predict antigenic regions in a given protein was by:

1. The use of hydrophilicity parameters of individual amino acids derived from the HPLC retention times to predict surface residues on the protein antigens.
2. The use of hydrophilicity-recognition profiles of proteins (Margalit, 1987).
3. Predicting local secondary structure, *i.e.* helix, pleated or random structure.
4. Looking for an amphipathic structure, *i.e.* a structure in which the hydrophobic residues tend to occur on opposite faces. These two sides may serve to interact with the MHC molecule on the antigen-presenting cell and with the T-cell receptor (Berzofsky, 1987).

5. The isolation of the peptide sequence that is released during the proteolysis of the antigen-MAb (monoclonal antibody) complex.
6. Constructing peptide sequences that are comers of the folded polypeptide chain.
7. Sequence accessibility, antigenic index and hydrophilicity parameters.

Role of MHC in Peptide Vaccine Design

The ability of an individual to respond to a given antigen is controlled by immune response (Ir) genes. In the epitope or peptide based vaccines, the immune response is restricted since the peptide binds to a restricted MHC molecule. This allele specific nature of peptide binding to MHC molecules indicates that single peptide vaccine will be ineffective except in the most limited homogeneous population. In general, multiple peptides or proteins yielding peptide fragments will be necessary to ensure that all members of a heterogeneous population possessing diverse MHC alleles can capture and present to their T-cells at least one effective antigen. Normally the size of the peptide recognized by class II molecule are usually between 10–20 amino acids in length while the size for class I molecule is 9–10 amino acids in length. Peptide class II interaction has been analyzed in detail both at the structural and functional level and peptide-binding motifs have been proposed for various mouse/human class II molecules (Hammer, 1994). In some cases, the peptide produced from the proteins of an infective agent will not contain optimal motifs for binding to prevalent MHC molecules in the populations. Predictions based on these motifs appear to be less accurate for class I molecules. This is because the peptide binding groove for class II molecule are open on both the sides thereby allowing different motifs to bind to single MHC molecule (Alexander, 1998). This methodology helped to engineer non-natural T helper epitope by modulating either MHC binding affinity or alteration of TCR (T-cell Receptor) contact residue or both. One such epitope identified for class II binding is PADRE peptide (Rosa, 2004). This methodology helped to engineer non-natural T-helper epitope by modulating either MHC binding affinity or alteration of TCR (T-cell receptor) contact residue or both. In such cases, the introduction of suitable motif residues or the elimination of dominant negative residues (Boehncke, 1993) can make some improvement in the vaccine material. Under proper circumstance, such peptides will stimulate T-cells and still be able to recognize the native peptide bound to the same MHC molecule. This is because primed T-cells require lower levels of TCR ligand for stimulation, for the lower efficacy of the natural peptide in forming the complexes with MHC molecule will still permit activation of the T-cells previously primed by the modified peptide. It is also possible to identify the key residues controlling the T-cell specificity (epitope residues) rather than MHC molecule binding and produce the vaccine material with multiple substitutions at

such position to preclude escape from immune destruction due to pathogen sequence variation at these sites (Takahashi, 1992). With reference to class I pathway, the antigen/peptide should have the access to the cytoplasmic delivery. This can be accomplished either by using live vehicles or liposomal delivery. Another important point is, it is crucial to ensure that load of the distinct antigens in a combined vaccine does not exceed the capacity of the presentation system because help for B-cell antibody production involves recognition of peptide-MHC class II complex on the B-cell. Sequestering the circulating antibodies can minimize another level of peptide competition.

Predicitions of MHC Class I Binding Epitopes

BIMAS (Parker, 1994) programme has been used for binding of overlapping peptides of a given protein to 33 HLA-I alleles. This was normally done by binding of a given peptide to the class I molecule with good binding affinity. The binding affinity is based on the half-time of dissociation of the β-2 microglobulin from HLA. SYFPEITHI (Rammenser, 1999) and RANKPEP (Reche, 2002) are the two other algorithms which predict binding of nanomeric peptides to 14 and 72 MHC class I HLA alleles respectively.

Predictions for MHC Class II Binding Epitopes

The analysis for class II HLA binding was used to predict the binding affinity for those peptides which have shown class 1 HLA binding by use of PROPRED (Singh, 2001) and RANKPEP (Reche, 2004) algorithms of 51 and 49 MHC class II allele, respectively. About 80% of MHC II restricted epitopes are found among the ~ 5% top scoring peptides by PROPRED.

The ability to predict the likely population impact of a vaccination programme and to select the most appropriate strategy to achieve the desired disease out come has been considerably advanced with the development of sophisticated dynamic mathematical models that can simulate disease transmission in the population. The aim of building models is to simulate the key processes that underlie the interaction between the organism and the individual and that determine the behaviour of the disease at the population level. The aim of vaccination is to move individuals from the susceptibility to the immune state without entering the latent or infectious period. By giving vaccination the immunity can be life long or is vaccine induced immunity waves off it is boosted by natural exposure. Universal immunization appears to be the only way to eliminate most of the deadly diseases.

Despite the enormous success in current vaccine there is no effective vaccine for HIV, malaria and TB, which account for a substantial proportion of death worldwide. Moreover, the mortality and morbidity associated with some deadly viruses such as ebola and potential threat of bioterrorism

have made vaccine biology, a major priority of scientific and medical investigation.

Immunogenicity of Peptides

Despite considerable research over many years the only adjuvant currently approved as gold standard for use with vaccines is alum but comparative studies show that it is a relatively weak adjuvant for antibody induction and a poor adjuvant for the induction of cell-mediated immunity. There is an urgent need to supplement this adjuvant with improved delivery systems, which are potent and safe and can be used with new generation vaccines. In order to increase the immunogenicity of peptide vaccine two important criteria should be satisfied. Firstly, efficient presentation of the processed antigen to the T-cell receptor. Secondly, immune response to be uniformly generated in outbred population. In an attempt to achieve the above goal, we have used two approaches, in such a way present the antigen in a particulate form so that antigen can be released slowly into circulation in depot formulation more so with liposomes or ISCOMs (Immuno-stimulating Complex's) or microspheres (Johansson, 1999) or in continuous and pulsatile form (Johansen, 2000). The influence of immunoadjuvants on the qualitative aspect of immune response should not be ignored and hence, we have used panel of immunoadjuvants that are non-toxic, permissible and water soluble. Adjuvants include a polymer of tuftsin (Gokulan, 1999), bio-active fragment of IL-1β (Gokulan, 1997), MDP analog such as murabutide (Agrawal, 2003) and casein immunomodulatory sequence (Thomas, 2001). A combination of adjuvants with modified delivery vehicles undoubtedly increased the immunogenicity of otherwise non-immunogenic peptide fragments of CS protein of *P. vivax,* RESA antigen of *P. falciparum* (Bhavna, 1998) and envelope & core peptide sequences of HIV-I. Our lab had also worked on Ulex Europaeus-I, a plant lectin as M-cell target, with HIV synthetic peptides in the PLGA microparticles to enhance the mucosal and systemic immune response (Manocha, 2005). Such an approach had generated high titer and high affinity antibodies. The quality of the generated isotype is polarized towards IgG 2a/2b, which is known to be cytophilic in nature, activates complement, enhance phagocytosis and clear the pathogen through ADCC mechanism. Most importantly the generated antibodies inhibited the growth of the relevant pathogen during *in vitro* study. The influence of such an approach had also contributed to cell-mediated immunity through activation/expansion of splenic lymphocytes and generated cytokines, which are predominately of IL-2 and IFN-γ (CD4 + Th1) (Kumar, 1999; Thomas, 2001). As most of the studies were done in inbred mice with different genetic background as well as in outbred strains, the outcome of the study undoubtedly proves that there is no MHC linked immune response with any of the above strains. Our lab was first to map the B- and T-cell epitopes of F1 and V antigen of Y. pestis based on the competitive immunoassay using the T- and B-cell algorithms prediction. Secondly, when B-T chimera were made and tested for humoral, cellular and mucosal immune response

in nanoparticles through intranasal immunization showing encouraging results. Few selected conjugates showed *in vivo* protection during challenges (Khan, 2008; Tripathi, 2006).

One of the rationales for designing engineered vaccines is based on putting together individual defined epitopes as in HIV one can select epitopes that induce neutralizing antibodies, cytotoxic and helper T-cells that might be protective thereby avoiding epitopes that induce enhancing antibodies, autoimmune responses or suppressor response. One can combine the epitopes in various ways to make them potentially active. This can be achieved by producing multivalent constructs (Berzofsky, 1991) or combining some neutralizing sequences arising from different clades of pathogens (Berzofsky, 1991; Cease, 1994) or instead improvise on these epitopes by tinkering with the internal structure of the antigenic determinant or antigenic site. It is always mandatory that for generating neutralizing antibodies that cross-react with native protein, the selected peptide should assume the necessary requisite conformation adopted by the native protein. In such circumstances, the importance of physical parameters like free energy of conformation plays a major role in critical binding of antigen antibody complex. The hypothesis is that if MHC molecules can combine to host peptides and only a few specific side chains are necessary for positive interaction, then negative or adverse interactions at nonessential positions should play role in determining the specificity of peptide MHC binding. Therefore by identifying the different residues in a peptide may suggest ways to improve the antigenic activity and the nonessential residues might be replaced with residues that have contact with MHC within peptide grove to enhance the function (Rupert, 1993). In another study as in the case of VP1 protein of foot and mouth disease virus, it is observed that the most variable regions of VP1 would be those subject to immunological pressure for mutation, which would be the site of greatest antigenicity. The obvious property associated with genetic polymorphism of class I and class II molecules determine the specificity and affinity of peptide binding is in T-cell recognition. In other words, individuals with different haplotypes will vary in CMI response to the same antigen. It is therefore important to identify the peptides recognized by T-cells for efficient protection against the disease. In theory all these studies suggest that it is possible to map the specificity of CTL clones by using a panel of recombinants expressing the overlapping peptides to create targets but in practice it is however more common to use synthetic peptides in conjunction to computer predictions.

In one of the studies in cancer it was observed that identification of peptide sequences recognizing CTL has led to direct induction of CTL responses *in vivo* (Schulz, 1991). To stimulate the $CD4^+$ T-cells that respond to peptides presented by class II molecule, proteins must be delivered efficiently to the endosomal-processing compartment. Thus the delivery

vehicle is important in maximizing such delivery. Also particulate antigen and delivery in a concentrated form may help class II pathway. Another way is to use ligand conjugation so that cellular receptors are used to enhance endocytic uptake of the antigen. Another way is to regulate the stage in the endosomal pathway when the antigen is available during processing. In one of the study it was observed that a fusion peptide with an endoplasmic reticulum-signal sequence at the amino terminus was more effective in the generation of CTL response than the peptide itself (Minev, 1994). Numerous reports are available in the literature regarding peptide vaccination for cancer. It was demonstrated that there is tumor regression by immunisation with MAGE-3 derived peptide even in the absence of any adjuvant (Marchand, 1995). Furthermore there is generation of CTL specific responses for gp100 derived (Salgaller, 1995) peptides immunised with Incomplete Freunds adjuvant. Many peptide vaccines are being studied currently *e.g.* peptides derived from MART-I, tyrosinase, gpl00, MAGE-3 (Jaeger, 1996; Parkhurst, 1996), prostate specific antigen (Meidenbaur, 2000). Several strategies for the modification of these peptides such as lipidification or changing anchor residues that binds to HLA motif are also being attempted (Rosenberg *et al.,* 1998) to produce an efficacious vaccine against all types of cancers. Many studies have been coming up for a better design of peptide vaccine by exploring the immunological specificity using synthetic peptide combinatorial libraries (Pinilla, 1999). The use of this approach has four major effects: first, the definition of high affinity ligands for both T-cells and antibodies; second, the application of alternative means for identifying immunologically relevant peptides for use as potential preventive and therapeutic vaccines; third, a new appreciation of the requirement for TCR interactions with peptide-MHC complexes in immunogenicity; fourth, the establishment of new principles regarding the level of cross reactivity in immunological recognition with the native antigen. Though peptide based vaccines have enormous advantages, it has few disadvantages:

1. Synthetic peptides are poor immunogens.
2. They are mono specific in the induction of immune response.
3. Generate immune response which is not uniform in outbred populations.
4. As the length of the peptide fragment is short it may contain insufficient information to fold into the correct shape necessary to mimic conformationally dependent epitope.

Plausible Ways of Overcoming the Disadvantages

The above drawbacks can be circumvented by use of adjuvant or controlled delivery vehicles *i.e.* ISCOMs, liposomes or microspheres. Chemical conjugates with antigen derived viral or bacterial proteins controlled polymerization of some of the epitopes and lipopeptide conjugation or using MAP (Multiple peptide antigens) comprising of T- and B-cell epitopes coming

from same antigen or from different antigens also provide an effective method of synthesis of many epitopes in a well defined orientation using a branched oligolysine matrix. All these approaches produce a long lasting B-cell, T helper and CTL response provided there is no epitopic competition in between multiple antigens and thus leading to suppression of antibody production to otherwise dominant epitopes. Strategies to enhance immunogenicity of these candidate vaccines are therefore critical. Several types of immunoenhancers are under investigation. They work in a variety of ways by changing the conformation of the antigen thereby enhancing the antigen presentation, by preventing the proteolytic destruction of the antigen in the stomach thus allowing it to pass into the intestine for presentation to gut associated lymphoid tissues or by targeting the antigen directly to M-cells of the gut to induce mucosal immune response or by the induction of various immunomodulatory cytokines such as GMCSF, IL-12, TNF-γ that act directly on the thymus derived helper T-cells to stimulate specific arm of maintaining immune responses. The exact molecular or cellular mechanism required for the generation of an effective immune response *in vivo* depends on the co-injection with adjuvant, which needs proper understanding (Virgil, 1999). Therefore, they are still surrounded by obscurity and called as "immunologists dirty little secret" (Janeway, 1989). Therefore for the formulation of a highly effective subunit vaccine, the inclusion of strong immunoadjuvants and/or proper delivery vehicle has become essential to elicit optimal immune response in the host.

Adjuvant and Delivery System

In many cases, the antigen itself is very weakly immunogenic; therefore an adjuvant is needed to intensify the immune response. Adjuvant can also be included in vaccine to guide the type of immune response generated. This may be especially important when developing vaccine for cancer, HIV or mucosal immune system. In contrast, a more immunogenic antigen may benefit from a specific delivery vehicle. This component may facilitate targeting and/or controlled release of the antigen to antigen presenting cells. Recent studies utilizing Toll-Like receptor ligands have shown that antigens associated with their ligands can produce exceptionally high antibody and rapid immune responses. Adjuvants have also been shown to protect antigens from degradation, although this generally depends on the nature of adjuvant. For example chitosan-adjuvate nano-particles were found to stabilize ovalbumin, other studies have shown that model protein antigens are actually destabilized by traditional aluminium salt adjuvants. The word adjuvant promotes lectin "adjuvare" which means to help, and can be defined as any product (or association of components that increase or modulates the humoral or cellular immune response against an antigen). The adjuvants can be classified based on five potential modes of action (i) Immunomodulation (modification of cytokine networks), (ii) Presentation

(maintenance of antigen confirmation), (iii) CTL induction, (iv) Targeting specific cells, and (v) depot generation (Cox, 1997).

An adjuvant can act in more than one way contributing to elicit a productive immune response against an antigen. However, the combination of one or more adjuvant plus the antigen has been studied in detail (Degten, 2003). In the last few years, the adjuvant properties of immuno-modulation have been attributed to several macromolecular components of micro-organisms which are recognized by PAMPs present on cells of innate immune system. These components are called molecular patterns because they are structures frequently encountered in microorganisms that facilitate the innate immune response against them. Examples of immunomodulation by these components includes binding of compounds like LPS, lipopeptides and CpG motifs to distinct members from Toll-Like receptor family leading to macrophages and dendritic cell activation and the binding of glycoprotiens or glycolipids to mannose receptor on phagocytes (Agarwal, 2003; Verthelji, 2003; Seya, 2003). Thus many components of this class have been purified and tested in different vaccine formulations targeting to elicit a suitable immune response against a specific antigen. Yet to perform its adjuvant effect, the antigen and the adjuvant should be together at the same sites since the antigen-presenting cell that will process the antigen should also be activated for a posterior activation of a naïve T-cell. To solve these problems, several formulations and carrier systems have been developed such as emulsion, liposome, microspheres, immunostimulating complexes (ISCOMs). These carriers share some of the following properties: protection of antigen from degradation following its administration by different routes including mucosal, ability to sustain the antigen release over an extended period of time, intracellular delivery of antigen contributing to cytotoxic T-cell stimulation and targetting at APCs. Hence with the aim of eliciting broad immune response especially with strong cellular compounds, the trend has been to combine adjuvant or to formulate them in order to achieve depot formation, recruitment and activation of APCs in the presence of the desired antigen (Sasaki, 2003).

Currently, only very few vaccine adjuvants are licensed for use in humans. Although both MF59 and aluminium salts have been approved in Europe, only aluminium salts have been used in licensed human vaccines in the USA (Rabinovich, 1994). For years alum is the only adjuvant used as a gold standard. Billions of doses of vaccines containing aluminium salts have been shown to elicit early, high and long lasting antibody titre after a single immunization (Simon, 2006). Despite their frequent global use for many decades, the mode of action of aluminium salts is not well understood. At least three potential mechanisms are frequently described in literature (Lindblad, 2004; Gupta, 1998). One idea is that the formation of depot at the site of injection allows the antigen to be released gradually, thereby extending the time possible for the antigen to interact with the antigen

presenting cells and lymphocytes (HogenEsch, 2002; Glenny, 1931). Besides a bias in the type of immune response elicited by aluminium salt adjuvants; other disadvantages of their use include instability to freezing and drying (Vegel, 1995) and inconsistencies in producing humoral immunity. Additionally, despite maintaining a good safety profile for more than seven decades, there have still been safety concerns regarding the use of aluminum salts. Although the evidence is afflicting symptoms such as erythrema, allergic response, hypersensitivity to contact, granulomas, inflammation and subcutaneous nodules as well as macrophagic myofasaltris have been reported for patients who received an aluminium salt containing vaccine (Gupta, 1995; Gwrardi, 2001; Trost, 1985). Due to the infrequent occurrence of these side effects the verdict remains that aluminum salts exhibit a particularly good safety profile.

In 1936, Freunds developed an emulsion of water and mineral oil containing killed mycobacteria, thereby creating one of the most potent known adjuvants, Freunds Complete Adjuvant (FCA). Despite being positive gold standard adjuvant, FCA causes local reactions and is considered too toxic for human use. The oil in water emulsion without added mycobacterium is known as Freud's Incomplete Adjuvant (FIA) and being less toxic.

Adjuvant Selection

Some of the features involved in adjuvant selection are the antigens, the species to be vaccinated, the route of administration and the likelihood of side effects (Lindblad, 1995; Byars, 1990). Ideally, adjuvants should be stable with long shelf life, biodegradable, cheap to produce, induce immune response (*i.e.* cellular or antibody immunity depending on requirements for protection) (Edelman, 1980). There are marked differences in the efficiency of adjuvants depending on the administration route (*e.g.* between mucosal and parenteral route). Hence, new vector antigen delivery systems or adjuvant compounds need to take into account the characteristics of the proposed administration route (Nentra, 1996).

Adjuvant Classification

Adjuvants can be classified according to their source, mechanism of action or physiochemical properties. Edelman (Allison, 1991) classified adjuvant into three groups:

(i) Active immuno-stimulants or substances that increase the immune response to the antigen.

(ii) Carrier, an immunogenic protein that provides T-cell help.

(iii) Vehicle adjuvant being oil emulsion that serves as a matrix for antigens for stimulating the immune response.

An alternative adjuvant classification divides adjuvant according to administration route, namely: Mucosal or Parenteral. The other criterion devised adjuvants into alum salts and other mineral adjuvants, tenso-active agents, bacterial derivatives, vehicles and slow release materials and cytokines. A recently proposed system of classification divides adjuvant into following groups: Gel Based adjuvant, tenso-activation, bacterial products, oil emulsion, particulate adjuvants, fusion proteins or lipopeptides (Jennings, 1998).

Current Status of Adjuvants in Vaccine Research

Potent adjuvants can improve the effectiveness of vaccines by accelerating the generation of robust immune responses, sustaining responses for a longer duration, inducing local mucosal immune responses, generating antibodies with increased avidity/affinity and neutralization capacity, eliciting cytotoxic T lymphocytes response (CTLs), enhancing immune responses in individuals with weakened immune systems (for example, children, elderly or immuno-compromised adults), increasing the response rate in low-responder individuals and reducing the amount of antigen needed, thus reducing the cost of vaccination programms. Adjuvants are functionally defined as components added to vaccine formulations that enhance the immunogenicity of antigens *in vivo*. Adjuvants can be divided into two classes (delivery systems and immune-potentiators) based on their dominant mechanisms of action. Immune-potentiators activate innate immunity directly (for example, cytokines) or through PRRs (such as bacterial components), whereas delivery systems (for example, micro-particles) concentrates the antigen and display antigens in repetitive patterns, target vaccine antigens to APCs and help co-localize antigens and immune-potentiators. Thus, both immune-potentiators and delivery systems can serve to augment antigen-specific immune response *in vivo*. For subunit vaccines it is highly desirable that the combination of delivery systems, immune-potentiators and isolated antigens will be required to elicit optimal immune responses.

Currently licensed adjuvants were developed using empirical methods, thus they are not optimal for many of the challenges in vaccination today. In particular, the historical emphasis on humoral immune responses has led to the development of adjuvants with the ability to enhance antibody responses. As a consequence, most commonly used adjuvants are effective at elevating serum antibody titers, but do not elicit significant Th1 responses or CTLs. The ability of an adjuvant to qualitatively affect the outcome of the immune response is an important consideration, because the need for vaccines against chronic infections [for example, human immunodeficiency virus (HIV), hepatitis C virus (HCV), tuberculosis and herpes simplex virus

(HSV)] and cancer has shifted the focus to generation of cellular immune responses and adjuvants specifically geared towards eliciting this effect. To this end, many new and existing adjuvant formulations are being tested in various preclinical and clinical trials. An expanded understanding of the immunobiology of TLRs and other PRRs, immunoregulatory cells, dendritic cells and the importance of specific T helper cell responses (Th1 versus Th2) in resolving particular diseases provides a framework for their continued optimization.

Viral-vectored Vaccines

Viral vectored vaccines consist of a non-replicating virus that contains some defined genetic material from the pathogen to which immunity is desired. Such vaccines are also commonly referred to as live recombinant vaccines (Protkin, 2005). Since the immune system has evolved to respond to viruses, this would seem to be an ideal way to deliver an antigen. Advantages of virally-vectored vaccines include their ease of production, a good safety profile (at least in some cases), ability to potentiate strong immune responses, potential for nasal or epicutaneous delivery and mucosal immunization (Van Kampen, 2005; Shi, 2001; Santosuosso, 2005). In some cases virally-vectored vaccines have been shown to significantly enhance immunogenicity (Hanke, 2007; McConkey, 2003; Gothard, 2003; Moore, 2003).

Adenovirus which has been administered orally as its own vaccine for decades has also provided a frequent vector platform for many of these types of vaccines, including delivery systems for Alzheimer's disease (Kim, 2007), influenza, tetanus, and HIV (Cantazaro, 2006) based vaccines. Such systems are also being used for alternative routes of administration (*i.e.* not the parental route, which is typically used for immunization). A recent phase-I clinical trial of an adjuvant-vectored flu vaccine administered intranasally and epicutaneously was found to elicit high serum antibody levels with a good safety profile. This study was the first of its kind to show that adenovirus-vectored vaccines are safe for intranasal and epicutaneous administration in humans. Preclinical studies of an adenovirus-vectored tetanus vaccine reported similar results.

In addition to adenovirus, a variety of other vectors have shown success in both preclinical and clinical studies. A modified vaccinia virus Ankara (MVA) was well-tolerated and produced a good safety profile in humans infected with HIV-I who are undergoing highly active anti-retroviral therapy (HAART). Additionally, a canary pox vector was used for expression of a cytomegalovirus (CMV) antigen (Berenesi, 2001). The resultant clinical results manifested a specific cytotoxic T-cell (CTL) response, which is especially important for developing immunity to intracellular viral pathogens. This was the first vaccine to produce this effect for CMV.

Preclinical studies have also shown promise for use of CMV as a vector for an immuno-contraception vaccine and yellow fever virus as a vector for expression of dengue virus envelope genes (Li, 2006). Additional viral-vector technologies that are currently being pursued for vaccine delivery include proxy-viruses, measles virus, vesicular stomatitis virus, HSV and alpha-virus, and a few more.

Immune responses generated by virally-vectored vaccines have been found to increase when a prime-boost regimen is employed. Such a procedure involves priming the immune system with one vectored vaccine (often a DNA vector) and boosting with the same pathogen's genetic material in another type of vector or a recombinant protein. While this approach has been successful for HIV vaccines in monkeys, it has not worked well in humans (Amara, 2002). A clinical study published few years ago demonstrated that a prime boost regimen HIV vaccine employing DNA and MVA resulted in "Multifunctional HIV-1 specific T-cells capable of rapid proliferation in eight out of eight vaccine recipients" (McShane, 2001). A malaria vaccine administered using this dosing regimen also showed promise in the clinical trails. Preclinical trials of viral-vectored vaccines used to immunize against tuberculosis, Ebola and SIV using a prime-boost strategy also appear promising. In virtually all cases, the prime-boost regimen has resulted in strong T-cell and/or INF-γ responses, leading to its current status as an especially promising technology.

Virus-Like Particles and Virosomes

Virus-like particles (VLPs) and virosomes also use nature's own mechanism and structural principles to trigger the immune system for protective effects. Like viral-vectored vaccine, these macromolecular complexes stimulate an immune response by delivering a material that mimics certain viral properties. VLPs are essentially non-infective viruses consisting of self-assembled viral envelope proteins without the accompanying genetic material. In the case of virosomes, the envelop of one virus is used as a platform to which additional components of the virus or another virus or pathogen are attached or inserted (Grgacic, 2006). Both types of particles maintain a morphology and cell-penetrating ability similar to infective viral particles. VLPs and virosomes have also been shown to stimulate both cellular and humoral immunity (Bruijn, 2005).

A number of virosome-based vaccines have already reached the market. The first of these was Epaxal™, a hepatitis A vaccine registered in 1994 by Berna Biologists Ltd. (Bern, Switzerland) in several European, Asian and South American countries. The same company also licensed an influenza vaccine, Inflexal V®, in switzerland in 1997 which is now available in 25 countries. Another flu vaccine utilizing virosome is Invivac® which is registered in the Netherlands and Switzerland. Nasal flu®, an intranasal

flu virosome vaccine that was co-administered with native *E.coli* heat-liable enterotoxin (LT) as a mucosal adjuvant, was marketed in Switzerland by Berna in 2001, but was removed from the market after an increased occurrence of Bell's Palsy was observed in people who had recently received the vaccine.

Additionally several recombinant HBV VLP vaccines have been licensed. The first licensed recombinant HBV vaccines, Recombivax (MERCK) and Energix-B (GSK), were composed of the viral small envelope protein, which upon expression in yeast formed 22 nm VLPs (McAleer, 1984). While these were effective, they suffered from a lack of immunogenicity (5–10% non-responders), which was determined to be due to an absense of Pre-S epitopes on the surface of the VLPs. A more immunogenic VLP vaccine was subsequently described that contained Pre-S1, Pre-S2, and HBV surface antigen. This potential third generation HBV vaccine, BioHepB was found to elicit a strong antibody response and 100% seroconvertion and seroprotection rates, although it has yet to reach the market.

The most recently approved VLP vaccine is Gardasil® for immunization against human papilomavirus (HPV) and subsequent prevention of cervical cancer and genital warts. This vaccine is composed primarily of self-assembled particles of L1 (the major capsid protein) from HPV types 6, 11, 16 and 18 and also contains an aluminium salt adjuvant. It has been shown to reduce infection of HPV by 90% and is apparently almost 100% effective against these types (Bosch, 2003). Since two of the four antigens in the HPV vaccine (HPV types 16 and 18) are implicated in 70% of cervical cancers, this vaccine is expected to drastically reduce the occurrence of this life threatening disease in women and has subsequently generated significant excitement.

Several other VLP vaccines have been made and are in clinical trails. A Norwalk virus vaccine has shown humoral, mucosal and cellular immune responses when administered orally suggesting that VLPs may be useful for delivery as mucosal inmmunization. Another study showed that a small peptide of the Der pl allergn covalently attached to a Qb bacteriophage VLP was well tolerated and generated high antibody titres in humans (Nardin, 2004).

Additionally, a malaria vaccine composed of a VLP of HBV core antigen containing proteins from the circumsporozoite stage of the *Plasmodium* parasite was shown to produce significant humoral and cellular immune responses when formulated with Alhydrogel®. Further studies revealed that a single dose of this vaccine administered with Montanide ISA 720 was immunogenic by multiple immunization of the alhydrogel-adsorbed vaccine. Similarly, a recombinant hybrid p17/p24: Ty VLP used for HIV immunization was found to produce both cellular and humoral immune responses to both components included in the VLP (Ott, 1995).

Liposomes

Liposomes which are sometimes classified as VLPs, are spherical entities composed of a phospholipid bilayer shell with an aqueous core. For this review, liposomes are considered to be composed of non-viral lipids (*i.e.* lipids not obtained passively from host cells in viral budding processes). For vaccine delivery, an antigen (or adjuvant) may be either encapsulated in the core of liposomes, buried within the lipid bilayer or adsorbed on the surface for presentation to antigen presenting cells. These delivery vesicles are considered to be non-toxic when the phospholipids used in their preparations are found in mammalian cells, but the lipids themselves are relatively non-immunogenic. Thus, for vaccine purposes, these particles are considered most useful for delivering antigens and adjuvants.

In contrast, liposomes can be made immunogenic by modifying the surface of the particle by adding a ligand antigen or another type of lipid. Nakanishi *et al.* demonstrated that cationic liposomes are much more potent than anionic or neutral liposomes for generating a cell mediated immune response (Nakanishi, 1999). An interesting preclinical immune response generated for MUC1 therapeutic cancer vaccine. This study revealed that liposome-associated MUC1 peptide (BP25) produced a strong specific CTL response; however, an antibody response was only observed for the surface-associated BP25 formulations. Clinical studies have confirmed that L-BP25 also known as Stimuvax®, a lyophilized liposome formulation of BP25 lipopeptide, MPL® and three lipids is well tolerated and elicited cellular immune response in patients with lung cancer. Stimuvax® is being developed by Merck and Biomira for the treatment of non-small cell lung cancers (NSCLC) which accounts for about 80% of all lung cancer.

Despite there being a number of liposome based products in the market in the U.S., there are currently no liposome-based vaccines (Katre, 2004). Besides the NSCLC vaccine just described, additional liposomal vaccines that have been investigated in human trials include vaccines against malaria, HIV, hepatitisA, influenza (Alving, 1995), prostate cancer and colorectal cancer. These were all found to be safe and highly immunogenic in experimental animals.

Proteosomes

The most common forms of proteosomes used for vaccine applications are nanoparticles composed of the outer membrane proteins (OMPs) of *Nesseria meningitids*. OMPs have been used successfully in a marketed meningococcal vaccine since 1981 and are considered non-toxic and well-tolerated (Meidenbauer, 2000; Bilukha, 2005; Gluck, 1992). Due to the hydrophobic nature of the OMPs, this immunogenic delivery system is appropriate for delivering apolar and noncovalent interaction between the proteosomes and antigen to form appropriate complexes (Langley, 2006).

These delivery vehicles have further been qualified as safe and well-tolerated materials through various human clinical trials. In most cases, a novel adjuvant known as ProtollinTM consisting of proteosomes non-covalent complexed with LPS has been used (Borm, 2004). For example, a vaccine composed of *Shigella flexneri* 2a LPS conjugated to proteosome was found to elicit an immune response similar to that observed after immunization with live pathogen. Additionally monovalent and trivalent influenza/H1N1)-proteosomes (no LPS) vaccines administered IN, produced high antibody titres in the serum as well as in nasal secretions (Auger, 2006), thus vaccines may be able to produce both systemic and mucosal immunity. Furthermore, preclinical studies have shown that such a vaccine is capable of protecting mice upon challenge with the infected pathogen.

Another very similar category of vaccines is the conjugate vaccine. These vaccines are relatively non-immunogenic (especially in infants), antigen linked to a more immunogenic carrier such as a protein or toxoid. The world's best selling vaccine, Prevnar, is an example of such vaccine. Prevnar is a pneumococcal vaccine manufactured that consist of the polysaccharides of the capsular antigens of seven serotypes of *S. pneumoniae* conjugated to mutant diptheria toxoid CRM197. Additionally, the conjugate vaccine for *H. influenzae* type B (Hib) were developed using Hib polysacchride conjugated to either diptheria toxoid (PRP-D), OMP of N. meningitis (PRP-OMP), mutant diptheria toxoid CRM197 (HbOC) or tetanus toxoid (PRP-T) to render the Hib antigen immunogenic in humans. The meningococcal conjugate vaccine, quadrivalent vaccine manufactured by Sanofi Pasteur and marketed as Mencata R contains four meningococcal polysaccharides conjugated to diptheria toxoid to enhance the immune response.

Montanide™

There are several different types of Montanide™, including ISA, 50V, 206 and 720. ISA 50 V, 51 and 720 are water-in-oil-in-water emulsion while ISA 206 and 50 V have been used in veterinary vaccine formulations while the other two are under investigation for use in humans. Emulsions of Montanide™ ISA 51 and 720 are composed of metabolizable sequence based oil with a mannide mono-oleate emulsifier. The ISA 720 formulation is slightly different from that of ISA and permits antigen to be released more rapidly into the circulation. Similar to IFA in physical character, the biodegradable nature of the Montanide™ eliminates many of the cytotoxic properties of IFA. The immune enhancement produced by the Montanide™ emulsion is believed to be due to site of injection.

ISA51 and 720 have been shown to induce high antibody titres and CTL responses in a variety of animal species. In many cases, the response was greater than achieved using other types of adjuvants. These emulsions

have been in phase I trial of a trivalent malaria peptide vaccine, a Wilm's tumor protein vaccine against various malignancies and melanoma vaccine containing either ISA 51 or 720 all showed strong CTL responses in humans.

Immunostimulating Complexes

Another vaccine delivery vehicle with potent adjuvant activity studied widely in the clinic is the immunostimulating complexes (ISCOMs). These are 40 nm cage-like particles produced by combining a protein antigen, cholesterol, phospholipid and the saponin adjuvant Quil A, which is derived from the bark of South American *Quillaia saponaria* Molina tree (Miller, 1996; Drane, 2006; Rimmelzwann, 1994). The matrix that is formed traps the protein antigens (typically hydrophobic membrane proteins) through a polar interactions (Barr, 1996; Hu, 2001). A similar vaccine delivery vehicle and adjuvant has also been developed that uses the same material minus the antigen and is referred to as ISCOMATRIX® (Pearse, 2005). The antigen can be added later to the ISCOMATRIX® during the formation of vaccine. This material seems to work similarly to ISCOMs, but provides for more general applications by removing the requirement for hydrophobic antigens.

A clinical study that compared a classical trivalent flu vaccine with an ISCOM adjuvanted version composed of the same three virus strains revealed a stronger immune response with the ISCOM vaccine eliciting rapid antibody responses as well as T-helper and some CTL responses (Rimmelzwaan, 2000). A separate study of an ISCOM based flu vaccine showed that virus-specific CTL memory was achieved in 50–60% of the patients, compared to only 5% who received the standard flu vaccine (Ennis, 1999). Additional ISCOM/ISCOMATRIX® vaccines have been in the clinic for HIV, HSV, HPV (Quinn, 2004), HCV and cancer (utilizing NY-ESO-I as the antigen) (Davis ID, 2004). In all cases, the studies have shown a good safety and tolerability profile in humans (Ronnberg, 1995; Rimmelzwann, 2001) as well as induction of both humoral and cellular immune responses. Despite these successes, the actual use of ISCOMs in human vaccines has been deferred by concerns regarding safety since some saponins are toxic at elevated levels. Nevertheless, certain saponins, such as Quil A and QS-21 have not shown major signs of toxicity at the doses administered in human.

When administered intranasally in mice, flu ISCOM vaccines were found to elicit strong mucosal (IgG and IgA) responses as well as systemic and cellular responses (Sjolander, 2003). A similar result was also observed in sheep and baboons, but the titres were much lower than those detected in mice. Oral administration of ISCOM vaccines has also been shown to be effective, but this route requires the use of high and frequent booster to maintain peak antibody titre. A study in sheep also indicated that ISCOM vaccines may be able to elicit strong mucosal immune responses when administered in the pelvic presacral space, which could be useful for

immunization against viral infections of the female genital tract (Thapar, 1999).

MF59

While other vaccine delivery vehicles have been included in the licensed vaccine formulations, MF59 is the only nano-sized vaccine adjuvant approved for human use thus far, although it is not yet licensed in the United States. MF59 is an oil-in-water emulsion composed of <250 nm droplets formed when squalene (4.3% v/v) and two surfactants, polysorbate 80 (0.5 v/v Tween 80) and sorbitan triolate (0.5% v/v, Span 85) are emulsified in citrate buffer (Ott, 1995; Podda, 2006). The strong immunogenicity enhancement of MF59 is clearly seen in preclinical data published by Ott *et al.* (1995), they reported that guinea pigs showed a 34-fold increase in antibody titres when immunized with glycoprotein D of herpes simplex virus (HSV) type 2 in the presence of MF59 compared to aluminium hydroxide, while goats and baboons showed 5–9 fold increase in antibody levels respectively. The mechanism of adjuvanicity of MF59 is believed to be through direct cytokine production (Dupuris, 1998).

Similar results have also been observed in the clinical trails too. For vaccines against HIV, HSV and CMV, antibody titres measured in seronegative patients were often greater than those of infected, seropositive patients (Kahn, 1994; Langenberg, 1995; Mitchell, 2002). Additionally strong helper T-cell responses were also detected in seronegative patients as a result of the vaccination (Podda, 2001). An MF59 adjuvant influenza vaccine, Flaud®, licensed in Europe (Gasparim, 2001; Frey, 2003; Atmar, 2006) as well as experimental vaccines for avian influenza A/H9N2 virus and HBV (Heineman, 1999) produced similar behavior in the clinical trails. Based on these and other studies, the safety, tolerability and adjuvanicity of MF59 in humans seem to be well established.

While these studies evaluated the vaccine delivered parentally, another clinical study evaluated the immunogenicity of an MF59-adjuvanted flu vaccine administered intranasally (IN). This vaccine also was well tolerated. The results indicated that a mucosal immune response may be generated upon IN administration, but this route may not be optimal for eliciting a humoral immune response. This study, however, found no enhanced potency of the vaccine in the presence of MF59 compared to unadjuvanted vaccine when administered IN (Boyce, 2000).

Polymeric Nanoparticles

A variety of polymers exist from which nanoparticles for drug delivery can be synthesized; however, the most commonly studied polymers are poly (D, L-lactide-co-glycolide) (PLG) and polylactide(PLA) (Panyam, 2003). These biodegradable, biocompatiable polymers have been approved for use in humans (*e.g.* sutures, bone implants and screws as well as implants for

sustained drug delivery) and have been extensively studied for use in the formulation of vaccine antigens (*i.e.* proteins, peptides, DNA, etc.) (Lutsiak, 2006; Wedorf, 2006). In these formulations, antigen can be either entrapped or adsorbed to the surface of the particles. Furthermore, these particles can be tailored to degrade over the range of rates. They can therefore act as depot from which the encapsulated antigen is gradually released. Additionally, the polymeric particles may offer protection to encapsulated antigens delivered orally and facilitate uptake by M-cells in the NALT/MALT when administered nasally, thus serving as a vehicle for mucosal immunization (Jung, 2001; Illum, 2007). Adsorbed antigen, however, offer improved stability and activity over unencapsulated antigen by avoiding formulation and acidic pH conditions caused by degradation of the polymer (Duncan, 2005).

Preclinical studies have shown that PLG nanoparticles can induce systemic antibody levels comparable to those of aluminium salts. Additionally, a study using tetanus toxoid (TT) found that a synergistic immune response (*i.e.* four-fold higher mean serum anti-TT IgG response) could be achieved by injecting TT bound to an aluminium salt along with TT-loaded nanoparticles (Raghuvanshi, 2001). Another study showed that PLG nanoparticles loaded with MPL® and a cancer-associated antigen (MUC1 mucin peptide) was efficiently taken up by dendritic cells (Elamanchili, 2004).

Non-Degradable Nanoparticles

In contrast to biodegradable nanoparticles, various non-degradable nanoparticles are being evaluated for their use as vaccine adjuvants and delivery systems. Among the materials that are being examined are gold, latex, silica and polystyrene. Since these materials may remain in the tissues for extended period of time, it is thought that the antigen may be presented to the immune system over similar time periods thereby enhancing immunogenicity. Gold particles have frequently being described for vaccine delivery both with and without the aid of electroporation, which has shown to often dramatically enhance the potency of DNA vaccine by improving delivery into cellular interiors (Zhang, 2003). Combining electroporation with intradermal delivery of DNA and gold particles, an enhanced and accelerated immune response has been observed in mice (Zhang, 2004), however electroporation may not be applicable in human clinical setting due to cell mortality resulting from the high voltage electrical pulses (Patil, 2005). A study in humans using these particles without electroporation produced a relatively low immune response after vaccination with DNA-gold particle-granulocyte-macrophage colony-stimulating factor (GM-CSF), transfected analogous tumor cells (Madhvi, 2002).

An alternative approach to delivering DNA vaccines employing non-degradable nanoparticles is through particle bombardment, also referred

to as particle mediated epidermal delivery (PMED) or the "gene gun" approach (Fuller, 2000; Luo, 2000). This method involves ballistically firing the DNA coated gold particles into the epidermis (Yang, 1990). While the delivery efficiency of this technique is quite low, only small amounts of DNA are required to achieve a significant immune response (Fyman, 1993). Clinical trials have shown that this approach can elicit both humoral and cellular immune responses, making it one of the consistently successful DNA vaccine delivery approaches. Success based on ballistic methods has been observed for vaccines against HBV, influenza, and malaria, the latter of which involved a prime-boost regimen.

Cholesterol- Bearing Hydrophobized Pullulan Nanoparticles

Cholesterol can be conjugated to a variety of carbohydrates, including pullulan, dextran and mannose, rendering the molecules amphiphillic. Such molecules have been shown to self assemble with and without proteins into 30–40 nm colloidally stable nanoparticles whose size and density can be modified by altering the degree of substitution of cholesterol on the polysacchride. Pullulan is the most popular polysaccharide, to which cholesterol has been conjugated, with numerous reports published for studies conducted *in vitro* but only a single one in humans.

Currently, there is only one report of cholesterol-bearing hydrophobized pullulan nanoparticles (CHP) being evaluated in the clinic. A complex of CHP and NY-ESO-1 was shown to enhance the humoral immune response. In this study, the cellular immune response was not evaluated due to seropositive patients possessing activated $CD8^+$ T-cells. Previously, an *in vitro* study showed that dendritic cells loaded CHP/NYESO-1 complexes induced both $CD8^+$ and $CD4^+$ T-cells. A preclinical study in mice showed that immunization with a complex of the HER2 oncoprotien and CHP induced both humoral and $CD8^+$ responses. In all studies, vaccination with CHP seems to be both safe and well-tolerated.

Calcium Phosphate Nanoparticles

Nanoparticles can be generated by combining calcium chloride, sodium phosphate and sodium citrate (He, 2000, 2002). Since calcium phosphate is naturally occurring in the body, issues surrounding the safety of these materials are reduced (Goto, 1993). Not to be confused with calcium phosphate gel adjuvant used in European diphtheria-pertusis-tetanus (DPT) vaccine formulations (Jiang, 2004), calcium phosphate nanoparticles are less than 1.2 micrometer in diameter. Bio-Pharmaceuticals has been developing this "CaP" technology. A phase-I study in healthy volunteers showed that CaP technology was safe and non-toxic when administered subcutaneously (BioSante Pharmaceuticals, 2007). Preclinical studies indicated that vaccines containing CaP resulted in immune responses

similar to or greater than those adjuvanted with aluminium salts and the duration of the response was longer. Additionally, CaP has shown promise as a mucosal adjuvant. Studies in mice utilizing an HSV-2 antigen suggest that CaP administered intranasally or intravaginally can elicit protective systemic and mucosal immunity. Vaccines utilizing CaP in preclinical studies include anthrax, HBV, Flu, (H5N1 avian and seasonal) and HSV-2 (BioSante Pharmaceuticals, 2007).

Genetic Adjuvants

Genes that encode cytokines, chemokines, costimulatory factors and a few other molecules. Used alone with antigens for immunization, they may represent a means of modifying the magnitude, duration and nature of the immune response. Usually, genetic adjuvants are co-administered with antigens, and on occasion bicistronic plasmids co expressing the adjuvant and antigen have been used.

Adjuvants work in part by signaling the innate immune system to orchestrate the type and magnitude of the subsequent adaptive immune response. Many of these stimulatory and modulating effects are mediated by triggering toll like receptors (TLRs) on innate immune cells of the 10 or more TLRs so far identified, most vaccine studies have focused on the immunostimulatory unmethylated CpG sequences recognized by TLR9.

Such stimulation results in enhanced antigen presentation, upregulation of costimulatory molecules, and cytokine production. Synthetic CpG sequences have been widely used with experimental vaccines, especially in the allergy field. It seems that a principal effect of CpG given along with protein or even peptide antigen is to direct a type 1 cytokine pattern response. This response is achieved by inducing upregualtion of IL-12 as well as an increase in the expression of costimulatory molecules on antigen-presenting cells (APCs) such as CD80, CD86, and CD40 (Sridevi *et al.*, 2005). Such stimuli result in the maturation of the APCs, allowing sustained as well as focused microbial or vaccine antigen presentation. A recent application of CpG was described in a prime-boost strategy of vaccination, which showed that when admixed with HIV-1 gag protein it elicited potent humoral, Th1 and $CD8^+$ T-cell responses. These responses were further enhanced by boosting with recombinant adenovirus expressing gag. Some of the peptide antigens derived from different stages of *P. vivax* or peptides derived from different proteins of HIV-1 when co-entrapped with CpG ODN in nanoparticles produced high titre and high affinity antibodies and able to inhibit parasite/virus growth *in vitro* (unpublished data).

Genetic Adjuvants Encoding Cytokines

Genetic adjuvants have been prepared encoding a large number of cytokines and have been evaluated alone or in combination for their effects on

immunity. The most notable molecular adjuvants studied to date include granulocyte/macrophage colony stimulating factor (GM-CSF), IL-2, IL-12, IL-15, IL-18, chemokines and bacterial endotoxins.

GM-CSF: The mechanism of the adjuvant effect of GM-CSF is only partially known. Presumably, it mobilizes dendritic cells (DCs) into the tissues after injection, thus enhancing the ability of the co-injected DNA vaccine antigen to be presented to the cells of the immune system. DCs generated in the presence of GM-CSF and IFN-α expressed higher levels of MHC class 1 molecules and produced equally high levels of IL-12. For example, systemic co-administration of a DNA vaccine encoding the env gene of HIV with GM-CSF expressing plasmids into mice induced both vaginal and fecal IgG and IgA, with levels of IgA exceeding those of IgG in both the vaginal wash fluids and feces. The virus specific IgG and IgA responses were enhanced even more when IL-12 DNA was co-administered with GM-CSF DNA.

IL-2: IL-2 is a lymphoproliferative cytokine that is mainly produced by $CD4^+$ cells. IL-2 enhances the immune response evoked by DNA vaccines. The mechanism of action appears to be through upregulation in the expression of CD48 and CD80 (B7.1) on DCs as well as upregulation of their respective ligands, CD2 and CD28, on CD^+ T-cells. Increased expression of the high affinity IL-2 receptor α-chain is also observed.

IL-12: is a proinflammatory cytokine that is heterodimeric in structure and is produced by phagocytes and DCs in response to infection by pathogens. Initially, IL-12 was known as a stimulator of natural killer (NK) cells, and it binds to IL-12 Rβ1 and IL12-Rβ2 on NK- and T-cells. IL-12 plays a significant role in the modulation of the CTL response and is central to immunity against pathogens that are controlled by cell mediated mechanisms driven by Th1 cells. IL-12 biases the naïve T-cells to the Th1 pheonotype both alone and through directed IFN-γ production by NK-cells.

IL-18: IL-18, formerly known as IFN-γ inducing factor, is produced by macrophages and kupffer cells and is a potent pleiotropic cytokine. It induces the production of IL-2 or IL-12 and enhances proliferation and activity of NK and $CD8^+$ T-cells. Overall, this cytokine is promoter of a Th1 immune response. Although, it does not itself induce differentiation of Th1 cells, it influences Th1 cells to produce IFN-γ.

Genetic Adjuvants Encoding Chemokines

Chemokines represent small (8–12 KDa) structurally related molecules that regulated trafficking of various types of leukocytes through interactions with a subset of seven transmembrane G-protein coupled receptors. At least 40 chemokine have been identified in humans. They mainly act on neutrophils, monocytes, lymphocytes and eosinophils and play a pivotal role in host defence mechanisms. Vaccination against HIV by gene

delivery of DNA encoding gp120 fused to proinflammatory chemokines CCL7 (MCP-3), CCL22 [macrophage-derived chemoattractant (MDC)], or β–defensin 2 induced both systemic and mucosal immune responses, with particularly higher titres of neutralizing antibodies. Moreover, the study showed that targeting the APCs with chemokines allowed conversion of a weak immunogen into a potent one that elicited robust mucosal immune responses.

For mucosal vaccination the most potent and presumably the most intensively studied adjuvants are protein enterotoxins of *Vibrio cholerae* (CT) and the heat labile toxin (LT) of enterotoxigenic *Escherichia coli*. The toxins act through interaction with M-cells and the GM1-ganglioside receptors on the mucosal epithelium, which causes adenosine diphosphate ribosylation and cyclic adenosine monophosphate induction, leading to various effects on lymphoid cells. They generally increase mucosal IgA responses to antigens administered orally.

Another strategy to enhance musosal immune responses to plasmid DNA vaccines involves targeting of the plasmid DNA to specific cell of the mucosa. Musosal immune responses (CTL and clearance of virus) could be enhanced significantly by coupling a plasmid DNA-encoding HIV gp160 to a protein that targets M-cells, the reovirus δ-1 protein. Targetted delivery of DNA to M-cells, which transport antigen from the lumen to immune cells of the mucosa residing the lamina propria represents an interesting approach to enhance genetic vaccine delivery in mucosal tissue (Manocha *et al.*, 2005).

Cell-Based Adjuvants and Delivery Systems

Dendritic cells, as described earlier, are able to prime potent lymphocyte responses and are increasingly being tested for their ability to act as adjuvants in therapeutic vaccines against various cancers. To effectively use dendritic cells in this way, they must be of the appropriate phenotype to optimally present antigenic peptides and express costimulatory molecules. Various TLR ligands, such as lipopolysaccharide and CpG DNA, have been tested for their capacity to condition *ex vivo* dendritic cells so that they express elevated levels of costimulatory molecules and surface molecules associated with cell migration. TLR-activated dendritic cells increase T-cell responses and can block the inhibitory effects caused by T_{reg} cells. Moreover, mouse dendritic cells have been shown to aid migration and recruitment of natural killer cells to the lymph nodes to provide an early source of IFN-γ necessary for T_H1 polarization. These insights may be modeled to develop functional or targeted high-throughput screening to identify synthetic compounds that optimally activate dendritic cells for use as cell-based adjuvants.

Search for Novel Peptide Ligands of PRRs

Most naturally occurring PAMPs are based on polysaccharides, lipids or nucleic acids. However, the peptide ALTTE, derived from bacterial fimbrae, was shown to activate TLR2. Another peptide isolated from an experimentally infected insect was able to stimulate natural killer cells to produce IFN-γ. Thus, immunomodulators can serve to potentiate the immune response based on peptides. Notably, the peptide sequence ALTTE is found in several human genes, thus peptide-based PAMPs are not necessarily limited to sequences of microbial origin. These observations suggest another dimension to immune potentiator discovery and open the possibility of screening peptide libraries generated by combinatorial peptide synthesis. However, barriers to the use of peptides as immune potentiators for vaccines are their poor pharmacokinetics and, in particular, their susceptibility to degradation *in vivo*, which may restrict their widespread use.

Adjuvant Safety Issues

The benefits from adjuvant incorporation into any vaccine formulation have to be balanced with the risk of adverse reactions (Warren, 1986; Eldelman, 1992). Adverse reactions to adjuvants can be classified as local or systemic. Important local reaction include pain, local inflammation, swelling, injection site necrosis, lymphoadenopathy, granuloma formation, ulcers and the generation of sterile abscesses. Systemic reactions include nausea, fever, adjuvant arthritis uveitis, eiosinophillia, allergy, anaphylxis, organ specific toxicity, immunoosupression or autoimmune diseases and liberation of different cytokines (Allison, 1991; Waters, 1986). Unfortunately potent adjuvant action is often correlated with increased toxicity as exemplified by the case of FCA which although potent is too toxic for human use. Thus, one of the major challenges in adjuvant research is to gain potency while minimizing toxicity. The difficulty of achieving this objective is reflected in the alum despite being initially discovered over 80 years ago, remains the dominant human adjuvant in use today.

Adjuvant Regulatory Requirement

Regulations of the human use of adjuvant are far more rigorous than those applied to veterinary vaccines. In addition to preclinical studies on the adjuvant itself, the combined antigen-adjuvant formulation also need to be subjected to toxicology prior to commencement of phase-I clinical trials (Goldenthal, 1993). The toxicological evolution is normally conducted in small animal species such as mice, rats or rabbits and should use the same administration route proposed for human use. The dose and frequency of vaccination for preclinical toxicology should be similar to or higher than the proposed human dose to minimize the ability to identify potential safety

problems (Stewart-Tull, 1989). Preclinical studies may also help in selecting the optimal vaccine dose (Edelman, 1990).

Adjuvant Limitations

In spite of progress in the identification of mechanisms of adjuvant action, alum remains the dominant adjuvant for the human vaccines. Although many other adjuvants have been proposed over the years, these have failed to be successful in human largely because of toxicity, stability, bioavailiablity and cost. Because of effects of size, electric charge and hydrophobicity which regulate the incorporation of proteins into the adjuvant formulation, it is difficult to predict on an empirical basis which adjuvant will work most effectively with a particular protein or peptide. Moreover epitope modification may occur during formulation or conjugation. In the case of carrier proteins, a pre-existing immunity to the carrier protein is the major limitation (Jennings, 1998). Furthermore, each adjuvant generates a characteristics immune response profile. For example the inability of alum based adjuvant to induce Th1 antibody isotype or cellular immune responses and their poor adjuvant effect on polysaccharide antigens limits their applicability to many vaccines.

Future Perspectives

Several forces are converging to drive increased research and development efforts in adjuvant design and discovery. First and foremost are the recent and dramatic breakthroughs in theoretical and mechanistic understanding of innate immunity and how it drives antigen-specific responses and the generation of immunological memory. This new appreciation of innate defense mechanisms provides a solid foundation for rational approaches to immune-potentiator discovery and optimization. Several first-generation candidates (for example, CpG, monophosphoryl lipid A and imidizo-quinolines) have shown some efficacy in experimental animals and in phase 1 studies in humans. Second, the trend in vaccine development away from traditional whole-cell or virus vaccines to subunit vaccines has shown that isolated antigens often lack sufficient immunogenicity, thus requiring the addition of potent adjuvants. Finally, the lack of vaccines for important disease targets such as HIV, HCV, HSV, *Neisseria meningitides* and others increases the need for improved vaccine adjuvants capable of boosting the antigen-specific immune response to protective levels against these insidious pathogens.

Although there is a growing acceptance by regulatory agencies and commercial vaccine producers that improved vaccine adjuvants are needed to meet the infectious diseases. At present, the safety and regulatory hurdles that will be encountered with the addition of novel immune-potentiators and delivery systems to final vaccine formulations may be significant and are still largely ill defined. The key focus should be on separating the

potential increases in immune toxicity from improved immunogenicity provided by vaccine adjuvants. It is likely that improved formulation and controlled release of potent immune-potentiators will limit toxicities while increasing efficacy. In addition, the growing number of immune-potentiators, targeting diverse innate immune mechanisms, should allow for the identification of candidates with improved therapeutic indices. Thus, the long-term goal should focus on selection of the optimal platforms and identification of key innate immune targets for induction of potent, but safe, immune responses. The mechanistic understanding of the innate immune system and the tools to manipulate it are growing, and together these will make a significant impact on vaccine development in the near future.

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7

Recent Advances in the Use of Medicinal Plants as Immunostimulants

NAYAK SMITA[1] AND MENGI SUSHMA[1,*]

ABSTRACT

The host defense against invading microbial pathogens is elicited by two components of the immune system namely innate immunity and acquired immunity. Augmentation of these components of the immune system forms the key to prophylaxis against infectious diseases. Several natural or synthetic agents are available to counter these infections. However, their drawbacks far outweigh their benefits. Against this background, the potential of medicinal plants as immunostimulants is an interesting alternative to conventional therapy for chronic and recurrent bacterial and fungal infections. The ancient Indian traditional system of medicine, 'Ayurveda', abounds in many examples of such useful plants which serve to strengthen the host's immunological barriers against infectious diseases. Such plants are labeled as 'Rasayana' and include plants like Allium sativum, Tinospora cordifolia, Glycyrrihza glabra, Azadirachta indica, Withania somnifera, Ocimum sanctum, Aloe vera and Morinda citrifolia. The present review focuses on the efficacy of the commonly used herbs and presents evidence of their therapeutic status.

Key words : Immunity, major histocompatibility complex, phagocytosis, toll-like receptors, T-cell receptor, B-cell receptor, cytotoxic T-lymphocytes

1. Department of Pharmacology, C U Shah College of Pharmacy, S.N.D.T. Women's University, Juhu-Tara Road, Santacruz (West), Mumbai - 400 049, Maharashtra, India.

* *Corresponding author* : E-mail : sushmamengi@yahoo.co.in

INTRODUCTION

The phrase '**immunity**' was derived from the Latin term 'immunitas' which means exemption from paying taxes and was used to describe the condition wherein infection of the host leads to a exemption from subsequent infection by the same organism (Doherty & Robertson, 2004). The host defense against invading microbial pathogens is elicited by two components of the immune system namely innate immunity and acquired immunity (Pathak & Palan, 2005). Augmentation of these components of the immune system forms the key to prophylaxis against infectious diseases. Infections can be countered by immunostimulant drugs which act on innate or acquired immune systems or both. Several natural or synthetic agents such as levamisol, isoprenosine, Bacillus Calmette Guerin (BCG), glucans, cytokines such as interleukin-1 (IL-1) and IL-2 have been used to stimulate host resistance against infections (Patwardhan & Gautam, 2005). However, these agents share severe limitations. One of the major drawbacks is the necessity to administer them parenterally, which often leads to granuloma formation. Indiscriminate use of antimicrobials has led to the development of resistance thus severely limiting their usage in clinical practice (Raskin *et al.*, 2002). The high cost of therapy is also another limiting factor leading to the search for suitable alternatives (Mungantiwar & Phadke, 2003).

Against this background, the potential of medicinal plants as immunostimulants is an interesting alternative to conventional therapy for chronic and recurrent bacterial and fungal infections. Since ancient times, the practitioners of Ayurveda, the ancient Indian traditional system of medicine, have been using complex combinations of botanical remedies to treat patients (Raskin *et al.*, 2002). Ayurvedic scriptures abound in many examples of such useful plants which serve to strengthen the host's immunological barriers against infectious diseases. Such plants are labeled as 'Rasayana' and include plants like *Allium sativum, Tinospora cordifolia, Glycyrrihza glabra, Azadirachta indica, Withania somnifera, Ocimum sanctum* and *Aloe vera* (Thatte & Dahanukar, 1995; Mungantiwar & Phadke, 2003). Using modern tools and techniques, these plants are being explored for their role in the prophylactic and therapeutic intervention of diseases whose underlying pathophysiology involves the immune system. A large number of medicinal plants have shown great promise with regard to the prevention and treatment of a wide range of disorders and are emerging as forerunners in the rapidly developing field of immunology (Labadie, 1993; Yan, 1998).

The objective of this review is to summarize the currently available information on medicinal plants commonly used as immunostimulants and describe the scientific evidence regarding their therapeutic status.

Generation of an effective immune response involves two major mechanisms of immunity—the non-specific or innate immune mechanism

and the adaptive or specific mechanism. An understanding of the immune system requires a description of the two arms of the immune system.

INNATE IMMUNITY

Innate immunity forms the first line of defense against infection and is the combined effect of a number of factors which can be divided into two main groups namely—the physiological and chemical barriers that prevent the entry and proliferation of pathogens and the cellular components that are actively involved in the phagocytosis of pathogens (Pathak & Palan, 2005). Further, dendritic cells, Major Histocompatibility Complex (MHC) molecules and molecules of the complement system form accessory molecules of the innate immune system.

Physiological and Chemical Barriers

The skin and the epithelial surfaces form the major physiological barriers to the entry of foreign bodies into the system. Saliva, tears and mucus secretions wash away microbes. In addition, the epithelial cells lining the skin, gastrointestinal tract and bronchi express antimicrobial peptides which include defensins, cathelicidins, dermicidin and melanin (Pathak & Palan, 2005). These peptides act via disruption of cell membranes, inhibition of the synthesis of DNA and RNA and activation of antimicrobial enzymes that lyse the components of the microbe. Microorganisms that gain entry into systemic circulation are countered by chemical barriers that include microbicidal factors in tissues and in blood such as spermine, spermidine, histones, protamine as well as lysozyme, lactoferrin, haeme compounds, complement, properdin and cytokines such as IFN-α, IFN-β and IFN-γ (Thatte & Dahanukar, 1995).

Another important class of proteins that contribute to the innate mechanism of immunity is the acute phase proteins. These proteins are secreted by the liver in response to cytokines released by phagocytic cells and include the mannose-binding lectin (MBL) and C-reactive protein (CRP) (Robertson, 1998). Both MBL and CRP can recognize bacterial cell wall components called as pathogen-associated molecular patterns (PAMP) leading to the activation of the complement system (Lin & Karin, 2007). This promotes the opsonization of the pathogens making it more susceptible to phagocytosis (Fig 7.1) (Kindt *et al.*, 2007).

Phagocytic Cells

Phagocytic cells such as macrophages, neutrophils and dendritic cells are involved in elimination of pathogens that enter the body. They engulf, internalize and destroy foreign particles by the process of phagocytosis. Further, they also secrete hydrolytic enzymes, plasma proteins and cytokines involved in the inflammatory response (Pathak & Palan, 2005).

The internalized antigens are broken down to smaller peptide fragments which are then displayed on major histocompatibility complex (MHC) molecules. Macrophages are present in the gastrointestinal tract, the lungs, liver and spleen. Neutrophils that circulate in blood are the first cells to be recruited at the site of infection and play a major role in inflammation (Capsoni *et al.*, 1988). Phagocytic cells express a series of phagocytic receptors which recognize, bind and trigger the ingestion of pathogens. The receptors present on phagocytic cells are termed Pattern Recognition receptors (PRRs) and are capable of recognizing and reacting with cell wall components called as pathogen-associated molecular patterns (PAMP) (Fig 7.1). PRRs secreted on the surface of phagocytic cells include C-reactive protein, LPS binding protein (LBP), Mindin, Collectins, endocytic PRRs and signaling receptors such as Toll-Like receptors (TLRs) (Poeta, 2004).

Figure 7.1 Pathogen elimination by innate immune system.

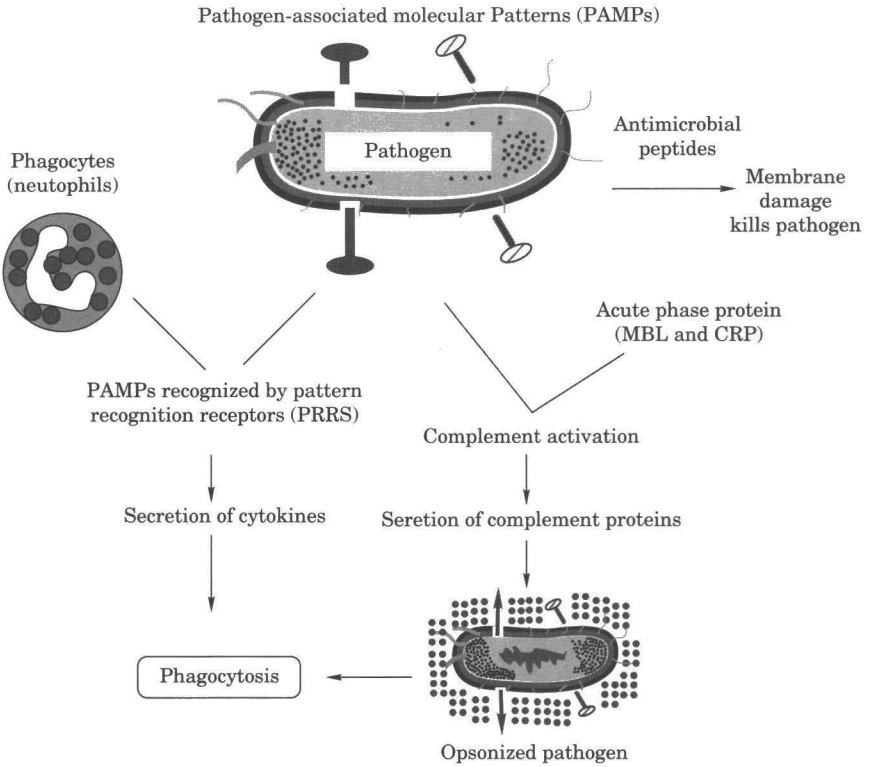

Fig 7.1. On entering the host, pathogens first encounter the chemical barriers such as the antimicrobial peptides and acute phase proteins resulting in their destruction. Pathogens that evade this route of destruction are acted upon by phagocytic cells. The pattern recognition receptors (PRRs) on the surface of these cells bind to pathogen-associated molecular patterns (PAMPs) on the surface of the pathogens. Thus the combined efforts of the cells and the peptides and proteins of the innate system lead to destruction of the pathogen

Activation of TLRs is a prerequisite to antigen elimination by phagocytic cells. These receptors have therefore been the focus of intensive research in the recent years leading to insights into their mechanisms (Bhattacharjee & Akira, 2005; Krishnan *et al.*, 2007). The nine human TLRs identified so far (TLR1 to TLR9) are membrane bound molecules which can recognize different microbial components on the surface or within extra cellular compartments of cells and propagate stimulatory signals to the immune system (Desjardins *et al.*, 2005). They are located within as well as on cell surfaces of phagocytic cells and aid in the detection of extracellular as well as intracellular pathogens (Fig 7.2).

Figure 7.2 the localization of TLRs in the cell.

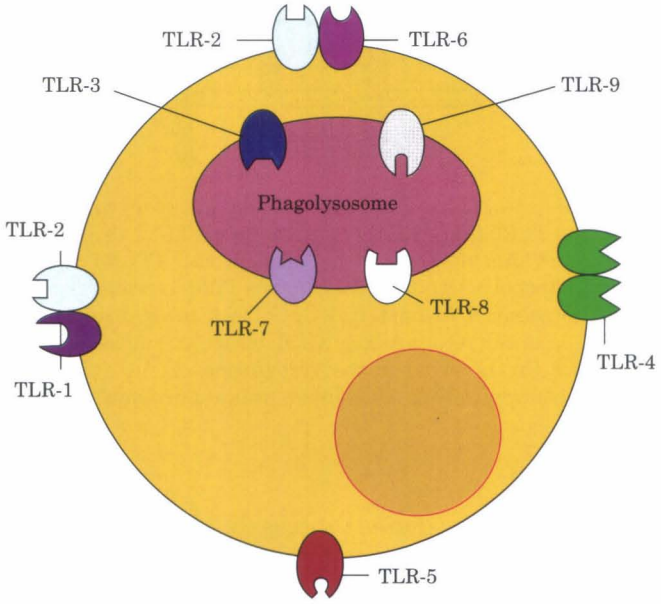

Fig 7.2. TLRs on the plasma membrane include TLR 1, 2, 4, 5 and 6. TLRs which are present on the endosomal membrane include TLR 3, 7, 8 and 9

TLR1/TLR2/TLR6 subfamily and TLR5 are specific to the recognition of bacterial and fungal components, whereas TLR3 and TLR7/TLR8 subfamily are responsible for viral recognition (Takeda, 2005). TLR4 and TLR9 are involved in the recognition of both bacterial and viral components (Fig 7.3) (Medzhitov, 2000).

Binding of TLRs on the surface of the APCs to antigenic peptides leads to the secretion of co-stimulatory molecules such as cluster of differentiation (CD) 28, CD40, CD80 and CD86 which further leads to T-cell differentiation and activation (Kanzler *et al.*, 2007). Thus TLRs serve as a bridge between the innate and the adaptive arms of the immune system.

Figure 7.3 Role of TLRs in elimination of pathogens

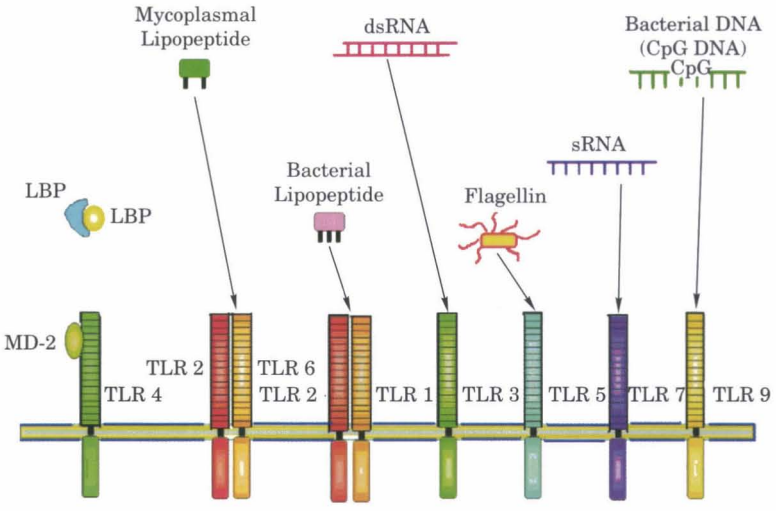

Fig 7.3. Toll like receptor (TLR) 2 recognises and binds to mycoplasmal lipopeptides. TLR1 and TLR6 functionally cooperate with TLR2 to discriminate between triacyl and diacyl lipopeptides respectively. TLR4 is the receptor for bacterial lipopolysaccharide (LPS) while TLR5 recognizes bacterial flagellin. TLR9 is essential in bacterial CpG DNA recognition, whereas TLR3 is implicated in the recognition of viral double stranded DNA (dsRNA). TLR7 and TLR8 are involved in the recognition of viral single-stranded RNA (sRNA). (Adapted from www.biken.osaka-u.ac.jp/act/images/akiraA.png)

Phagocytosis

Phagocytosis is a receptor-mediated process that leads to the internalization of foreign particles into phagocytic cells (Klaas *et al.*, 2005; Pathak & Palan, 2005). The cell membrane of the phagocyte extends projections, called pseudopods that engulf the microorganism. Once the microorganism is surrounded, the pseudopods meet and fuse, enclosing the microorganism within a vesicle called a phagosome. The phagosome extends the cytoplasm and merges with the lysosome to form a single, larger structure called a phagolysosome. The lysosome contributes lysozyme, which breaks down microbial cell walls and digestive enzymes, which degrade carbohydrates, proteins, lipids and nucleic acids. The phagocytes also form lethal oxidants in a process called as an oxidative burst. Within a phagolysosome, the chemical onslaught provided by lysozyme, digestive enzymes and oxidants quickly digests many types of microbes into peptides which associate with class II Major Histocompatibility Complex (MHC) molecules. These peptide-MHC class II molecules move to the surface of the phagocyte membrane where they are presented to the cells of the adaptive immune system such as T-cells for further processing (Fig 7.4).

Figure 7.4 Steps involved in phagocytosis of pathogens by phagocytic cells.

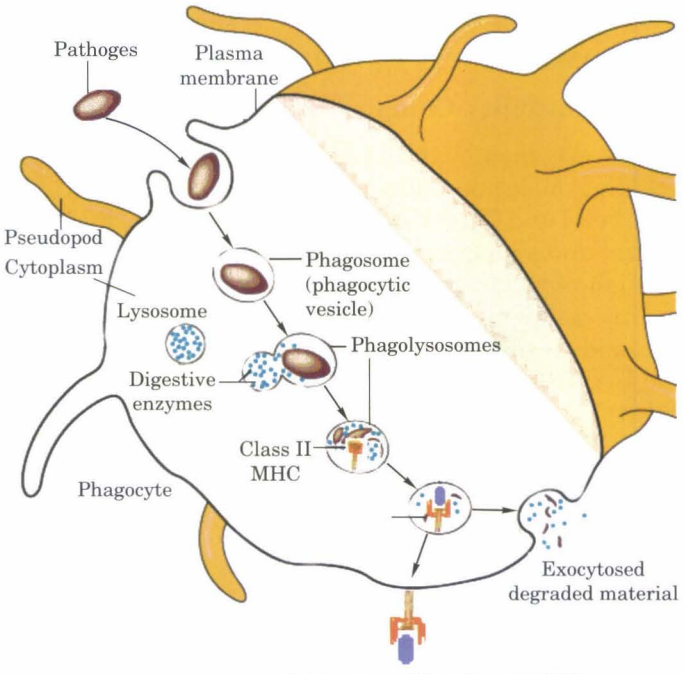

Fig 7.4. The phagocyte engulfs pathogens into vesicles to form phagosomes. Phagosome fuses with the lysosome to form phagolysosome. The pathogens are degraded within the phagocyte by the action of lysosomal enzymes and oxidants. The resultant peptides interact with Class II MHC molecules to form complexes that are displayed on the cell surface. (Adapted from www.utc.edu/Faculty/Becky-Bell/1-210/210-outlines/210-outline10.html)

Although phagocytosis proceeds by invagination of the plasma membrane, in some instances, the amount of plasma membrane needed for phagocytosis exceeds the surface area of the phagocytes leading to the conclusion that other organelles such as the endoplasmic reticulum (ER) may be involved in phagocytosis. A study carried out by Gagnon *et al.* (2002) to determine the mechanism of phagocytosis has confirmed the involvement of ER in phagocytosis. It has been proposed that the ER fuses with the phagocytic membrane underneath the phagocytic cups to provide part of the membrane needed for the formation of the phagosomes. The ER is also supplied intermittently during different stages of the phagosome maturation (Gagnon *et al.*, 2005).

The degradation of pathogens in phagolysosomes results in the formation of peptides for presentation on the surface of the MHC molecule. Thus the

phagocytic cells are the link between the generation of degradative products and antigen presentation to the cells of the adaptive immune system (Ackerman & Cresswell, 2004).

ACCESSORY COMPONENTS OF THE INNATE IMMUNE SYSTEM

Major Histocompatibility Complex (MHC) Molecules

MHC molecules are membrane-bound glycoproteins that function as antigen-presenting molecules (Kindt *et al.*, 2007). They are functionally classified into Class I and Class II molecules. Class I MHC molecules present endogenous viral and tumor peptides to the cytotoxic T-cells. This results in the destruction of the infected cells (Fig 7.5). Class II MHC molecules are expressed by antigen-presenting cells such as macrophages, mature dendritic cells and B-cells. Phagocytosed proteins of bacteria and viruses are presented by MHC Class II molecules to helper T-cells (Ackerman & Cresswell, 2004).

Figure 7.5: Role of Major histocompatibility complex (MHC) in elimination of pathogens.

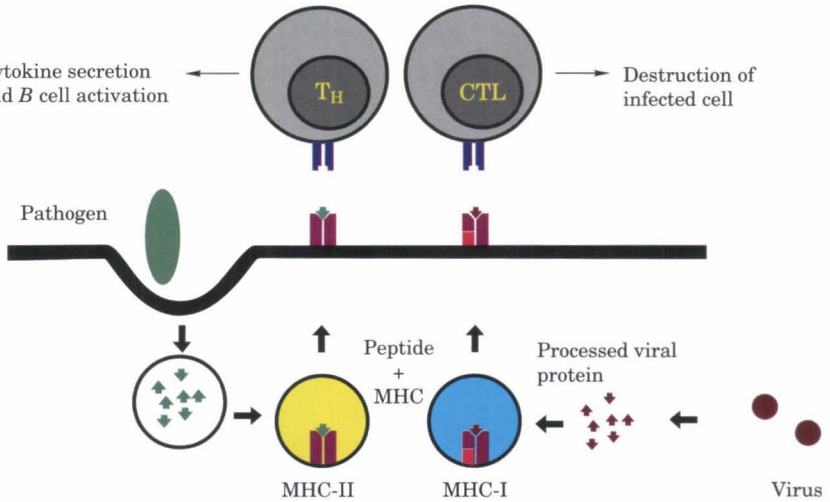

Fig 7.5. Major Histocompatibility complex (MHC) class I molecules bind to peptides that are fragments of viral or tumor proteins. MHC class II molecules present peptides degraded by the process of phagocytosis. The resultant MHC: peptide complex is transported to the cell surface and the peptides are presented to the T-cell receptors (TCR) of cytotoxic T-lymphocytes and T-helper cells respectively for destruction

Dendritic Cells

Dendritic cells are members of the innate immune system and function as antigen presenting cells (APC) to both T helper (Th) cells and cytotoxic

T-lymphocytes (CTL). On encountering an antigen, the dendritic cells bind to the antigen by means of toll like receptors (TLRs) present on their surface and internalize it by phagocytosis. They then migrate to lymph nodes where they present the antigenic peptide to the T-cells (Kindt *et al.*, 2007). The B7 family of co-stimulatory molecules on the surface of dendritic cells in conjunction with MHC class I and class II molecules are responsible for T-cell stimulation (Fig 7.6) (Woodhead *et al.*, 2000; Ulevitch, 2004).

Figure 7.6: Role of dendritic cell in T-cell activation.

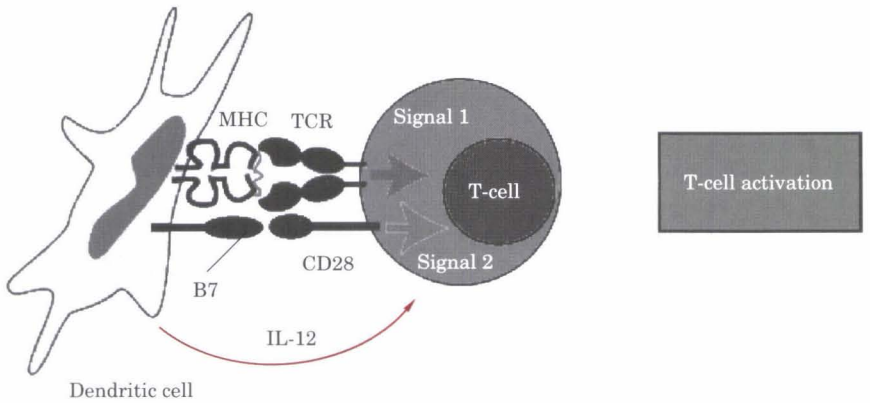

Fig 7.6. The dendritic cells mediate the activation of T-cells by three signaling pathways. The stimulatory signal (Signal 1) is generated by the binding of MHC: peptide complex to the T-cell receptor (TCR). The co-stimulatory signal is generated by binding of the ligand B7 with the T-cell co-receptor CD28. Dendritic cells also secrete interleukin (IL) 12 which acts on T-cells leading to their activation, expansion and differentiation

Complement System

The proteins and the glycoproteins that constitute the complement system are important accessory components of the innate immune system. Its functions include cell lysis, participation in the inflammatory response, opsonization of antigen, viral neutralization and clearance of immune complexes. Complement activation occurs by one of the three pathways, the classical pathway, the alternate pathway and the lectin pathway. Complement activation by any of these pathway leads to the formation of the membrane-attack complex (MAC). This complex forms a large channel through the membrane of the target cell, enabling ions and small molecules to diffuse freely across the membrane (Fig 7.7). The cell loses its osmotic stability and is killed by an influx of water and loss of electrolytes (Carroll, 2004). Thus MAC can lyse gram-negative bacteria, parasites, viruses, erythrocytes and nucleated cells.

Figure 7.7: Pathways involved in elimination of antigens by complement system.

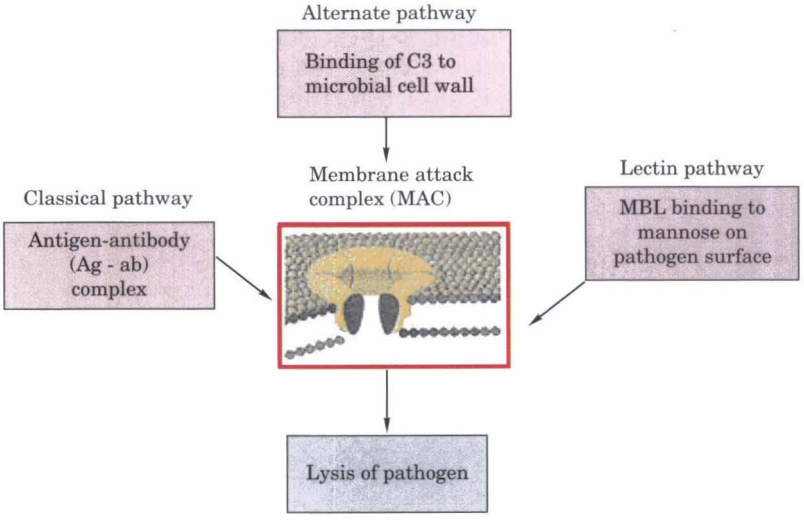

Fig 7.7. Activation of the complement system takes place via one of the three pathways, classical pathway, alternate or lectin pathway. A membrane attack complex is formed leading to lysis of the target cell

Adaptive Immunity

Adaptive immunity involves a cell-mediated and humoral immune response mediated by T-cells and B-cells respectively (Fig 7.8). In addition, natural killer cells also contribute to the adaptive arm of the immune system (Pitcher & Van Ooers, 2003).

Cell-Mediated Immunity

Cell-mediated immunity is mediated by two different types of lymphocytes namely T-helper (Th) cells and T cytotoxic (T_c) cells. In addition, T regulatory (T_{reg}) cells have also been defined. T_{reg} cells have the capacity to differentiate into either Th or T_c cells (Kuby, 2007). T-cells which display the peptide, Cluster of Differentiation 4 (CD4) on their surface function as T helper (Th) or CD4 positive ($CD4^+$) cells while those displaying CD8 function as cytotoxic T (T_c) or $CD8^+$ cells. $CD4^+$ cells are functionally classified into Th1 cells which activate macrophages to kill the intravesicular bacteria and Th2 cells which are responsible for stimulating antibody secretion from B-cells. Th cells are differentiated into two subtypes—the Th1 cells which secrete IL-2, IFN-γ and TNF-β and are responsible for mediating delayed-type hypersensitivity and activation of cytotoxic T-lymphocytes; the Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 and mediate B-cell activation (Kindt *et al.*, 2007).

Figure 7.8: Schematic representation of the adaptive immune response to antigen.

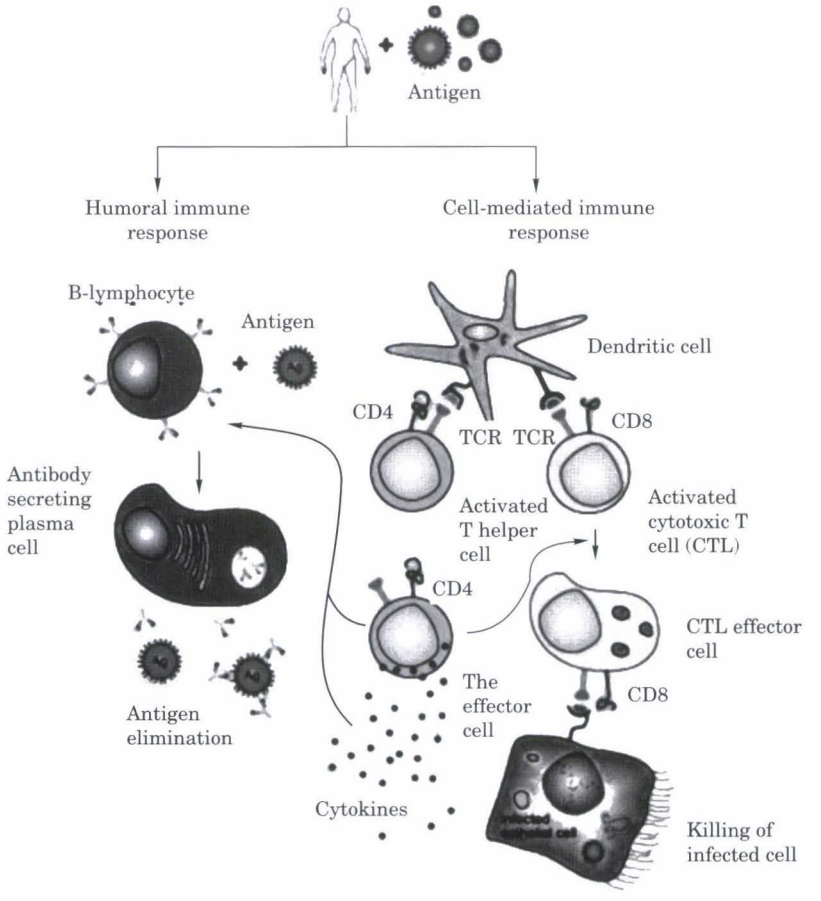

Fig 7.8. The processed antigens are presented to the adaptive immune system resulting in activation of T-cells and B-cells. Activated T-cells secrete cytokines leading to proliferation and differentiation of the T-cells into cytotoxic T-cells and helper T-cells. Cytotoxic T-cells bind to and eliminate the pathogens. Cytokines released by helper T-cells lead to B-cell activation and subsequent production of antibodies. These antibodies complex with the antigens resulting in the destruction of antigens (Adapted from www.influenzareport.com/ir/pathogen.htm)

T-cells possess receptors known as T-cell receptors (TCR) which consist of a disulphide-linked heterodimeric glycoprotein that enables T-cells to recognize a diverse array of antigens in association with MHC molecules. It consists of γ and δ subunits or occasionally α and β subunits. It is associated at the T-cell surface with a complex of polypeptides known collectively as

CD3 which is required for activation of T-cells (Fig 7.9) (Krogsgaard & Davis, 2005).

Figure 7.9: Schematic representation of the T-cell receptor (TCR).

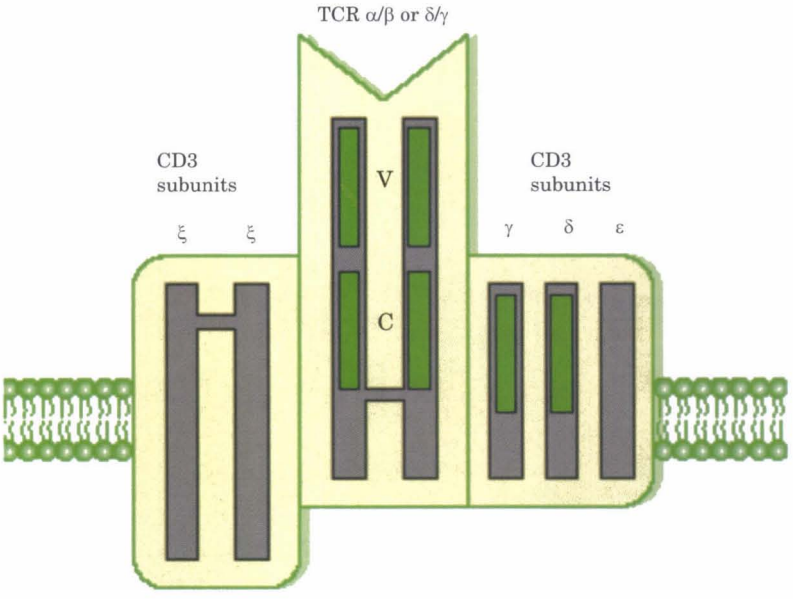

Fig 7.9. The T-cell receptor (TCR) consists of α and β subunits and occasionally γ and δ subunits in association with CD3

Binding of TCR to MHC: antigen complex provides the stimulatory signal for the activation of T-cells. However, activation occurs only after several accessory membrane molecules, including co-receptors such as CD28, CD2, LFA-1, CD28 and CD45R bind to other ligands on APCs or target cells (Fig 7.10). The T-cells thus activated form effector cells and memory cells (Pitcher & Van Ooers, 2003). The effector T-cells includes the T helper (Th) cells and the cytotoxic T (T_c) cells. Memory T-cells are long-lived quiescent cells that respond with heightened reactivity to subsequent infection with the same antigen. They result in a rapid secondary response to the antigen (Janeway & Travers, 1997a).

T_c cells do not directly act on the target cells. Rather they are activated to form cytotoxic T-lymphocytes (CTL). CTL contain cytoplasmic granules that secrete soluble molecules such as the pore forming protein perforin and granzyme (Anjum & Khar, 2002). When CTL conjugates with the target cell via its TCR and CD28 receptors, perforin and granzyme are released into intracellular space. Perforin monomers are inserted into the target cell membrane and polymerize to form cylindrical channels. This mediates the entry of the protease granzyme (Fig 7.11). Granzyme acts on target cell DNA leading to its fragmentation and the target cell undergoes apoptosis (Mahajan *et al.*, 2003). Alternatively, the cytotoxic cells can initiate apoptosis

in the target cells via the death receptor ligand pathway (Kluck, 1997). Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific ligands and play an important role in apoptosis.

Figure 7.10: Antigen presentation by major histocompatibility complex (MHC) to the T-cell.

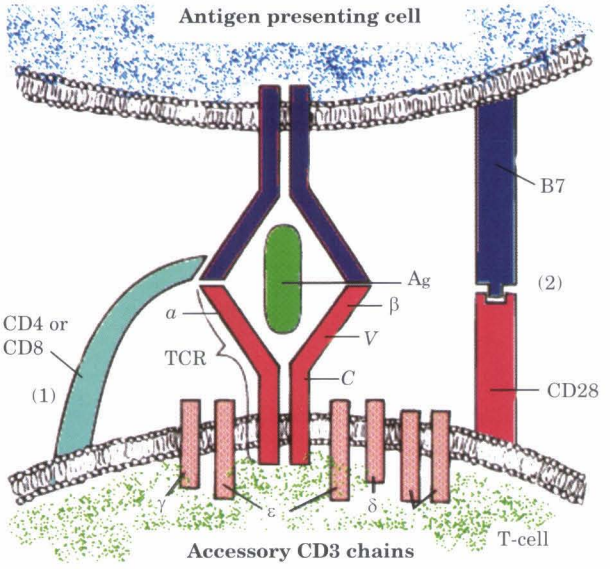

Fig 7.10. Binding of the T-cell receptor (TCR) to the antigenic peptide displayed on the surface of the Major Histocompatibility Complex (MHC) and of the co-receptor CD28 to the ligand B7 present on the surface of the MHC mediates the activation of the T-cell (Adapted from www.merck.com/mmpe/sec13/ch163/ch163b.html)

Another protein responsible for T-cell activation is the urokinase plasminogen activator (uPA). uPA is a serine protease that catalyses the conversion of plasminogen to plasmin. Following bacterial infection, uPA is detected in circulation. In the absence of uPA, T helper cells, Th1 and Th2 fail to differentiate in response to pathogen challenge *in vivo*. Recent evidence accumulated revealed an elevation in the levels of uPA and uPA receptor (uPAR) in the tissues or fluids of patients affected by AIDS and cancer (Mondino & Blasi, 2004). Rapid upregulation of uPA and uPAR levels following T-cell activation has been observed (Handley, 1996).

Humoral Immunity

Humoral immunity is mediated by antibodies which are antigen-specific products of B-cells. Naïve B-cells bear membrane bound antibodies which can bind to antigens. The binding of the antigen to the B-cell causes the cell to divide rapidly into memory cells and effector plasma cells (Fig 7.12).

Memory B-cells express the same membrane bound antibody as the parent B-cell while plasma cells produce antibodies that circulate freely in the blood. These secreted antibodies are the major effector molecules of humoral immunity (Janeway & Travers, 1997b).

Figure 7.11: Destruction of target cell by cytotoxic T-lymphocytes (CTLs) Granzyme.

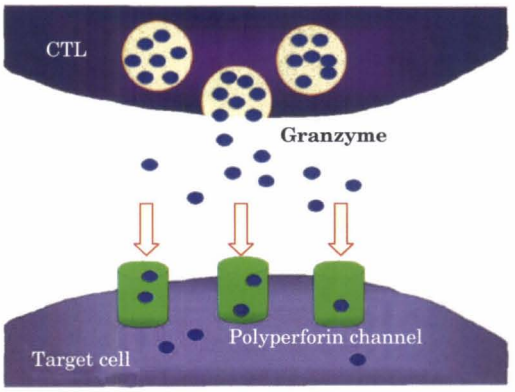

Fig 7.11. Perforin granules released from CTL form perforin channels in the target cell membrane. Granzyme that is released from CTL enters the cell through these channels and brings about fragmentation of DNA

Figure 7.12: Role of B-cells in humoral immune response.

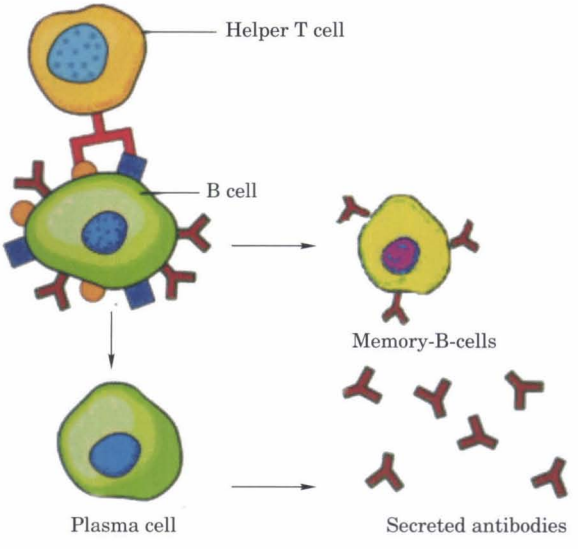

Fig 7.12. B-cells are activated by helper T-cells and divide rapidly into plasma cells which secrete antibodies and memory B-cells bearing membrane bound antibodies

Antibody molecules, also referred to as immunoglobulins are Y shaped molecules consisting of three equal sized segments, loosely connected by a flexible tether. Two types of chains make up the antibody molecule, two heavy chains and two light chains. Each heavy chain is linked to a light chain and the two heavy chains are linked to each other. The fragment consisting of the light chain and the portion of heavy chain bound to it is responsible for binding the antigen and is termed Fragment antigen binding (Fab) region. The two Fab regions correspond to the two arms of the antibody molecule. Five different types of immunoglobulins exist namely, IgM, IgD, IgG, IgA and IgE. They differ in their Fab regions. The third fragment contains no antigen binding activity but crystallizes readily and therefore called Fragment crystallizable (Fc) region (Fig 7.13) (Notkins, 2004). The antibody binds via its Fc region to Fc receptors on accessory effector cells. These accessory cells include phagocytic cells such as macrophages and neutrophils which can ingest antibody-coated bacteria and kill them and other cells such as natural killer cells, eosinophils and mast cells which are triggered to secrete stored mediators when their Fc receptors are engaged (Janeway & Travers, 1997c).

Figure 7.13: Schematic representation of an antibody molecule.

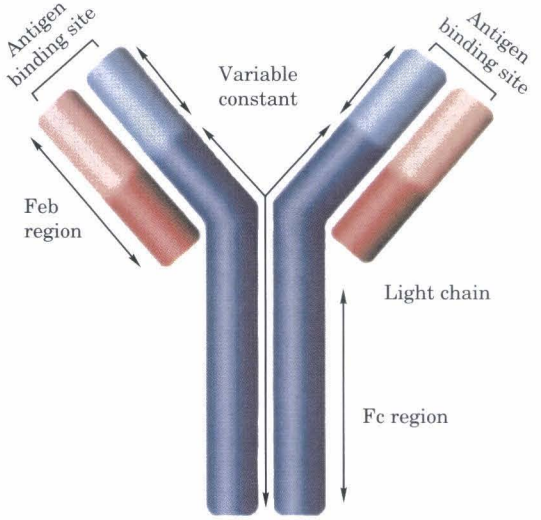

Fig 7.13. The Fab region of the antibody molecule binds to the antigen while the Fc region binds to effector molecules resulting in the generation of an immune response (Adapted from www.biology.arizona.edu/.../immunology/05t.html)

B-cells express antigen-binding receptor on their membrane. The BCR is a trans-membrane protein complex composed of immunoglobulin molecules and disulfide-linked heterodimers called Ig-α and Ig-β (Fig 7.14) (Janeway & Travers, 1997c).

Figure 7.14: Schematic representation of a B-cell receptor.

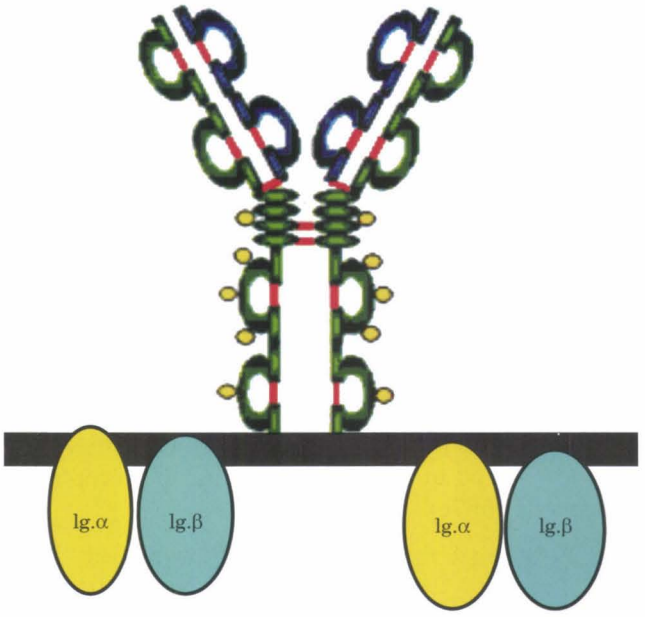

Fig 7.14. B-cell receptor contains membrane bound antibodies and disulfide-linked heterodimers called Ig-α and Ig-β

Natural Killer (NK) Cells

NK-cells are large lymphoid cells with prominent intracellular granules. NK-cell activity is stimulated by the cytokines IFN-α, IFN-β and IL-12. The destruction of antigen-antibody complexes by NK cells is known as antibody-dependant cell-mediated cytotoxicity (ADCC). ADCC is triggered when bound antibody interacts with Fc receptors (FcRs) on NK-cells (Kindt *et al.*, 2007). The Fc receptor (FcR) on NK-cells triggers the release of antimicrobial peptides perforin and granzyme. NK-cell mediates a perforin-mediated membrane damage and granzyme-mediated cytotoxic attack on target cells resulting in apoptosis of the target cell (Rao, 2002).

Cytokines play a predominant role in the pathways of both the immune as well as the adaptive immune system. These cytokines have emerged as important biomarkers in the evaluation of agents modulating immune functions.

CYTOKINES

Cytokines are potent regulatory proteins which are produced in minute quantities by the body and are capable of inducing their effects at nanomolar to picomolar concentrations (Walsh, 1998; Cruse & Lewis, 1999).

Cytokines act as chemical communicators between various cells and induce their effects by binding to specific cytokine receptors thereby triggering various intracellular signal transduction events (Kotenko & Langer, 2004). Based on their binding affinities, cytokine receptors are classified as Class I and Class II receptors. Class I receptors include those that bind a number of interleukins including IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-11, IL-12 and IL-15. Class II receptors bind interferon IFN α, β and γ and IL-10 (Walsh, 1998).

Binding of the cytokine with its receptor induces a complex signal transduction pathway leading to the destruction of the target cell (Fig 7.15). The cytokines produced by Th cells have a well-defined role to play in immunity. Th1 derived cytokines promote cell-mediated immune functions, whereas Th2 derived cytokines primarily elicit a humoral immune response (Notkins, 2004).

Figure 7.15: Elucidation of action by cytokines.

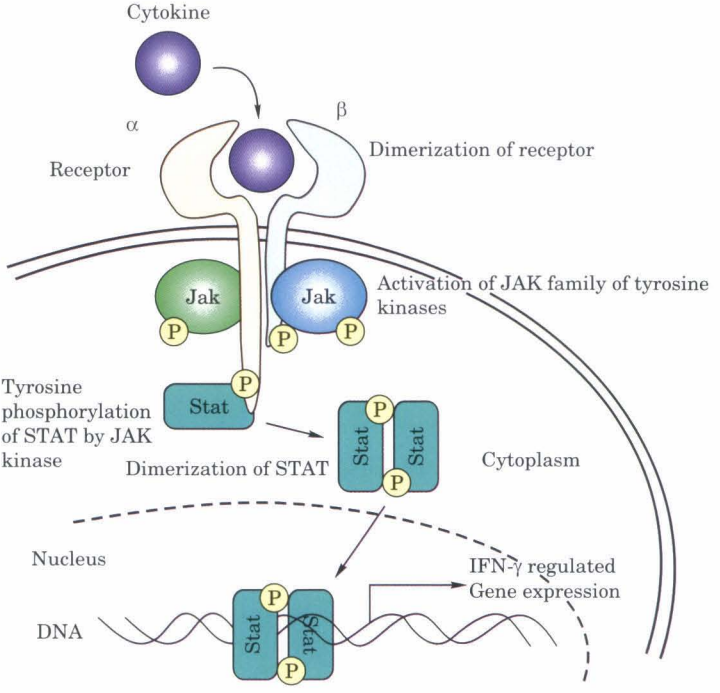

Fig 7.15. Cytokines bind with specific receptors and induce signal transduction resulting in triggering of gene expression in target cells (Adapted from genomebiology.com/2004/5/12/253)

The cytokine environment in which the Th cell differentiates determines whether Th1 or Th2 cells will proliferate. The cytokine IFN-γ is generated by activated T-cells and antigen presenting cells (APCs). It upregulates

IL-12 production by macrophages and dendritic cells and activates IL-12 receptor on T-cells. IL-12 is a key mediator of Th1 differentiation and this leads to the formation of a positive feedback loop (Fig 7.16).

In contrast, if the cytokine environment is dominated by the cytokine IL-4, Th2 subset dominates leading to eosinophil activation and synthesis of IgE. Thus cytokines produced by each subset positively regulate the subset that produces it and negatively regulate the other subset (Fig 7.16). This phenomenon is known as cross-regulation. Two transcription factors, have been found to be responsible for this phenomenon (Kindt *et al.*, 2007). T box expressed in T-cells (T-bet) is a novel protein belonging to the T-box family of transcription factors (Szabo *et al.*, 2000). Expression of T-Bet stimulates the differentiation of cells into Th1 cells and suppresses the differentiation of cells into Th2 cells. Expression of another factor, GATA binding protein 3 (GATA-3) promotes the development of naïve T-cells into Th2 cells and suppresses their differentiation into Th1 cells (Zheng & Flavell, 1997; Hodge *et al.*, 2005).

Figure 7.16: Cytokine-mediated generation and cross-regulation of Th subsets.

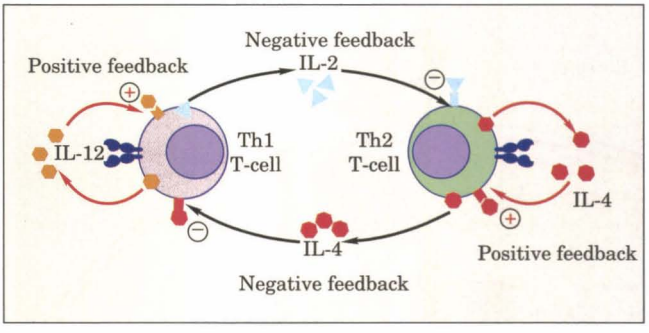

Fig 7.16. Secretion of IL-12 by macrophages drives the differentiation of naïve T-cells to Th1 type. Th1 cells secrete IFN-γ thus providing a positive feedback for further secretion of IL-12. IL-4 provides a positive feedback loop for Th2 cells. ⊕ Indicates a positive feedback loop. ⊖ Indicates negative feedback mechanism (Adapted from www.ermm.cbcu.cam.ac.uk)

ROLE OF NITRIC OXIDE IN IMMUNITY

Nitric oxide (NO) is an important bioregulatory molecule, having a number of physiological effects on the immune system (Bogdan, 2001; Jagetia *et al.*, 2004). It is produced by a large number of immune cells including dendritic cells, NK-cells, mast cells, phagocytic cells such as monocytes, macrophages, microglia, Kupffer cells, eosinophils, and neutrophils, T-cells as well as other cells involved in immune reactions such as endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblasts,

keratinocytes, chondrocytes, hepatocytes, mesangial cells and Schwann cells (Moncada *et al.*, 1991; Blesson *et al.*, 2002).

In the immune system, NO regulates the differentiation, proliferation and apoptosis of immune cells, production of mediators such as cytokines and the expression of costimulatory and adhesion molecules. It upregulates the secretion of IFN-γ by macrophages and superoxide anion (O_2^-) release by neutrophils. It also induces apoptosis of activated T helper cells once the immune response is generated. Nitric oxide synthesized by inducible nitric oxide synthase in activated murine macrophages is an important component of host defense mechanism (Fig 7.17). It kills pathogens including leishmania, *Mycobacterium tuberculosis*, malaria parasites, and certain fungi and is toxic to tumor cells (Coleman, 2001).

Figure 7.17: Role of NO in mediating cytotoxicity.

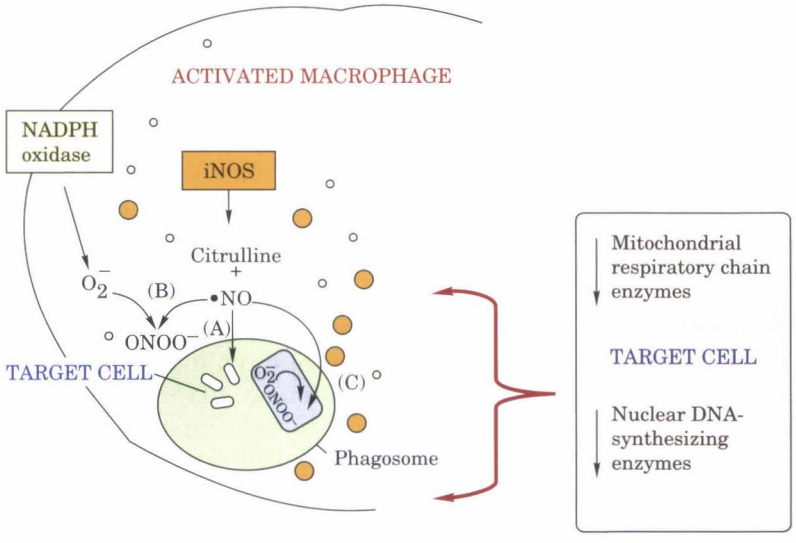

Fig 7.17. Target cells (tumor cells, bacteria, fungi and helminthes) are destroyed by NO radicals which mediate cytotoxicity by (A) acting directly on the target cell or by the peroxynitrite formed by (B) reaction of NO with O_2^- generated by the NADPH oxidase of the macrophage or (C) produced within the microbe itself

Under aerobic conditions, NO is oxidized to reactive nitrogen oxide species (RNOS) such as nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3) and trinitrogen tetraoxide (N_3O_4). The RNOS induces cell cytotoxicity by nitrosating thiols to modify key signaling molecules such as kinases and transcription factors. RNOS also affects DNA repair proteins like DNA alkyl transferase, formamopyrimidine-DNA glycosalase and DNA ligase that are responsible for the maintenance of genetic integrity. Another mechanism by which RNOS elicits cytotoxicity is by combining with O_2^- to

form the peroxynitrite anion ($ONOO^-$). Peroxynitrite inhibits mitochondrial respiration and mediates toxic effects of NO. RNOS enhances or inhibits signaling proteins such as mitogen-activated protein kinase (MAPK), Erk, Jun N-terminal kinase (JNK), stress-activated protein kinase (SAPK) and src family kinases. RNOS inhibits the expression of cytokine genes including those that encode IL-1, IL-6, IL-8, IFN-γ and TNF-α by various cells of the immune system (Esch *et al.*, 2003).

Thus NO elicits antiviral, antimicrobial, immunostimulatory, cytotoxic and cytoprotective effects. However, in some cases, NO-mediated cytotoxicity and tissue damage, inhibition of T-cell proliferation, T-cell apoptosis, and stimulation of viral and microbial growth lead to detrimental effects on the host cells. Depending on the concentration of NO released, the type of NO pathway activated, disease state and severity of disease, NO can exert a beneficial or detrimental effect on the immune system (Mukherjee & Wahile, 2006).

ROLE OF PLANT BASED THERAPEUTIC AGENTS IN STIMULATION OF IMMUNE SYSTEM

Agents geared to target disease conditions having an immune component are mainly symptomatic and fail to address the underlying depressed immunity. There is a lacunae in the availability of effective immunostimulants which are required in a wide variety of conditions ranging from infectious diseases to cancer. The traditional Indian system of medicine, 'Ayurveda' specifies a category of plant based therapeutic modalities termed as 'Rasayana'. The 'Charaka Samhita', an ancient treatise on Ayurveda mentions that 'Rasayanas' help to attain longetivity, enhance memory, intelligence, freedom from disease, youthfulness, lustrous complexion, excellent voice, physical strength, respectability and brilliance. In the modern context, 'Rasayanas' are plant drugs that prevent infections and strengthen the host against other stressors (Katiyar *et al.*, 1997). Ayurveda abounds in many examples of such useful plants. Plants like Lahsuna (*Allium sativum*), Guduchi (*Tinospora cordifolia*), Yashtimadhu (*Glycyrrihza glabra*), Nimba (*Azadirachta indica*), Ashwagandha (*Withania somnifera*), Tulsi (*Ocimum sanctum*) and Ghritakumari (*Aloe vera*) find a mention in 'Charaka Samhita' (Gupta, 1994).

Ayurveda is backed by centuries of research and clinical experience. This clinical data serve as the starting point for research in Ayurvedic medicine. Such an approach is termed as Reverse Pharmacology and is a powerful tool in man's quest for newer drug discovery by the natural products route. Reverse Pharmacology is the science of converting available clinical data into leads using exploratory studies and developing these leads into candidates for experimental and clinical research (Vaidya & Devasagayam, 2007). In this approach, Ayurvedic preparations with proven

abilities are subjected to well-designed scientific exploratory studies comprising of *in vitro/ex vivo* and *in vivo* components. Such an approach has also been termed as the 'Clinics to Laboratories' approach (Patwardhan, 2005).

Reverse Pharmacology approach comprises of three phases (Vaidya, 2002). In the first stage, documentation of clinical observations of plant-based drugs from the Ayurvedic database and follow up of treatment is done. In the second stage, exploratory *in vitro* and *in vivo* studies for establishing safety and evaluating dose response of the plants are carried out. In the final stage, elucidation of targets on which the drugs act and the pathways by which they act is carried out (Fig 7.18). In addition, detailed investigations to elucidate the structure of the active moieties are also conducted (Patwardhan *et al.*, 2004).

Figure 7.18: Reverse pharmacology approach for Ayurvedic drug discovery.

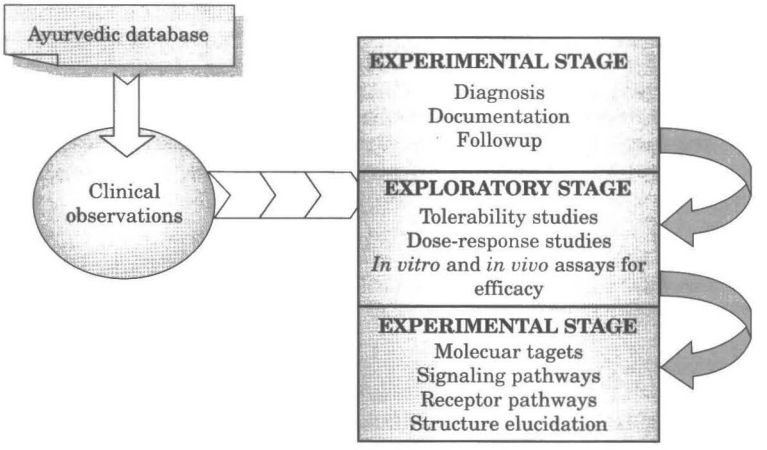

Fig. 7.18.

The tradition of using plants to treat diseases was revived in India by Sir Ramnath Chopra who conducted extensive research on indigenous medicinal plants. Immunostimulant activity, the modern equivalent of Rasayana was evaluated in plants claiming to be antibacterial, antiviral, antifungal or cytotoxic (Wagner, 1995). Plants that were active at low doses were selected for evaluation as the low dose suggested that immune stimulation rather than direct cytotoxic effect was responsible for its therapeutic activity (Balachandran & Govindarajan, 2007). Evaluation of these plants by modern techniques revealed that these plants have a vast potential to biosynthesize chemicals that can fight against diseases. Thus the inability of modern medicines to contain diseases, increasing incidences of emerging infections and the promise shown by plants in the treatment

of infections has propelled researchers to study plants as alternatives in the treatment of diseases related to the immune system. Initial screening for activity was performed with crude extracts that closely resembled traditionally used medicinal products. However, crude extracts are usually composed of constituents displaying a wide range of molecular weights. Hence fractionation of the extracts and bioactivity guided assays were carried out to get an insight into the components that were responsible for the activity. In the context of immunostimulant herbal drugs, the major classes of compounds that have demonstrated immunomodulatory activity included carbohydrates, proteins, alkaloids and phenolics (Labadie, 1993).

Carbohydrates

Investigation of the immunomodulatory activities of carbohydrates from plant sources has focused mainly on polysaccharides isolated from fungi, lichens, algae and higher plants. Polysaccharides from a number of plants including *Echinacea purpurea, Calendula officinalis, Aloe vera, Viscum album, Picrorhiza kurroa* and *Panax ginseng* have demonstrated immunostimulant properties (Labadie, 1993). Polysaccharides are termed as biological response modifiers and trigger immune responses on binding to pattern recognition receptors (PRRs) (Leung *et al.*, 2006). The polysaccharides stimulate various immune functions including phagocytosis, T-lymphocyte proliferation, cytokine release and activation of complement factors (Ooi & Liu, 2000). They are believed to upregulate gene expression of various cytokines and cytokine receptors (Wagner, 1987).

Proteins, Peptides and Glycoproteins

The mediators of immune reactions are proteins, peptides or glycoproteins. Hence this class of compounds is believed to interact with functional immunofactors and immunosystems. Many peptides have been found to exert their effect by binding to the MHC Class I and MHC Class II molecules and serving as ligands for T-cell receptors (Murugan & Dai, 2005; Rajapakse *et al.*, 2007).

Alkaloids

Alkaloids have antimicrobial properties and have been found to be useful against HIV and intestinal infections associated with AIDS (Witchers & Maes, 2002).

Phenolics

Phenolics are a widely studied class of phytochemicals. They are products of secondary metabolism of plants. Due to their radical scavenging activity, they are involved in the defense mechanisms of the plants (Liu, 2004). Several

studies have shown a correlation between the phenolic content of plants and their antioxidant potential (Pellegrini *et al.*, 2003; Lasikova *et al.*, 2007).

A reverse pharmacology approach has led to the discovery of new leads and confirmation of activity of plants mentioned in traditional literature. Several laboratories have carried out basic and clinical research on the positive health benefits of medicinal plants. Newer approaches that combine traditional knowledge with scientific research are being used to establish the therapeutic efficacy of plants. In line with this, we have outlined below investigational studies leading to the establishment of immunostimulant potential of plants.

ASHWAGANDHA

Botanical name: *Withania somnifera* (L. Dunal).

Family: Solanaceae.

Common name: Winter cherry, Indian ginseng, Withania, Varahakarni, Askandhatili. Ashwagandha is an ayurvedic plant that finds a mention in the ancient ayurvedic text 'Vedas' and is purported to be a herbal tonic. Ashwagandha is reported to have antibacterial, anti-inflammatory, antiarthritic and antitumor activities. Many pharmacological investigations have been conducted to investigate and authenticate the use of Ashwagandha as an immunostimulant (Dikshit *et al.*, 2000; Ben-Erik & Wink, 2004).

Ziauddin *et al.* (1996) conducted extensive studies on mice whose immune system was suppressed by cyclophosphamide, azathioprine, or prednisolone to evaluate the effect of Ashwagandha on the immune system. The results revealed that Ashwagandha successfully blocked drug-induced immunosuppression and restored bone marrow activity. This was reflected by a significant increase in hemoglobin concentration, RBC count, platelet count and antibody titre. In the same study, a significant increase in hemolytic antibody responses towards human erythrocytes was also seen. Another study by Davis and Kuttan (1998) provided supporting evidence for the efficacy of the plant in countering drug induced immunosuppression. The immunosuppressive effect of cyclophosphamide in mice was successfully reversed by treatment with Withania extract. This was evident by the increase in WBC count of treated rats. Due to its usefulness in immunosuppressed conditions, the plant extracts are being viewed as candidates for supportive treatment with cancer chemotherapeutic agents (Diwanay *et al.*, 2004). The immunomodulatory effect of Ashwagandha was also demonstrated in a study by Agarwal *et al.* (1999). The results of the study revealed a potentiation of cell-mediated immunity as evidenced by increase in foot pad thickness induced by Ashwagandha in immuno-

suppressed rats. A significant increase in antibody titre of the immunosuppressed rats was also seen indicating that Ashwagandha enhanced the humoral arm of the immune system (Agarwal *et al.*, 1999). The anti-infective potential of the plant was evaluated against experimental aspergillosis induced in mice (Dhuley, 1998). The results indicated that Ashwagandha conferred protection against aspergillosis by stimulating phagocytic activity of macrophages. Davis and Kuttan (2000) demonstrated the immunostimulant potential of the extract of Ashwagandha root by evaluating its effect on hematologic parameters in mice. The extract significantly enhanced total WBC count and increased the antibody titre. Additionally it also enhanced the phagocytic activity of macrophages. Further investigations into its mechanism of action revealed that Ashwagandha stimulated nitric oxide production by macrophages (Iuvone *et al.*, 2003). Ashwagandha is also a component of many multi component herbal mixtures used as immunostimulants. Further, studies conducted on such preparations have also confirmed immunostimulant activity of the herbal mixtures (Babu, 2001; Chatterjee, 2001).

Aloe Vera

Botanical name: *Aloe barbadensis* Mill.

Family: Asphodelaceae.

Common name: Aloe, kumari, ghritakumari, kanya, paktikandadala, pichchasambhruta.

Aloe gel has been used in traditional Ayurvedic and Thai medicine for treatment of peptic ulcers (Barner *et al.*, 2002). Phytochemical investigations of the gel have yielded the presence of constituents such as polysaccharides (acemannan is the principal polysaccharide), aloins such as barbaloin, isobarbaloin and betabarbaloin, tannins, sterols, organic acids, enzymes such as cyclooxygenase, saponins, vitamins and minerals (Bone, 2003; Turner *et al.*, 2004).

Lau *et al.* (1994) conducted studies to evaluate the immunostimulant potential of acemannan. The studies indicated that short-term exposure of peritoneal macrophages to acemannan upregulated their respiratory burst, phagocytosis and killing of pathogens. Acemannan also enhanced cell-mediated responses and was found to be beneficial in treating and preventing tumors. Acemannan also demonstrated an increase in splenic and peripheral blood cellularity and levels of hematopoeitic progenitors in the spleen and bone marrow. Due to its immunostimulant activity, acemannan has shown promise in clinical studies carried out on for treatment of HIV infections (Mukhtar *et al.*, 2008). The biological mechanisms underlying the immunostimulant activity of Aloe were elucidated by Zhang and Tizard (1996). Macrophages that were incubated *in vitro* in the presence of acemannan demonstrated an increase in the

production of nitric oxide. In addition, IL-6 and TNF-α levels were also significantly elevated.

Aloe gel is widely used in cosmetic products as a hydrating ingredient in creams, sunlotions, shaving creams, lip balms, healing ointments, face packs and creams (Jin *et al.*, 1999). Studies carried out in the laboratory indicate that aloe cream demonstrates benefit in first and second-degree burns which could be a result of inhibition of thromboxane B_2 and prostaglandin F_{2a} formation, thereby preserving dermal circulation and decreasing burn wound tissue (Barner *et al.*, 2002).

ASTRAGALUS

Botanical name: *Astragalus membranaceus*.

Family: Fabaceae.

Common name: Astragalus, Huang-qi, Milk-Vetch root.

Astragalus is a traditional Chinese herbal drug whose immunostimulant effects have been widely studied. Astragalus roots have been used in traditional medicine for several years to promote the discharge of urine, lower blood pressure and increase endurance. It is purported to have immune-enhancing properties, especially for the prevention and treatment of common cold and chronic hepatitis (Zhen *et al.*, 1999). The major components of the roots of Astragalus are saponins, polysaccharides, isoflavanoids and astragaloside (Jin *et al.*, 1999). Other phytoconstituents isolated from the root include phytosterols, volatile oil, and amino acids that include gamma-aminobutyric acid (GABA) and L-canavanine. Studies conducted on the extracts and fractions isolated from Astragalus have revealed the immunostimulant effect of the herb.

In a study conducted to elucidate the mechanism of action of Astragalus, mice implanted with renal cell carcinoma revealed a reduction in tumor progression. Investigation of the underlying mechanism revealed an abolition of tumor associated supression of macrophage function (Chu, 1988). An *in vitro* study by Lee and Jeon revealed that macrophages were stimulated to produce nitric oxide by the NF-κB pathway (Lee & Jeon, 2005). The effects of the polysaccharides of Astragalus were evaluated on antibody levels in both normal as well as immunocompromised rats (Zhao *et al.*, 1990). T-dependent antigens were used in this study. The results revealed elevated antibody levels and an increase in T helper cell activity. This implied that Astragalus polysaccharide stimulated both the cell-mediated as well as the humoral arm of the immune system. Another study by Chu *et al.* (1988) revealed a similar mechanism of action. In this study, administration of the partially purified fraction (F3) of Astragalus in mice resulted in rejection of grafts due to a heightened response of the immune system. Further, Astragalus extracts have also demonstrated stimulatory

effects on T helper and T-cytotoxic cells as evidenced by the increase in expression of CD69 marker on T-cells (Brush *et al.*, 2006).

Flow cytometric analysis and confocal laser scanning microscopy was used to elucidate the effect of Astragalus polysaccharides (APS) on macrophages and B-cells (Shao *et al.*, 2004). The study revealed that mice whose toll like receptors (TLR4) were dysfunctional responded positively to Astragalus and demonstrated a significant proliferation of splenic B-cells. Further, they also enhanced the activity of macrophages.

Mice that had been dosed with Astragalus demonstrated an increase in IL-2 receptor expression on mouse splenocytes and concurrent splenocyte proliferation both *in vitro* and *in vivo*. In the same study, augmentation of the antibody response to sheep red blood cells was seen indicating that Astragalus stimulated the cell-mediated as well as the humoral arm of the immune system (Cho & Leung, 2007).

ECHINACEA

Botanical name: *Echinacea purpurea.*

Family: Asteraceae.

Common name: Echinacea, purple coneflower, American coneflower.

Among the chemical constituents of *Echinacea*, the alkamides, caffeic acid derivatives such as chicoric acid and the polyphenols are considered important for biological activity.

The polysaccharides isolated from the plant have also been shown to augment immunity (Brousseau & Miller, 2005).

Echinacea is a herbal drug used widely for the prevention and treatment of cold. Studies to elucidate its molecular mechanism of action have revealed a significant and sustained increase in circulating white blood cells, monocytes, neutrophils and natural killer cells (Goel *et al.*, 2005). This has renewed the interest in this herb as a potential immunostimulant. The ability of Echinacea and its components to alter the immune response was examined *in vitro* in a macrophage cell line (Barrett, 2003). Another study by Matthias *et al.* (2007) convincingly confirmed the macrophage stimulatory activity of Echinacea by both *in vitro* and *in vivo* methods. In a study conducted by Zhai *et al.* (2007), Echinacea has been reported to modulate both T-cell and B-cell functions. In this study, Echinacea was found to increase T-cell proliferation in the spleen and potentiate the antibody response to sheep red blood cells. Investigation of cytokine levels revealed an increase in IFN-α, IL-4 and IL-1 levels. Echinacea treated macrophages exhibited increased production of tumor necrosis factor-alpha (TNF-α) and enhanced cytotoxicity against tumor target cells WEHI 164 as well as against the intracellular parasite *Leishmania enrietti.* Immunosuppressed

mice when treated with Echinacea restored their resistance against lethal infections caused by the predominantly macrophage-dependent pathogen *Listeria monocytogenes* and predomi-nantly granulocyte-dependent *Candida albicans* (Farcas *et al.*, 2004).

Studies conducted on the alkamides of Echinacea have revealed their immunostimulant potential. The alkamides effectively stimulated alveolar macrophage function in healthy rats. They also demonstrated inhibitory activity in *in vitro* cyclooxygenase (sheep microsomes) and 5-lipoxygenase (porcine leukocytes) assays. The alkamides demonstrated a synergistic antioxidant effect when combined with cichoric acid (Thygesen, 2005). Regular intake of Echinacea has been proposed as a prophylactic against spontaneous-developing tumors since it maintains Natural Killer cells in an elevated state (Steinmuller *et al.*, 1993). These pharmacological studies have established a scientific basis for therapeutic use of this plant as an immunostimulant.

OCIMUM

Botanical name: *Ocimum sanctum.*

Family: Lamiaceae.

Common name: Tulsi, holy basil, Ajaka, brinda, manjari, parnasa, Krishna tulsi.

Ocimum is a shrub widely grown throughout the world. Numerous preclinical studies have been carried out to establish the immunostimulant property of this herb (Gupta *et al.*, 2002). The chief constituents of Ocimum include volatile oil containing chiefly eugenol and β-caryophyllene. A number of sesquiterpenes and monoterpenes namely bornyl acetate, β-elemene, methyleugenol, β-pinene, camphene and α-pinene are also present. Ursolic acid, campesterol, cholesterol, stigmasterol, β-sitosterol and methyl esters of common fatty acids are also found in the plant extracts (Umadevi, 2001).

Evaluation of the immunostimulant mechanism by Mediratta *et al.* (2002) revealed that *Ocimum sanctum* seed oil (OSSO) (3 mL/kg, *i.p.*) modulates both cell-mediated and humoral immune responsiveness. This was evidenced by the increase in antibody titre to sheep blood cells (humoral response) and elevated levels of leukocyte migration inhibitory (cell-mediated) response. The effect was seen in normal as well as immuno-compromised rats.

Studies to determine the immunostimulant potential of the plant have also been carried out in other animal models. Leaf extract of *Ocimum sanctum*, when administered intraperitoneally, stimulated both antibody response and neutrophil activity in the finfish *Oreochromis mossambicus*

(Logambal *et al.*, 2000). Dietary intake of *Ocimum sanctum* also enhanced the antibody response and disease resistance against *A. hydrophila* in the finfish (Mukherjee *et al.*, 2005). The aqueous extract of *Ocimum sanctum* treatment in bovine subclinical mastitis reduced the total bacterial count and increased neutrophil and lymphocyte counts with enhanced phagocytic activity and phagocytic index. The antioxidant and wound healing properties of Ocimum have been amply demonstrated in a study by Shetty *et al.* (2008). These activities of Ocimum complement its immunostimulant potential and further the use of the plant in immune related diseases.

GUDUCHI

Botanical name: *Tinospora cordifolia.*

Family: Menispermaceae.

Common name: Giloya, Guduchi, Gulvel.

Guduchi is a herb widely used in the ayurvedic system of medicine as a general tonic. The constituents that have been isolated from *Tinospora cordifolia* plant belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides (Singh *et al.*, 2003). Leaves of this plant are rich in protein (11.2%) and are fairly rich in calcium and phosphorus. The polysaccharide isolated from the plant was found to consist chiefly of $1 \rightarrow 4$ linked glucan with occasionally branched points (Desai *et al.*, 2002). The dry stem crude extract contains a polyclonal B-cell mitogen which is chemically a polysaccharide (Thatte *et al.*, 1992).

The anti-infective potential of *Tinospora cordifolia* was confirmed in a study wherein the plant was found to protect rats against mixed bacterial abdominal sepsis and mice against *E. coli* induced peritonitis (Thatte *et al.*, 1992). Cyclophosphamide induced myelosuppression was also overcome by *Tinospora cordifolia* in mice (Nayamalli *et al.*, 1986). Nair *et al.* (2004) isolated and evaluated polysaccharides from *Tinospora* for their immunostimulant activity in rats. Their study revealed a significant elevation in the levels of IL-1, IL-6, IL-18, IFN-γ and TNF-α. This cytokine profile confirmed the activation of Th2 pathway of cell-mediated immunity. The polysaccharides were also responsible for the activation of natural killer cells and B-cells. They also demonstrated a dose-dependent killing of tumor cells *in vitro.* In another study, rats whose immune system was suppressed by administration of carbon tetrachloride demonstrated a reduction in phagocytic activity and chemotactic migration of the neutrophils. This was successfully reversed by oral administration of Tinospora extract (Bishayi *et al.*, 2002). Also a significant increment in the functioning capacity of rat peritoneal macrophages was seen. The anti-infective potential of *Tinospora cordifolia* was also confirmed in a study wherein mice pretreated with the

polysaccharide fraction of the stem were protected against lipopolysaccharide induced mortality (Desai *et al.*, 2007). The underlying mechanism was an increase in serum IL-1, IL-6 and IFN-γ levels and a reduction in IL-10 levels. Increase in nitric oxide production by macrophages was also evident. Administration of two bioactives isolated from the plant, syringin and cordiol demonstrated significant increase in serum IgG levels in rats (Kapil & Sharma, 1997). Tinospora was found to contribute to immunostimulant activity in a multicomponent herbal mixture when evaluated for experimental amoebic liver abscess in golden hamsters and in immunomodulation studies. The herbal formulation demonstrated enhanced humoral immunity as evidenced by the elevated haemagglutination titre. Additionally, leukocyte migration inhibition (LMI) tests revealed that cell-mediated immune response was also stimulated (Sohni & Bhatt, 1996).

The antitumor activity of Tinospora was evaluated by Thippeswamy and Salimath (2007). The hexane fraction of Tinospora induced apoptosis in tumor cells implanted in mice by stimulating the expression of caspase activated DNAse, an enzyme that is responsible for the destruction of cellular DNA. The anti-stress and tonic property of the plant was clinically tested and it was found that it brought about good response in children with moderate degree of behavioral disorders and mental deficit. It also significantly improved the I.Q. levels in these children (Desai *et al.*, 2002).

GARLIC

Botanical name: *Allium sativum.*

Family: Alliaceae.

Common name: garlic, lahsuna.

Allium sativum commonly known as garlic has been used throughout recorded history for both culinary and medicinal purposes. Around 200 components have been identified from garlic. However, the organosulfur compounds such as allicin, ajoenes, diallyltrisulfide and s-allylcysteine have been identified as the most potent phytoconstituents (Amagase & Milner, 1993; Patya *et al.*, 2003; Eikai, 2001).

The immunostimulant potential of the extracts and bioactives of garlic have been explored using various models. In a study conducted by Burger *et al.* (1993) the components of garlic increased the production of IL-1 and IL-2 by isolated human peripheral blood mononuclear cells. The plant bioactives also stimulated natural killer cell activity. In the psychological stress model, aged garlic extract (AGE) significantly prevented the decrease in spleen weight and restored the reduction of anti-sheep RBC hemolytic plaque-forming cells caused by the electrical stress (Hassan *et al.*, 2003). The glycoprotein isolated from garlic was found to stimulate natural killer

cell activity against K562 tumor cell line (Ludtke *et al.*, 2001). A protein fraction (F4) isolated from AGE enhanced the cytotoxicity of human peripheral blood lymphocytes (PBMC) against natural killer sensitive and resistant tumor cell lines. Allicin induced proliferation of murine splenocytes and enhanced cytotoxicity of human peripheral blood mononuclear cells (Patya *et al.*, 2003). In the murine bladder carcinoma model, garlic demonstrated significant anti-tumor activity (Riggs *et al.*, 1997). *In vitro* studies indicated that the extracts and bioactives of garlic significantly attenuated chemically induced carcinogenesis (Amagase & Milner, 1993).

AGE contains organosulfur compounds such as S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC) which have been evaluated for their hydrogen peroxide scavenging effects. A potent antioxidant activity was demonstrated by these indicating that they may be useful for the prevention of diseases associated with oxidative damage (Ichikawa *et al.*, 2006). In another study conducted on AGE, AGE was found to exert antioxidant action by scavenging reactive oxygen species (ROS), enhancing the cellular antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, and increasing glutathione in the cells (Borek, 2001). Thus the anti-tumor potential of garlic is mainly elicited by its immunostimulant action. Additionally, it has potent antioxidant activity. This indicates that garlic is a candidate for further studies leading to the elucidation of its immunostimulant properties.

MISTLETOE

Botanical name: *Viscum album* L.

Family: Loranthaceae.

Common name: mistletoe, Bird Lime, All-heal.

Mistletoe is a semi-parasitic plant that grows attached to and within the branches of a tree or shrub. The extracts and bioactives of this plant have been evaluated for their immunostimulant activity using different animal models. Tumor bearing mice when treated with mistletoe increased the circulating antibody titre. Also the survival time of skin grafts in these mice was reduced due to a potentiation of the immune response (Jurun, 1997). Studies carried out to allocate the activity of the plant extract to a class of compounds revealed that the *Viscum album* agglutinin (VAA) I and II had a cytostatic effect. VAA I and II enhanced mRNA expression and thereby enhanced the secretion of proinflammatory cytokines. They also stimulated NK cells (The Wealth of India, 1962). Another class of compounds, the β-galactoside-specific mistletoe lectin (ML-1) induced the expression of genes encoding for IL-1α, IL-1β, IL-6, TNF-α, IFN-γ, GM-CSF and IL-10. ML-1 also increased NK cytotoxicity and increased the phagocytic activity of granulocytes in cancer patients (Hajto *et al.*, 1999;

The Wealth of India, 1962). Lectins isolated from mistletoe were evaluated for their effect on macrophages *in vitro*. The study revealed an increase in the anti-tumor activity of macrophages and a potentiation of its nitric oxide producing capacity (Mossalayi, 2006).

Mistletoe extracts demonstrated normalization of the suppressed immune system when tested in rats. In conjunction with radiation therapy, it prolonged the lifespan of tumorogenic mice (Burger *et al.*, 2001). The extract was found to be active against Renca renal cell carcinoma, C8 colon 38 carcinoma and F9 testicular carcinoma (Braun *et al.*, 2002). It upregulated thymus weight and peripheral blood leukocyte counts in tumor bearing mice (Timoshenko & Gabius, 1995). Another immunostimulant mechanism reported by Schmidt *et al.* (1989) is the capacity of the mistletoe extract to stimulate chemotaxis of human polymorphonuclear granulocytes.

Recurrent respiratory infections (RRIs) in children exposed to the radioactive fallout from Chernobyl were treated with *Viscum album* (Chernyshov *et al.*, 2000). This resulted in a significant reduction of clinical symptoms. Investigation of the mechanism revealed a normalization of reduced immune indices (Hajto, 1986). Studies carried out in breast cancer patients who had been administered *Viscum album* revealed an increase in neutrophils count and enhanced phagocytic activity. In addition an increase in NK count and ADCC activity and augmentation of lymphocyte counts was also reported (Hajto *et al.*, 1999). These studies reveal that the mechanism underlying the anti-tumor and anti-infective potential of mistletoe is stimulation of the immune system.

NONI

Botanical name: *Morinda citrifolia* L.

Family: Rubiaceae.

Common name: Morinda, noni, Indian mulberry, hog apple, Cheese fruit.

Morinda citrifolia is a fruit bearing shrub whose parts have been widely used in traditional medicine for the prophylactic and therapeutic treatment of a wide range of diseases. The fruits of *Morinda citrifolia* have been reported to contain polysaccharides, fatty acid glycosides, iridoids, anthraquinones, coumarins, flavonoids, lignans, phytosterols, carotenoids and essential oils consisting mainly of hexoic and octoic acids with small quantities of paraffin and esters of ethyl and methyl alcohols (Liu *et al.*, 2001). Phytochemical investigation of the fruit juice of *M. citrifolia* revealed the presence of two glycosides, 6-O-(β-D-glucopyranosyl)-1-O-octanoyl-β-D-glucopyranose and asperulosidic acid (Wang *et al.*, 2000). Purification of a butanol-soluble fraction of the methanol extract of *Morinda citrifolia* fruits led to the isolation of two new iridoids glycosides, 6α-hydroxyadoxoside and 6β,7β-epoxy-8-*epi*-splendoside (Takashima *et al.*, 2007). Recent studies

focusing on potential chemopreventive constituents of the fruits of *Morinda citrifolia* have led to the isolation of a new anthraquinone, 1, 5, 15-tri-O-methylmorindol and two new saccharide fatty acid esters, 2-O-(β-D-glucopyranosyl)-1-O-hexanoyl-β-glucopyranose and 2-O-(β-D-glucopyranosyl)-1-O-octanoyl-β-D-glucopyranose (Potterat & Hamburger, 2007; Dalsgaard *et al.*, 2006; Wang *et al.*, 2002).

Morinda fruits have been used traditionally by native Polynesians for the treatment of diabetes, high blood pressure, cancer, injury, arthritis, digestive distress, arteriosclerosis, pain relief and senility (Johnson *et al.*, 2002). The aqueous and ethanolic extracts of *M. citrifolia* fruit, pulp, peel and seed have demonstrated cytotoxicity against Clonetics Human Mammary Epithelial cells (HMEC) and breast carcinoma cell lines MCF-7 and MCF-7i (Hiramatsu *et al.*, 1993). In another *in vitro* study conducted by Japanese scientists, the extract of *Morinda citrifolia* was found to be the most effective in inhibiting Ras cells. Ras cells are precursors to certain types of cancer cells (Hirazumi *et al.*, 1994). Mice suffering from Lewis Lung Carcinoma (LLC) when treated with the ethanol-precipitable polysaccharide fraction of noni fruit juice were observed to survive for a longer time as compared to untreated mice. Several mediators such as tumor necrosis factor-α, (TNF-α), interleukin-1β, IL-10, IL-12 p70, interferon-γ (IFN-γ) and nitric oxide were released from the effector cells in response to the polysaccharide fraction in the above study. Suppression of IL-4 levels was also recorded. The above studies indicate that scientists have so far investigated the plant for its anti-tumor potential. However, the underlying mechanism involved is that of immunostimulation. This has opened up a world of opportunity for immunologists. A recently reported study carried out by Palu *et al.* (2008) revealed that *Morinda citrifolia* fruit juice modulates the immune system by activating cannabinoid receptors (CB_2) and elevating IFN-γ levels and inhibiting IL-4 levels. This plant has the potential for being exploited in the treatment of a host of diseases whose etiology involves the immune system.

CONCLUSIONS

At present, the current modalities for treating diseases having an immune component are symptomatic and do not address the issue of the underlying suppression of the immune system. Attempts made by scientists to unravel mechanisms underlying the anti-tumor activity of medicinal plants have led to the discovery that several plants have tremendous immunostimulant properties. The concept of 'Rasayana' is slowly being rediscovered and plant based medicines are being evaluated for their immunostimulant activity. Identification, isolation and characterization of active principles from crude extracts of the plant parts accompanied by the evaluation of the preclinical activity have gained momentum. Efforts have also been directed towards the investigation of the underlying mechanisms responsible for the

immunostimulatory effect of medicinal plants and isolated components. Discovery of newer druggable targets and cytokines that influence the immune pathways is fast gaining momentum. This will aid in the faster and more effective screening of plants and their bioactives for immunostimulant potential. The reverse pharmacology approach has provided the impetus necessary for research leading to the discovery of efficacious plant based therapeutic agents in this area. This will ultimately result in newer and better modalities for the treatment of acute and chronic infections.

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8

Balance of Pro-/Anti-Inflammatory Cytokines Release in Spleen Cells from Mice Treated with *Crotalus durissus terrificus* Venom

GARDUÑO-RAMÍREZ M.L.[2], HERNÁNDEZ CRUZ A.[1], ZUCATELLI MENDONÇA R.[3] AND PETRICEVICH V.L.[1]

ABSTRACT

Envenomation caused by Crotalus durissus terrificus is a constellation of clinical signs and symptoms resulting from excessive systemic host inflammatory response are largely mediated by cytokines. An in vitro study was conducted to examine their immunoregulatory effects on murine T-cells, including splenocyte proliferation, cytokines and NO production. The results showed that Cdt had proliferation activity and induced the cytokines and NO production in spleen cells. Significant differences were observed in the time-course of cytokine levels and Cdt induced increments of all cytokines here studied. The maximum levels of IL-2, TNF, IL-6 and IL-10 were observed 6 h post-exposition. The more pronounced levels of IL-5 were observed 24 h after exposure. The maximum levels of IL-4 and IFN-γ were observed 72 and 96 h post-exposure, respectively. NO levels peaked at 96 h. The present study also showed that it could markedly increase the production of pro-inflammatory cytokines such as IFN-γ, TNF and IL-2. High levels of anti-inflammatory cytokines IL-4, IL-5 and IL-10 were

1. Facultad de Medicina de la Universidad Autónoma del Estado de Morelos. Universidad Autónoma del Estado de Morelos (UAEM) Avenida Universidad 1001, Cuernavaca, Morelos CP. 62210, Mexico.
2. Centro de Investigaciones Químicas de la Universidad Autónoma del Estado de Morelos. Universidad Autónoma del Estado de Morelos (UAEM) Avenida Universidad 1001, Cuernavaca, Morelos CP. 62210, Mexico.
3. Laboratorio de Imunologia Viral, Instituto Butantan. Anevida Vital Brasil 1500, CEP 05555, São Paulo–Brazil.

* *Corresponding author* : E-mail : vera.petricevich@uaem.mx

also observed. The TNF/IL-10 ratios suggesting a major participation of anti-inflammatory over pro-inflammatory cytokines. All these demonstrated that Cdt could increase pro-inflammatory cytokine production, thereby shifting the pro-/anti-inflammatory balance toward an anti-inflammatory dominant phenotype. Nevertheless, a direct correlation between cytokine of pro-inflammatory (IFN-γ and TNF) and anti-inflammatory (IL-4 and IL-10) indicating a mutual participation.

Key words : Anti-inflammatory cytokines, cell number, *Crotalus durissus terrificus* venom, nitric oxide, pro-inflammatory cytokines, proliferative response

INTRODUCTION

The accidents caused by snake bites represent a serious public health problem in developing countries due to their high incidence, severity and sequelae (Kamiguti *et al.*, 1989). In Brazil, fatal cases of bites involving *Crotalus durissus terrificus* (Cdt) and its venom contains, a variety of toxic proteins, including crotoxin, crotamine, gyroxin, convulsium and thrombin-like enzyme, and produces serious complications, such as neurotoxicity, coagulation disorders, systemic myotoxicity and acute renal failure (Oshima-Franco *et al.*, 1999; Rodríguez *et al.*, 2000), with possibly additional heart and liver damage (Chippaux *et al.*, 1991; Pulido-Mendez *et al.*, 1999; Rodríguez Acosta *et al.*, 1999). The multiple organ failure represent effects in endothelial cell injury, edema formation, and an excessive systemic host inflammatory response are largely mediated by complex immunologic cascade that ensures a prompt protective response to venom in humans and experimental animals. The envenomation process is consequence of the excessive or poorly regulated immune response to the injury organism. The consequence of imbalanced immune response is caused by the released of endogenously mediators. A successful immune response agent on constructed using some of their structural components is dependent on the activation of an appropriate set of immune effectors function and load may determine the differentiation of precursor T-helper (TH0) lymphocytes into TH1 and TH2 cells. T-helper type 1 cells (TH1) and TH2 cells play important roles on the immunoregulation (Abbas *et al.*, 1991; Mosmann *et al.*, 1997). TH1 cells are characterized by the prevalent production of pro-inflammatory cytokines such as, interleukin-2 (IL-2), interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), which can potential the disease (Romagnani, 1997). By contrast, TH2 cells produce anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13, which have been associated with remissions of the disease. TH1/TH2 cytokines are responsible of the crossregulate the development and function of each subset (Cohen *et al.*, 1997). It has been reported that abnormalities in TH1/TH2 balance may account for the pathophysiology of human autoimmune diseases and in envenomation processes.

In the mechanisms that are involved in the pathogenesis of envenomation, including release of mediators such as cytokines and nitric oxide. However, their clinical significance and prognostic value have not been elucidated (Gerard *et al.*, 1993; Howard *et al.*, 1993; Jo *et al.*, 1995; Stein & Gordon, 1991; van der Meide & Schellekens, 1996). It seems that a complex network of interactions between different cytokines and possibly other components of the immune response takes place during severe infections. These are accumulating data suggesting that equilibrium between the TH1 and TH2 cytokines responses is important for the final outcome of victims with severe envenomation (Gerard *et al.*, 1993; Howard *et al.*, 1993).

The nitric oxide (NO) is another inflammatory mediator that is an important free radical serving as a second messenger in processes including neurotransmission, maintenance of vasodilator tone and arterial pressure and it has been suggested that cytokine-mediated circulatory shock is caused by activation of the inducible isorform (type II) of NOS (Kaufmann, 1993). In severe envenomations the cytokine-mediated increases in concentrations of nitrite/nitrate (Petricevich, 2004).

The purposes of the present study are: (a) to compare the spleen cell proliferation in cultures exposed to mitogenic agents and/or Cdt venom, (b) to investigate the levels of pro- and anti-inflammatory cytokines in spleen cells from mice exposed to Cdt venom and (c) to determine the ratios of pro-/anti-inflammatory in supernatant from spleen cells mice exposed to Cdt.

MATERIALS AND METHODS

Chemicals, Reagents and Buffers

Actinomycin D, orthophenyldiamine (OPD), sodium nitrate, *N-N*-(1-naphthyl) ethylene-diamine dihydrochloride), sulfanilamide, RPMI-1640 medium, fetal calf serum (FCS) were purchased from Sigma (St. Louis, Mo), murine anti-IL-6 (clones MP5-20F3 and MP5-32C11), recombinant IL-6, murine anti-IFN-γ (clones XGM1.2 and R4-6A2), recombinant IFN-γ, murine anti-IL-10 (clones JES5-16E3 and SXC-1), recombinant IL-10, murine anti-IL-4 (clones 11B11 and BVD6-24G2), recombinant IL-4, murine anti-IL-5 (clones TRFK5 and TRFK4), recombinant IL-5 were purchased from BD Biosciences Pharmingen and recombinant TNF was purchased from Boehring Mannheim (Mannheim, Germany).

Venom

Lyophilized venom of *Crotalus durissus terrificus* (Cdt) was obtained from the Laboratory of Herpetology, Instituto Butantan, São Paulo, Brazil, and stored at –20°C. The venom was dissolved in sterile physiological saline [0.85% (w/v) NaCl solution], immediately before use.

Animals

Female BALB/c mice with 13–15 g of body weight were obtained from an established colony maintained by the Bioterio of Instituto de Biotecnología (UNAM, Mexico). The animals were maintained and used under strict ethical conditions according to international recommendations for animal welfare set by Committee Members, (International Society on Toxicology, 1992).

Spleen Index (SI)

Groups of female mice with 13–15 g were injecting by intraperitoneally (*i.p.*) via with 5, 10, 15 and 20 µg of Cdt and after three h the control or injected animals were weighed, sacrificed by cervical dislocations and their spleens were removed and weighed. The splenic index was calculated as follows: [(spleen weight/body weight of treated mice)/(spleen weight/body weight of control mice)] × 100 (Hygen & Palfliet, 1985).

Spleen Cells

In brief, the spleens were pressed through a wire mesh, the red blood cells were removed by buffer lysis containing: 155 mm NH_4Cl (pH 7.4), 10 mm $NaHCO_3$ and 0.1 mm EDTA. The cells were collected and washed two times by centrifugation at 290 × g for 5 min. The cells were seeded in 96-well microtiter plates at a concentration of 5 × 10^6 cells/mL, and cultured in RPMI-1640 medium supplemented with 10% FCS and 20 mmol/L *N*-[2-hydroxyethyl]piperazine-*N'*-[2-etanesulfonic acid] (HEPES) buffer. The spleen cells were independently exposed to lypolysaccharide (LPS – 10 µg/mL), concanavalin A (ConA – 1 µg/mL) and 10 µg/mL of Cdt venom. After incubation at 37°C for 0, 6, 12, 18, 24, 48, 72, 96 and 120 h in a humidified atmosphere of 5% CO_2, the supernatants were collected and stored at –20°C until assayed for the presence of the mediators, such as nitric oxide and cytokines.

Cytotoxicity Assay

Cytotoxicity of Cdt was evaluated by conventional 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Cho, 2007). Briefly, 0.5 × 10^6 cells/mL were cultured in 96 well plates. After 2 h of incubation at 37°C with 5% CO_2 different concentrations of Cdt were added for each well and cultured for 48 h. Cell viability was measured by the MTT method.

Cell Viability and Proliferation Assay

Cell proliferation was measured by MTT assay. At 4 h prior to culture termination, 10 µl of MTT solution (10 ng/mL in phosphate buffered-saline)

was continuously cultured until termination. The reaction was stopped by addition of 15% sodium dodecyl sulphate in 1.5 N HCl into each well for solubilization of formazan and the optical density at 570 nm was measured by a microplate reader.

MEDIATORS PRODUCTION

Nitric Axide Assay

The levels of NO in supernatants from spleen cells control or exposed to LPS or ConA and/or Cdt were assayed by adding 100 µl of freshly prepared Griess reagent (Keller *et al.*, 1990) to 100 µl of the sample in 96-well plates, and then reading the absorbance at 540 nm 10 min later by comparison with the absorbance curves of serial dilutions of sodium nitrate in complete culture medium. The minimum level of NO detectable under the assay conditions was 1 nm.

Cytokines

The levels of cytokines IL-2, IL-4, IL-5, IL-6, IL-10 and IFN-γ in the supernatant from spleen cells of BALB/c mice were assayed by a two-site sandwich enzyme-like immunosorbent assay (ELISA) (Schumacher *et al.*, 1988). In brief, ELISA plates were coated with 100 µl (1 µg/mL) of the monoclonal antibodies anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-6, anti IL-10, or anti-IFN-γ in 0.1 M sodium carbonate buffer (pH 8.2) and incubated for 6 h at room temperature. The wells were then washed with 0.1% phosphate-buffered saline (PBS/Tween-20) and blocked with 100 µl of 10% fetal calf serum (FCS) in PBS for 2 h at room temperature. After washing, duplicate supernatants samples of 50 µl were added to each well. After 18 h of incubation at 4°C, the wells were washed and incubate with 100 µl (2 µg/mL) of the biotinylated monoclonal antibodies anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-6, anti-IL-10, or anti-IFN-γ as second antibodies for 45 min at room temperature. After a final wash, the reaction was developed by the addition of orthophenyldiamine (OPD) to each well. Optical densities were measured at 405 nm in a microplate reader. The cytokine content of each sample was read from a standard curve established with the appropriate recombinant cytokines and expressed in pg/mL. The minimum levels of each cytokine detectable in the conditions of the assays were 50 pg/mL for IL-2, IL-4, IL-5, IL-6 and IL-10 and 300 pg/mL for IFN-γ.

To measure the cytotoxicity of TNF present in the supernatant from spleen cells of BALB/c mice, a standard assay with L-929 cells, a fibro-blast continuous cell line was used as described previously by Ruff and Gifford (1988). The percentage cytotoxicity was calculated as follows: $(A_{control} - A_{sample}/A_{control}) \times 100$. Titres were calculated as the reciprocal of the dilution of the sample in which 50% of the cells in the monolayers were

lysed. TNF activity is expressed as pg/mL, estimated from the ratio of a 50% cytotoxic dose of the test to that of the standard mouse recombinant TNF.

Statistical Analysis

Data are expressed as the mean ± standard deviation. Statistical analyses were performed by Student *t*-test and the level of significance was set at $p<0.05$.

RESULTS

In vivo: Effect of Cdt on Spleen Index and Cell Number

In order to determine the spleen index immediately after the mouse sacrifice, the spleen was removed and its net weight was measured. As a consequence of assessments, the spleen index showed a clear correlation the spleen weight. Cdt injection caused spleen decrease, for groups injected with 10, 15 and/or 20 µg/mL of Cdt the spleen index were significantly lower when compared with those obtained for control groups ($p<0.0001$) (Fig 8.1).

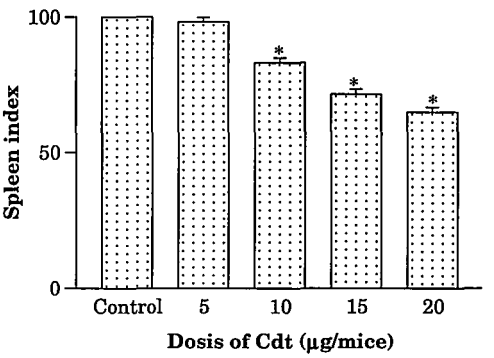

Fig 8.1. Spleen index. Groups of female BALB/c mice were injected intraperitoneously with saline solution and the other groups were injected (*i.p.*) with different amounts of Cdt by three h. The splenic index was calculated as described in Materials and Methods. Each bar represents the mean values of samples from four experiments in different groups of two mice. Statistical differences between the treatments were $p<0.0001$

The number of spleen cells from mice injected with different concentrations of Cdt by three h is illustrated in Fig 8.2. No difference was observed in cell number between control groups and groups of mice injected with 5 µg/mL of Cdt. As described in Fig 8.2 the number of spleen cells significantly diminished when the animals were injected with 10, 15 and 20 µg/mL.

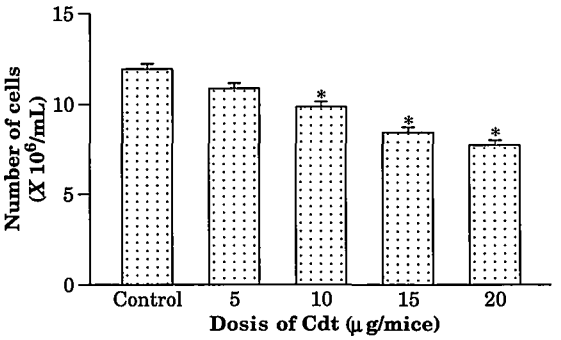

Fig 8.2. Number of spleen cells. Groups of BALB/c female mice intraperitoneously injected with different concentrations of Cdt by three h. After this time the animals were sacrificed and their spleens were removed. The spleen cells were obtained as described in Materials and Methods. The cells were counted with a hemocytometer, and viability was determined by Trypan blue exclusion. Data are reported as mean ± standard deviation values for 5 mice per group. Statistical differences between the treatments were $p<0.001$

In vitro: Effect of Cdt on Spleen Cytotoxicity and Splee Proliferation

Control groups of spleen cells and groups of cells exposed to assayed amounts of Cdt were incubated in the same conditions and, after 24 h, a dose-dependent change occurred in spleen cells exposed to Cdt, spleen cells treated with 5 and 10 µg/mL no lyses was detected, whereas 15 µg of Cdt caused around 1% of the lyses and 20 µg of Cdt caused effect cytotoxicity around 1.4% (Fig 8.3).

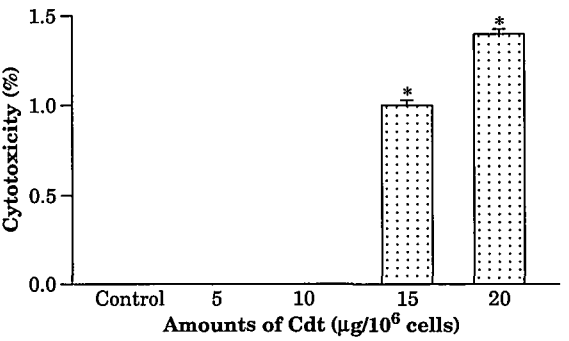

Fig 8.3. Cytotoxicity. Groups of BALB/c female mice were sacrificed and their spleens were removed and exposed *in vitro* with different concentrations of Cdt. After 24 h of incubation of cultured spleen cells, the cytotoxicity percentages were determined. Each bar represents the mean values of samples from three experiments in different groups of three mice. Statistical differences between the treatments were $p<0.0001$

To verify whether Cdt is also able to stimulate the proliferation of splenic lymphocytes harvested from mouse spleen was determined and the

proliferation, as evaluated by MTT assay. Fig 8.4 shows that the Cdt is a potent inhibitor of splenocytes proliferation when used a concentration of 15 and/or 20 µg/mL. The proliferation in these cultures was significantly reduced (over 20 and 40%, respectively).

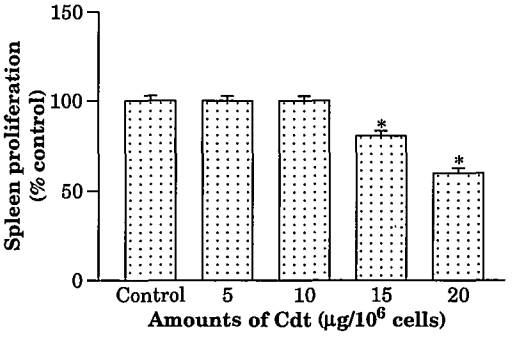

Fig 8.4. Spleen proliferation. *In vitro* proliferation of spleen cells harvested from BALB/c female mice. Samples of spleen cells were exposed to different concentrations of Cdt by 24 h at 37°C. Cell proliferation was assessed by MTT assay as described in Materials and Methods. Each bar represents the mean values of samples from three experiments in different groups of three mice. Statistical differences between the treatments were $p<0.0001$

In order to compare proliferation, mice were sacrificed and their spleen collected and stimulated in separates groups, in absence or presence of 10 µg/mL of Cdt, 10 µg/mL LPS, and 1 µg/mL of ConA. As shown in Table 8.1, the mitogenic significantly stimulated proliferative responses of mouse spleen cells induced by LPS and/or ConA. The stimulatory activity is to be greater in the ConA-treated culture than LPS-treated culture. Ten µg/mL of Cdt was able to stimulate the proliferation in mouse spleen cells, the

Table 8.1. Comparative spleen cells proliferation

H	ConA 1 µg/mL	LPS 10 µg/mL	Cdt		
			10 µg/mL	15 µg/mL	20 µg/mL
6	178 ± 8.4	143 ± 7.2	100 ± 6.0	80 ± 6.0	60 ± 6.0
12	168 ± 7.9	141 ± 6.4	100 ± 6.1	80 ± 6.1	60 ± 6.1
18	165 ± 7.6	140 ± 6.9	100 ± 5.8	75 ± 5.8	58 ± 5.8
24	160 ± 7.5	130 ± 6.6	95 ± 5.2	73 ± 5.2	50 ± 5.2
48	150 ± 6.2	125 ± 6.2	93 ± 5.1	71 ± 5.1	48 ± 5.1
72	120 ± 5.7	100 ± 5.2	90 ± 4.8	69 ± 4.8	45 ± 4.8
96	100 ± 6.3	85 ± 4.3	70 ± 3.9	65 ± 3.9	39 ± 3.9
120	80 ± 4.5	65 ± 3.6	50 ± 2.6	35 ± 2.6	30 ± 2.6

Spleen cells were harvested from BALB/c female mice, and exposed to 10, 15 and 20 µg/mL of Cdt, 10 µg/mL of LPS and/or 1 µg/mL ConA. The spleen cells were incubated by different intervals of time and the cell proliferation was assessed by MTT assay as described in Materials and Methods. Each bar represents the mean values of samples from three experiments in different groups of three mice.

maximum proliferative response was observed in cultures exposed by 6 h, decaying thereafter (Table 8.1). The proliferative response of mouse spleen cells induced by Cdt was significantly lower when compared with than those obtained in cultures stimulated with the mitogenic ($p<0.0001$) (Table 8.1).

To compare mediators production, groups of mice were sacrificed and their spleen collected and stimulated in separated groups in absence or presence of 10 µg/mL of Cdt and 1 µg/mL of ConA. The levels of pro- and anti-inflammatory cytokines released by spleen exposed to Cdt were significantly lower when compared with those obtained in groups of cells exposure to ConA (Table 8.2).

Table 8.2. Comparison of cytokine released after Cdt or ConA stimulation

Mediators 10 µg/mL	Cdt 1 µg/mL	ConA
IL-2	600 ± 30	870 ± 43.5[(b)]
IL-4	621 ± 31.1	910 ± 45.5[(b)]
IL-5	1975 ± 98.7	2870 ± 143[(b)]
IL-6	3309 ± 165.5	4810 ± 240.5[(b)]
IL-10	9266 ± 463.3	10435 ± 521.7[(a)]
TNF	3624 ± 182	5255 ± 262.7[(b)]
IFN-γ	2011 ± 100.6	2100 ± 120[(a)]
NO	21.4 ± 1.1	32 ± 1.6[(b)]

Groups of mice were sacrificed and their spleens were collected. Spleen cells were obtained and stimulated with 10 µg/mL of Cdt or 1 µg/mL of ConA, as described in Materials and Methods. The levels of IL-2, IL-6, IL-10 and TNF were determined after 6 h, for IL-5 after 18 h, for IL-4 after 72 h, and for IFN-γ and NO after 96 h. TNF levels were determined by a standard assay with L929 cells, and others cytokines were assayed by ELISA assay using antibodies as the probe. NO levels were detected by the Griess colorimetric reaction. [(a)]No significant and [(b)]$p<0.005$.

Effect of Cdt on Mediators Production

To compare the mediators production and determine the ability of Cdt to induce the production pro-, anti-inflammatory cytokines and NO, groups of mice were sacrificed and their spleen removed and exposed to 10 µg/mL of Cdt, for different time periods analyzed. As shown in Fig 8.5, the levels of NO present in supernatants of spleen cells exposed to Cdt were significantly higher than those obtained from control cultures ($p<0.0001$). The levels of NO of the spleen cells exposed to Cdt increased up to 96 h, decreasing thereafter.

The kinetics of pro-inflammatory cytokines is shown in Fig 8.6, the levels of IL-2 and TNF of the spleen cells exposed to Cdt increased up to 6 h, decreasing thereafter. The levels of IL-2 and TNF in cultures of spleen cells exposed to Cdt were significantly higher than those obtained from control cultures ($p<0.0001$). The levels of IFN-γ started to appear after 48 h in groups of spleen cells exposed to Cdt. Fig 8.6 also shows that the

more high levels of IFN-γ were observed in cultures exposed to Cdt for 96 h, decaying thereafter.

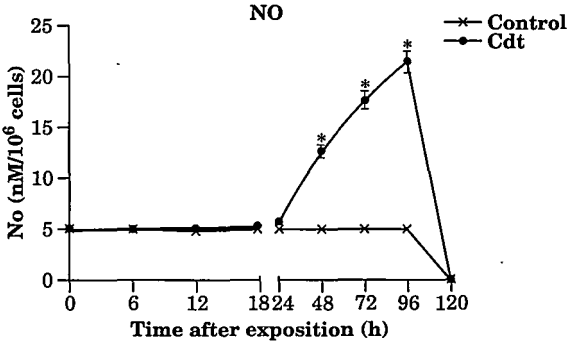

Fig 8.5. NO production. Groups of mice were sacrificed and their spleens were harvested as described in Materials and Methods. After different times the supernatants were collected and NO levels determined by the Griess colorimetric reaction. Each bar represents the mean values of samples from three experiments in different groups of three mice. Statistical differences between the treatments were $p<0.0001$

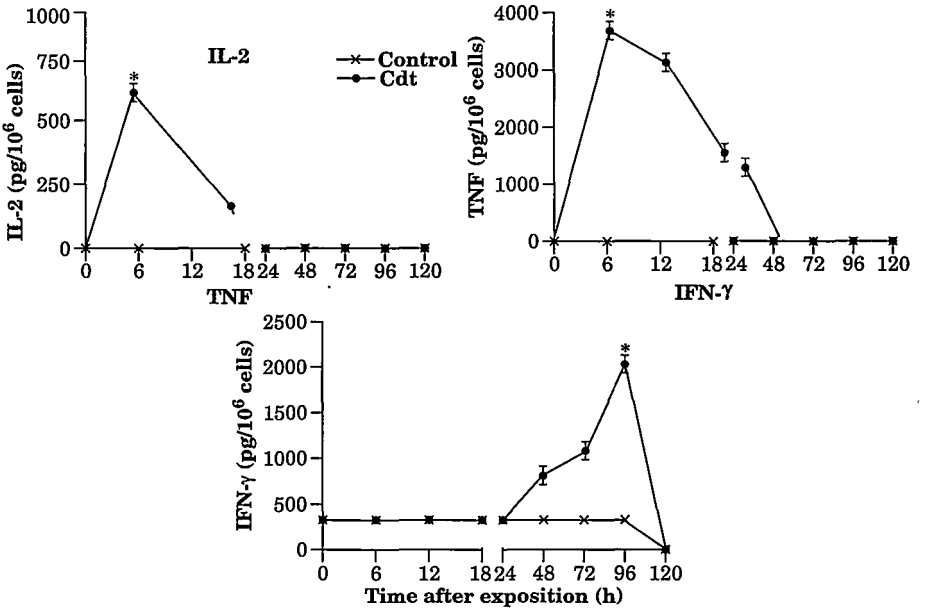

Fig 8.6. Pro-inflammatory cytokines. Groups of mice were sacrificed and their spleens were harvested as described in Materials and Methods. Spleen cells were obtained and exposed *in vitro* to 10 μg/mL of Cdt for different periods of time. IL-2 and IFN-γ were assayed by ELISA using monoclonal antibodies as the probe. TNF levels were determined by standard assay with L929 cells. Each bar represents the mean values of samples from three experiments in different groups of three mice. Statistical differences between the treatments were $p<0.0001$

The levels of anti-inflammatory cytokines are shown in Fig 8.7. The levels of IL-4 started to appear after 48 h in groups of spleen culture exposed to Cdt, and the maximum levels was observed in cultures exposed for 72 h, decaying thereafter. Fig 8.7 also shows that the levels of IL-5 started to appear after 6 h up to 24 h, decaying thereafter. The maximum production of IL-6 and IL-10 were detected for 6 h, decaying thereafter (Fig 8.7).

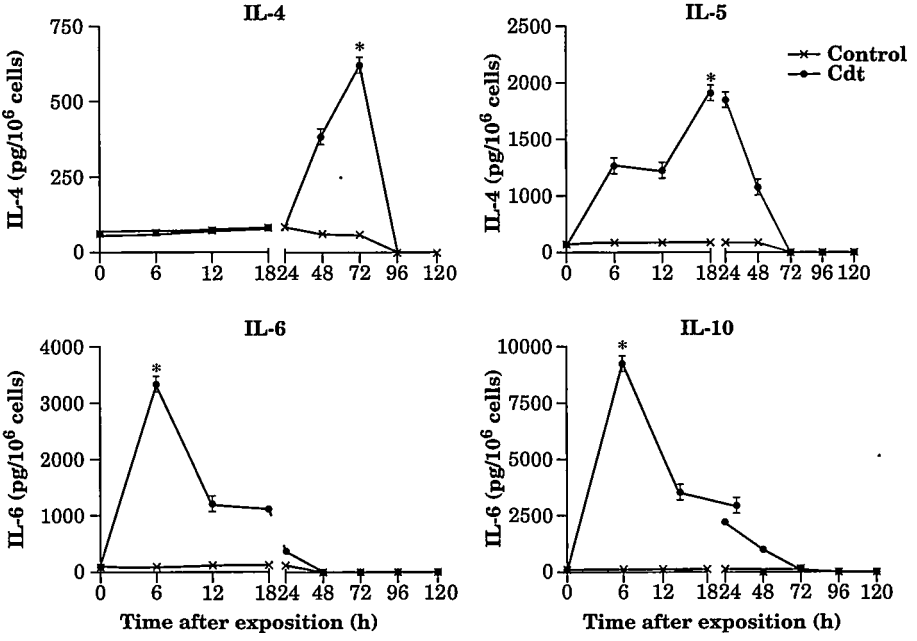

Fig 8.7. Anti-inflammatory cytokines. Groups of mice were sacrificed and their spleens were harvested as described in Materials and Methods. Spleen cells were obtained and exposed *in vitro* to 10 μg/mL of Cdt for different periods of time. After different times the supernatants were collected and the anti-inflammatory cytokines were determined by ELISA using monoclonal antibodies as the probe. Each bar represents the mean values of samples from three experiments in different groups of three mice. Statistical differences between the treatments were $p<0.0001$

Effect of Cdt on Pro-/Anti-Inflammatory Ratios

To determine the pro-/anti-inflammatory balance, the cytokines were determined in supernatant of spleen cell exposed to 10 μg/mL of Cdt for different times and the ratios were determined (Fig 8.8). Cdt induce increment in IFN-γ/IL-10 and IFN-γ/IL-4 ratios increasing up to 48 and 72 h, respectively. In contrast, spleen cells exposed to Cdt a marked decrease in TNF/IL-10 and IL-6/IL-10 ratios were observed. In these cultures exposed to Cdt the pro-/anti-inflammatory balance showed toward a anti-inflammatory dominant (Fig 8.8).

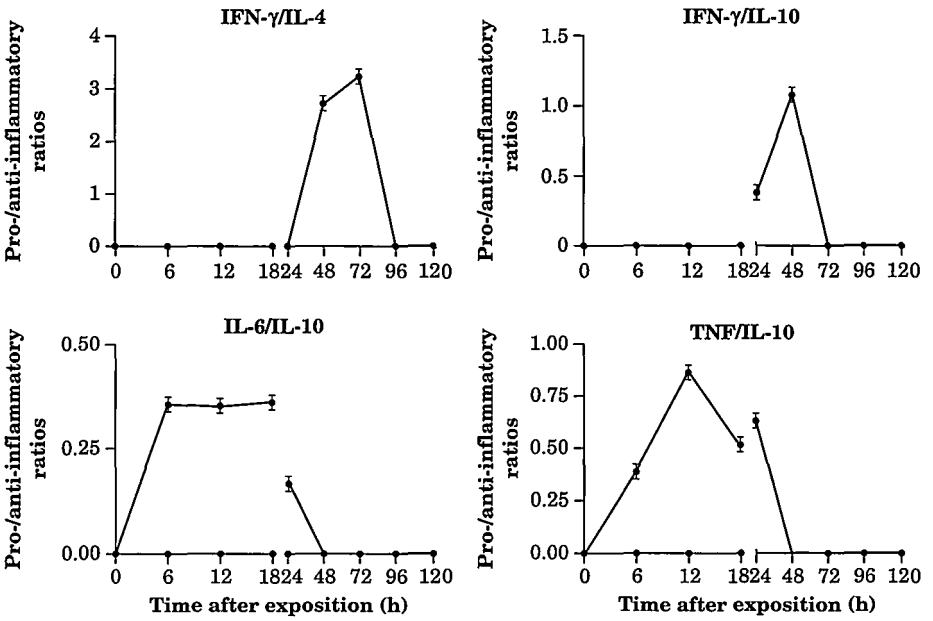

Fig 8.8. **Pro-/Anti-inflammatory ratios.** Groups of mice were sacrificed and their spleens were harvested as described above. Cytokine levels were determined as described in Materials and Methods. IFN-γ/IL-4, IFN-γ/IL-10, IL-6/IL-10 and TNF/IL-10 ratios were calculated. Each bar represents the mean values of samples from three experiments in different groups of three mice. Statistical differences between the treatments were $p<0.0001$

DISCUSSION

Crotalus durissus terrificus snake is responsible for the majority of accidents among humans in Brazil. It's venom have been shown to consist of a mixture of many toxic proteins and enzymes, with diverse and complex pharmacological effects. Despite increasing knowledge concerning the pathophysiology events following snake envenoming, the inflammatory response has scarcely been investigated.

The major secondary lymphoid organ is the spleen that contains a variety of immunocompetent cells, such as B- and T-cells. The contact of these cells with a specific antigen or mitogens induces a proliferation and differentiation into plasma cells or immunoregulatory cells (Otani *et al.*, 2007).

In order to establish optimal conditions for spleen-venom interactions, the effects of Cdt were evaluated *in vivo* and *in vitro*. For *in vivo* studies we showed that spleen index were significantly lower in mice injected with Cdt when compared with those obtained from control spleen. No difference was observed in cell number between control groups and groups of mice

injected with 5 μg/mL of Cdt. In contrast, the number of spleen cells was significantly diminished when the animals were injected with 15 and/or 20 μg/mL.

For *in vitro* studies the spleen-venom interactions, the effect of Cdt on cytotoxicity and proliferation percentages were studied. This study showed that 5 and/or 10 μg/mL of Cdt did not induce cytotoxicity in spleen cells from BALB/c mice. The highest cytotoxicity percentage was observed in cultures of spleen cells exposed to 15 and/or 20 μg/mL of Cdt.

LPS and ConA are B-cell and T-cell mitogens, respectively (Nakamura *et al.*, 1986). This study shows that Cdt induced percentages significantly lower of spleen proliferation when compared with those obtained from cultures exposed to LPS and/or ConA (Table 8.1). Among the spleen cultures exposed to Cdt the highest percentage of proliferation was observed in groups of cells treated with 5 and/or 10 μg/mL. Thus in subsequent experiments the spleen cells were exposed to 10 μg/mL of Cdt were used and inflammatory responses in this model were investigated.

Various studies have been revealed that most cytokines are a group of regulatory and immunomodulatory proteins involved in a number of physiological processes. The role of cytokines during inflammation is both initiation and fine-turning of the whole process: some cytokines initiate and amplify the response, others sustain or attenuate it, and some of them cause it to resolve. Cytokines are direct mediators of inflammation and influence the progress and direction of many immunological reactions (Romagnani, 1994). They may be divided into pro-inflammatory cytokines such TNF, IL-1, IL-6 and IL-8, that include the mobilizing immune system cells to proliferate and produce more cytokines creating an inflammatory cascade (Akira *et al.*, 1990; Beutler, 1995; Dinarello, 1991), and as anti-inflammatory cytokines, *e.g.* IL-10 which function to dampen or control the inflammatory response (Petricevich, 2004).

In this study we compare the mediators production in spleen cells exposed to Cdt versus ConA. The lowest levels of all cytokines were observed in spleen cells treated with Cdt. These results agree with previous studies that carried out crotoxin that is the major neurotoxin present in *Crotalus* venom, demonstrated the activities such as immunosuppresor and immunomodulatory in experimental animals (Cardoso *et al.*, 2001; Rangel-Santos *et al.*, 2004).

Under the conditions used in the present study, we observed that the exposure of spleen cells to Cdt may alter pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ production. IL-2 and TNF-α production peaked after 6 h, decaying thereafter. The increment of TNF-α levels has also been observed by others authors (Barros *et al.*, 1998; Cardoso & Mota, 1997; Petricevich *et al.*, 2000; Petricevich, 2004; Petricevich *et al.*, 2007).

IFN-γ is a key cytokine in host defences against intracellular organism (Kaufmann, 1993). The present study shows that Cdt is capable of stimulating spleen cells to release IFN-γ. The levels of IFN-γ started to appear after 48 h up to 96 h, decaying thereafter.

IL-10 exerts anti-inflammatory effects on macrophages and dendritic cells by suppressing production of inflammatory cytokines. The present study shows a markedly increment of IL-10 production in spleen cells exposure for 6 h to Cdt. IL-10 is an anti-inflammatory cytokine that acts in part by blocking monocyte/macrophage production of inflammatory cytokines such as IL-6, TNF-α, and IL-1 (de Waal Malefyt *et al.*, 1991).

IL-4 cytokine is principally responsible for the production of IgE in mice in response to a variety of stimuli that elicit Ig class switching to the expression of this Ig class (Paul, 1987). The present study shows that Cdt has the ability to stimulate the IL-4 production that certainly is exerting a modulatory effect of host inflammatory response.

IL-5 is a cytokine that regulates hematopoiesis and inflammation (Mosmann & Coffman, 1989). In this study it shows increment levels of IL-5 in spleen cells exposure to Cdt.

NO is known to be involved in multiple biologically important reactions (Gazzinelli *et al.*, 1992; Nathan & Xie, 1994). The present study shows that the Cdt has the ability to stimulate the NO production in spleen cells exposed to Cdt. These results agree with previous reports which showed that different cells treated to a variety of snake venoms have significantly increment NO production (Petricevich, 2004; Petricevich *et al.*, 2000).

In conclusion under the conditions used for this study the cellular proliferation was studied. With respect of cytokines levels we shows that the levels of IFN-γ and TNF-α were higher in spleen cells exposure to Cdt than in control group. Nevertheless, a direct correlation between IFN-γ and TNF-α and IL-4 and IL-10 cytokines was observed in spleen cells treated with Cdt indicating a mutual pro-/anti-inflammatory participation. In brief, Cdt is regulated by both pro- and anti-inflammatory cytokine responses and the deviation of the pro-/anti-inflammatory balance toward anti-inflammatory predominant type.

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9

Bioactive Agents from Herbal Sources with Immunopharmacological Properties Abating Inflammation and Malignancy

CHAKRAVARTY ASHIM K.[1,*], MAZUMDER TAMAL[1] AND YASMIN HADIDA[1]

ABSTRACT

Herbal products remain interested in man since inception of civilization. Ayurveda in India is one of the old and most systematic compilations of uses of plants for human cure. The plants remain the source for many modern drugs; nearly about thirty percent of the worldwide sales of drugs are based on natural products. The new millennium with its developed technology has provided much opportunity for validation of the uses of natural products. Our laboratory is engaged for many years to find out efficacy of certain plant products for activating immunity and immunotherapeutic measures towards malig-nancy. In this article, we presented our findings with natural products from two different sources; rhizome of Curcuma longa L. and leaves of Eupatorium adenophorum. The product from rhizome of C. longa is commonly known as turmeric. The ethanolic turmeric extract (ETE) has been used in the study. Other laboratories mostly used the curcumin constituent of turmeric. We observed that ETE performed as a better agent in certain immunological events, such as abating delayed type hypersensitivity reactions. Turmeric extract could activate T lymphocytes and drive them all the way to cytotoxic T-cells which could effectively mount cytotoxic response against tumor cells. Turmeric extract as such could also induce apoptosis to the malignant cells. This double edged activity of turmeric qualifies itself as an effective immuno-therapeutic agent. It has also been found to be scavenger for reactive oxygen species (ROS); the far reaching consequences of this has been discussed. The activities of the

1. Immunology and Cell Biology Lab., Department of Zoology, School of Life Sciences, University of North Bengal, Siliguri 734 013, India.

* *Corresponding author* : E-mail : prof_ashim_chakravarty@rediffmail.com

ethanolic extract of leaves of Eupatorium adenophorum, a shrub prevalent at the altitude of 1400 mt of the Eastern Himalayas was also tested for its ability to activate immunocompetent cells, to subside DTH reaction and anti-tumor activity. The degree of immunopharmacological activities of Curcuma longa and Eupatorium adenophorum were compared with certain other plant extract having similar activities as reported in the literature. A discussion has been made at the end to highlight the active ingredients in the plant extracts; works in this area are yet to be completed. The present investigation intended to come up with a definitive approach to validate an herbal product with immunotherapeutic and antitumor activity.

Key words : Immunopharmacological agent, turmeric, *Eupatorium adenophorum,* T-cell activation, inflammation, malignancy

INTRODUCTION

Indigenous medicines including Ayurveda of India are getting much attention nowadays for treating various diseases. This is due to their potency and less side effect (Mitchell, 2003). During the past two decade, traditional systems of medicine have become a topic of global importance and about thirty per cent of the worldwide sales of drugs are based on natural products. Among the natural products around 80% drugs are of plant origin; their sales exceeded US$ 65 billion in 2003 worldwide. US$ 14 billion a year was just for the market of the United States (Vaidya *et al.,* 2003; Patwardhan *et al.*, 2006).

Natural product extracts of therapeutic relevance are of paramount importance as reservoirs of structural and chemical diversity. A recent report reveals that at least 120 distinct chemical substances from different plants have utility as lifesaving drugs (Patwardhan, 2003; Patwardhan *et al.,* 2006; Kala *et al.*, 2006; Chattopadhyay & Naik, 2007). This has been achieved through chemical and pharmacological screening of only 6% of the total plant species. Over 60% of currently used anti-cancer drugs are derived in one way or other from natural sources (Cragg *et al.*, 2005). These include vinblastine and vincristine isolated from the *Madagascar periwinkle*, paclitaxel (taxol) from the bark of *Taxus brevifolia* Nutt., homoharingtonine, isolated from *Cephalotaxus harringtonia* var. *drupacea*, armamentarium from *Camptotheca acuminate* Decne and many others (Newman *et al.*, 2003).

In India, Ayurveda still remains dominant compared to modern medicine, particularly for treatment of a variety of chronic disease conditions among masses, more than 75% of the population. India has about 17,000 species of higher plants, 7500 are known for medicinal uses (Shiva, 1996; Kala *et al.*, 2006). This is the highest proportion of plants known for their medical purposes in any country of the world.

The basis of the ancient wisdom of Ayurvedic medicine was that a system as complicated as the human body, could not easily be cured by single compounds, rather to reset harmony of the spirit and body by the administration of combinations of medicines. With the arrival of modern chemistry in the mid-18th century, the ability to isolate, to purify specific compounds and to synthesize them in large quantities became the foundations of the current pharmaceutical industry, *i.e.* chemistry based focus on achieving purity of single compounds. Interestingly Ayurvedic crude extracts sometime produce better results than a single isolated compound from it, the detailed discussion on it will be included in the text.

We entered in the arena of herbal medicine in search of compounds able to activate T-cells polyclonally. Concanavalin A (Con A) was found successful in stimulating the murine T-cells for cell division and cytotoxic differentiation (Chakravarty & Clark, 1977). Even the compound was found effective in *in vivo* situation (Chakravarty & Chowdhury, 1983; Chowdhury & Chakravarty, 1983). The treatment was found to generate clones of killer cells against malignant cells bearing non-self antigens particularly tumor associated antigens (TAAs) (Chakravarty, 1978). This approach was undertaken to mount tumor specific response without immunizing the host with tumor cells; it was expected that out of multiple clones activated by a polyclonal stimulator, statistically one or two would respond to TAA bearing tumor target cells.

Subsequently we were looking for bioactive substances from herbs with multiutility, such as turmeric which could stimulate immunity against malignant cells. Turmeric initially was known as anti-inflamamtory and anti-bacterial agent, was found to be stimulatory for the lymphocytes and apoptotic for the tumor cells (Chakravarty & Yasmin, 2003, 2005, 2008). Stimulation of lymphocytes by ethanolic turmeric extract (ETE) was judged in reference to blastogenesis, DNA synthesis in ^{3}H-thymidine incorporation and FACS analysis. Both Scanning and Transmission electron microscopy revealed the healthy nature of the lymphocytes and apoptotic condition of the tumor cells with the treatment of ETE. Furthermore, the total extract of turmeric (ETE) was better in activating the T-cells than commercial curcumin. The two way efficacy of turmeric, stimulation of lymphocytes against tumor target cells and directly apoptotic to the tumor cells, suggest for its use in immunotherapy of tumors and encourage for screening other herbal agents with this kind of activities.

Our institute is located at the foothill of the Eastern Himalayas which is a treasure house for many medicinal plants yet to be documented. We are also carrying out works with *Eupatorium adenophorum* and *Leucas aspera,* two local shrubs. The experimental findings with *E. adenophorum* have been highlighted in this paper. The objective and experimental protocols are similar to those used to judge the stimulation of

immunocompetent cells and their cytotoxic differentiation by the treatment of turmeric. The effects of these herbal products to control delayed type hypersensitivity is also in our agenda. In recent time much interest has been generated in reactive oxygen species (ROS) in immunoregulation, cytotoxic differentiation and apoptosis. The study of ROS (OH^-, O_2^-, H_2O_2 and NO) in normal and treated lymphocytes is a part in our investigation. The bioactive agents of our interest were tested to find their ability to quench ROS.

Up and downregulation of certain specific genes in response to the herbal products are in current focus in the laboratory. Possible correlation, if any, between the chemical structure and function of the natural compounds will be taken into consideration. Our effort is on to find out any possible relation between the function and structure of the natural compounds. This may guide biochemical screening for new immunopharmacological drug development.

MATERIALS AND METHODS

Animals

Inbred adult Swiss mice of both sexes, 8–12 weeks of age, were used for all experiments. Breeding nuclei were obtained from Indian Institute of Chemical Biology, Kolkata and are maintained with food and water *ad libitum* in our animal house.

Tumor Induction

Ehlrich ascitic carcinoma cell lines were obtained from Chittaranjan National Cancer Research Institute, Kolkata and maintained in our laboratory by serial passages.

Maintenance of Ascitic Tumor Cell Line

Tumor cells were collected from peritoneal exudates of the mice bearing ascitic tumor by aspiration with a syringe fitted with 27-gauge needle. Tumor cells were then washed twice by centrifugation with phosphate buffered saline (PBS) and resuspended in PBS at a concentration of 10^6 cells in 0.1 mL PBS and injected intraperitoneally to the mouse for induction of ascitic tumor. After every 20 days the ascitic tumor cells were transferred to new mice following the same protocol as a routine for serial passage.

Solid Tumor Induction

To induce a solid tumor, 10^6 tumor cells suspended in 0.1 mL PBS were injected subcutaneously at the base of thigh of left leg of a normal mouse.

EXTRACT PREPARATION

Turmeric

Turmeric a rhizomatous perennial herb was described as *Curcuma longa* by Linnaeus. The important constituent of *Curcuma longa* L. is curcumin a diferuloylmethane (3–4%), responsible for the yellow colour. Curcumin comprises of curcumin I (94%), curcumin II (6%) and curcumin III (0.3%). Demethoxy and bisdemethoxy derivatives of curcumin have also been isolated. In addition to this turmeric also contains protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%) and moisture (13.1%). The abundant starch is largely gelatinized. A complex acidic arabinogalactan, ukonan A, is also present. It also contains 2–7% essential oil, comprising mainly bisabolane, guaiane, and germacrane sesquiterpenes: turmerone, ar-turmerone, zingiberene, curlone, etc.; the high content of bisabolane derivatives distinguishes turmeric from other *Curcuma* species (Tomoda *et al.*, 1990).

In initial experiments, water extract of turmeric was found not much effective in terms of blastogenesis and activation of lymphocytes as much it was with alcoholic extract. We continued our experiments with alcoholic extracts.

Ethanolic Turmeric Extract (ETE) Preparation

Fresh rhizomes of turmeric (*Curcuma longa* L.) were obtained from the local market. After cleaning properly with water; 10 g of sample was crushed to a paste with mortar and pestle and 10 mL absolute alcohol was added to the paste and kept in a refrigerator at 4°C for overnight. The extract was then filtered through Whatman filter paper 1 and the filtrate was refiltered again through cellulose acetate Millipore filter paper (0.2 μm porosity, Sartorius) for sterilization and the final solution obtained was aliquoted and stored at 4°C. To ascertain the dry weight of ETE a volume of ETE in each batch was evaporated to dryness under reduced pressure (Rotary Vaccum, EYELA, Japan) at 55°C. The average dry weight of ETE was 0.435 ± 0.032 mg/mL. As suggested by our earlier study (Chakravarty & Yasmin, 2003, 2005) 25 μl dose of ETE was used in the present study. The equivalent amount of ethanol (25 μl) was used for control, and this protocol was maintained for all the experiments.

Eupatorium adenophorum

Eupatorium adenophorum Spreng. is a shrub of Astaraceae family and found in the Terai belt of the Eastern Himalayas, predominantly between 800–2050 meters of altitude. The leaves of this plant are used by some local people to treat sores, suggesting its possible role in subsiding inflammation and wound healing.

Eupatorium adenophorum Leaf Extract Preparation

Fresh leaves of the plant, *Eupatorium adenophorum* Spreng. were collected from their natural habitat at about 1400 mt high slope of the Eastern Himalayas around Kurseong, district of Darjeeling. The leaves were cleaned thoroughly with water and allowed to air dry. Ten gms of leaves were crushed to a paste with a mortar and pestle. An amount of 10 mL of absolute alcohol (ethanol) was added to the paste and kept in refrigerator for overnight. The mixture was then filtered first through Whatmann filter paper and then through cellulose acetate filter paper (0.2 µm porosity, Sartorius) for sterilization and finally stored in an airtight sterilized vial at 4°C for further use.

SEPARATION OF T LYMPHOCYTES

Lymphocytes from spleen and lymph node were separated in Ficoll and Hypaque solution (Type IV, Sigma Co., USA). The ficoll-hypaque purified lymphocytes were finally resuspended in prewarmed RPMI 1640 supplemented with 10% goat serum (Chaudhuri & Chakravarty, 1983) and the lymphocyte preparation was poured on a nylon wool fiber (Robins' Scientific Corporation, USA) column for separation of T-cells (Julius *et al.*, 1973). The columns were loaded with cell suspensions (6×10^6 lymphocytes in 1 mL) and incubated at 37°C for 1 h. Non adherent T-cells were eluted out with an excess amount of warm RPMI and re-suspended in fresh medium. The column was then filled up with chilled RPMI and further incubated in ice for 10 min. Thus Nylon wool adherent B-cells were eluted out with an excess amount of cold RPMI by agitation of the wool and then re-suspended in fresh medium. T- and B-cells were counted with the help of haemocytometer.

IN VITRO CELL CULTURE MEDIUM

The cells were suspended in RPMI-1640 or MEM (Hi-Media) supplemented with glutamine, HEPES buffer, 200 mg $NaHCO_3$/100 mL, 100 U of penicillin/mL, 100 µg/mL streptomycin, 50 µg/mL nystatin and 10% heat-inactivated sterile goat serum.

MEASURE OF *IN VITRO* BLASTOGENESIS

The transformation of T-and B-cells into blasts after *in vitro* activation by ethanolic extract of turmeric rhizome (ETE) and *E. adenophorum* leaves (EEA) were studied. Blastogenic activity of turmeric was also compared with that of Concanavalin A (Con A), a polyclonal activator. Con A type IV (Sigma Chem. Co., USA) at a concentration of 5 µg/mL was used for blastogenic transformation of murine T-cells (Chakravarty & Maitra, 1990; Chakravarty & Chaudhury, 1989). The percentage of blast was enumerated

with a haemocytometer in presence of trypan blue under the microscope fitted with micrometer in eye piece. Cells with diameter over 6 μm were considered as blast.

MEASURE OF DNA SYNTHESIS

Blast transformation is usually accompanied by DNA synthesis and cell proliferation. DNA synthesis at different hours of turmeric treatment was measured by incorporation of ^{3}H-thymidine (^{3}H-TdR) into DNA. Cells were obtained from mice injected (i.v.) earlier with turmeric extract and suspended at a concentration of 2×10^6 cells/ml in culture medium (RPMI-1640), of which 200 μl of cell suspension was aliquot in each well of a 96-well micro-culture plate. The micro-culture plate was incubated for 8 h at 37°C in humidified atmosphere containing 5% CO_2 in air in the presence of 1 μCi of ^{3}H-thymidine (Sp. Act. 18.5 Ci/mm, Board of Radiation and Isotope Technology, Mumbai) per well. At the end of the culture period, cells were harvested with a PHD Cell Harvester (Cambridge, MA) onto glass fiber filters, washed with methanol, dried and kept in Standard Scintillation vials (Beckman, USA). At the time of radioactivity count, 5 ml of scintillation fluid (6 g PPO, 0.5 g POPOP/lt of Toluene) was added into each vial. Radioactivity was counted in β scintillation counter (LS 1800 BECKMAN, USA). All assays were done in triplicate and the level of ^{3}H-TdR incorporation was expressed as counts per min.

CELL CYCLE ANALYSIS BY FACS

For cell cycle analysis, normal as well as tumor bearing mice were injected intravenously with turmeric extract and at different intervals (16 and 24 h), spleen cell and ascitic tumor cell suspensions were prepared in PBS separately. Cells were fixed overnight at 4°C in 80% ethanol. Fixed cells were then centrifuged, the supernatant was decanted off, and 0.5 μl of 500 μg/mL (Standard 250 μg/mL) RNAse A was added, followed by incubation for 45 min at 37°C. The cells were centrifuged and suspended in 0.5 mL of 69 mm ethidium bromide (a fluorochrome which stains nuclear DNA) at room temperature for 30 min. Finally the cell cycle analysis was done in fluorescence activated cell sorter (FACS, Caliber, Becton Dickinson), in which a laser beam and light detector were used to count the DNA content of single intact cells in suspension.

SCANNING ELECTRON MICROSCOPY (SEM)

Cells treated with turmeric extract *in vitro* and *in vivo* were fixed in Karnovsky fixative for 3 to 4 h at 4°C. A drop of cell suspension was taken on clean glass stubs, (approximately 18 × 18 mm) and was air dried. The

cells were washed in cocodylate buffer, followed by dehydration with an ascending grade of acetone. After dehydration the cells were dried by critical point drying method. Finally cells were coated with gold in a fine coat ion sputter (J.C.F. 1100) with electro-conducting paints. Cells were then examined and photographed under Scanning electron microscope (Leo 435 VP) at AIIMS, New Delhi.

^{51}CR-RELEASE ASSAY (CYTOTOXICITY ASSAY)

Cytotoxic ability of T lymphocytes was determined by using ^{51}Cr release assay (Chakravarty & Maitra, 1983). This assay is based on the fact that radioactive chromate ions ($^{51}Cr_3O_4^-$) diffusing into a cell are retained in the cytoplasm for a considerable period of time. This internal ^{51}Cr is released into supernatant fluid, following cell membrane damage caused by cell mediated cytotoxic response of the effector lymphocytes.

In this study, 10^7 tumor target cells (Ehlrich ascitic carcinoma cells) in 1ml suspension labeled with 200 µCi sodium chromate ($Na_2\ ^{51}CrO_4$, Sp. Activity 50 µCi/mg, BRIT, Mumbai), by incubating for 90 min at 37°C in humidified atmosphere containing 5% CO_2 in air. The tube containing the cells was shaken thrice during incubation for proper labeling and after incubation, the cells were washed three times with PBS and the number of cells was adjusted to 1×10^3 cells in 0.25 mL. These radioactive chromium labeled cells were used as target cells for cytotoxic assay.

Effector T lymphocytes were collected from mice treated earlier with 25 µl of ETE or EEA for 48 h *in vivo*. To 1 mL of effector cells, 250 µl of ^{51}Cr labeled target cell suspension was added in three different targets: effector ratios (1: 100, 1: 50 and 1: 10) and the mixture were incubated for 6 h at 37°C in humidified atmosphere containing 5% CO_2 in air.

The aliquots of 250 µl containing 10^3 target cells only were taken in the tubes for spontaneous and maximum release. In the tubes for spontaneous release of isotope from labeled target cells, no effector cell was added and instead 1 mL of culture medium was added to keep the volume same with the experimental tubes. In the set of tubes for maximum release of radioactivity from the target cells, 1 mL of distilled water (keeping the volume same with experimental tubes) was added instead of medium. After 6 h of incubation, culture tubes were centrifuged at 1000 rpm and 1 mL of supernatant was collected from each tube and the amount of ^{51}Cr released into the supernatant was assessed by using gamma-ray spectrometer (Model no. GR532A, ECIL, India). The percentage of cytotoxicity was calculated in following manner:

$$\% \text{ of Cytotoxicity} = \frac{\text{Experimental} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

DELAYED TYPE HYPERSENSITIVITY ASSAY WITH 2, 4-DINITRO-FLUORO BENZENE (DNFB)

The delayed hypersensitivity type reaction depends on overall T-lymphocyte function. Primary sensitization of the mouse was carried out by applying 0.05 mL of 0.0001% DNFB made in acetone in the right foot pad subcutaneously, and then resensitization was made with 0.05 mL of 0.000001% DNFB in the left foot pad on 8^{th} day. The extract (ETE or EEA) was administered intravenously 1 h prior to resensitization. The degree of erythema and induration was measured in terms of the diameter of the resensitized paw in cm from the ninth day onward. Size of the reaction spot was measured twice at right angles by a slide caliper and the average was taken as index for the reaction. The day of resensitization has been considered as 0 day for easy representation of the data.

One additional experiment was carried out with EEA where 5 µl of the extract or ethanol was applied topically on the resensitized paw from the 1^{st} day onward.

SEPARATION OF CD4⁺ HELPER T-CELLS FROM THE DTH MICE THROUGH MAGNETIC ASSORTED CELL SORTER (MACS) AND THEIR ESTIMATION

MACS (Miltenyi Biotech, Germany) technology is an extremely efficient magnetic separation method, for cells with specific antigenic markers. With MACS technology, cells of interest were specifically labeled with super paramagnetic MACS microbeads. Then the cells were passed through a MACS column, placed in a strong permanent magnet. The magnetically labeled cells are retained in the column and separated from the unlabeled cells, which passed through the column. After removing the column from the magnetic field, the retained cellular fraction was eluted.

Briefly, lymphocytes isolated from the spleen of DTH mice after 24 and 48 h of resensitization were suspended in PBS for separating $CD4^+$ helper T-cells. Cell suspension was then centrifuged at 1000 rpm for 10 min and the cell pellet was resuspended at a concentration of 10^7 cells in 80 µl of fresh PBS. To this cell suspension, 20 µl of $CD4^+$ (L3TH) microbeads (130-049-201, Miltenyi Biotech, Germany) were added and refrigerated for 15 min at 4 to 8°C for conjugation with specific cell. Cell suspension containing the microbeads was poured into the column. After collecting the total effluents of the unlabeled cell fractions, the magnetic separator (MS) column was removed from the separator and placed in a collection tube. PBS was pipetted onto the MS column and the magnetic labeled cells were immediately flushed out from the column by firmly pushing a plunger into the column. And thus the magnetic labeled cells were collected which were then counted with the help of haemocytometer.

EFFECT OF ETE ON TUMOR GROWTH AND SURVIVALITY OF HOSTS

Efficacy of ETE in controlling the growth of tumors was also investigated. For this, the lymphocytes in tumor bearing mice were stimulated by repeated intravenous injections as well as by oral administration with ETE (25 µl) as per following schedules:

Schedule I—Two doses of ETE was administered intravenously (i.v.) with an interval of 1 week followed by subcutaneous injection of tumor cells (10^6 cells in 0.1 mL of PBS for initiation of solid tumor) on the 4^{th} day after 2^{nd} dose of ETE.

Schedule II—Oral administration of ETE was done thrice on 0 day, 3^{rd} day and 6^{th} day, and tumor cells were injected subcutaneous on 10^{th} day.

Solid tumor growth of individual mice was measured at every 7^{th} day using a slide caliper. Size of a tumor was determined as an average of two readings at right angles by the slide caliper and expressed in cm^2. Each schedule consisted of 6 mice and the experiments were repeated thrice.

ESTIMATION OF TNF-α BY ELISA

Solid phase sandwich ELISA kit (PharMingen, USA) was utilized for the evaluation of serum TNF-α in DTH mice. Mouse TNF-α was assayed on microplates precoated with affinity purified polyclonal antibody specific for mouse TNF-α. The optical density of the plate was read at 450 nm. Concentration of TNF-α was extrapolated from the standard curve of TNF-α (Paul *et al.,* 2001).

BIOCHEMICAL ESTIMATION OF FREE RADICAL

Superoxide Scavenging Assay

Superoxide radical (O_2^-) was generated from autoxidation of hematoxylin and was detected by an increasing absorbance at 560 nm wavelength in a UV-visible spectrophotometer (ELICO, S L164). The reaction mixture contained 0.1 M phosphate buffer (pH-7.4), 0.1 mm EDTA, 50 µm hematoxylin, 25 µl of ethanolic turmeric extract (ETE)/ethanolic leaf extract of *Eupatorium adenophorum* (EEA). The final volume of the reaction mixture was adjusted to 2.5 mL by adding double distilled water (Martin *et al.*, 1987). The inhibition of autoxidation of hematoxylin in presence of extract over the control was calculated.

Hydroxyl Ion Generation

Hydroxyl radical was generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fentons' reaction) which attacks the deoxy D-ribose and a series of reaction

that eventually resulted in the formation of malonaldehyde (MDA). The reaction mixture contained 2.8 mm 2-deoxy D-ribose, 20 mm of KH_2 PO_4-KOH (pH-7.4), 100 mm $FeCl_3$, 104 µm EDTA, 1 mm H_2O_2, 1 mm ascorbic acid and 25 µl of ETE/EEA. In control, 25 µl of ethanol was added instead of the extracts. The reaction mixture was incubated at 37°C in humidified atmosphere containing 5% CO_2 in air for 1 h. Then 2 mL of thiobarbituric acid–trichloroacetic acid (TBA-TCA) reagent was added in each tube and boiled for 15 min. The color of the reaction mixture changes to a pink MDA-TBA chromogen which was finally measured at 532 nm in UV-spectrophotometer (ELICO, S L164). The level of hydroxyl radical generation in presence of extract over the control was calculated (Halliwell *et al.*, 1987).

Lipid Peroxidation

Lipid peroxidation of lymphocytes and tumor cells with the influence of turmeric extract was estimated separately, according to Miller and Aust (1989). The reaction mixture containing 1×10^6 packed cells in 0.2 M phosphate buffer pH (7.4), with 20 mm Tris-HCl, 2 mm $CuCl_2$, 10 mm ascorbic acid and 25 µl of ETE/EEA and were incubated for 1 h at 37°C in humidified atmosphere containing 5% CO_2 in air. In control, 25 µl of ethanol was added instead of the extracts. Lipid peroxidation was measured as malonaldehyde (MDA) equivalent using TBA-TCA reagent (0.375% w/v TBA, 15% w/v TCA and 0.25 N HCl). OD value was determined spectrophotometrically at 535 nm. Result of lipid peroxidation in presence of extract over the control was calculated.

Nitric Oxide Synthase (NOS) Activity

NOS activity was determined by measuring the conversion of oxyhemoglobin to methemoglobin according to Jia *et al.* (1996). Briefly 1×10^6 packed cells (lymphocytes or tumor cells) were incubated for 2 h with 50 mm Tris-HCl buffer (pH 7.4), 10 mm L-arginine, 64 mm hemoglobin, with 25 µl of ETE/EEA at 37°C in humidified atmosphere containing 5% CO_2 in air. After incubation reaction mixture was centrifuged at 1000 rpm for 5 min and the optical density of supernatant was measured in UV-spectrophoto-meter (ELICO, S L164) at 535 nm. Results of NO production were expressed as pmol of NO produced/h.

To confirm that the production of NO was actually due to the activation of NOS, a competitive inhibitor of nitric oxide synthase (NOS), 10 µm N^G methyl-L-arginine acetate ester (NAME) was added in a separate set of experimental tubes.

RESULTS AND DISCUSSION

Activities of Ethanolic Turmeric Extract

***Immunostimulatory Property*:** One immediate goal of research in cancer immunology is to come up with methodologies to enhance the body's natural defense against malignant tumors. This was an objective of our laboratory for long. And thus, we got interested in turmeric. Initially we investigated activation of immunocompetent cells in presence of turmeric which we thought would be the basis of boosting the immune system. The survival indices of lymphocytes with 25 µl of ETE was better (Chakravarty & Yasmin, 2003, 2005) and found to be stimulatory in reference to the different criteria such as *in vitro* blastogenesis, DNA synthesis and cell cycle analysis by FACS.

Blastogenic Response and DNA Synthesis of Lymphocytes with ETE Treatment

The treatment of ETE caused increase in number of blasts over the control (alcohol) with peak at 24 h (Fig 9.1a). Stimulation of blastogenesis by ETE showed better participation of T-cells in comparison to B-cells (Fig 9.1b). However the percentage of blast transformation was lower when compared with that induced by a polyclonal activator like Con A. The h related response was also different from that induced with Con A.

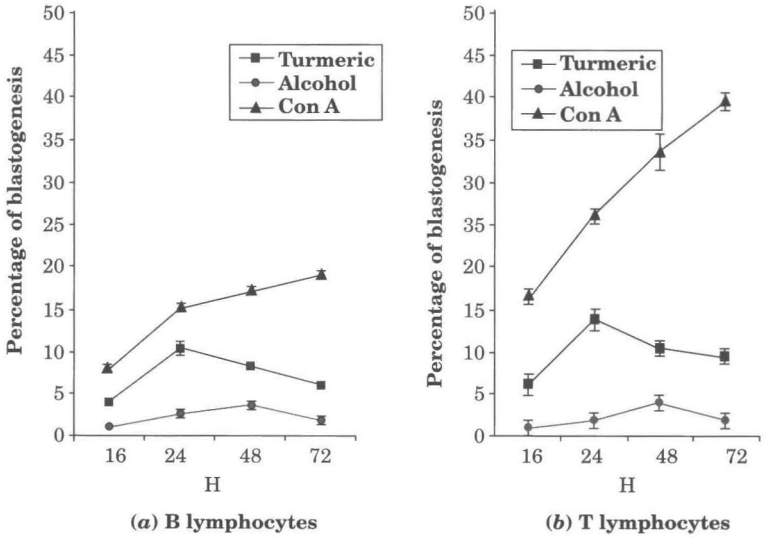


Fig 9.1. Blastogenic responses of lymphocytes from spleen treated with ETE, alcohol and Con A: (a) B lymphocytes, (b) T lymphocytes. Results are expressed as mean ± SD, Two way ANOVA revealed all the treatment values are significant compared to control ($p<0.01$)

The kinetics of DNA synthesis was also in agreement with the blastogenesis. At 24 h, the ^{3}H-TdR incorporation was higher in lymphocytes than control (Fig 9.2a). Lymphocytes from lymph node showed higher 3H-TdR incorporation (Fig 9.2b), suggesting more participation of T-cells than B-cells, as lymph node is known to harbor more of T-cells than they are in spleen.

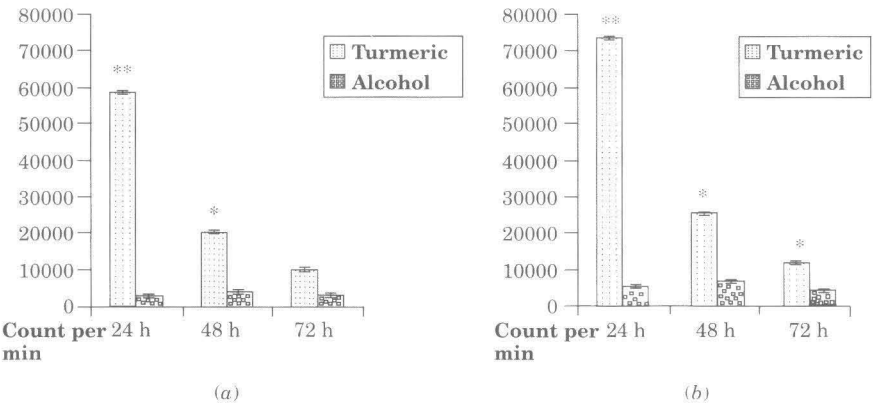


Fig 9.2. Pattern of incorporation of ^{3}H-thymidine by lymphocytes treated for different h with ETE: (a) lymphocytes from spleen, (b) lymphocytes from lymph node. Results are expressed as mean ± SD, *p<0.05 & **p<0.01 compared to respective controls

CELL CYCLE ANALYSIS WITH ETE TREATMENT

Flourescent activated cell sorter (FACS) analysis corroborated that ETE treatment had driven majority of the lymphocytes towards mitotic cycle at 24 h. The M2 peak indicating the G_0-G_1 phase showed lower percentage of lymphocytes in the ETE treated group in comparison to the control, suggesting turmeric possibly had driven the cells quickly into the next phase, *i.e.* S-phase. At G_2-M phase (*i.e.* M4 peak), ETE treated lymphocytes showed a percentage of 12.63 cells in comparison to 3.34 percentages in the control (Table 9.1 & Fig 9.3). This indicated that ETE had driven majority of the lymphocytes towards mitotic cycle.

Table 9.1. FACS analyses indicating percentage of lymphocytes at different stages of cell cycle after 24 h treatment with ETE and alcohol (control)

Cell cycle stages (Peak)	Lymphocytes (%)	
	ETE-treated	Control
Sub G_0–G_1 (M1)	0.96	2.36
G_0–G_1 (M2)	67.83	73.88
S (M3)	2.39	1.25
G_2-M (M4)	12.63	3.34

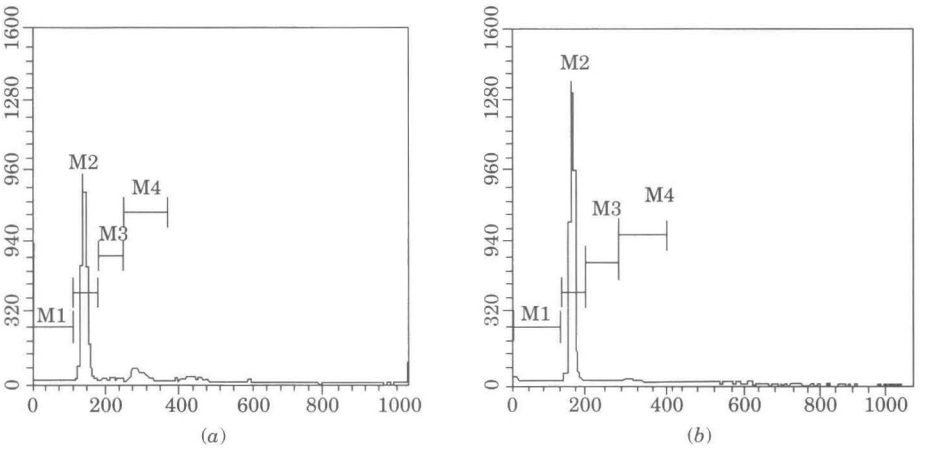


Fig 9.3. Cell cycle analysis by FACS on the basis of percentage of the lymphocytes at different stages after 24 h treatment (a) with 25 ml ETE and (b) 25 µl alcohol (control) [Cell cycle stages: M1 represents sub G_0-G_1; M2, G_0-G_1; M3, S; M4, G_2-M]

SCANNING ELECTRON MICROSCOPIC STUDY

Blastogenic response, DNA synthesis and cell cycle analysis of lymphocytes indicated stimulatory nature of ethanolic extract of turmeric. The results made us to judge the gross morphology of ETE treated cells. Observations were carried out with scanning electron microscopy.

Scanning electron micrograph of murine lymphocytes after 16 h of *in vitro* ETE treatment shows overall normal morphology of the cells and no detrimental changes in the cell surface topography (Fig 9.4).

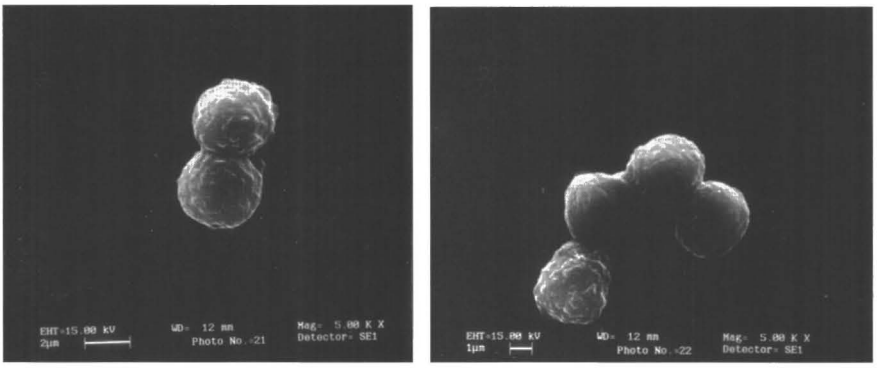


Fig 9.4. Scanning electron micrographs of murine lymphocytes from spleen after 16 h of *in vitro* treatment, (a) ETE treatment, lymphocytes showing no significant changes on its surface topography, (b) Alcohol treated (control)

AUGUMENTATION OF CELL MEDIATED IMMUNE RESPONSE

^{51}Cr-Release with ETE Treatment

In consequence of activation of T lymphocytes by ETE, as measured by DNA synthesis and division of the cells, some of the cells differentiate to effector cells capable of inducing death to the tumor target cells. ETE-treated T-lymphocytes showed significantly higher percentage of cytotoxicity towards tumor target cells than the alcohol-treated and normal lymphocytes with the three different target: effector cell ratios (1: 100, 1: 50 and 1: 10). The cytotoxicity indices with different ratios (Fig 9.5) produced a graded response, indicating ETE-generated effector T-cells performed just like the effector cells activated by specific antigen or lectins (Milas *et al.*, 1974; Purnell, *et al.*, 1975; Ray *et al.*, 1979; Waterfield & Waterfield, 1976; Chakravarty & Clark, 1977; Chakravarty & Maitra, 1983, 1990).

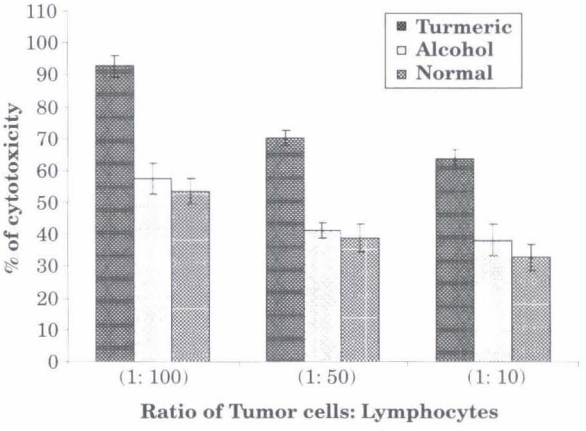


Fig 9.5. Percentage of cytotoxic response mounted by ETE treated lymphocytes against the tumor target cells at three different ratios of target: effector cells. Results are expressed as mean ± SD, Two way ANOVA revealed all the treatment values are significant compared to control ($p<0.01$)

INHIBITION OF DELAYED TYPE HYPERSENSITIVITY REACTION

Possible relation of inflammation and cancer under certain circumstances is sometimes raised (Arias *et al.*, 2007; Lotze & Finn, 1990). It is now clear that proliferation of cells alone does not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA damage promoting agents, together certainly potentiates and further promotes neoplastic risk. So in a way it can be said that tumors acts as wound that fail to heal (Dvorak, 1986). So any drug which can heal the inflammatory wound faster can act as a wonderful antidote to cure malignancy. And in this regard we found ETE to be an excellent inhibitor of delayed type hypersensitivity reaction induced in mouse paw by 2, 4 DNFB and also healed the wound very faster compared

to control (Figs 9.6, 9.7). Oliver and Nouri (1991) proposed DTH types of reactions are critical for host resistance to cancer. Inhibition of DTH response by ETE as shown by us might help it to get access in the list of anticancer agents.

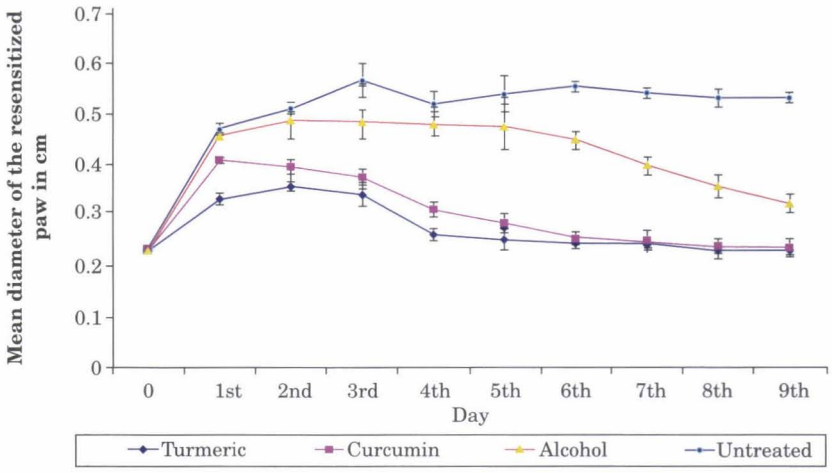


Fig 9.6. Change in paw size of DTH mice with ETE and curcumin treatment given intravenously, 1 h prior to resensitization (Day of resensitization indicated by 0); alcohol treated and untreated controls were maintained. Results are expressed as mean ± SD. Two way ANOVA revealed all the treatment values (ETE and curcumin were significant compared to controls ($p<0.01$)

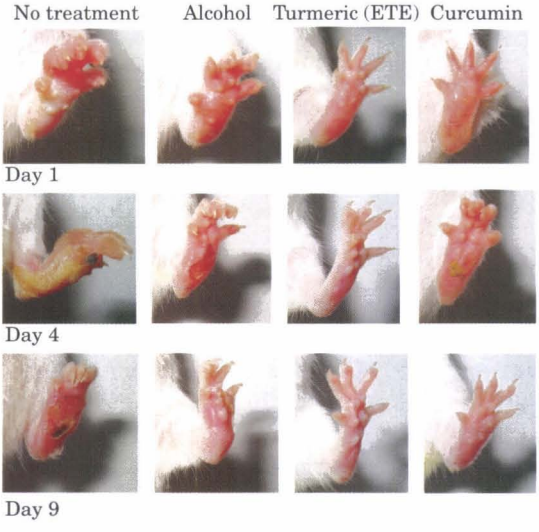


Fig 9.7. Photograph showing the DTH reaction of paw in untreated, alcohol, ETE and curcumin treated mice in course of 9-day period of study. The recovery from the reaction is notable in the cases treated with turmeric

ESTIMATION OF CD4⁺ T-CELLS DURING DTH RESPONSE

CD4⁺ T-cells are known to play significant role in terms of providing necessary cytokines during DTH response, estimation of the cells in course of the reaction was carried out with the help of Magnetic Assorted Cell Sorter (MACS). CD4⁺ T-cells in the DTH mice increased considerably over the control with ETE treatment (Fig 9.8). This raises the question about the role of ETE in inflammation. Besides several other virtues, the CD4⁺ T-cells have so far been depicted as source of pro-inflammatory cytokines, such as gamma interferon (IFG), and the interleukin-6 (Black, 2000). The ETE treatment is increasing their number but simultaneously diminishing the degree of inflammation, leading to repair and regaining normalcy. That suggests ETE must be performing some critical functions in abatement of inflammatory reactions in spite of increasing the CD4⁺ T-cell population.

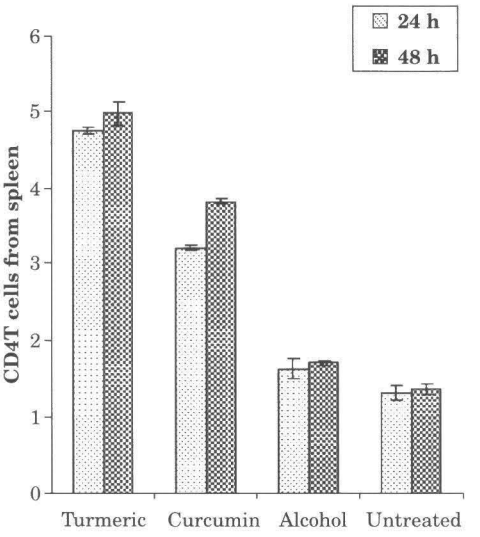


Fig 9.8. Total count of CD4⁺ T-cells (10^6 in 10^7 lymphocytes) isolated from spleen of DTH mice with ETE and curcumin treatment (10 μm) after 24 and 48 h of resensitization. Results are expressed as mean ± SD. Two way ANOVA revealed all the treatment values (ETE and curcumin) were significant compared to controls ($p<0.01$)

The estimation of TNF-α, a known proinflammatory cytokine, with the ETE treatment, was a necessary corollary from our findings so far.

INHIBITION OF TNF-α WITH ETE TREATMENT DURING DTH RESPONSE

The pleiotropic cytokine TNF-α (Mace *et al.*, 1988; Torisu *et al.*, 2000) play important roles in the immune regulation such as lymphoid cell

development, activation, cell proliferation, cell death and in pro-angiogenic activities (Rossi & Zlotnik, 2002; Balkwill & Mantovani, 2001). In the present investigation ETE could significantly inhibit TNF-α production in the DTH mice (Fig 9.9). The curcumin from commercial source produced response in ditto. Thus, inhibiting a proinflammatory cytokine might contribute in abating inflammation in mouse paws undergoing DTH reaction and helping in early recovery (Figs 9.6, 9.7).

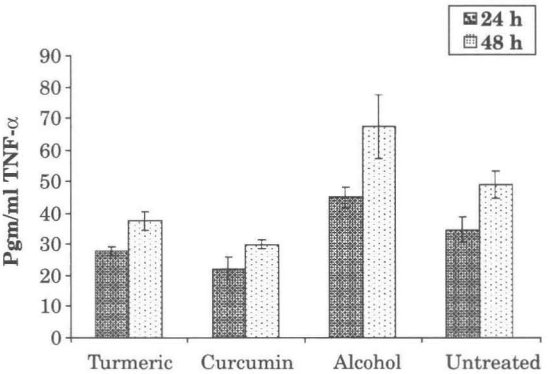


Fig 9.9. Inhibition of serum TNF-α level in pgm/mL quantity with ETE and curcumin treatment after 24 and 48 h of resensitization as judged by ELISA. Two way ANOVA revealed all the treatment values (ETE and curcumin) were significant compared to controls ($p<0.01$)

ANTITUMOR ACTIVITY

Curcumin, a diferuloyl methane, the major pigment in turmeric inhibits proliferation of a wide variety of transformed cells such as HeLa cells, (Huang *et al.*, 1997), Jurkat cells (Piwocka *et al.*, 2001), prostrate cancer cells (Mukhopadhyay *et al.*, 2001) MCF-7 cells (Henry *et al.*, 1998), AK-5 tumor cells (Khar *et al.*, 1999) and many others. Several studies in recent years have also shown the inhibitory effect of turmeric and curcumin in different experimental tumorigenic models (Huang, 1994) and it has been found to be a potent inhibitor of the initiation and promotion of chemical carcinogen (12-O tetradecanoyl-phorbol-13 acetate (TPA), 1, 2-dimethyl-hydrazine dihydrochloride (DMH), 20-methylcholanthrene, dimethyl benathracene (DMBA), benzo[a]pyrene, 7, 12-dimethylbenz [a] anthracene etc.) induced tumor formation in animals (Huang *et al.*, 1988; Kim *et al.*, 1988; Soudaminin & Kuttan, 1989; Deshpande *et al.*, 1997, 1998).

In our study the antitumor activity of ethanolic turmeric extract (ETE) on Ehlrich ascitic carcinoma cells was studied. ETE showed propensity to induce cell death in Ehlrich ascitic carcinoma cells *in vitro* as judged through ^{51}Cr release assay (Fig 9.10). Fig 9.11 shows formation of multiple apoptotic bodies of tumor cells after *in vitro* ETE treatment.

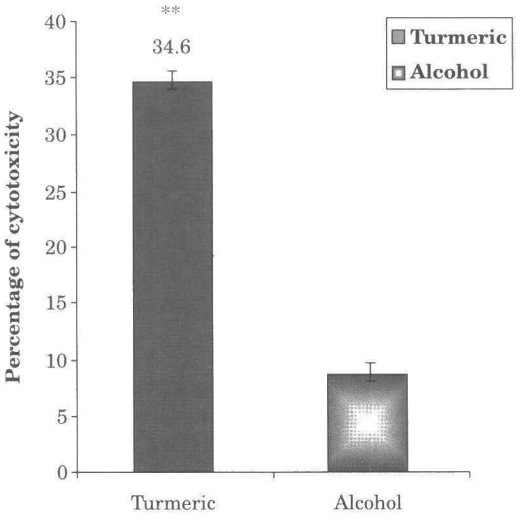


Fig 9.10. Percentage of cytotoxicity towards tumor cells after 6 h of *in vitro* ETE treatment. Results are expressed as mean ± SD, **p<0.01 compared to respective controls

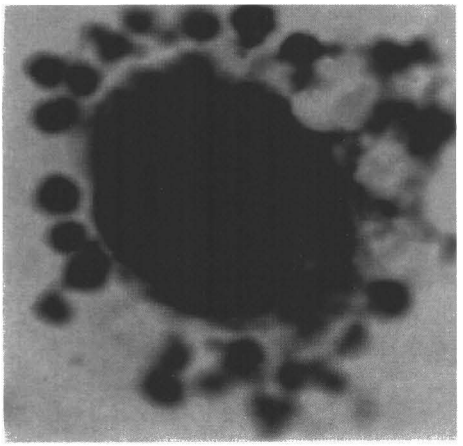


Fig 9.11. Photomicrographs of Ehlrich ascitic carcinoma cells stained with Giemsa after 16 h ETE treatment, formation of multiple apoptotic bodies

ETE showed its antiproliferative effect on tumor cells during cell cycle progression. 24 h *in vivo* ETE treatment significantly reduced the proliferation rate of tumor cells, resulting in higher percentage of tumor cells at sub G_0–G_1 phase (apoptotic phase), in comparison to the control (Table 9.2 & Fig 9.12). The comparable trend remained for the next G_0–G_1 phase. Accumulation of arrested cells in this phase and decline in the percentage of cells at S-G_2-M phase indicated that tumor cells were arrested from entering the mitotic cycle.

Table 9.2. FACS analyses indicating percentage of Ehlrich ascitic carcinoma cells at different stages of cell cycle after 24 h treatment with ETE and alcohol (control)

Cell cycle stages (Peak)	Ehlrich ascitic carcinoma (%)	
	ETE-treated	Control
Sub G_0–G_1 (M1)	5.04	0.30
G_0–G_1 (M2)	47.84	38.34
S-G_2-M (M4)	41.80	57.80

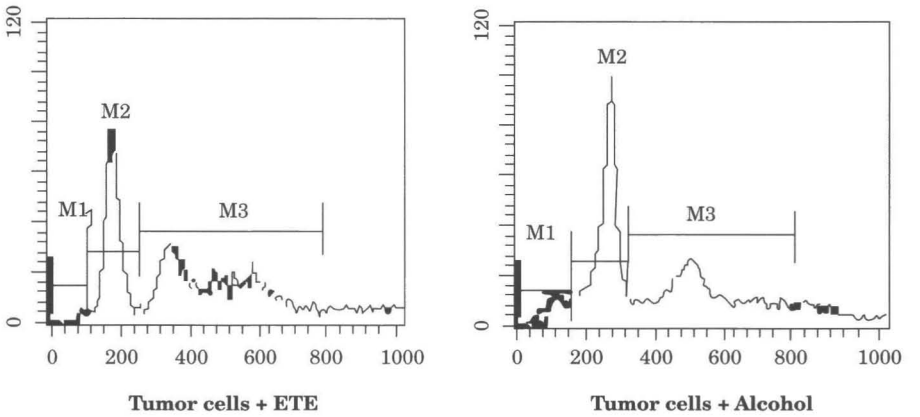


Fig 9.12. DNA histograms by FACS for cell cycle analysis of Ehlrich ascitic carcinoma cells indicating S-phase arrest. [Sub G_0–G_1 (M1); G_0–G_1 (M2); S-G2-M (M3 peak)]

SCANNING ELECTRON MICROSCOPY OF TUMOR CELLS WITH ETE TREATMENT

The process of apoptosis in tumor cells in presence of ETE has also been documented with scanning electron microscopy (Fig 9.13a). Initiation of apoptosis was within 10 min of *in vitro* ETE treatment (Fig 9.13b). The changes accentuated, and after 8 h of turmeric treatment, the blebs on cell surface became numerous and the cell volume tends to decrease indicating set in of disintegration of cell structure (Fig 9.13d). The loss of cellular organization with disintegration of plasma membrane was more pronounced at 16 h of treatment (Fig 9.13e), the state of cells indicate the final phase of apoptosis. Whereas tumor cells treated with alcohol (control) remain unchanged (Fig 9.13f).

Thus, the direct application of ETE to the tumor cells could initiate apoptosis of tumor cells. This corroborates in detail our observation with light microscope (Fig 9.11). Cytotoxic effect of direct application of ETE to tumor cells was also analyzed by using ^{51}Cr labeled tumor cells in *in vitro*

cytotoxicity assay. The results, indeed, established the cytotoxic property of ETE towards tumor cells (Fig 9.10).

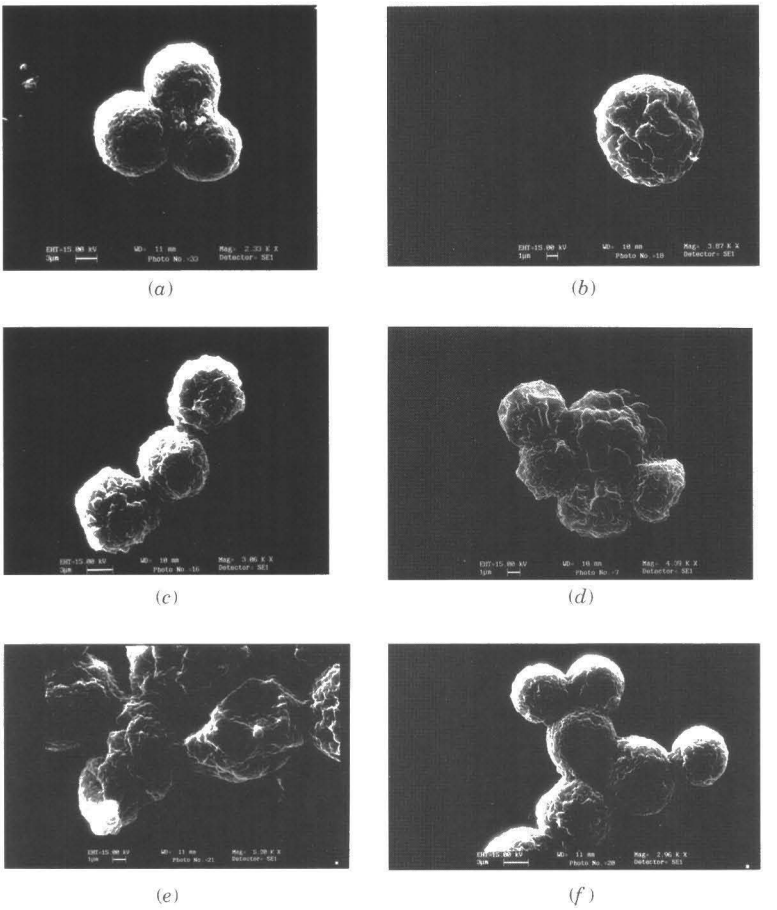


Fig 9.13. Scanning electron micrographs of Ehlrich ascitic carcinoma cells, (a) Tumor cells prior to turmeric treatment, showing ruffles distributed all over the surface; (b) after 10 min of turmeric treatment, cells showing formation of cytoplasmic blebs; (c) after 4 h of turmeric treatment, cytoplasmic blebs were broader and conspicuous; (d) After 8 h of treatment, cytoplasmic blebs, became numerous, cell volume tends to decrease; (e) after 16 h of turmeric treatment, loss of cellular organization with disintegration of plasma membrane; (f) control, after 16 h of culture tumor cells remains unchanged

INHIBITION OF TUMOR *IN SITU*

These observations so far suggested the activation of lymphocytes in the host as a poly to combat the malignant growth. *In vivo* application of ETE through intravenous and oral routes was likely to affect tumor cells by

direct means and activating cytotoxic T-cell. ETE was found to reduce the tumor growth and thereby increased longevity of the tumor bearing hosts (Fig 9.14). The results with intravenous application were superior to that with oral treatment the tumors were palpable but not measurable upto 4th week in mice receiving ETE intravenously; the survivality of these mice were upto 20th week (Fig 9.14a). Whereas tumors became palpable on 3rd week and measurable from 5th week when ETE was administered orally; the tumor bearing mice survived up to 18th week (Fig 9.14b).

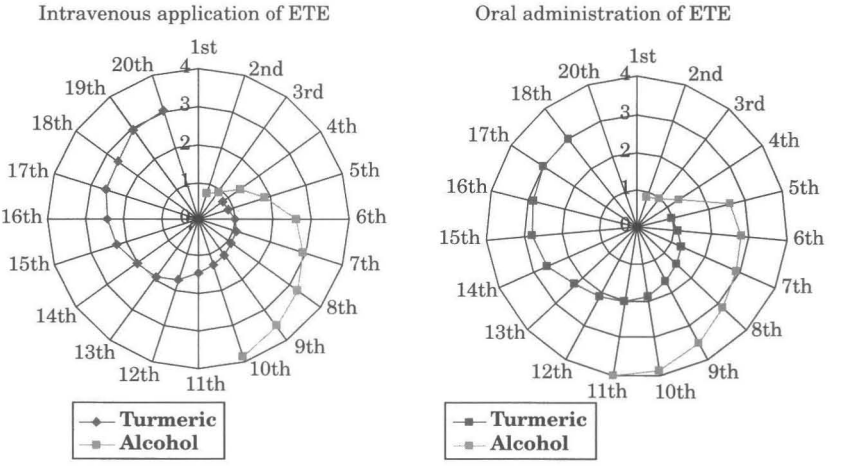


Fig 9.14. Rate of solid tumor growth (in cm^2); (a) with two intravenous injections of ETE twice before tumor induction. (b) orally administered ETE thrice (on 0, 3rd and 6th day) before tumor induction. Tumor growth was measured every week after induction; Each concentric circle denotes 1 cm^2 growth as indicated by 1–4

This could be due to poor absorbtion of curcumin by the gastrointestinal tract (Ammon & Wahl, 1991). Evidences suggest that curcumin is biotransformed in the intestinal tract of humans and rodents and its systemic availability is also poor in them (Holder *et al.*, 1978; Ravindranath & Chandrasekhara, 1982).

Suggested molecular mechanisms for this inhibition of tumor growth are varied. Liu and coworkers (1993) showed that curcumin exerts inhibition through the serine/threonine protein kinase C pathway. Reddy and Aggarwal (1994) opined inhibition through protein tyrosine kinase transduction pathways. Others considered that the antiproliferative property of curcumin is partly mediated through inhibition of c-myc, c-jun, c-fos mRNA expression and even bcl-2 mRNA expression (Kakar & Roy, 1994; Lu *et al.*, 1994; Chen & Huang, 1998).

Curcumin affects the tumor cells not only being antiproliferative, our observations showed that it can also induce apoptosis as presented in the present investigation. Curcumin induced apoptosis correlated with the

activation of caspase-3 and caspase-8 and the downregulation of the expression of anti-apoptotic proteins, Bcl-2 (Mukhopadhyay *et al.*, 2001). Sikora *et al.* (1993) found that curcumin activates the downstream caspase-9 and inhibited the matrix metalloproteinase-9 (MMP-9) enzymatic activity by inhibiting DNA binding activities of NF-κB and AP-1 transcription factors in course of induction of apoptosis. Curcumin downregulates Bcl-XL, release of cytochrome c, through blockade by N-acetylcysteine, indicating role of ROS in Caki cells' death (Woo *et al.*, 2003). Curcumin also downregulates p^{53} gene expression, by modulating intracellular Ca^{++} (Chen *et al.*, 1996).

SCAVENGING OF FREE RADICALS

One of the recognized features of curcumin is its antioxidant property. Superoxide is the most abundantly produced free radical which dismutases into molecular oxygen and hydrogen peroxide in the presence of proton. This hydrogen peroxide then induces cellular damage in the presence of ferrous ions by a Fenton reaction, resulting in the formation of OH^- free radicals and further aggravates the peroxidation of lipid membrane. It has been found in the present investigation that generation of superoxide and hydroxyl ion was inhibited by ethanolic turmeric extract (Fig 9.15). Furthermore, ETE inhibits copper-ascorbate induced lipid peroxidation effectively in lymphocytes than in tumor cells (Fig 9.16). This signifies better protection of lymphocytes from lipid peroxidation by ETE. Probably that is why survivality of lymphocytes was always better with turmeric treatment *in vitro* (Chakravarty & Yasmin, 2005).

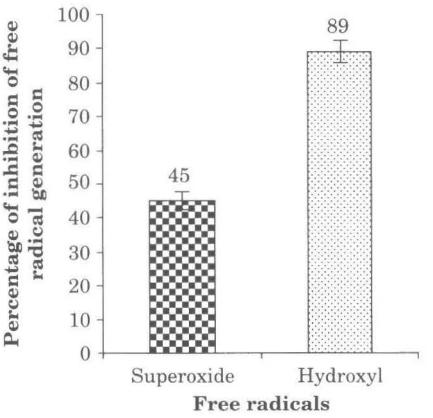


Fig 9.15. The percentage of inhibition of superoxide and hydroxyl ion generation by ETE treatment over the control (alcohol treated)

Lipid peroxidation is actually the oxidative deterioration of polyunsaturated lipids. This leads to decrease in membrane fluidity, increase the leakiness of the membrane to substances like inactive membrane-bound enzymes, Ca^{2+} etc. Turmeric, we found inhibits lipid

peroxidation by scavenging and neutralizing free radicals, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), contributing to cell membrane integrity. The phenolic and β-diketone (Tonnensen *et al.*, 1995) groups in curcumin play the role of the antioxidant.

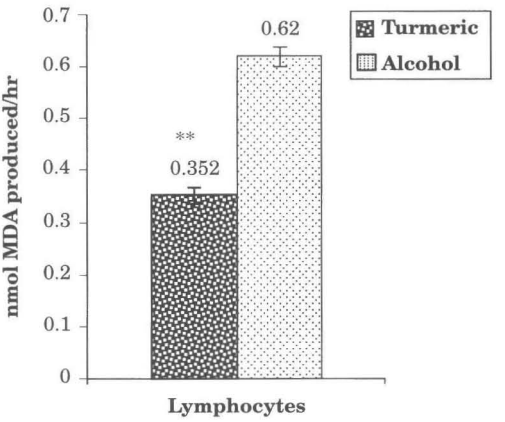


Fig 9.16. Copper ascorbate induced H_2O_2 generation in lymphocytes in presence and absence of ETE. ETE treatment caused lower production of MDA than the control. Results are expressed as mean ± SD, $^{**}p<0.01$ compared to respective controls

NITRIC OXIDE SYNTHASE ACTIVATION

NO is pleiotropic molecule and mediates diverse functions by acting in most of the body cells through interaction with different molecular targets from superoxide anion to protein macromolecules, which can either be activated or inhibited through oxidation of thiols, hemes, Fe-S clusters, and other nonheme iron prosthetic groups of macromolecules (Fleming, 1999; Ignarro, 1996; Moncado & Higgs, 1995; Furchgott & Jothianandan, 1991; Nathan, 1992; Moncado, 1999). This NO being designated as a messenger molecule of different biological functions (Bredt & Snyder, 1994; Gladwin *et al.*, 2004) can also act as protector from cytotoxicity associated with oxyradical (Ignarro, 1989). We observed L-arginine derived NO production in both lymphocytes as well as tumor cells with turmeric treatment. Lymphocytes collected from tumor bearing mice produced more NO then the normal lymphocytes (Fig 9.17). Increase in the NO production in lymphocytes possibly leads the cells towards cytotoxic differentiation (Nagy *et al.*, 2003). The higher NO level in lymphocytes from tumor bearing mice supports the contention to an extent. We showed that the tumor infiltrating lymphocytes were in a sensitized state (Das & Chakravarty, 1998). Thus, in all likelihood the lymphocytes in tumor bearing host were poised to mount response towards tumor cell and ETE hightened the response.

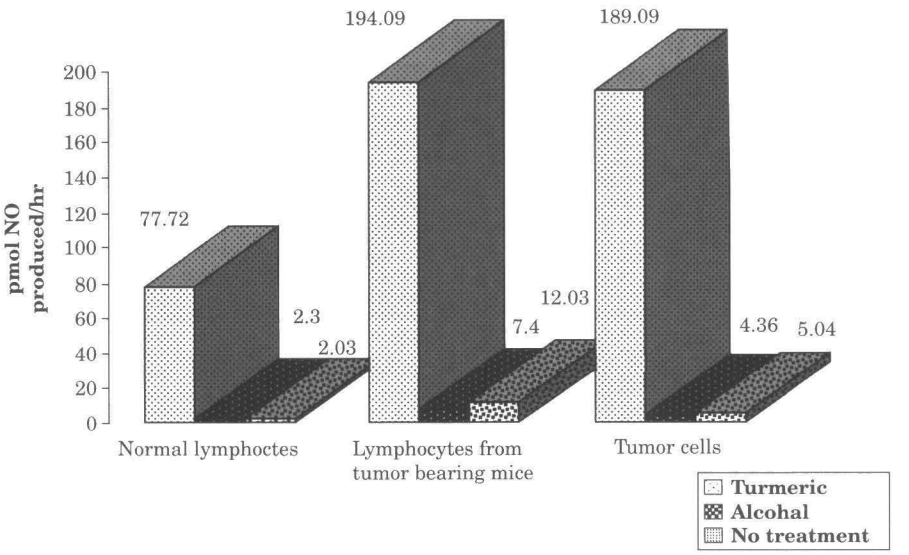
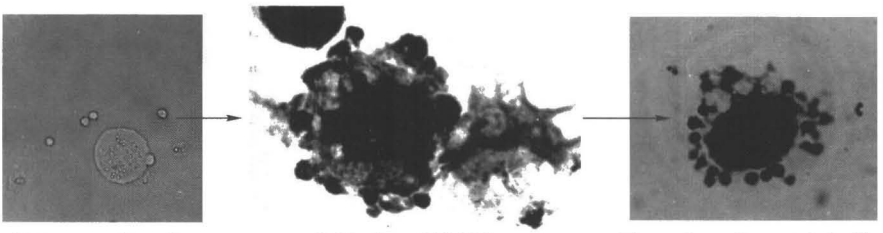


Fig 9.17. L-arginine derived NO production in both lymphocytes as well as tumor cells. Activation of NOS by ETE treatment, as suggested by higher production of NO than the control

On the other hand, L-arginine derived NO production is also critical for tumor cells (Hibbs *et al.*, 1987; Xie & Fidler, 1998; Jyothi & Khar, 1999) which was more with ETE treatment (Fig 9.17). Excessive NO results in limitation of angiogenesis and in some tumor cells increases apoptosis (Hung & Xie, 2003; Hofseth *et al.*, 2003). This is another manifestation of pleiotropic nature of NO molecule. So, it can be suggested that, increase in NO in tumor cells with ETE treatment logistically might inducing apoptosis. The phenomenon of apoptosis has already been documented in Figs 9.11 and 9.13 and ^{51}Cr release from ETE treated labeled tumor cells *in vitro* (Fig 9.10).

SUMMARY OF THE RESULTS WITH TURMERIC

- The effect of turmeric was judged at cellular level for both lymphocytes and malignant cells. Blastogenesis (Fig 9.1), DNA synthesis (Fig 9.2), cell cycle study (Fig 9.3) and cytotoxicity towards tumor cells were investigated (Fig 9.5). By all these counts turmeric seems to be stimulatory for the lymphocytes and causing cytotoxic differentiation of T-cells towards malignant cells. Ethanolic turmeric extract (ETE) itself was capable of setting apoptosis in the malignant cells (Figs 9.10, 9.11 & 9.13). Thus, turmeric plays diabolically opposite role for lymphocytes and malignant cells in murine model and in some total detrimental to tumor cells.



T-cell: tumor cell conjugate Initiation of blebbing Formation of apoptoic bodies

- The present investigation establishes that by activating cytotoxic T-cells towards tumor cells and directly setting apoptosis in the tumor cells, turmeric qualifies itself as a strong immunotherapeutic agent for cancer. The intravenous injection and oral administration of ETE could delay the tumor induction and tumor growth (Fig 9.14). Thus the life span of the tumor bearing host notably increased with ETE treatment. Although the tumor growth was affected but total elimination of tumor could not be achieved in present experimental protocols. Devising further experiments including combination of some other agents with turmeric will be attempted in future.
- Furthermore we documented the anti-inflammatory role of turmeric in 2, 4 DNFB induced DTH reaction in mouse paw (Figs 9.6, 9.7). Activation of $CD4^+$ cells (Fig 9.8) and inhibition of serum TNF-α level in DTH mice (Fig 9.9) probably allowed faster wound healing. Ethanolic turmeric extract (ETE) was better than the commercial curcumin as anti-inflammatory agent. We are comparing the efficacy of ETE and curcumin for blastogenesis and cytotoxic differentiation of lymphocytes and setting apoptosis to tumor cells. In this future project, the fractionation of ETE on thin layer chromatography (TLC) and characterization of the fractions will continue to draw equivalence of some fraction with commercial curcumin and to establish the reason of superiority of ETE over curcumin.
- Generation of free radicals by univalent reduction of O_2 is fundamental to any biochemical process and involved in cellular metabolism. Simultaneously cellular antioxidant quenches the free radicals to maintain a balance and not allow them to affect the system deleteriously. We observed a strong antioxidant role of turmeric inhibiting generation of O_2^-, H_2O_2 and OH^- (Figs 9.15, 9.16). This explains maintenance of integrity of cell membrane especially of the lymphocytes which possibly conducive for activation and differentiation of cytotoxic T-cells.
- ETE stimulates NO production in both lymphocyte & tumor cell significantly (Fig 9.17), allowing the first to be activated and induction of apoptosis for the second one.

The strong quenching and NO generating ability of turmeric allow it to be promoting for lymphocytes and apoptotic for tumor cells. The double edged effectiveness of turmeric supports further the immunotherapeutic value of turmeric.

ACTIVITIES OF ETHANOLIC EXTRACT OF LEAVES OF *E. adenophorum* (EEA)

The ethanoloic extract of the leaves of *Eupatorium adenophorum* has been tested for its efficacy in activating lymphocytes and for its anti-tumor and anti-inflammatory activities. There is sparingly one or two reports in the literature about the activity of extract from the plant. The analgesic property of the methanolic extract of leaves of *E. adenophorum* has been reported by Mandal *et al.* (2005). To screen the toxicity is a foremost requirement for introducing any use of an extract from herbal source. To begin with the hematological parameters, such as percentage of haemoglobin, RBC count and WBC count have been looked into after injecting the leaf extract in mice.

EFFECT OF EEA ON HAEMATOLOGICAL PARAMETERS

The Blood is important for pulmonary and tissue respiration, as a medium of endocrine and neurohumoral transmissions, biotransformation and metabolic excretion (Adebayo *et al.,* 2005), nutritional and immunological processes, as well as homeostatic responses (Oze, 2008). Therefore, any natural product or plant extract is subjected to test for its any toxic effect towards the hematological parameters prior to investigating any pharmacological properties (Gupta *et al.*, 1994; Iranloye, 2002; Senthilkumar, 2008). Irrespective of their target organs, the extracts are carried to their sites of action *via* the blood stream. This makes it imperative to study the influence of this extract on blood parameters. All the parameters were measured everyday upto 16^{th} day.

HAEMOGLOBIN PERCENTAGE

In course of the experiment the percentage of haemoglobin of both experimental and control mice did not show any marked variation from the normal counts on 0 day (Fig 9.18). Apparently EEA has no notable effect on haemoglobin and thus is not likely to interfere with gaseous exchange at tissue level.

RBC COUNT

The RBC count performed in course of the treatment for 16 days did not show any marked variation from normal count of 15.95×10^6 RBCs/mm^3, again indicating no apparent adverse effect to the RBCs.

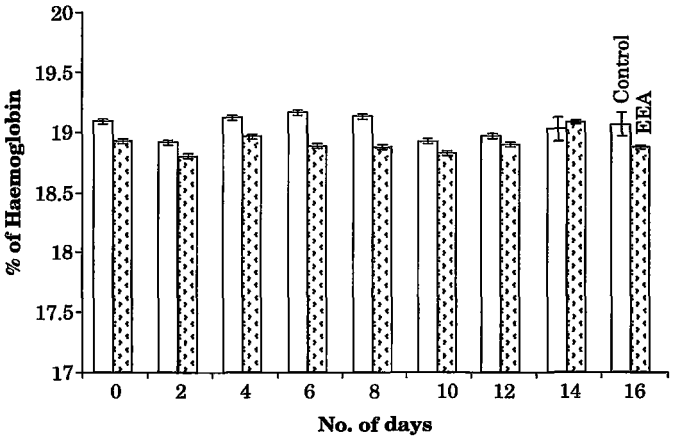


Fig 9.18. Percentage of haemoglobin in blood remains unaffected after intravenous application of EEA and ethanol

WBC COUNT

On the other hand EEA treated mice showed higher WBC count up to 10 days in comparison to the control group (Fig 9.19). For our study this was an important observation as lymphocytes carrying out the immune response belong to leukocytes.

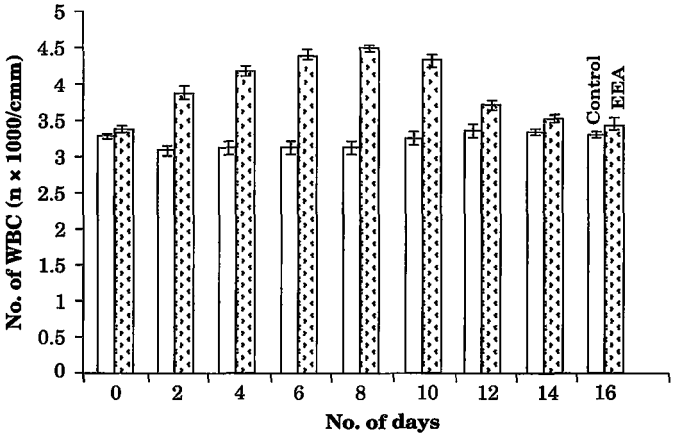


Fig 9.19. EEA promotes total count of leukocytes in blood (no./cmm) after intravenous application

IN VITRO BLASTOGENIC TRANSFORMATION OF T- AND B-CELLS

Blastogenic transformation in a lymphocyte population is normally indicative of stimulation of immune system. Hence, the quantum of blasts after treatment with extract is likely to reveal the immunostimulatory property of the extract. The blastogenic transformation of T- and B-cells has

been studied *in vitro* with both 10 and 25 µl doses of EEA. The cell types were separated by passing through nylon wool column following the standard procedure (Julius & Coworker, 1973). Cells with diameter over 6 mm were considered as blasts as per methodology standardized over the years.

EEA treatment caused blastogenic transformation of both T- and B-cells. For T-cells, the higher dose was more effective in induction of higher percentages of blasts at both 24 and 48 h. The maximum percentage (46.67%) of blasts was recorded in T-cells treated with 25 µl EEA after 48 h of treatment (Fig 9.20). The blastogenic indices with EEA treatment were slightly more for T-cells in comparison to the B-cells at both the h. The peak of blastogenic response with EEA treatment was attained at 48 h of treatment, whereas, ethanolic turmeric extract (ETE) could induce the maximum response after 24 h of treatment (Fig 9.1) for both T- and B-cells.

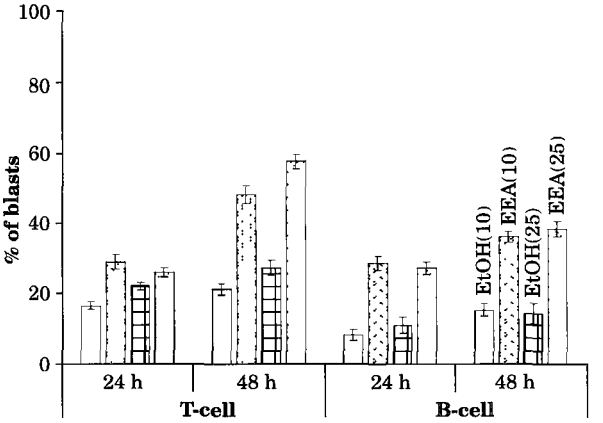


Fig 9.20. Blastogenic transformation of T- and B-cells upon *in vitro* treatment with EEA and ethanol control. Results are expressed as mean % ± SD

Currently the stimulatory role of EEA is being investigated by cell cycle analysis with FACS and ^{3}H-thymidine incorporation. The initial experiments indicate that EEA drives the lymphocytes towards S phase after 16 h of treatment and G_2-M phase after 48 h of treatment. ^{3}H-thymidine incorporation assay also shows higher level for DNA synthesis in lymphocytes with EEA treatment than the control (unpublished).

EEA INDUCES CELL MEDIATED CYTOTOXICITY TOWARDS TUMOR TARGET CELLS

^{51}Cr-release assay was performed to judge whether EEA can drive the T-cells towards cytotoxic effector ones against tumor target cells.

The effector cells from the mice treated *i.v.* with EEA elicited a higher degree of cytotoxic response in comparison to the control cells from alcohol treated mice (Fig 9.21). The degree of cytotoxicity maintained a correlation with the concentration of effector cells.

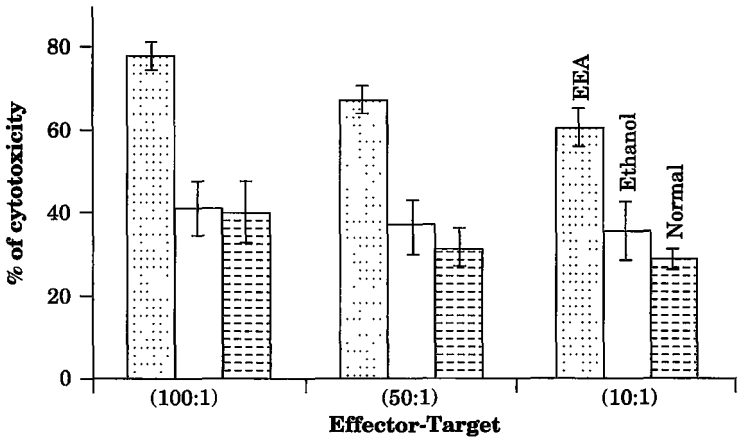


Fig 9.21. Lymphocytes from EEA treated mouse mounts cytotoxicity against tumor target cells in ^{51}Cr release assay

EFFECT OF EEA IN DELAYED TYPE HYPERSENSITIVE REACTION

Inhibition of DTH by Intra Venous Application of EEA

Intravenous application of *E. adenophorum* leaf extract before resensitizing the paw can inhibit the reaction to a good extent. In case of EEA application the swollen foot regained the normal size on 13th day when it was double in control mice (Fig 9.22).

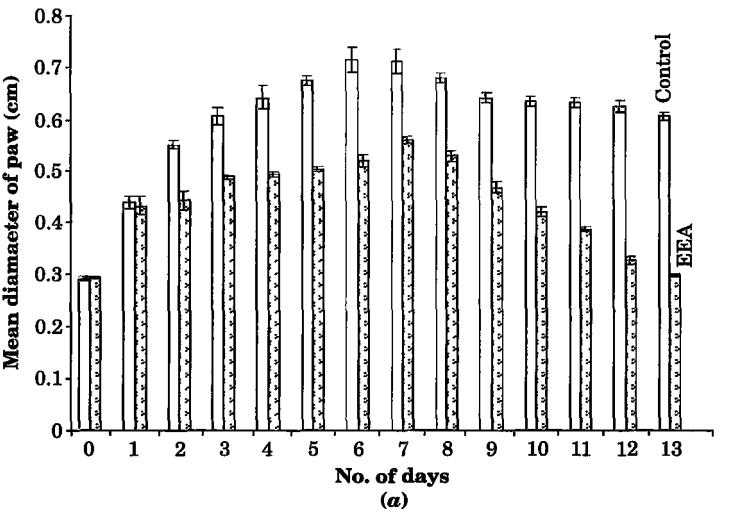
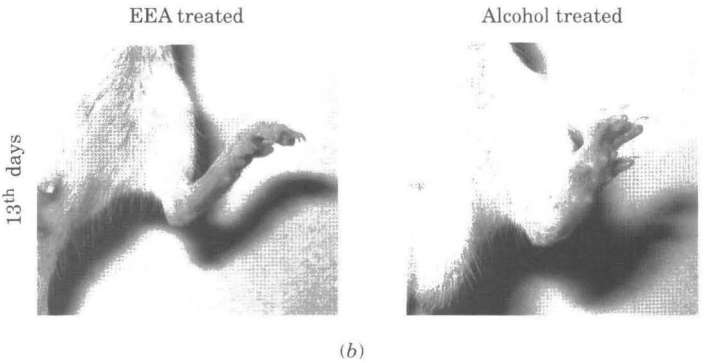


Fig 9.22. (a) Inhibition of DTH reaction by ethanolic leaf extract of *E. adenophorum* upon intravenous application. Results are expressed as mean ± SD



(*b*)

Fig 9.22. (b) Photograph showing the DTH reaction of paw in EEA and alcohol treated mice on 13th day. The recovery from the reaction is notable in the case of EEA treated mice

Inhibition of DTH by Topical Application of EEA

5 mL topical application of EEA extract everyday on the swollen foot significantly inhibited the swelling over control (Fig 9.23). The paw of treated mice became almost normal after 9 days from resensitization; the paw size in control mice was still more than double.

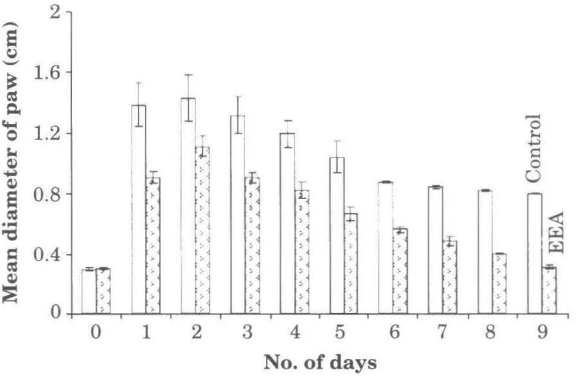


Fig 9.23. Inhibition of DTH reaction by EEA upon topical application on the resensitized swollen foot. Results are expressed as mean ± SD. Two way ANOVA revealed the EEA treatment values on 7th, 8th and 9th days were significant compared to controls ($p<0.01$)

EEA INHIBITS GENERATION OF ROS

Scavenging of Superoxide Anion

EEA was found to scavenge superoxide anions, generated from autoxidation of hematoxylin, at an average of 49.94% at 10 µl dose, whereas the same dose of ethanol in control could scavenge 19.57% only (Fig 9.24). Superoxide

is the first and most abundantly produced reactive oxygen species during the chain propagation reaction. Therefore inhibition in the generation of O_2^- by EEA may suppress the generation of other ROS as well.

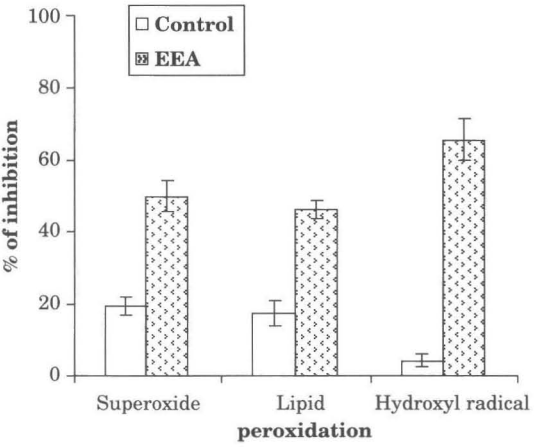


Fig 9.24. Inhibition of three deleterious ROS by EEA *in vitro*. Data have represented as percentage of inhibition ± SD calculated over no treatment samples

INHIBITION OF LIPID PEROXIDATION

EEA also could inhibit lipid peroxidation induced by copper-ascorbate system in murine lymphocytes when incubated with 10 mL of the extract. Percentage of inhibition by EEA was about 2.5 fold more than that by the same dose of ethanol in controls (Fig 9.24). EEA seems to play a protective role for the murine lymphocytes as suggested by the inhibition of lipid peroxidation by the extract.

INHIBITION OF HYDROXYL RADICAL GENERATION

A notable inhibition in generation of hydroxyl radical was also observed with EEA treatment in comparison to ethanol control (Fig 9.24). Hydroxyl radical is also a deleterious free radical and mainly leads to DNA damage. Many of the potent anti-inflammatory agents inhibit hydroxyl radical generation as well. Therefore, the inhibition of hydroxyl radical generation by EEA might have a role in course of subsiding the inflammatory swelling.

SUMMARY OF THE RESULTS WITH *E. adenophorum*

- The haematological assays apparently indicate that EEA doesn't have any deleterious effect on any of the parameters rather possibly plays some promotional role for the leukocytes, particularly inducing blastogenesis.

- The extract from leaves of *E. adenophorum* could also stimulate murine lymphocytes for cytotoxic transformation against tumor target cells as revealed by ^{51}Cr release assay. In sum total the extract is not that effective as turmeric was against tumor cells *in vitro* and tumor growth *in vivo*.
- Intravenous and topical application of the extract could inhibit delayed type hypersensitive response induced by 2, 4-dinitrofluorobenzene; indicating the anti-inflammatory property of the extract. The production of cytokines including TNF-α will be looked into for explaining the anti-inflammatory role of the substance.
- EEA has been found with anti-oxidant property towards three deleterious reactive oxygen species.

Immunopharmacological Properties of *Curcuma longa* and *Eupatorium adenophorum* Compared with Certain Other Plant Extract or Compounds

A recent estimation shows that only 5–15% of the 2,50,000 higher plant species have been tested both phytochemically and pharmacologically (Monks, 2002). Thus exploring novel therapeutic agents from rest of the plant species is a mammoth task and may take another century to compile the database. With progression of our work we tried to compare our results of anti-tumor, anti-inflammatory and anti-oxidant activities by ethanloic extract of *C. longa* rhizome (ETE) and *E. edonophorum* leaves (EEA) with other plant extracts as we have come across in the available scientific literature (Table 9.4). The effects of different plant extracts on various physiological processes have been compiled in Fig 9.25. The experimental values have been devided by control values and the ratios have been plotted as histogram. Any index ratio of 1.2 or more has been considered to have positive effect. ETE and EEA seem to have more effective anti-tumor and anti-inflammatory activities in comparison to other plant extracts (Fig 9.25). It is interesting to note the coupling of anti-tumor property with anti-inflammatory.

Table 9.4. Immunopharmacological activities of certain plant extracts

Name of plant	Part used	Active consti-tutent	Activity	Experiment conducted	Reference(s)
Curcuma longa	Rhizome	Terpenoid	Anti-tumor	Tumor regression assay	Chakravarty & Yasmin, 2003, 2005
			Anti-inflammatory	Inhibition of DTH reaction	Kim *et al.*, 1998; Khar *et al.*, 1999; Srimal & Dhawan, 1973
			Anti-oxidant	Inhibition of ROS	Brouet & Ohshima, 1995

Table 9.4. *Contd.*

Name of plant	Part used	Active constituent	Activity	Experiment conducted	Reference(s)
					Cohly *et al.*, 1998 Kunchandy & Rao, 1989, 1990; Ruby, *et al.*, 1995; Chakravarty & Yasmin, 2008
Eupatorium adenophorum	Leaves		Anti-tumor	Tumor regression assay	Present investigation
			Anti-inflammatory	Inhibition of DTH reaction	
			Anti-oxidant	Inhibition of ROS	
Hemidesmus indicus	Roots		Anti-inflammatory	Carrageenan induced mice paw edema	Atal *et al.*, 1986
			Anti-oxidant	Inhibition of ROS	Karnick, 1977
Aconium heterophyllum	Rhizome	Steroidal alkaloid	Anti-inflammatory	Carrageenan induced mice paw edema	Atal *et al.*, 1986
			Anti-oxidant	Inhibition of ROS	
Anisomeles indica		Falvone	Anti-inflammatory	Carrageenan induced mice paw edema	Dharmasiri *et al.*, 2002
			Anti-oxidant	Inhibition	Atal *et al.*, 1986
Radix glycyrrhizae			Anti-inflammatory	Carrageenan induced mice paw edema	Atal *et al.*, 1986
			Anti-oxidant	Inhibition of ROS	Singh *et al.*, 1984; Singh *et al.*, 2003
Tylophora indica	Leaves	Alkaloid	Anti-inflammatory	Carrageenan induced mice paw edema	Atal *et al.*, 1986
			Anti-oxidant	Inhibition of ROS	Shivapuri, Singhal & Prakash, 1973
Ocimum gratissimum	Leaves	Pinenes, limonene	Anti-inflammatory	Carrageenan induced mice paw edema	Atal *et al.*, 1986
			Anti-oxidant	Inhibition of ROS	

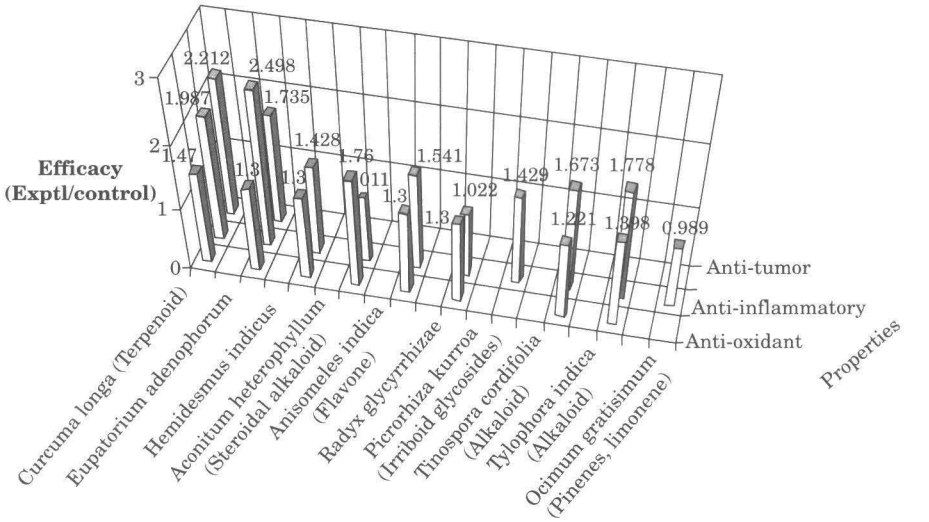


Fig 9.25. Immunopharmacological properties of ETE and EEA compared with certain other plant extracts. Indices represent the level of efficacy of the extracts as ratio over control; the indices presented at the top of the column for easy representation. Data for the natural products from the plant species other than *C. longa* and *E. adenophorum* compiled on the basis of data in published literature (indicated in Table 9.4). The chemical nature of the active substance is in the parenthesis

In course of comparing the extracts from different plants we tried to find out the active component present in the different extracts. But the report in this regard is not complete yet. Alkaloids are the effective compounds present in most of the extracts. Alkaloids such as tylophorine and tinosporine impart anti-inflammatory property to *Tylophora indica* and *Tinospora cordifolia* extract respectively (Rai & Gupta, 1966; Singh, 2003; Atal, 1986). Steroidal alkaloids, present in the extract of *Aconitum heterophyllum* and *Holarrhena antidysenterica*, also show anti-nflammatory properties. Mathew *et al.* (2004), showed that both ethanolic and petroleum ether extract of *Gentiana lutea* rhizome inhibits xylol and carrageenan induced mouse paw edema. The rhizome of the plant also has been reported to contain alkaloids along with glycosides and xanthones (Bruneton, 1995). But the mechanism of their action is not known in most of the cases except for tylophorine which inhibits degranulation of mast cells (Geetha *et al.*, 1981). We are yet to explore the chemical nature of the compound present in the ethanolic leaf extract of *E. adenophorum*. Apart from alkaloids several other compounds are also known to have anti-inflammatory properties. The active compound present in *C. longa* extract is curcumin, a terpenoid, whereas, the extract of *Anisomeles indica* contains a flavone, 5, 6-dimethoxy-7, 3′, 4′-trihydroxyflavone, as the active anti-inflammatory agent. Ineterestingly, most flavonoids from plant origin show anti-inflammatory

activity *in vitro* and *in vivo* (Middleton, 2000; Havesteen, 1983). Fisetin (3, 7, 3′, 4′-tetrahydroxyflavone), a dietary ingredient in the smoke tree (*Cotinus coggyria*) and widely distributed in fruits and vegetables, is a potent anti-inflammatory agent (Sung *et al.*, 2007; Haddad *et al.*, 2006).

REACTIVE OXYGEN SPECIES (ROS) AS TARGET OF MANY NATURAL COMPOUNDS

About 5% or more of the inhaled oxygen in body is converted to reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl (^-OH) radical by univalent reduction of O_2 (Bandyopadhyay, 1999). Thus free radicals play an important role both in health and disease. Formation of free radicals is implicated in pathophysiology of a wide range of diseases including inflammation and cancer (Fig 9.26) (Halliwell, 1997). Therefore, many natural products have been subjected for screening of their anti-oxidant properties. The natural products of plant origin provide structural diversities that far surpasses which can be synthesized in the laboratory. Moreover, natural products are small molecules (<1000 daltons) with existing drug like properties (Harvey, 1990; Wagner, 1993). Affinity and efficacy are the prime characters of a successful drug action (Clark, 1933, 1937). Affinity of a drug for its target strictly depends on the chemical nature of the drug, and a successful binding with its ligand leads to an effective drug action. Most plant products with potent immunostimulatory and immunomodulatory activities explored so far belong to terpenoids, flavonoids, isoflavonoids, alkaloids, quinones, isobutylamides and simple phenolic compounds (Wagner, 1990; Katyiar, 1983; Atal, 1986). Majority of the above mentioned compounds bear phenolic hydroxyl groups in their structure. Phenolic compounds are proton donor and e^- receiver, and thus they can quench free radicals (Tiwari, 2001) and their generation can be inhibited by neutralization of O_2^- radical, the first generated reactive oxygen species (ROS). Most of the anti-oxidant compounds isolated so far are either phenolic or polyphenolic compounds. Among the dietary polyphenols, flavonoids are the most extensively reviewed for their anti-oxidant activity (Lairon & Amiot, 1999; Chan *et al.*, 2003; Pulido *et al.*, 2000; D'Archivio, *et al.*, 2008). Silymarin, a 3-hydroxy flavone, present in *Silybum marianum* inhibits peroxide formation and protects rat liver (Gazak, 2007). Quercetin and other flavonoids are effective inhibitors of O_2^- production by cells (Kondratyuk & Pezzuto, 2004). Wegener and Fintelmann (1999) proposed that the anti-oxidant functions and enzyme modifying actions of flavonoids could account for many of their pharmacological activities.

In our laboratory we have shown turmeric can also inhibit generations of deleterious ROS such as O_2^-, H_2O_2 and OH^- (Chakravarty & Yasmin, 2007). Natural *o*-methoxyphenols of curcumin {1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dion} are not only anti-oxidants but they have also been found to inhibit oxidative stress in red blood cell

membranes (Grinberg *et al.*, 1996; Tonnesen *et al.*, 1995), induce detyoxification enzymes and seems to provide protection against degenerative diseases (Conney *et al.*, 1997). Ross *et al.* (2000) suggested that curcumin is a phenolic anti-oxidant and neutralizes the peroxyl radicals by electron delocalization. Reactive hydroxyl and alkoxyl radicals of curcumin participate in neutralization of peroxyl radicals (ROO^2). Neutralization of the peroxyl radicals breaks the chain reaction of autoxidation.

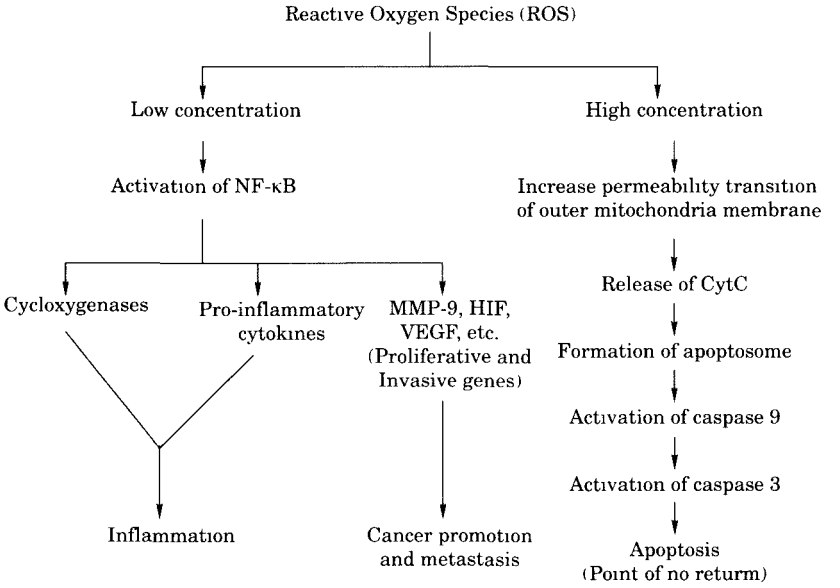


Fig 9.26. Differential activity of variable concentrations of reactive oxygen species (ROS) generated for cell fate (Arch & Thompson, 1999)

Toda and Shirataki (2002) investigated inhibitory effects of isoflavones from *Sophora moorocrotiana* on lipid peroxidation. Among the three different isoflavones from the plant, licoisoflavone B seemed to be more effective to inhibit lipid peroxidation with IC_{50} (50% inhibitory concentration) value of 2.7 μm. The other two, licoisoflavone A and sophoraisoflavone A, could inhibit lipid peroxidation in less effective manner with IC_{50} values of 7.2 and 27.0 μm respectively. The authors have demonstrated that the effective inhibition of lipid peroxidation by licoisiflavones A and B are due to presence of 22′ hydroxyl group which has stronger effects than sophoraisoflavones A. Hu *et al.* (1995) have also demonstrated that superoxide anion scavenging activities of isoflavones increase with an increasing number of hydroxyl radicals in the B ring. This can be related to the finding that the flavonoids and catechins having orthodihydroxy or phenyl group in ring A or B, or a 5, 7-dihydroxy substitution pattern as in galangin were found to be good inhibitors of

cycloxygenase (Wagner, 1993). Therefore, it seems that number and position of hydroxyl groups on a phenolic structure plays a major role in determining the efficacy of the compound as an anti-oxidant one.

Table 9.5. Some natural compounds and their active functional groups with probable mechanism of their activities

Natural compound	Functional group/ chemical nature	Immunopharma-cological properties	Probable mechanism	Reference(s)
Curcumin	Phenolic OH and β-diketone/ Terpenoid	Anti-tumor	• Suppresses NF-κB regulated gene products	Aggarwal, Kumar & Bharti, 2003
			• Activates caspases	Bharti, *et al.*, 2003
			• Downregulates p53	Lin *et al.*, 2007
			• Inhibits angiogenesis	Shishoida *et al.*, 2005
			• Suppresses AP-1	Shankar & Srivastava, 2007
			• Inhibits COX	Ireson *et al.*, 2007
		Anti-inflammatory	• Downregulates TNF	Manna *et al.*, 2000
Resveratrol	Phenolic OH/Stibene	Anti-tumor	• Suppresses NF-κB	Baur *et al.*, 2006
			• Suppresses AP-1	
			• Activates caspases	
		Anti-inflammatory	• Inhibits COX-2 and lipoxygenase	Tseng *et al.*, 2004
Celastrol	Phenolic OH/Terpenoid	Anti-tumor	• Inhibits NF-κB and related gene products	Sethi *et al.*, 2007
Fisetin	Phenolic OH/Flavone	Anti-tumor	• Inhibits NF-κB and related genes	Sung, Pandey & Aggarwal, 2007
Genistein	Phenolic OH/Isoflavone	Anti-tumor	• Inhibits NF-κB • Inhibits Akt	Wang *et al.*, 2007

SOME CELLULAR TARGETS FOR NATURAL COMPOUNDS

From the phytochemical database, it is obvious that immunopharmacological agents belong to different classes according to their structure and chemical nature. The chemical nature may be a defining factor in determining the target of the agent. Some agents can traverse the lipid bilayer and therefore may have their target in the cytoplasm and even on the nuclear membrane. And those which are not allowed to penetrate the

cell membrane may have their receptor or target on the cell surface. Some natural products can bind to other conventional cellular receptors. For example, isoflavones possess a stereochemical similarity with estrogen and can therefore bind with mammalian estrogen receptors. Ligand binding allows the natural products to regulate gene expression profile of immunocompetent cells (Sung *et al.*, 2007; Sethi *et al.*, 2007; Lin *et al.*, 2007). Again inhibition of ROS generation by these compounds may play a central role in regulating the downstream cascade reactions.

Among different cellular targets of the immunopharmacological agents, NF-κB seems to be affected by most of the agents. Most carcinogens, inflammatory agents and tumor promoters including cigarette smoke, phorbol ester, okadoic acid, H_2O_2 and tumor necrosis factor (TNF) have been shown to activate NF-κB (Sung *et al.*, 2007). NF-κB is the transcription factor that regulate expression of several genes involved in tumorigenesis (Aggarwal, 2004) and inflammation (Aeurle, 1998). These include anti-apoptotic genes, proliferation and invasion genes, genes encoding adhesion molecules and cell cycle regulatory genes. Natural products such as fisetin, celastrol and curcumin have found to suppress NF-κB activation and thus can modulate the pathway in course of their anti-tumor and anti-inflammatory activities.

DNA damage is considered to be one of the hallmark events in initiation of cancer. Histone acetyl transferases (HATs) play a pivotal role in manintaining the homeostasis of the genome. Two groups of enzymes, *viz.*, histone acetyl transferases (HATs) and deacetylases (HDACs) balance the acetylation level as required for cellular function (Roth *et al.*, 2001). The first known cell permeable small molecule HAT inhibitor is garcinol, an isoprenylated benzophenone derivative from *Garcinia indica* fruit (Balasubramanyam *et al.*, 2003). Garcinol was found to be highly potent nonspecific HAT inhibitor that inhibits histone acetylation *in vivo* and induces apoptosis (Balasubramanyam *et al.*, 2004).

CONCLUSIONS

The foliar extract of *Eupatorium* showed common immunomodulatory properties with ethanolic extract of turmeric. But degree wise it is lesser effective than turmeric except in its anti-inflammatory property. The results with *Eupatorium* extract vis-à-vis ETE indicated some correlation among blastogenesis, anti-inflammatory, anti-oxidant and anti-malignancy events. This correlation might provide some clue to validate immunotherapeutic properties of some plant products. Of course, chemical nature of the effective substance must also be included in the validation process. We are now contemplating to include the data of expression of some specific genes known to participate in immunological reactions, in response to the herbal extracts. This will be our objective of continuation of the study in near future.

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10

Differential Effects of Subchronic and Chronic Oral Treatments with *Orbignya phalerata* Mart. Mesocarp on the Inflammatory Response

Flávia R.F. Nascimento[1,*], Elizabeth S.B. Barroqueiro, Ana Paula S. Azevedo, Márcia C.G. Maciel, Wanderson S. Pereira, Susanne C. Ferreira, Jardel C. Farias, Mayara T. Pinheiro, Lucilene A. Silva and Rosane N.M. Guerra

ABSTRACT

Orbignya phalerata Mart. (Arecaceae) is traditionally known as babassu palm. The babassu mesocarp has been used in Brazil for the treatment of inflammatory diseases. However, there are few and conflicting results focusing the real effect of this natural product on the inflammatory response. We evaluated here the effect of subchronic and chronic treatments, by oral route, with an aqueous suspension of babassu mesocarp (BM) on the inflammatory reaction. C57Bl/6 mice (2 months old) were treated ad libitum by 30 days (subchronic) or by 120 days (chronic) with BM. The control received water by the same time (n = 20/group). Four inflammatory models were tested: the carrageenan-induced paw edema, the early (6 days) and the delayed (17 days) cotton pellet granuloma, the wound lesion healing and the BCG-induced peritonitis. The results showed divergent effects according to the time of BM treatment. The subchronic treatment reduced the carrageenan-induced paw edema and the delayed granuloma, but it increased the early granuloma. On the other hand, the chronic treatment increased the paw edema but had no effect on early or delayed granuloma.

1. Laboratório de Imunofisiologia, Centro de Ciências Biológicas e da Saúde, Universidade Federal do Maranhão, Av dos Portugueses s/n, Campus do Bacanga, CEP: 650 85-580, São Luís, MA, Brazil.

* *Corresponding author* : E-mail : nascimentofrf@yahoo.com.br

Both treatments decreased the time of wound healing and increased the BCG-induced nitric oxide, despite they had no effect on both BCG-induced cell influx and hydrogen peroxide release. We conclude that the BM oral treatment has a modulatory effect on inflammation inducing either pro- or anti-inflammatory effects depending on both the time of treatment and the kind of inflammation. A common data between the treatments were the healing property and the nitric oxide production improvement. Therefore, it is crucial to point that the use of babassu mesocarp in inflammatory diseases must be restricted to some specific situations and it can not be considered as a universal anti-inflammatory treatment

Key words : Arecaceae, babassu, inflammation, mice, *Orbignya phalerata*, oral treatment

INTRODUCTION

Orbignya phalerata Mart. (syn. *Attalea glasmanii* Zona Palms) (Arecaceae) is a palm typical from northeastern, Brazil, known by the popular name of babassu. This palm has been used by Apinaye and Guajajara Indians, yielding a variety of important products. Babassu palms provide food, fuel, shelter, fiber, construction materials, medicine, magic and other basic necessities of life for these people (Balick, 1988).

The babassu mesocarp is extracted, powdered and suspended in water for use by the Brazilian people as food supplement, since it is rich in carbohydrates and mineral salts (Gaitan *et al.*, 1994). In the same way, it is also used in the folk medicine in the treatment of dysmenorrheal, constipation, obesity, rheumatism, ulcerations, leukemia and tumors, venous diseases and inflammatory related diseases such as colitis and arthritis (Silva & Parente, 2001; Caetano *et al.*, 2002).

The main focus of researchers has been the anti-inflammatory and healing properties of babassu mesocarp. It was shown that acute treatment with chloroform extract of babassu mesocarp by oral or intraperitoneal routes inhibits the paw edema, the cotton pellet granuloma, the formalin-induced arthritis in rats and the carrageenan-induced peritonitis (Maia & Rao, 1989). In fact, Silva and Parente (2001) reported the isolation and chemical characterization of a bioactive polysaccharide MP1 responsible for the anti-inflammatory and immunomodulator activities.

However, we have previously shown that the intraperitoneal treatment with an aqueous suspension of babassu mesocarp had no anti-inflammatory properties since it did not inhibit the carrageenan-induced paw edema, the BCG-induced peritonitis and the cotton pellet-induced granuloma (Azevedo *et al.*, 2003; Ferreira *et al.*, 2004) and besides, this treatment activates the peritoneal macrophages triggering the secretion of some pro-inflammatory

metabolites, as hydrogen peroxide (H_2O_2), nitric oxide (NO) and tumor necrosis factor (TNF) (Nascimento *et al.*, 2006).

Another group of researchers showed that the intraperitoneal treatment with an aqueous suspension of babassu mesocarp has a favorable effect in the healing using different surgical processes (Brito-Filho *et al.*, 2006; Martins *et al.*, 2006; Ferreira *et al.*, 2006; Batista *et al.*, 2006; Baldez *et al.*, 2006). Moreover, the topical treatment with this suspension was also efficient in the healing of skin lesions (Amorim *et al.*, 2006).

The use of babassu mesocarp flour as medicine is completely empiric and it is based on popular knowledge that recommends the suspension of about two soup spoons of babassu mesocarp flour in one liter of water and using it twice a day by topical or oral routes, depending on the disease, until the cessation of symptoms. Despite, the popular use occurs mainly by oral route there are few works investigating the biological effects induced by babassu mesocarp taken by this route.

Recently, we showed that the chronic oral treatment with babassu mesocarp has a significant anti-thrombosis activity (Azevedo *et al.*, 2007), what seems to be related to a reduction of some components from the coagulation cascade (Farias *et al.*, unpublished results). These results indicate that the oral route can be useful in the prospecting of babassu mesocarp-derived products.

Therefore, considering that: the Brazilian people use babassu mesocarp, mainly by oral route, and sometimes by long periods of time; the healing effect of babassu mesocarp in different kind of lesions; and the conflicting data about the effect of babassu mesocarp in the inflammatory response; the aim of this work was to evaluate the effect of a subchronic (30 days) or chronic (120 days) oral treatments with an aqueous suspension of babassu mesocarp on the edema induction, on the cell recruitment induced by either an inert or an infectious agent and on the healing process.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (10/group), eight weeks-old, weighing 20–25 g in the beginning of the treatments, have been maintained for many generations in the Animal Breeding Unit (Biotério Central da Universidade Federal do Maranhão, São Luís, MA, Brazil) under standard conditions. The animals were kept in well cross ventilated room at 26 ± 2°C, relative humidity 44–56%, light and dark cycles of 12 h. The animals had free access to sterilized food and acidified water. All procedures described were reviewed and approved by the Animal Ethics Committee in accordance with COBEA (Brazilian College of Animal Experimentation).

Plant Material

Babassu mesocarp (BM) flour was purchased from Hensa Farma, São Luís-MA (Brazil). This commercial product was previously submitted to analysis of authenticity, integrity and purity, by physical-chemical tests such as standard chromatographic techniques. Similarities in all the botanical and phytochemical aspects were demonstrated when it was compared to the mesocarp flour obtained in our laboratory (Batista & Ribeiro, 2003). In these tests, *O. phalerata* fruits were collected from Pedreiras-MA, Brazil, and an authenticated voucher specimen is kept on file in Herbário Ático Seabra under the number 1135.

Treatment

Two grams of the babassu mesocarp (BM) flour were suspended in one liter of filtered distillated water according to the described by Sousa *et al.* (2003) and Azevedo *et al.* (2007). This solution was offered to the mice *ad libitum* as an optional drink for either 30 or 120 days. The amount of liquid ingested was quantified daily using metabolic cages and the mean oral intake of the BM per mouse was 500 mg/Kg/day. The animals from control and BM groups has drunk almost the same total amount of liquids and the treatment with BM did not induce death, alterations in the mice body weight and other toxicity signs (Lopes *et al.*, 2004).

Carrageenan-Induced Paw Edema

After 30 or 120 days of BM treatment the paw edema was induced by injecting 0.05 mL of carrageenan (1% *w/v*) in distilled water into the subplantar region of the right hind paw (Winter *et al.*, 1962). The paw volume was measured initially and after 3 h of carrageenan injection using a digital caliper. The delta was calculated by the subtraction of the final volume minus the initial volume of the paw.

Cotton Pellet Granuloma

The method described by Ismail *et al.* (1997) was used with modifications. After 30 or 120 days of BM oral treatment the sterile cotton pellets (9 ± 1 mg) were implanted subcutaneously on the back of mice. After 6 or 17 days the mice were sacrificed and the cotton pellet removed, dried overnight at 37°C and weighed.

Wound Healing Induction

After 30 or 120 days of BM oral treatment the animals were anesthetized and the excision procedure was performed as previously described by our group (Gomes *et al.*, 2005). The control group received only water *ad libitum*. The measurement of the wounds area (cm^2) was carried out each two days until the total healing using a digital caliper and applying the following

equation as previously described by Marques *et al.* (2004): $A = p \pi x (R \times r)$, where "A" represents the area, "R" represents larger radius and "r" the smaller radius of the lesion. The number of days necessary to total healing was also evaluated to calculate the mean and perform the statistical analysis.

BCG-Induced Peritonitis

After 30 or 120 days of BM oral treatment the mice were injected by intraperitoneal route with Onco BCG (Bacilo Calmette-Guérin), (Instituto Butantan–SP/Brazil), in a dose of 100 mg/kg to induce the peritoneal inflammation according to the described by Nascimento *et al.* (2003). After 7 days of BCG inoculation the animals were sacrificed and the peritoneal cells were aseptically collected by washing the peritoneal cavity with 5 mL sterile ice-cold phosphate buffered solution (PBS), devoid of calcium and magnesium ions. For total cell determination, nine volumes of peritoneal cells were added to 1 volume of 0.05% crystal violet dissolved in 30% acetic acid and counts were performed using a bright-line hemacytometer (Sigma, St. Louis, MO, USA). After the counting, the cells were used to evaluate the macrophage metabolites release.

Sequential Analyses of Hydrogen Peroxide and Nitric Oxide on a Single Sample of Macrophages

We used a method described previously by Nascimento *et al.* (2003) that permits to determine hydrogen peroxide (H_2O_2) release and nitric oxide (NO) production in a single macrophage sample. Two million peritoneal cells from mice treated or not with BM were suspended in 1 mL freshly prepared phenol red solution to evaluate H_2O_2 release by using the horseradish peroxidase-dependent phenol red oxidation microassay. The phenol red solution consisted of ice-cold Dulbecco's PBS containing 5.5 mm dextrose, 0.56 mm phenol red (Sigma) and 8.5 U/mL horseradish peroxidase type II (Sigma). One hundred microliters of the cell suspension were plated onto each well and incubated for 1 h at 37°C in a humid atmosphere containing 5% CO_2-95% air. The plates were centrifuged once at 150 g for 3 min and the supernatants were collected and transferred to other plate. The reaction was stopped with 10 µl 1 N NaOH. Absorbance at 620 nm was measured with a microplate reader (MR 5000, Dynatech Laboratories Inc., Gainesville, VA, USA). Conversion of absorbance to µm H_2O_2 was deduced from a standard curve obtained with known concentrations of H_2O_2 (5 to 40 µm) as described previously.

For NO determination the same plates were washed three times with PBS and the remaining adherent macrophages were cultured in 100 µl of complete RPMI 1640 medium supplemented with 10 mm HEPES, 11 mm sodium bicarbonate, 100 U/mL penicillin, 100 µg/ml streptomycin, 2 mm L-glutamine, 23 mm L-asparagine, 1 mm folic acid, 0.1 mm pyruvic acid and 5% Fetal Calf Serum (FCS) for 48 h at 37°C in an humid atmosphere

containing 5% CO_2—95% air. After this time, the supernatants were collected and transferred to other plate. The accumulation of nitrite (a stable end product of NO) in supernatants was determined with the standard Griess reagent. Briefly, 50 µl of the supernatants were incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthalene diamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min and absorbance at 550 nm was determined. Conversion of absorbance to µm NO was deduced from a standard curve using known concentration (5–60 µm) of sodium nitrite diluted in RPMI medium.

Statistical Analysis

The significance of differences between control and treated groups was analyzed using Student's *t*-test. Differences were considered significant at $p \leq 0.05$ and significant values were represented by an asterisk. All experiments were repeated at least two times. Data are expressed as means ± SEM.

RESULTS

BM Treatment Effect on Carrageenan-Induced Mice Paw Edema

There was a significant reduction in carrageenan-induced paw edema after 30 days of oral treatment with BM. The edema induced in the control mice with 6 months old was significantly decreased when compared to that observed in the young mice, however, the chronic treatment with BM (120 days) increased the edema to an amount similar to that one observed in the young mice (Fig 10.1).

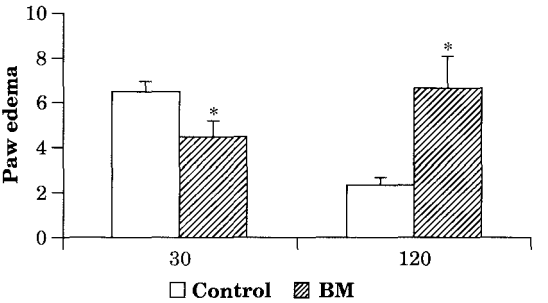

Fig 10.1. Effect of subchronic and chronic oral treatment with babassu mesocarp on carrageenan-induced paw edema. C57Bl/6 mice were treated by oral route *ad libitum* with an aqueous suspension of babassu mesocarp (BM) (500 mg/kg/day) or water (control) *ad libitum* by 30 or 120 days. After these times the mice were inoculated with carrageenan (1%) in the right paw. The paw thickness was measured before and 3 h later the carrageenan injection. The difference between both measures represents the edema formation. The data represent the mean ± SEM of 10 animals/group. *$p \leq 0.05$ when compared to control group

BM Treatment Effect on the Cotton Pellet-Induced Granuloma

The subchronic treatment with BM induced opposite results according to the time of the granuloma formation. The subchronic treatment significantly increased the 6-day granuloma (Fig 10.2A), but in the opposite way it decreased the 17-day granuloma (Fig 10.2B). On the other hand, the chronic treatment had no effect in the granuloma formation.

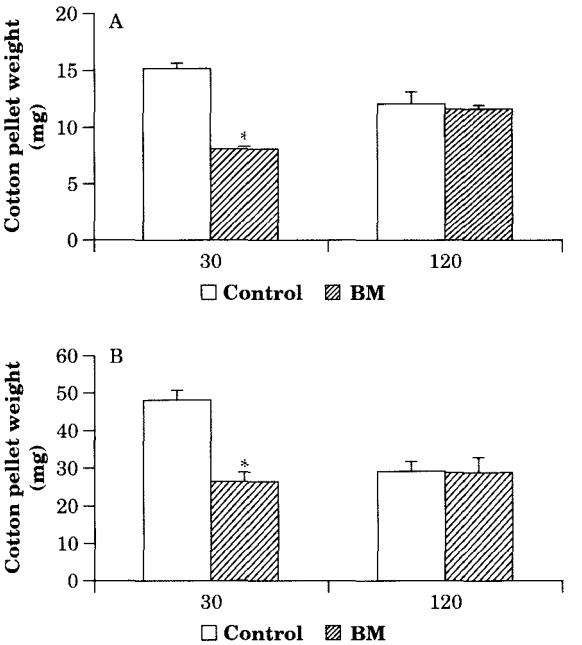

Fig 10.2. Effect of subchronic and chronic oral treatment with babassu mesocarp on cotton pellet granuloma formation. C57Bl/6 mice were treated by oral route *ad libitum* with an aqueous suspension of babassu mesocarp (BM) (500 mg/kg/day) or water (control) *ad libitum* by 30 or 120 days. After these times the mice were anesthetized and received a cotton pellet subcutaneous implant in the dorsal region. After 6 (A) or 17 (B) days the mice were killed and the implants were recovered, dried and weighed. The difference between the final weight and the initial weight of the cotton pellet was considered as the granuloma weight. The data represent the mean ± SEM of 10 animals/group. * $p \leq 0.05$ when compared to control group

Wound Healing

The results showed that the subchronic and chronic treatments accelerated the healing process (Figs 10.3A, B). The chronic treatment with BM restored in the aged mice the ability to heal in a similar way then that observed in the young mice (group 30 days) (Fig 10.3C).

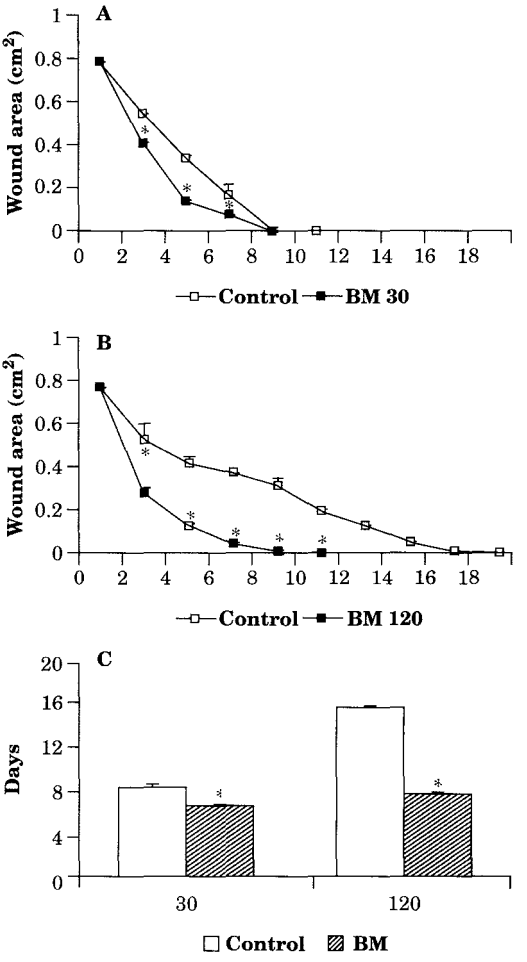

Fig 10.3. Effect of subchronic and chronic oral treatment with babassu mesocarp on wound healing. C57Bl/6 mice were treated by oral route *ad libitum* with an aqueous suspension of babassu mesocarp (BM) (500 mg/kg/day) or water (control) *ad libitum* by 30 or 120 days. After these times the mice were anesthetized and a wound was induced in dorsal region and it was measured each two days (A, B). Figure 10.10C shows the mean of days necessary to complete healing. Data represent the mean ± SEM of 10 animals per group. *$p<0.05$ when compared with the control group

BM Treatment Effect on BCG-Induced Peritonitis

BM treatment did not alter the cellular migration to peritoneal cavity even after BCG inoculation (Fig 10.4A). Additionally, the BM treatment did not interfere in the BCG-induced H_2O_2 release (Fig 10.4B). However, there was a time dependent increasing in the BCG-induced NO production (Fig 10.4C).

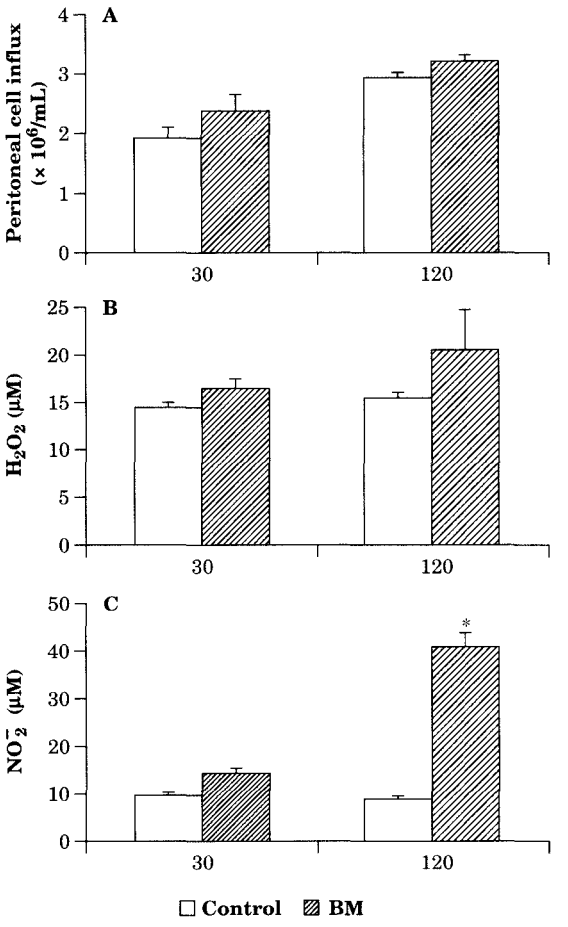

Fig 10.4. Effect of subchronic and chronic oral treatment with babassu mesocarp on BCG-induced peritonitis. C57Bl/6 mice were treated by oral route *ad libitum* with an aqueous solution of babassu mesocarp (BM) (500 mg/kg/day) or water (control) *ad libitum* by 30 or 120 days. After these times the mice were inoculated with BCG (100 mg/kg) and after 7 days they were sacrificed to quantify the peritoneal cellular influx (A), the hydrogen peroxide release (B) and the NO production (C). The data represent the mean ± SEM of 10 animals/group. *$p \leq 0.05$ when compared to control group

DISCUSSION

The oral consumption of an aqueous suspension of babassu mesocarp is a common ethnobotanical practice in northeast of Brazil for the treatment of inflammatory diseases. In the present study, the effectiveness of this practice was examined by treating mice, *ad libitum,* with an aqueous suspension of babassu mesocarp and by using classical models of experimental inflammation.

The first inflammatory model evaluated was the carrageenan-induced paw edema which is a useful model to investigate systemic anti-inflammatory agents. Carrageenan produces an acute inflammatory response of a two phase type. The initial phase of edema (0–1 h), which is not inhibited by non-steroidal anti-inflammatory drugs such as indomethacin or aspirin, has been attributed to the release of histamine, 5-hydroxytryptamine (5-HT) and bradykinin. In contrast, the second accelerating phase of swelling (1–6 h) which has a gradual increase until the 3rd hour, when the edema begins to decay, is correlated with the elevated production of prostaglandins (Di Rosa *et al.*, 1971).

It was shown here that the subchronic oral treatment with babassu mesocarp inhibited the paw edema. It could be reasonable to suppose that this treatment may decrease the release of some inflammatory mediators as: histamine, serotonin and other kinins in early phase, or arachidonate metabolites such as prostaglandins, which are related with late phase of edema (Just *et al.*, 1998). In fact, the edema inhibitory property of babassu mesocarp was also showed by Maia and Rao (1989) using an acute treatment with a chloroform extract from babassu mesocarp. In the same way, Silva and Parente (2001), using a model of vascular extravasation, showed that a bioactive polysaccharide, called MP1, found in babassu mesocarp was related with this anti-inflammatory property. So, the edema inhibition by subchronic treatment with babassu mesocarp could explain, at least partially, the popular use of this natural product as anti-inflammatory.

However, we observed that the chronic treatment with babassu mesocarp, in an opposite way, increased the paw edema. Interestingly, the pro-edema effect induced by this treatment restored the inflammatory ability which seems to be partially lost in the aged mice (6 months old). In the same way, we have previously shown that the carrageenan-induced paw edema was not inhibited after the intra-peritoneal treatment with an aqueous suspension of babassu mesocarp (Azevedo *et al.*, 2003). These results indicate that besides the time of treatment, the route of application and the kind of extract can severely influence the results obtained with the babassu mesocarp, suggesting a time- and a route-dependent effect on the edema formation.

To verify if these controversial results were related only to the initial phase of inflammation or if it would be also extensive to the late phases, we evaluate the effect of babassu mesocarp on the cellular recruitment of inflammatory cells, using the cotton pellet granuloma, the wound healing and the BCG-induced peritonitis models.

The cotton pellet-induced granuloma has three initial phases: the transdudative which occurs at the first 3 h, the exsudative which occurs between 3 and 72 h and the proliferative which occurs between 3 and 6 days after the cotton pellet implantation (Swingle & Shideman, 1972).

All these events involve the release of pre- or post-formatted inflammatory mediators, as well as the recruitment of inflammatory cells such as neutrophils and macrophages. After 6 days of cotton pellet implantation there is an intense immunological activity that results in the accumulation of lymphocyte and modified macrophages, including giant cells and epithelioid cells, which are organized in concentric stratums. This process is mediated by some cytokines as tumor necrosis factor (TNF), Interferon-γ (IFN-γ) and chemokines. In another phase, after 17 days, the granuloma is completely organized, and the presence of fibroblasts and T lymphocytes can be detected (Kunkel *et al.*, 1989).

The results obtained here with the cotton pellet granuloma model were different to that ones found in the carrageenan-induced paw edema model. The subchronic treatment with BM increased the early granuloma formation (6 days), but decreased the late granuloma (17 days). Taking account the phases of granuloma formation it is possible to suggest that the subchronic BM oral treatment exacerbates the transdudative, exudative and proliferative phases of granuloma since it induced a significant increased on the cotton pellet weight after 6 days of implantation. Nevertheless, the late phase, which is T-dependent and characterized by the influx of fibroblasts, was inhibited, suggesting a faster resolution of the inflammatory process, what can be due the stimulation of anti-inflammatory cytokines production or a deviation to an anti-inflammatory pattern of T-mediated immunological responses.

On the other hand, the chronic treatment had no effect on 6 or 17 days granuloma. It was observed that the young mice had a stronger ability to mount the inflammatory response against the cotton pellet then the aged mice. Therefore, it is likely that the chronic BM treatment was not enough to interfere in this response, or maybe there was a kind of adaptation of the organism to this treatment.

Nevertheless, this adaptation was not true to the wound healing model. It was also noted that aged mice have a slower healing process then the young mice, what is according to a delayed or a weak inflammatory response observed in the granuloma model. Nonetheless, the treatment with BM accelerated the healing process. The wounds from the mice treated with BM healed as fast as that from the young mice. Taken together, the data obtained here indicate that the pre-treatment with BM restore the inflammatory ability of mice, which seems to be partially lost with the age progression.

The healing property of babassu mesocarp observed after the oral treatment seems to be independent of the route or the time of use since the same property was showed by some authors using the topical (Amorim *et al.*, 2006) or intraperitoneal routes of treatment in rats (Brito-Filho *et al.*, 2006; Martins *et al.*, 2006; Ferreira *et al.*, 2006; Batista *et al.*, 2006; Baldez *et al.*, 2006).

In fact we have previously shown that the intraperitoneal treatment with babassu mesocarp directly activates the macrophages, inducing large amounts of hydrogen peroxide and nitric oxide production (Nascimento *et al.*, 2006) and it is possible that it also stimulates the secretion of different growing factors and chemokines that could be involved in the faster healing observed. On the other hand the intraperitoneal treatment inhibits the BCG-induced cell influx (Ferreira *et al.*, 2004), which is constituted by macrophages, neutrophils and lymphocytes and is dependent on the release of distinct inflammatory mediators, cytokines and adhesion molecules (Menezes-de-Lima-Junior & Henriques, 1997). Thus, the babassu mesocarp given by intraperitoneal route seems to inhibit the exacerbated inflammation, what could consequently to avoid the tissue damage and to facilitate the regeneration.

The BM consumption by oral route did not activate *per se* the peritoneal macrophages (data not shown) and also did not inhibit the BCG-induced cell influx. Besides to induce the cellular migration, the BCG inoculation induces the activation of peritoneal macrophages, increasing the production of reactive oxygen and nitrogen metabolites, generated via NADPH oxidase and inducible nitric oxide synthase, respectively (Bogdan *et al.*, 2000; Nascimento *et al.*, 2003). In this context the BM oral treatment seems to prime the macrophage to a secondary stimulation with BCG. So, the BM and the BCG seem to act synergistically, in a time-dependent manner, to induce the triggering of NO production by macrophages. Nonetheless, the BM oral treatment did not affect the hydrogen peroxide release. These results are not contradictory, since those metabolites are secreted by independent routes during macrophage activation. The secretion of hydrogen peroxide occurs immediately after the phagocytosis or a similar process, while the nitric oxide production occurs later and is dependent on a more elaborated mechanism, which requires at least two signals to trigger the iNOS activation (Moncada *et al.*, 1991).

The increased nitric oxide, a potent vasodilator, and consequently, essential to the edema induction (Moncada *et al.*, 1991) could seems to be conflicting with the decrease of paw edema induced by subchronic BM treatment. Nonetheless, Salvemini *et al.* (1996) showed that the NO produced by cNOS is involved in the development of inflammation at early time points following carrageenan administration and that NO produced by iNOS is involved only in the maintenance of the inflammatory response at later time points. Therefore, the results of inhibition of edema and the increasing of iNOS-dependent nitric oxide induced by BM subchronic treatment are not contradictory.

It seems clear that the anti-inflammatory effect of babassu mesocarp is related to the kind of extract and via of treatment since it was previously demonstrated by our group that the treatment with BM by intraperitoneal route inhibits the BCG-induced peritonitis (Ferreira *et al.*, 2004) but have

no effect on carrageenan-induced edema (Azevedo *et al.*, 2003). By the way, the treatment with BM by the intraperitoneal route has a pro-inflammatory effect since it increases *per se* the cellular influx and the secretion by macrophages of inflammatory mediators such as tumor necrosis factor, nitric oxide and hydrogen peroxide (Nascimento *et al.*, 2006). Moreover the anti-inflammatory effect of oral BM treatment seems to be true only to the acute inflammation, such as the carrageenan-induced paw edema that involves pre-formed mediators, but not to the delayed inflammatory responses that involves very complex reactions which include cellular migration, molecules expression and the secretion of several mediators.

Altogether, those results showed that the BM treatment has a modulator effect on the inflammatory process, inducing anti- or pro-inflammatory effects depending on the time and the route of treatment as well of the kind of inflammatory response. The exception is the healing property which seems to be true independent on the route of treatment. As a result it is necessary to emphasize that the babassu mesocarp consumption as anti-inflammatory must be restricted to some specific situations and this consideration has to be taking account in the prospecting projects using this natural product.

ACKNOWLEDGEMENTS

We thank Mr. Wicliff Eric for revising the manuscript. This work was supported by CNPq (Proc. N° 478351/2006-2 and N°477714/2007-2); FINEP (Proc-N° 0105039400) and BNB. F.R.F.N. was supported by researcher fellowship from CNPq (Proc. N° 311339/2006-9). R.N.M.G. was supported by post-doc fellowships from CNPq. A.P.S.A. was supported by master fellowship from CAPES. S.C.P.F., W.S.P., J.C.F. and M.T.P. were supported by fellowships from CNPq.

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A Mistletoe Lectin-Containing Preparation for Oral Use Provokes an Immune Response and Induces an Increase in the Population of Activated Natural Killer Cells

WINGE I.[1,2], DALE T.M.[1], TILREM J.P.P.[1], AND PRYME I.F.[2,*]

ABSTRACT

Mistletoe lectins are major bioactive components in extracts of European mistletoe (Viscum album). A preparation enriched in mistletoe lectins produced free of the molecules that cause nausea has become available. The aim of the investigation was to evaluate whether or not the preparation taken orally affects the immune system in healthy adults. Eight healthy individuals took the preparation on a daily basis for three months. Blood samples were taken before and during the course of the study. All individuals had anti-lectin antibodies in the blood at day 84, showing that an immune response had indeed occurred. During the observation period four cell types showed a marked response: activated IL-2 helper cells, memory cells, activated T-cells and activated natural killer (NK) cells. In general the levels of activated IL-2 helper cells and memory cells were elevated on day 28 with a return to normal levels on day 84. The levels of activated T-cells and the activated AEFNK-cells, however, were still elevated on day 84. At the onset of the investigation four of the individuals had levels of NK-cells that were well below normal values. Three of these, however, at 84 days had values that were now within the normal range. Taken together it is evident that the mistletoe preparation is not only an unspecific immunogenic trigger but is also able to activate the specific immune system. In a pilot study it was

1. Palm Research, Lungegaardsbakken 1, N-5015 Bergen, Norway.
2. Department of Biomedicine, University of Bergen, Jonas Liesvei 91, N-5009 Bergen, Norway.
* *Corresponding author* : E-mail : ian.pryme@biomed.uib.no

shown that there were increases in endostatin, TNF-α and β-endorphin values in the blood within a few days of taking the preparation. The oral mistletoe preparation described here is proposed as being an extremely effective dietary supplement for immunomodulation.

Key words : β-endorphin, endostatin, IL-2 helper cells, immunomodulation, memory cells, T-cells, TNF-α, *Viscum album*

INTRODUCTION

Mistletoe (*Viscum album*), a member of the Loranthacea family, is a semi-parasitic plant that has been used since ancient times in treatment of a variety of human ailments like epilepsy, rheumatism, arthritis and cancer. Mistletoe preparations have been reported to have a series of medicinal properties including the ability to lower blood pressure, slow the heart beat, stimulate the immune system, relax muscular spasms and exert both sedative, diuretic and anti-cancer effects (Bown, 1995; Briggs, 2003). In the West Indies a tea prepared from mistletoe leaves has been used for many years in the treatment of diabetes (Peters, 1957). Hajtó *et al.* (2005) have recently reviewed recent observations where mistletoe lectin has been used in *in vitro* experiments and in animal model systems, with particular respect to the potential use in the clinic. In addition to mistletoe lectins a series of other lectins will almost certainly have an impact in biomedicine in forthcoming years (Pusztai *et al.,* 2008).

The components responsible for the biological effects of mistletoe extracts have been suggested to be the alkaloids, the viscotoxins, and the lectins. It has been known for 20 years that a mistletoe lectin (ML) preparation when injected subcutaneously in humans results in an immune response (Hajtó *et al.,* 1989). An initial strong proliferation of peripheral blood mononuclear cells has been observed (Stein *et al.,* 1998), this being accompanied by increased levels of both TNF-α and IL-6 in the blood and somewhat lower levels of IFN-γ and IL-4. Hajtó *et al.* (1990) demonstrated an induced increase in the secretion of TNF-α, IL-1, and IL-6 in cultures of human peripheral blood mononuclear cells treated with mistletoe lectin. Männel *et al.* (1991) detected an increased production of TNF-α by peripheral blood monocytes incubated with ML-I, this being accompanied by a strong expression of TNF-α mRNA. An increased activity of peripheral blood natural killer cells (NK) in response to the lectin has been reported (Baxevanis *et al.,* 1998; Schink, 1997; Heiny *et al.,* 1998). A series of recent studies have clearly demonstrated that NK-cells play a major role in immune surveillance (see review, Takeda & Okumura, 2004). Their activity, furthermore, is greatly influenced by a series of agents including environmental factors, stress, food components and drugs. Yoon *et al.* (2003) have shown that the anti-tumour effect of the lectin from Korean mistletoe can be attributed to the activation of macrophages and NK-cells.

Heiny *et al.* (1998) have observed increased β-endorphin plasma levels occurring simultaneously with a stimulation of T-lymphocytes exhibiting expression of CD25/interleukin-2 receptors and HLA/DR-antigens. Baxevanis *et al.* (1998) provided clear evidence that mistletoe lectins preferentially stimulate and expand a population of $CD8^+$ T-cells, known to mediate cytotoxic effects. Gabius *et al.* (1992) obtained clear evidence of the immunomodulatory effects of mistletoe lectins in their studies on the immunophenotyping of blood samples collected from lectin-treated patients; increased numbers of NK, amplified activity of peritoneal macrophages cells and also a response of splenic T-cells to mitogens were reported. A combined effect of both cytotoxic and immunomodulatory properties have been associated with anti-tumoural effects of mistletoe lectins (Hajtó *et al.*, 1990; Burger *et al.*, 2001). In addition to promoting immunomodulation mistletoe lectins have a number of interesting properties including induction of apoptosis and anti-angiogenic effects (see review, Pryme *et al.*, 2007).

Following ingestion the mistletoe plant can occasionally cause vomiting and thus incorrectly has a history of being classified as toxic. Two studies where the outcome of over 2000 exposures to accidental intake of the plant has been examined did not show any evidence of toxicity in humans (Hall *et al.*, 1986; Krenzelok *et al.*, 1997). It is clear, therefore, that the unfortunate reputation that mistletoe has of being toxic following oral intake has no clinical support. Even when large doses of mistletoe lectins were added to diets fed to rats no toxic reactions were recorded (Pusztai *et al.*, 1998). On the other hand mistletoe lectins, supplied as dietary supplements, have actually shown to exert growth-stimulatory effects on crypt cells of the small intestine in rats and mice (Pusztai *et al.*, 1998; Pryme *et al.*, 2002). Purified ML-I and ML-III when added to diets fed to mice (Pryme *et al.*, 2002, 2006) have been shown to reduce growth of a non-Hodgkin's lymphoma in mice and even result in complete ablation of the tumour (Pryme *et al.*, 2004).

Using a method based on extraction from dried mistletoe Eifler *et al.* (1993) purified three mistletoe lectins, termed ML-I, ML-II and ML-III, respectively, ML-I was found to be by far the most dominant lectin amounting to 68% of the total, followed by ML-III (21%) and ML-II (11%). All three lectins consist of an A-chain that has N-glycosidase activity (causing inactivation of ribosomes–*i.e.* RIP-function) and a B-chain possessing galactose—(ML-I) or galactose/N-acetylglucosamine specific (ML-II and III) binding properties. A disulphide bridge connects the A chain to the B-chain (Eschenburg *et al.*, 1998). It has become evident that neither the A-nor the B-chain individually possess cytotoxic activity, both chains have to be present for the molecule to be able to exhibit RIP activity (Vervecken *et al.*, 2000). Following internalisation of the A chain it unfolds (Agapov *et al.*, 1999a) and is translocated from the membrane compartment into the cytosol (Agapov *et al.*, 1999b). In many transformed cells this process

is accompanied by an activation of the inherent N-glycosidase activity causing an expression of cytotoxic properties *in vitro* (see review, Pryme *et al.,* 2006). Mistletoe lectins also cause reduced growth of tumours *in vivo* (see review, Pryme *et al.,* 2007), where other properties than the direct cytotoxic effects of the lectins on tumours are involved.

Yoon *et al.* (1995) reported that the anti-metastatic effect of an extract of Korean mistletoe on a number of murine tumour cell lines (B16-BL6 melanoma, colon 26-M3.1 carcinoma and L5178Y-ML25 lymphoma cells) was partly due to an inhibition of tumour-induced angiogenesis. *In vivo* analysis for tumour-induced angiogenesis showed that the number of blood vessels oriented towards the tumour mass was considerably reduced. Their results suggested that the extract of Korean mistletoe reduced tumour metastasis by the inhibition of tumour-promoted angiogenesis through the induction of TNF-α production. Similar results were obtained by Park *et al.* (2001). Pryme *et al.* (2006) reported that NHL tumours in mice fed ML-I showed a greatly reduced incidence of tumour vascularisation when compared to mice not fed the lectin. The results would suggest that one of the responses following the binding of ML-I to receptors in the gut is an induction of the production of anti-angiogenic factors.

Lavelle and colleagues (Lavelle *et al.,* 2000, 2001, 2002) have clearly demonstrated that when delivered by the oral route in mice ML-I stimulated the production of specific serum IgG and IgA antibody. These observations were compatible with the selective induction of Th2-type immune responses and provided conclusive evidence that when provided orally then the mistletoe lectin was able to induce an immunomodulatory response in the host.

The intention of the present study was to perform a pilot study in order to evaluate whether or not the mistletoe lectin preparation could function as an immunomodulatory agent when taken orally by humans. Furthermore, it was of interest to investigate if endostatin, TNF-α and β-endorphin values in the blood were affected by short-term ingestion of mistletoe lectins.

MATERIALS AND METHODS

Preparation Containing Mistletoe Lectins

An aqueous extract of dried mistletoe leaves and bark was made using 0.2 M acetic acid. This was subjected to chromatography on a Sephadex C-50 column as described by Eifler *et al.* (1993). After extensive washing of the column with buffer, the lectins were eluted with high salt and the material was dialysed against PBS. Lectins were present in this preparation at a concentration of approx. 12 ng/mL. The chromatography step results in a concentrated lectin preparation free of nausea-inducing components (termed Palm) that can be taken orally.

Western Blot Analysis

SDS polyacrylamide gel electrophoresis was carried out in a 12% separation gel following the method described by Laemmli (1970). Mistletoe lectin (ML-I from Sigma) was used at amounts of 1 μg per lane; and 30 μl of Palm was applied per lane. After transferring the antigens onto PVDF membranes (Millipore, Bedford, USA) (Towbin *et al.,* 1979), the sheets were incubated with sera obtained from the eight individuals (84 days) at a dilution of 1:50. Peroxidase-conjugated rabbit anti-human immunoglobulin (Bio-Rad) was used at a dilution of 1: 2000.

ELISA

Blood samples were taken from the control persons (N = 5) and endostatin, TNF-α and β-endorphin values in the serum were measured by ELISA using kits from R&D Systems. The ELISA was performed according to the supplier's protocol. Serum was used at a dilution of 1: 50.

Subjects

Eight healthy subjects (6 females and 2 males, mean age 47, range 29–63 years) that had to their knowledge never been exposed to mistletoe or preparations thereof, participated in this pilot study. They took 1 mL Palm daily, diluted in 200 mL water, for a period of 84 days. In the text they are designated as CP1, CP2-CP8. Blood samples were collected on day 0, day 28 and day 84 and analysed in Dr. Bayers laboratory, Stuttgart, Germany.

RESULTS

ML Antibody Production

Serum prepared from blood samples taken from the 8 individuals on day 84 was analysed for the presence of ML antibodies. As shown in Fig 11.1, antibodies that recognized ML were identified in the serum of all individuals, however, there was some variation concerning antibody specificity. Individual factors apparently influence antibody production. Mistletoe antibodies were not detected in serum from individuals who had not ingested Palm (results not shown).

Effect of Mistletoe Lectin Preparation on Cells of the Immune System

The complete results of the immunoprofile analysis are presented in Tables 11.1 and 11.2. Only minor fluctuations were seen in the values in Table 11.1. Thus the values for cell types including thrombocytes, leukocytes, lymphocytes, monocytes and granulocytes essentially remained within the normal range during the 3-month period.

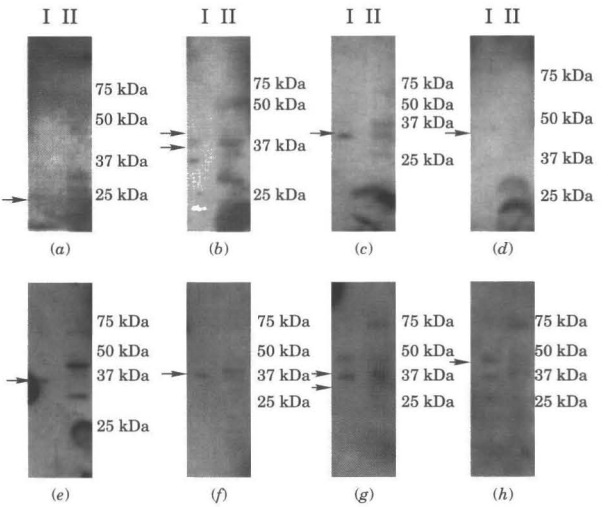

Fig 11.1. Western blot analysis of blood serum obtained from eight individuals who had ingested Palm orally for 84 days. Recognition of different mistletoe epitopes following 12% SDS-PAGE under reducing conditions. Antigen concentration, 1 µg/lane: Lane I (ML-1 Sigma) and Lane II (Palm). (a) CP1, (b) CP2, (c) CP3, (d) CP4, (e) CP5, (f) CP6, (g) CP7 and (h) CP8. The arrows indicate ML epitopes

Table 11.1. Thrombocytes and leucocytes measured in blood samples of control persons (CP) at 0 day (before intake) and after 28 and 84 days of intake of Palm

Thrombocytes, absolute number (× 1000)/µl (normal values 200000–300000)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	184	215	293	192	222	313	294	274
28	195	245	293	216	225	301	357	263
84	171	253	333	214	231	335	291	263

Leucocytes, absolute number (×1000)/µl (normal values 4000–10000)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	7.5	6.2	5.9	5.5	6.3	5.9	7.9	6.8
28	6.7	5.8	6.8	5.9	6.0	6.3	6.9	6.9
84	6.2	5.3	5.1	5.6	6.9	7.8	6.4	6.2

Lymphocytes, relative % of total leucocytes (normal values 20–35%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	38	33	26	31	32	40	33	40
28	43	29	30	30	30	36	33	34
84	37	35	42	24	27	23	31	40

Table 11.1. *Contd.*

Monocytes, relative % of total leucocytes (normal values 4–9%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	6	5	8	5	7	5	6	8
28	6	5	8	4	7	6	7	6
84	5	6	7	4	7	5	5	7

Granulocytes, relative % of total leucocytes (normal values 58–72%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	56	62	67	63	60	55	61	52
28	51	67	63	66	63	59	60	59
84	58	59	51	72	65	72	64	53

Table 11.2. Lymphocytes measured in blood samples of control persons (CP) at 0 days (before intake) and after 28 and 84 days of intake of Palm

T-Lymphocytes CD3, relative % of total lymphocytes (normal values 59–75%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	71	77	75	71	77	66	74	73
28	74	77	76	64	74	71	71	77
84	79	82	78	73	70	73	71	74

B-Lymphocytes CD19, relative % of total lymphocytes (normal values 7–15%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	9	13	11	8	9	9	9	10
28	8	10	14	8	11	13	8	10
84	9	8	13	10	9	13	9	11

T-helper cells CD4, relative % of total lymphocytes (normal values 40–50%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	50	45	52	56	53	44	61	38
28	51	44	52	47	51	48	58	45
84	51	44	50	55	45	51	57	37

T-supressor cell CD8, relative % of total lymphocytes (normal values 27–37%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	33	40	32	30	44	33	19	42
28	37	38	31	35	41	32	21	38
84	28	41	29	31	46	34	21	45

Table 11.2. *Contd.*

Natural Killer cells CD56/16, relative of total lymphocytes (normal values 9–21%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	15	8	9	16	10	21	13	13
28	14	8	6	25	10	14	17	8
84	7	5	4	13	16	10	15	10

Cytotoxic T-cell CD3+/CD56/CD16, relative % of total lymphocytes (normal values 2–8%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	2	5	6	6	14	3	2	10
28	2	4	10	4	14	3	2	7
84	2	4	9	5	17	4	2	9

Activated T-cell HLA-DR, relative % of total lymphocytes (normal values 5–10%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	7	9	8	6	8	6	7	22
28	11	8	9	5	8	7	5	12
84	9	12	11	7	8	10	9	25

Activated Killer-cells, relative % of total CD56+natural killer cells (normal values 18–40%)

Days	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
0	12.5	33.3	20	6.7	10.5	6.7	18.2	44.4
28	15.4	33.3	20	8	10	7.7	15.4	33.3
84	33.3	43.5	33.3	24	9.5	20	20	66.7

From Table 11.2 it is evident that 4 cell types showed a similar time-response in 7 of the 8 individuals: Activated helper cells (IL-2), memory cells, activated T-cells (HLA-DR) and activated NK-cells. The individual CP5 appeared to respond poorly to oral intake of Palm as far as changes in these cell types was concerned. For activated helper cells, 7 out of 8 individuals showed an increase at day 28, compared with day 0. The 2 individuals that exhibited the smallest effect were those with the highest normal values. The memory cells, as for the helper cells, were increased in all individuals at day 28. Values were decreased on day 84. The activated T-cells in samples from all individuals, gave approximately the same pattern. Levels of activated T-cells showed a slight reduction before an increase at day 84.

The profile of activated NK-cells presented as a scatter diagram (Fig 11.2) shows that 4 individuals (CP1, CP4, CP5 and CP6) had values at

day 0 which were well below the lowest level of the normal range (18%). Three of these (CP1, CP4 and CP6) demonstrated small increases on day 28, although still remaining below normal limits. On day 84, however, these individuals showed marked increases in cell numbers. For CP1 the percentage value increased from 15.4 on day 28 to 33 on day 84, for CP4 from 8 to 24 and for CP6 from 7.7 to 20, such that all three now had values within the normal range. CP5 showed no increase in activated NK-cells throughout the period of analysis and in fact all values remained well below normal. Interestingly, throughout the study this individual had the highest level of memory helper cells but the lowest level of naïve helper cells and thus a very high ratio of memory: naïve helper cells. The individual CP7 only responded poorly and demonstrated exactly the opposite behaviour to CP5, exhibiting namely the lowest level of memory helper cells but the highest level of naïve helper cells and consequently by far the lowest ratio of memory: naïve helper cells of all 8 individuals in the study. The ratio of 0.49 being well below the normal range (0.7–1.6).

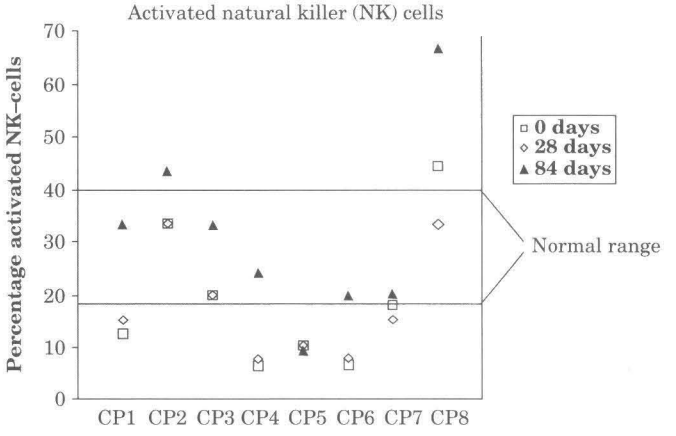

Fig 11.2. Scatter plot of activated NK-cells in the blood obtained from eight individuals who had ingested Palm orally for 84 days. Blood samples were taken at day 0 (before intake), 28 and 84. Data from Table 11.2

Endostatin, TNF-α and β-endorphin Values

Figure 11.3a shows that the level of endostatin did not change during the first day after oral intake of the mistletoe preparation but showed an increase on day 2, increasing slightly on the two following days. TNF-α values showed an increase already on day 1 but the highest increase was observed on day 4 after commencing oral intake of the lectin preparation (Fig 11.3b). Similar to TNF-α, β-endorphin values also showed a small increase on day 1 (Fig 11.3c) and a large increase in the blood was observed after 4 days. Interestingly, when percentage increases above initial levels (day 0) for TNF-α and β-endorphin were plotted pair-wise for each individual

(Fig 11.3d) then there appeared to be a clear correlation between the values *i.e.* an increase in β-endorphin was mirrored by an increase in the value of TNF-α.

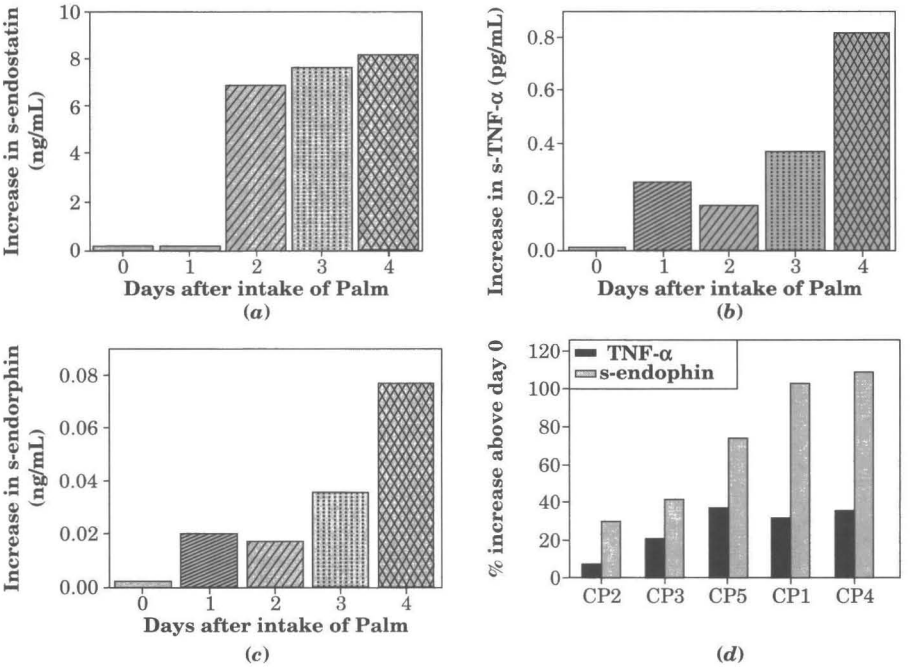

Fig 11.3. Changes in serum levels of (a) endostatin, (b) TNF-α and (c) β-endorphin following the ingestion of Palm. Blood samples (n = 5) were taken at day 0 (before intake) and then on the following 4 days. Measurements were made by ELISA as indicated in Materials and Methods and the results presented as means of the values obtained from the 5 (CP 1-5) individuals. (d) Serum levels of TNF-α and β-endorphin for each individual calculated as a percentage increase above day 0 values and arranged pairwise

DISCUSSION

Many approaches have been made to evaluate the potential medicinal use of the mistletoe plant. Teas and different extracts of mistletoe are commercially available, however, an evaluation of the biological properties of such preparations following their use by humans is generally lacking. Recent intense research efforts have been directed to examining the effect of oral mistletoe lectins on tumour growth *in vivo* (Pryme *et al.*, 2002, 2004, 2006, 2007). Reduced growth of a non-Hodgkin lymphoma (mice), an F-98 glioma (rat), colon carcinoma (mice), B16-BL6 melanoma (mice) and MB49 urinary bladder carcinoma (mice) have been observed following various treatment regimes with mistletoe lectins (for review, see Pryme *et al.*, 2006). In human studies positive effects of treatment with ML have been observed

both for malignant glioma (Lenartz *et al.*, 2000) and urothelial bladder cancer (Elsasser-Beile *et al.*, 2005).

It is evident that the orally presented lectin-enriched mistletoe preparation used in this study results in the stimulation of peripheral blood lymphocytes. As expected, the mistletoe preparation did not stimulate as many cell types as was observed following subcutaneous administration, although important sets of cells *i.e.* T-helper cells, memory cells, cytotoxic T-cells and NK-cells were all stimulated.

Surprisingly, at the onset of the investigation, 4 of the healthy individuals (CP1, CP4, CP5, CP6) had NK-cell levels of between 6.7 and 12.5% *i.e.* well below normal values (18–40%). Three of these showed increases after drinking the mistletoe lectin preparation for 28 days, although the levels had not reached normal limits. At 84 days, however, the values for CP1, CP4 and CP6 had all risen to within the normal range. It was thus evident that in order to "prime" the immune system 3 of 8 individuals had to drink the preparation for a period of one to three months to bring their NK-cell levels to within the normal range. Keeping the NK-cell level at normal values may be important for maintaining a potent first line of attack against virus and bacterial infections. Since it has been shown that in animals with low levels of NK-cell activity an increased number of both spontaneous and experimental tumours develop (Talmadge *et al.*, 1980; Gorelik *et al.*, 1982; Okumura *et al.*, 1982), then this would strongly suggest another good reason for keeping the number of activated NK-cells within normal limits.

Compared with the values at the onset of the study two individuals showed a three-fold increase in NK-cells at 84 days. The fourth individual (CP5) with an initial low level of NK-cells (10.5%) did not respond during intake of Palm for 84 days (final value 9.5%). This subject, however, had a level of memory helper cells of 76, which was clearly above the normal range (37–56), but exhibited a low level of naïve helper cells (27, normal range 38–61) and thus a very high ratio memory: naïve helper cells of 3.18 (normal 0.7–1.6). The reason for this is unclear.

Antibodies to mistletoe lectins have been detected in humans after subcutaneous administration (Stettin *et al.*, 1990; Stein *et al.*, 1997; Klein *et al.*, 2002). We show here that the consumption of a mistletoe lectin preparation by adults results in the induction of antibody production against mistletoe lectins. After drinking the preparation for 84 days all 8 individuals were observed to have produced ML antibodies. This is in line with previous observations where oral presentation of ML to mice resulted in the appearance of specific serum IgG and IgA antibody after three oral doses (Lavelle *et al.*, 2000). As expected different epitopes were detected since ML-I antibodies cross-react for example with ML-III.

There are many dietary supplement preparations commercially available where claims are made that they are immunostimulatory when taken orally. Documentation of these claims, however, is in on the whole sadly lacking. In this study we present two lines of convincing evidence that the mistletoe preparation described here has potent immuno-stimulatory properties, firstly, four cell types of the immune system showed marked proliferation, and secondly, all individuals produced antibodies to the lectins present.

By using the oral, as opposed to the subcutaneous route, the possibility of an allergic reaction is reduced and a much more far-reaching, long-term effect is observed. About 80% of immunoreactive tissue in the body is associated with the gastrointestinal tract. Following consumption of Palm the lectins work by binding to specific galactose/N-acetylglucosamine receptors on M-cells of Peyer's patches associated with the MALT/GALT system (Sharma *et al.,* 1996). Subepithelial lymphoid follicles provide the most important sites for antigen sampling in the small intestine and constitute an effector mechanism for secretory immune responses of GALT (Roy *et al.,* 1987). The importance of this mechanism is two-fold, firstly an immune response is vital such that protection of host tissues against invading microorganisms can operate, and secondly that the extent of an infection can be curtailed as much as possible (Kraehenbuhl & Neutra, 1992). Lymphoid follicles are overlayed by the follicle-associated epithelium (FAE) that includes a number of cell types such as lymphocytes, enterocytes, goblet cells, M-cells and pre-M-cells (Gebert & Hach, 1993). In studies on specimens of human small intestine containing Peyer's patches Sharma *et al.* (1996) showed by histochemistry that mistletoe lectin bound to both enterocytes and M-cells of the FAE. It is therefore evident that the surface of the small intestine in humans contains receptors that bind mistletoe lectins (see Table 11.3 in Sharma *et al.,* 1996).

Following binding to receptors lectins causing a release of signal molecules to the blood (Bardocz *et al.,* 1995) and these are then able to stimulate the immune system and also induce an anti-angiogenic response (Pryme *et al.,* 2002, 2006). Helper cells show a fast initial response, whereas cells involved in immuno-memory show a slower but prolonged increase. This, however, was as expected since populations of memory cells are quite stable and do not need to be activated to have an impact on the immune system as do the helper cells.

Interestingly, it was observed that the individuals who in general showed the weakest reaction were those who had initial values above normal limits, whilst those who demonstrated the highest response had values below normal at the onset of the study. The results suggest that the higher the starting (normal) values, the lower the response and *vice versa*, which is in agreement with other observations (Takeda & Okumura, 2004).

The results presented here demonstrate that mistletoe lectins taken orally are effective as immunomodulators and thus a mistletoe preparation does not have to be injected (*e.g.* subcutaneously) in order to modulate the immune system in humans. The lectin-enriched mistletoe preparation (Palm) described here appeared to effectively function as an immunostimulating dietary supplement for the healthy individuals who took part in the study. Of special interest was the increase in activated NK-cells that was observed in several of the individuals who participated in the study. This is in line with Takeda and Okumura (2004) who state that toll-like receptors and receptors to lectins, including those from mistletoe, are among the most likely candidates responsible for NK-cell activation. It is interesting to note that lectins, also those from mistletoe, have recently been regarded as having great potential as anticancer agents (González De Mejía & Prisecaru, 2005).

Although present in many foodstuffs of plant origin (González De Mejía & Prisecaru, 2005) lectins are denatured by heat treatment and thus cooking, frying etc. will reduce their immunostimulating potential virtually to zero. Since there are many lectins which have specific receptors in the human small intestine (Sharma *et al.,* 1996) it is evident that our diet needs to contain a certain quantity of these molecules in an active form in order for them to trigger the immune system. Modern diets unfortunately provide limited amounts of active plant lectins, perhaps insufficient to achieve high-level immunosurveillance. This will mean that we will be more prone to infections and other disorders. There is thus a strong case for dietary lectin supplementation. The fact that of the 8 individuals that participated in the pilot study 4 had at the outset below normal values of activated NK-cells while a further 2 were boarder line cases would support this.

As mentioned above there are a series of observations that clearly indicate that mistletoe lectins can induce the induction of an anti-angiogenic response. It was therefore interesting to note that serum endostatin values showed increases within a few days of the oral intake of the lectin preparation. These results would suggest that oral intake of mistletoe lectin was able to induce an anti-angiogenic response. The observed increase in TNF-α values in the blood within a short time after oral intake of mistletoe lectins is in agreement with other findings showing induction of TNF-α production following subcutaneous administration of ML (Hajtó *et al.,* 1990; Männel *et al.,* 1991; Stein *et al.,* 1998). Elevated levels of β-endorphin in the blood as a result of the oral consumption of a mistletoe lectin preparation are in line with the results of Heiny and Beuth (1994) and Heiny *et al.* (1998) who showed increased plasma levels of β-endorphin after treatment of breast cancer patients with standardized mistletoe lectin extracts. It is not known from this pilot study whether the observation that TNF-α values increased in parallel with increased levels of β-endorphin values is a matter of coincidence or is indeed of some biological significance. Clearly further

work will be required to provide concrete evidence that the observed changes in endostatin, TNF-α and β-endorphin values in response to oral intake of the mistletoe preparation are important.

In conclusion the mistletoe preparation described here (termed Palm), can be envisaged as a useful dietary supplement in order to stimulate the immune system in subjects where this would be beneficial *e.g.* in immunodeficiency. Such a preparation could also be beneficial for the public at large in order to "prime" the immune system such that, for example, the unpleasant effects of common colds and flu may be greatly reduced. In addition the body will in general be more suited to combat other types of bacterial or viral infection, and, furthermore, be able to operate more effectively in the inactivation/destruction of abnormal cells in the body (*e.g.* cells of precancerous nature, or even tumour cells themselves).

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12

Flavonoids from Complementary and Alternative Medicine: Mechanism of Imunomodulation of Macrophages

PANDIMA DEVI K.[1,*], KIRUTHIGA P.V.[1] AND KARUTHA PANDIAN S.[1]

ABSTRACT

Traditional, complementary and alternative medicines are commonly used to treat or prevent disease and chronic illness and to improve quality of life. More than 50% of the Indian populations living in rural areas rely on medicinal plants for their health care needs. There has been renewed attention and interest in the use of traditional medicine globally in the past decade. Even in developed countries, such medicines are becoming more popular nowadays. There is emerging evidence that these medicines have the ability to treat many diseases like cancer, infections, ulcers etc., and many medicinal plants also have the ability to modulate the immune system and disrupt the proinflammatory cascade through a variety of mechanisms like antioxidant effects and alteration in Phospholipase C mediated cell signaling. Macrophages often referred to as scavengers or antigen-presenting cells (APC) are considered as the important cells in the regulation of immune responses. Together with cytotoxic T lymphocytes and natural killer cells, they serve as effector cells to provide immunosurveillance against various diseases. In addition, they also influence the immune system by secretion of cytokines in both an autocrine and paracrine manner to protect the host against infective and toxic agents as well as to modulate the behavior of cells in the environment of triggered cells. Many research reports envisage complementary and alternative medicines as modulators of the functions of macrophages. The present review gives an introduction about the immune

1. Department of Biotechnology, Alagappa University, Karaikudi 630 003, Tamil Nadu, India.

* *Corresponding author*: E-mail : devikasi@yahoo.com

system, importance of macrophages and further focuses on the immunomodulatory role of flavonoids from complementary and alternative medicines on the function of macrophages.

Key words: Macrophages, immunomodulation, flavonoids, respiratory burst, inflammatory pathway

INTRODUCTION

The immune system has a collection of defense mechanisms, which recognizes the invading agents like pathogens and initiates a response in the host to eliminate the invading agents. The immune system is remarkable for its specificity of the antigen, that it is able to recognize subtle chemical differences that distinguish one foreign pathogen from another. In addition, the system also discriminates between foreign molecules and the body's own cells and proteins. Once a foreign organism has been recognized, the immune system recruits a variety of cells and molecules to mount an appropriate response, called an effector response, to eliminate or neutralize the organism. In this way the system is able to convert the initial recognition event into a variety of effector responses, each uniquely suited for eliminating a particular type of pathogen. Later exposure to the same foreign organism induces a memory response, characterized by a more rapid and heightened immune reaction that serves to eliminate the pathogen and prevent disease. The immune response is mediated by several types of cells like T-cells, B-cells, dendritic cells, Natural killer (NK) cells, macrophages and a multitude of other participating proteins. The immune system has 2 types of immunity called innate (non-specific) and adaptive (specific) immunity. Macrophages are innate immune effector that is they are activated without antigenic specificity. Macrophages are recruited and activated by many signals and have an impressive armamentarium of molecules to promote tissue damage, tissue repair, coagulation and fibrinolysis and to communicate with the rest of the immune system. Macrophages often referred to as scavengers or antigen-presenting cells (APC) and are important cells in the regulation of immune responses and their role is to phagocytose cellular debris and pathogens either as stationary or mobile cells, and to stimulate lymphocytes and other immune cells to respond to the pathogen.

MACROPHAGES—THE IMPORTANT CELL OF THE IMMUNE SYSTEM

Macrophages are heterogenous population of cells ubiquitously distributed in various organs and tissues of humans and animals; they show different cell morphology and have diverse functions according to requirements of the tissues in which they occur. More than a century has already passed since Metchnikoff in 1892 first termed large phagocytic cells as

"macrophages" on the basis of his phylogenetic studies and described their presence in all invertebrates and vertebrates (Takahashi, 2001).

MACROPHAGES IN THE TISSUES

Macrophages present in the tissues includes macrophages in connective tissue (histiocytes), liver (Kupffer's cells), lung (alveolar macrophages), lymph nodes (free and fixed macrophages), spleen (free and fixed macrophages), bone marrow (fixed macrophages), serous fluids (pleural and peritoneal macrophages), skin (histiocytes, Langerhans's cell), kidney (Mesangial cells), brain (Microglial cells), bone (Osteoclast) and in other tissues (Golds *et al.,* 2000).

FUNCTIONS OF MACROPHAGE

Macrophages are derived from circulating precursors known as blood monocytes. In stimulated conditions, monocytes migrate into tissues and differentiate into exudate macrophages. The essential step is carried out by tissue macrophages, which alert innate immunity. Unlike T- and B-cells, they do not contain any specific receptors. During infectious conditions macrophages posses certain types of receptors that recognize differential carbohydrate patterns on foreign cells such as lipopolysaccharide (LPS) (endotoxin). Stimulated macrophages starts to secrete cytokines like IL-1, 6, 8, 12 and TNF-α, that aid in their functions. Therefore, the activation and stimulation of macrophages is a key event for effective innate and adaptive immunity. In addition, macrophages are essential for effective tissue regeneration as they regulate the recruitment, proliferation and differentiation of target cells, such as fibroblasts, osteoblasts, endothelial cells and keratinocytes during healing processes (Golds *et al.,* 2000).

Apart from the above-mentioned functions, the main functions of macrophages are respiratory burst (Iles & Forman, 2002) and involvement in inflammatory processes (arachidonic acid metabolism) (Mantovani, 2007).

Respiratory Burst/Oxidative Burst in Macrophages

The respiratory burst is one of the main killing mechanisms for phagocytic cells like macrophages and it is the first line of defense against pathogens. It involves a series of signaling molecules and enzymes in a coordinative regulation. Macrophages possess a diversity of plasma membrane receptors, which recognize and bind both particulate and soluble stimuli found in body fluids. After the exposure of the external stimulants, the initial steps in the signal transduction cascades are G proteins. G protein-coupled ligands in macrophages initiate the enzyme Phospholipase C (PLC), which is a key member of signaling pathways. PLC, a family of eukaryotic intracellular

enzymes performs a catalytic mechanism, generating inositol triphosphate (IP_3) and diacyl glycerol (DAG). These molecules further modulate the activity of downstream proteins important for cellular signaling. IP3 is soluble, and diffuses through the cytoplasm and interacts with IP3 receptors, causing the release of calcium and raising the level of intracellular calcium which leads to activation of PLA2. On the other hand DAG remains tethered to the inner leaflet of the plasma membrane due to its hydrophobic character, where it recruits protein kinase C (PKC). Phosphorylated PKC activates NADPH oxidase there by tiggering signal transduciton events (Iles & Forman, 2002; Hoyal *et al.,* 1998). Assembly and activation of NADPH oxidase which utilizes cytosolic NADPH to reduce extracellular O_2 to O_2^- (Schreck *et al.,* 2006). The superoxide anion generated is enzymatically converted to hydrogen peroxide by superoxide dismutase (SOD). From the superoxide anion, hydrogen peroxide generated can serve as a precursor for generation of other ROS. ROS also activates members of the MAPK family of signaling pathways, including ERKs, JNKs, and p38 MAPKs. Oxidant-mediated activation of the MAPK signaling pathway is well established. MAPKs manage many diverse cellular functions, and are controlled by a balance of phosphorylation/dephosphorylation of MAPK family members (Gwinn & Vallyathan, 2006).

NF-κB signaling pathway was one of the first major pathways shown to be activated by ROS. It is highly activated at sites of inflammation and can induce transcription of proinflammotry cytokines, chemokines, adhesion molecules, COX-2 and inducible nitric oxide synthase, TNF-α, IL-1β. These proinflamatory cytokines are responsible for increase in the levels of NO. and prostaglandins. In macrophages, NO is known to be synthesized from L-arginine by inducible NO synthase (iNOS), the high-output isoform, which is highly expressed in LPS-activated macrophages and this contributes to the pathogenesis of septic shock (Kang *et al.*, 2002). Because iNOS can be strongly induced by proinflammatory stimuli, it is often called a high-output source of NO (Pacher *et al.*, 2007). Thus, the inhibition of NO production by blocking iNOS expression may result in a useful strategy for the treatment of various inflammatory diseases. COX is the enzyme that converts arachidonic acid to prostaglandins (PGs), and exists as two isoforms (COX-1 and -2). COX-2, the inducible form is responsible for the production of large amounts of pro-inflammatory PGs at sites of inflammation.

Macrophage in Arachidonic Acid Metabolism

Macrophages are key orchestrators of chronic inflammation (Gordon, 2003). Most anti-inflammatory agents directly or indirectly inhibit the formation or the effects of arachidonic acid metabolites collectively known as eicosanoids (prostaglandins, leukotrienes, thromboxanes, endoperoxides, and other mediators). Research into eicosanoids holds enormous promise for the treatment of disease with various drugs that selectively inhibit or

stimulate the production of specific prostaglandins. Phospholipase A2 (PLA2), one of the most intensively studied membrane proteins which catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to liberate arachidonic acid (AA), a precursor of eicosanoids including prostaglandins (PGs) and leukotrienes (LTs). PLA_2 proteins are of high pharmaceutical concern since they are responsible for the release of arachidonic acid from membranes, and since the subsequent conversion of this fatty acid to leukotrienes and prostaglandins is part of the inflammatory response. The cytosolic PLA2 (cPLA2) family consists of 3 enzymes, among which cPLA2 alpha plays an essential role in the initiation of AA metabolism (Murakami & Kudo, 2002). The principal pathways of arachidonic acid metabolism are the 5-lipoxygenase pathway, which produces a collection of leukotrienes (LT) and the cyclooxygenase (COX) pathway, which produces prostaglandin H_2 (PGH_2). PGH_2 serves as the substrate for two enzymatic pathways: one leading to the production of several prostaglandins (PG); the other leading to the production of thromboxanes (Tx).

Despite decades of research, corticosteroids and NSAIDs remain the main pharmacological weapons to control inflammation in the clinic. Unfortunately, these drugs have significant side effects, especially when used chronically. Consequently, there is tremendous interest in the development of novel, safer, and more effective anti-inflammatory drugs.

IMMUNOMODULATION OF MACROPHAGE

Macrophages play an important role in regulating the immune responses. Because macrophages are vital cells in host defense, alteration of macrophage function by different agents (Fig 12.1) could have undesirable effects on the host. Consequently, there is tremendous interest in the development of novel, safer and more effective immunomodulatory drugs. Numerous ayurvedic medicines have been tested for their biologic (especially immunomodulation) and clinical potential using modern ethnovalidation, thereby setting an interface with modern medicine (Chopra & Doiphode, 2002). Immunomodulatory medicinal plants are believed to promote positive health and maintain organic resistance against infection by reestablishing body equilibrium and conditioning the body tissues. They are reported to restore the immunocompetency of impaired hosts without hyperstimulation by augmenting macrophage chemotaxis, phagocytosis, and promoting interaction with other immunoregulatory lymphoid cell (Devi *et al.,* 2004). The proper regulation of macrophage function by immunomodulatory molecules could also help to protect the host from various pathologic and cancerous attacks. Currently immunomodulation of macrophages using plant derived flavonoid has received more attention in the scientific community and have become a very important research focal point.

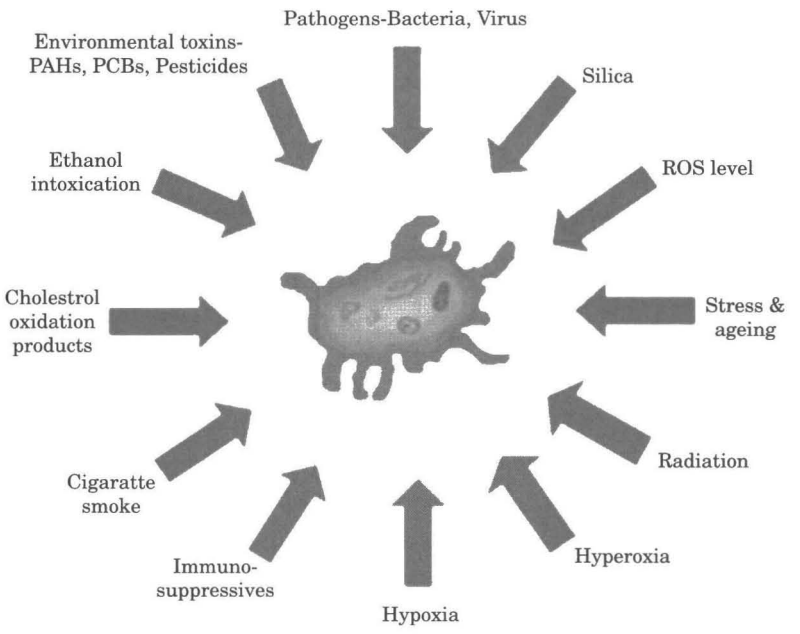

Fig 12.1. Diverse agents including pathogens, chemicals, radiation and other pathological conditions damage macrophages

FLAVONOIDS FROM COMPLEMENTARY AND ALTERNATIVE MEDICINES IN IMMUNOMODULATION OF MACROPHAGES

Flavonoids are naturally occurring polyphenolic compounds that are ubiquitous in plants. Over 4,000 different flavonoids have been described, and they are categorized into flavonols, flavones, catechins, flavanones, anthocyanidins, and iso-flavonoids. They have a variety of biological effects in numerous mammalian systems *in vitro* as well as *in vivo* such as free radical scavengers, antioxidants, and pro- or anti-mutagens, anti-inflammatory, antiviral, purgative effects etc., and some of them have been noted for their beneficial effect on cardiovascular diseases and cancer prevention. These effects could be explained by their abilities to inhibit the cell cycle, cell proliferation, or oxidative stress, improve the efficacy of detoxification enzymes, induce apoptosis, and stimulate the immune system. Because of its wide range of pharmacological properties flavonoids have been used as treatment for many diseases.

Currently, wide attention has been given to the molecular mechanism behind the immunomodulatory action of flavonoids. Many of the flavonoids have been described to modulate certain immune processes, such as the inhibition of enzymes and signaling molecules involved in respiratory burst, and inflammatory pathway (Fig 12.2).

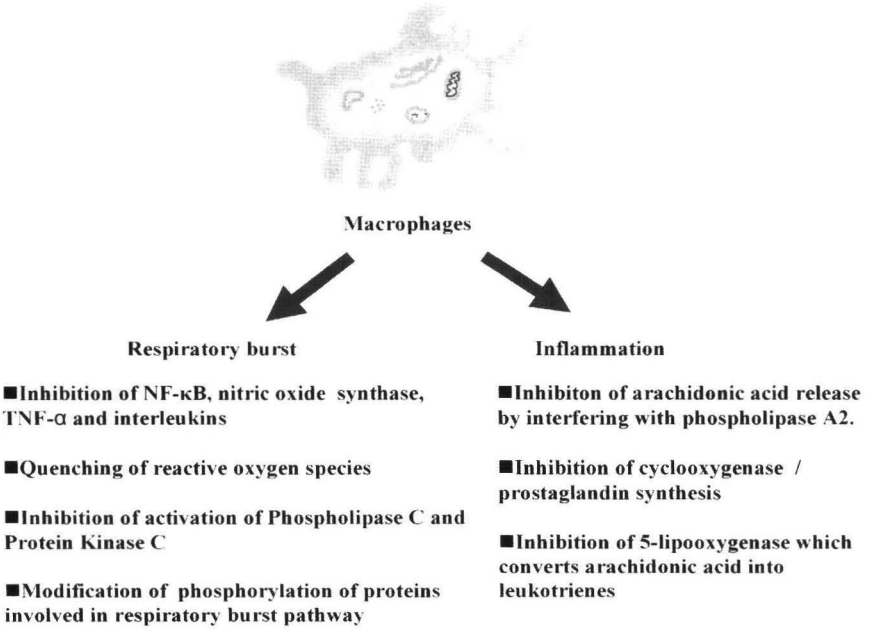

Fig 12.2. Mechanism of immunomodulatory action of flavonoids in macrophage

Mechanism of Immunomodulatory Action of Flavonoids in Respiratory Burst Pathway of Macrophages (Fig 12.3)

Inhibition of NF-κB, Nitric Oxide Synthase, and TNF-α and Interleukins by Flavonoids

More attention has been paid to the effect of flavonoids on the synthesis of proinflammatory cytokines against inflammatory conditions. Proinflammatory cytokines induce the formation of large amounts of nitric oxide (NO) by inducible nitric oxide synthase (iNOS), and compounds that inhibit NO production by blocking iNOS expression have anti-inflammatory effects. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are well known cytokines, secreted in great amounts by the activated macrophages. IL-1β and TNF-α may contribute to the development of bacterial sepsis, hypercholesterolemia, hypertension, and multiplex sclerosis.

- Studies in J774.2 macrophages has revealed that the flavonoids apigenin, kaempferol and resveratrol inhibited TNF-α gene, and apigenin and kaempferol suppressed IL-1β gene expression. These results indicate that anti-inflammatory action of these compounds, at least partly, may be mediated by transcriptional inhibition of IL-1β and TNF-α gene expression in macrophages (Kowalski *et al.*, 2005).

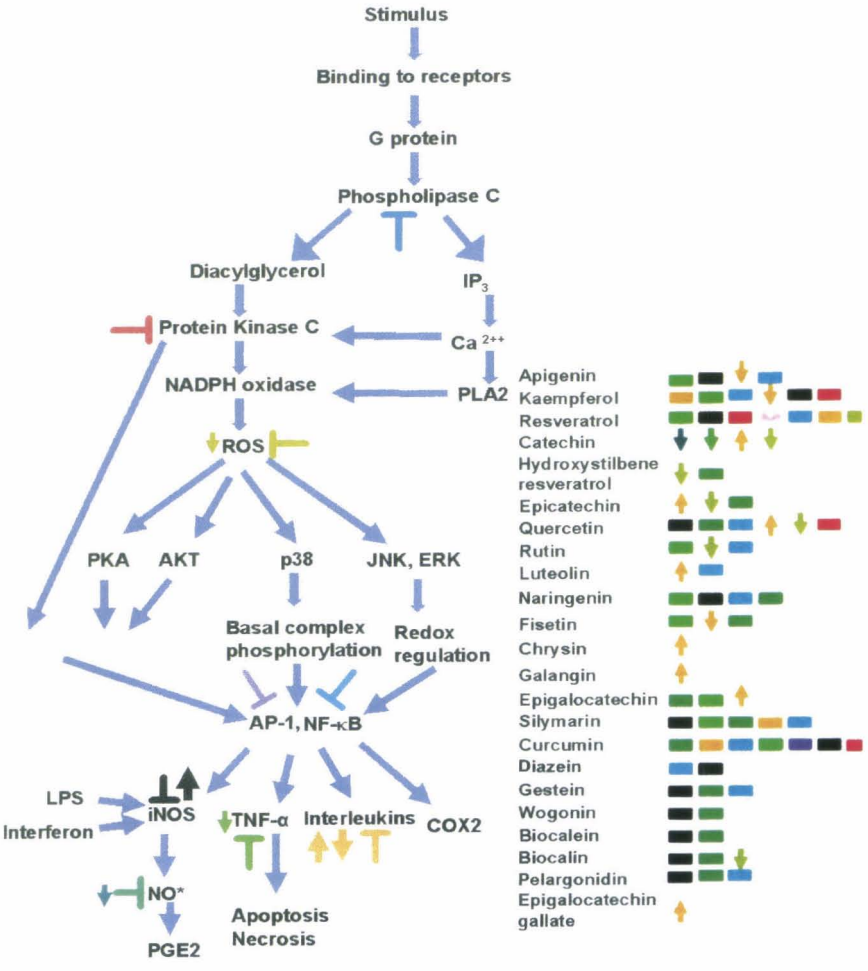

Fig 12.3. Immunomodulatory role of flavonoids in signaling pathway of macrophage respiratory burst (■—Inhibition, ↓—Decrease/Down-regulation, ↑—Increase/Upregulation)

- A glycoside of flavonol rutin, inhibits osteoclast formation induced by receptor activator of NF-κB ligand (RANKL) in bone marrow-derived macrophages. It reduces reactive oxygen species produced by RANKL and its inhibitory effect results from reduced levels of TNF-α. Rutin also lowers NF-κB activation in response to RANKL (Kyung *et al.,* 2008).
- The iNOS gene expression is regulated mainly at the transcriptional level in macrophages (Xie *et al.,* 1993), and the major transcriptional

regulators of iNOS gene are the NF-κB/Rel family of transcription factors that is also a key regulator of a variety of genes involved in immune and inflammatory responses. Saliou *et al.* (1998) reported that silymarin blocked the activation of NF-κB/Rel induced by okadaic acid and LPS, but not that induced by TNF-α in HepG2, a human hepatoblastoma-derived cell line. In contrast, TNF-α-induced NF-κB/Rel binding was inhibited by silymarin in U937, a human histiocytic lymphoma (Manna *et al.,* 1999), thus demonstrating pathway-dependent and cell type-specific inhibitory effect of silymarin. Silymarin also inhibits NO production and iNOS gene expression in macrophages, and these effects are mediated through the inhibition of NF-κB/Rel transcription factor. As stated earlier, NO plays an important role in the pathogenesis of various inflammatory diseases (Kleemann *et al.,* 1993). Therefore, the inhibitory effect of silymarin on iNOS gene expression suggests one of the mechanisms responsible for the anti-inflammatory action of silymarin (Kang *et al.,* 2002).

- Like NF-κB, STAT-1 (signal transducer and activator of transcription-1) is another important transcription factor for iNOS. A systematic investigation of the effects of 36 naturally occurring flavonoids and related compounds were carried out in macrophages exposed to the inflammatory stimulus LPS. The LPS-induced activity of STAT-1 was nearly totally (91% inhibition) inhibited by quercetin. Whereas, daidzein, genistein, and kaempferol had a moderate (32%–41% inhibition) inhibitory effect. These flavonoids also inhibited LPS-induced NF-κB activations, and iNOS expression (Hamalainen *et al.,* 2007). The mechanisms by which these flavonoids inhibit STAT-1 activation are not known, but may be associated with inhibition of phosphorylation of STAT-1 or its up-stream kinase JAK2 (Akiyama *et al.,* 1987).

- In addition, four compounds (flavone, isorhamnetin, naringenin, and pelargonidin) inhibited NF-κB activation and iNOS expression but had no effect on STAT-1 (Hamalainen *et al.,* 2007). Because NF-κB and STAT-1 are involved in the activation of several inflammatory genes, flavonoids that inhibit activation of NF-κB and/or STAT-1 are likely to down-regulate production of an array of inflammatory mediators in addition to iNOS.

- Another study to explore the action of quercetin, the most widely distributed flavonoids, on LPS and IFN-γ induced NO production was studied in BV-2 microglia cells. Quercetin could suppress LPS- and IFN-γ-induced NO production and iNOS gene transcription. LPS-induced inhibitor-κB kinase (IKK), nuclear factor-κB (NF-κB) and activating protein-1 (AP-1) activation, and IFN-γ-induced NF-κB, signal transducer and activator of transcription-1 (STAT1) and

interferon regulatory factor-1 (IRF-1) activation were reduced by quercetin. Moreover quercetin was able to induce heme oxygenase-1 expression and quercetin-mediated inhibition of NO production and iNOS protein expression were partially reversed by heme oxygenase-1. The involvement of signal pathways in quercetin-induced heme oxygenase-1 gene expression was associated with tyrosine kinase and mitogen-activated protein kinases activation (Chen *et al.,* 2005).

- Another set of flavonoids (catechin, epigallocatechin gallate (EGCG), epicatechin (EC), luteolin, chrysin, quercetin, and galangin) were examined for their effects on LPs induced NO production in RAW 264.7 macrophages. All those flavonoids except EGCG, apigenin and fisetin increased IL-2 secretion whereas EGCG, apigenin, and fisetin inhibited the secretion. These results indicated that flavonoids have the capacity to modulate the immune response and have a potential anti-inflammatory activity (Lyu & Park, 2005).
- Baicalin, baicalein and wogonin, the polyphenolic compounds isolated from the Chinese herb Huang Qui, inhibited LPS-induced NO production in RAW 264.7 macrophages through inhibition of iNOS gene expression, but not the activity of iNOS (Chen *et al.,* 2001).

Quenching of Reactive Oxygen Species by Flavonoids

The overproduction of reactive oxygen and nitrogen species (ROS and RNS, respectively) by phagocytes causes oxidative damage to membrane lipids, DNA, proteins and lipoproteins. These reactions have functional consequences, which may be deleterious to the cells and tissues. Thus, the inhibition of ROS and RNS production is a popular target for the attenuation of many inflammatory diseases (Shen *et al.,* 2002). Dietary polyphenols with antioxidative effects from fruit and vegetables play an important role in prevention of the oxidative stress.

- Flavonoid resveratrol exerted a strong inhibitory effect on superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) produced by macrophages which were stimulated by LPS or phorbol esters (PMA) (Martinez *et al.,* 2000).
- Baicalin (BA) exhibits anti-inflammatory effect in both *in vivo* and *in vitro* and is used to treat inflammatory diseases. BA inhibits the activation of macrophage and protects mice from macrophage-mediated endotoxin shock. It inhibited the production of reactive oxidative species (ROS) and also augmented the level of intracellular SOD. It inhibited the production of inflammatory mediators by macrophage and may be a potential target for treatment of macrophage-mediated diseases (Liu *et al.,* 2008).

- The polyphenols present in wine such as catechin, epicatechin, and quercetin, and the hydroxystilbene resveratrol prevented inflammation by decreased production of reactive oxygen species (ROS) in RAW264.7 macrophages (Ciz *et al.,* 2008). They exerted significant and dose-dependent scavenging effects against peroxyl radical and nitric oxide in chemical systems. Quercetin and resveratrol decreased the release of nitric oxide by these cells in a dose-dependent manner which corresponded to a decrease in iNOS expression in the case of quercetin. The higher number of hydroxyl substituents is an important structural feature of flavonoids in respect to their scavenging activity against ROS and nitric oxide, while C-2, 3 double bond (present in quercetin and resveratrol) might be important for inhibition of ROS and nitric oxide production by RAW 264.7 macrophages.
- Rutin also inhibited the osteoclast formation by inhibiting NF-κB by decreasing the production of ROS (Kyung *et al.,* 2008).
- ROS pathway might be another target of silymarin and is believed to be involved in NF-κB/Rel activation (Flohe *et al.,* 1997). Silymarin has a radical scavenging activity in RAW 264.7 cells, suggesting the possible mechanism for the inhibitory effect of silymarin on NF-κB/Rel activation. It is well known that NF-κB/Rel activation is regulated by the redox status of the cells (Cho *et al.,* 2000). However, the exact molecular targets responsible for the redox regulation of NF-κB/Rel activation remain unknown and need to be investigated.

Inhibition of Activation of Phospholipase C and Protein Kinase C by Flavonoids

- Quercetin and kaempferol also are reported to be inhibitors of protein kinase C (PKC) (Kantengwa & Polla, 1991).
- Resveratrol also inhibits PKC there by reducing the inflammatory processes (Sheu *et al.*, 2004).
- Because of multiple pharmacological properties curcumin act as promising anti-inflammatory agent. It also inhibits Phospholipase C, the important enzyme involved in the inflammatory pathway (Mahmmoud, 2007).

Modification of Phosphorylation of Proteins Involved in Respiratory Burst Pathway by Flavonoids

- Microglial cells are important protagonists in the cascade of events leading to tissue injury following neurodegeneration and other types of cerebral damage. Resveratrol was found to produce a potent suppressive effect on TNF-α and NO production induced by LPS in the mouse microglial cell line N9. These effects are mediated through

inhibition of nuclear factor κB (NF-κB) activation and p38 MAPK phosphorylation (Candelario-Jalill *et al.,* 2007).

- Quercetin blocked the phosphorylation of extracellular signal regulated kinase 1/2 (Erk1/2), p38, and c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) and also the activity of tyrosine kinases in LPS-stimulated RAW cells (Mu, 2001).

Mechanism of Immunomodulation of Flavonoids on Inflammatory Pathway in Macrophages (Fig 12.4)

Flavonoids have a wide range of biological effects, which include antihepatotoxic, antiallergic, anti-inflammatory, antiosteoporotic, and antitumor activities (Di Carlo *et al.,* 1999). Of all these effects, the anti-inflammatory effect is one of the most important properties of flavonoids that has been studied extensively. Landolfi *et al.* (1984) reported that many of the flavonoids are able to modify platelet function and arachidonic acid metabolism and showed that some flavonoids block both the cyclooxygenase and lipoxygenase pathways and also found to have a significant effect on both proliferative and exudative phases of inflammation (Agarwal, 1982).

Inhibiton of Arachidonic Acid Release by Flavonoids on Interference with Phospholipase A2

Most anti-inflammatory agents directly or indirectly inhibit the formation or the effects of arachidonic acid metabolites collectively known as eicosanoids (prostaglandins, leukotrienes, thromboxanes, endoperoxides, and other mediators). In phagocytic cells, activation of phospholipase A_2 (PLA_2) signalling pathways are amongst the earliest events triggered by inflammatory stimuli, and are believed to play a role in triggering or modulating chemotaxis, secretion, phagocytosis and superoxide anion release. It plays a key role in arachidonic acid metabolism. PLA_2 could be a better target in inflammatory pathway.

- Curcumin inhibits the cytosolic PLA_2 ($cPLA_2$) and further inhibit the release of arachidonic acid and its metabolites in LPS-stimulated RAW cells. Inhibition of phosphorylation of $cPLA_2$, inhibited the formation of prostaglandin E_2 (PGE_2) significantly and also inhibited LPS-induced COX-2 expression. This result suggested that curcumin affects arachidonic acid metabolism by blocking the phosphorylation of $cPLA_2$, decreasing the expression of COX-2 and inhibiting the catalytic activities of 5-LOX. These activities may contribute to the anti-inflammatory and anticarcinogenic actions of curcumin and its analogs (Hong *et al.*, 2004).
- Resveratrol also significantly decreased [3H] arachidonic acid release induced by LPS and PMA or by exposure to O_2^- or H_2O_2 (Martinez *et al.*, 2000).

Inhibitory Effect of Flavonoids on Cyclooxygenase/Prostaglandin Synthetase

COX-2 enzymes synthesis and prostaglandins biosynthesis is one of the processes in the inflammatory pathway. It is essential to target these processess to block the inflammation. Several past decades many flavonoids have been targeted to these processes (Fig 12.4).

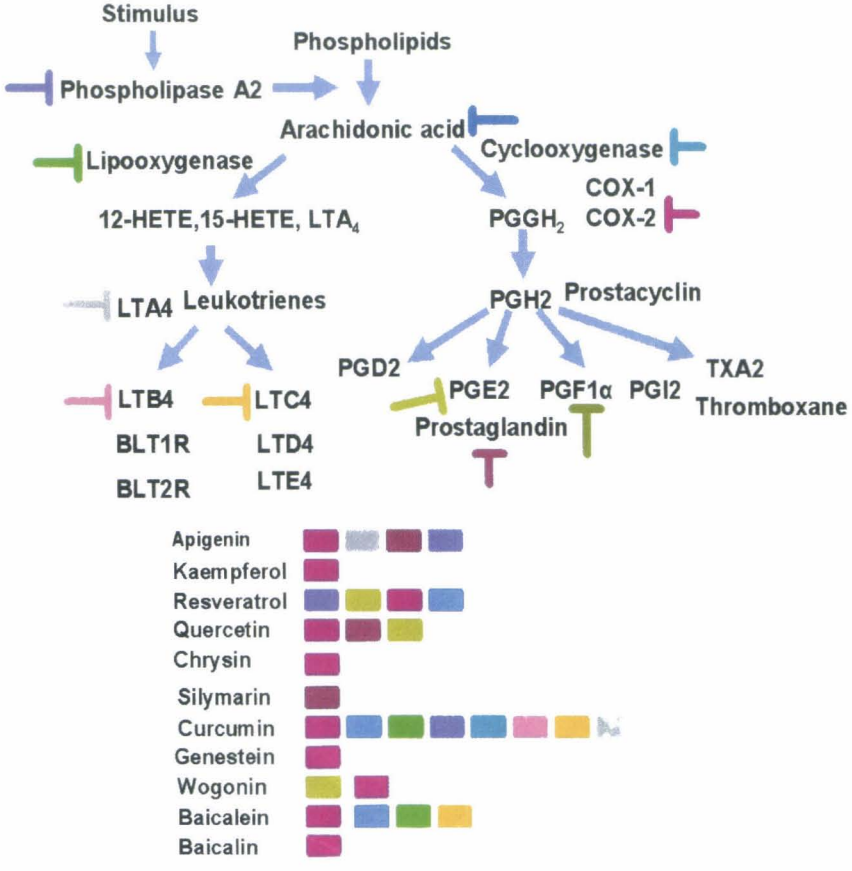

Fig 12.4. Immunomodulatory roles of flavonoids in macrophage inflammatory pathway (■—Inhibition, ↓—Decrease/Down regulation, ↑—Increase/Upregulation)

- Resveratrol (*trans*-3, 5, 4′-trihydroxystilbene) is a polyphenolic compound present in relatively large amounts in grapes and red wine. Study on LPS-activated primary rat microglia revealed that resveratrol is a prostaglandin E2 inhibitor which specifically prevents microsomal prostaglandin E synthase-1 (mPGES-1) expression which is the most important terminal synthase responsible for the synthesis of PGE2 by activated microglia. It potently reduced LPS-induced PGE2

synthesis and the formation of 8-*iso*-$PGF_2\alpha$, a measure of free radical production. Interestingly, resveratrol dose dependently reduced the expression (mRNA and protein) of mPGES-1 without affecting COX-2 levels (Candelario-Jalil *et al.,* 2007). Previous studies demonstrated that resveratrol readily crosses the intact blood brain barrier, suggesting resveratrol as one of the most promising immunomo-dulatory compounds against brain injury. The prenylated resveratrol derivative 4-(3-methyl-but-1-enyl)-3, 5, 3′, 4′-tetrahydro-xystilbene which was purified from fungally infected peanuts are of interest as inhibitors of cyclo-oxygenase-2 and as antiinflammatory agents (Patel *et al.*, 2005). Resveratrol treatment also caused a significant impairment of cyclooxygenase-2 (COX-2) induction stimulated by LPS and PMA or by O_2^- or H_2O_2 exposure (Martinez *et al.,* 2000).

- Flavonoids such as apigenin, genistein and kaempferol were markedly active inhibitors of transcriptional activation of COX-2 (Huang *et al.,* 1999).
- Flavonoids such as baicalin, baicalein, and wogonin, were examined for their effects on LPS-induced NO production and iNOS and COX-2 gene expressions in RAW 264.7 macrophages (Chen *et al.*, 2001). The results showed that the flavonoids protect cells by inhibiting COX-2. Another study regarding the effects of 5,7-dihydroxy-8-methoxyflavone (wogonin) in macrophages revealed that it inhibits COX-2-mediated prostaglandin E_2 production (Wakabayashi & Yasui, 2000).
- Lycopene and quercetin inhibited the expression of iNOS and COX-2 gene in RAW 264.7 macrophages stimulated by gliadin (De Stefano *et al.*, 2007).
- Flavonoids wogonin and quercetin attenuated LPS-induced prostaglandin E_2 production *in vitro* in RAW 264.7 and primary peritoneal macrophages (Shen *et al.,* 2002).

Inhibition of 5-Lipooxygenase by Flavonoids

Inhibitors of the 5-LOX pathway have chemopreventive abilities in animal lung carcinogenisis and block the oxidation of several potent carcinogens.

- Biacalein (5, 6, 7-trioxyflavone-7-O-beta-D-glucuronide) had a anti-inflammatory activity by inhibiting the 5-lipooxygenase (5-LO) pathway thereby inhibiting the biosynthesis of leukitriene C4 (LTC4) in rat resident peritoneal macrophages stimulated with the calcium ionophore (Butenko *et al.*, 1993).
- Extensive scientific research on curcumin, a natural compound present in the rhizomes of plant *Curcuma longa* Linn., have demonstrated its antiinflammatory action. Curcumin is a LO inhibitors and is also reported to inhibit the formation of leukotrines via inhibition of arachidonic acid pathway (Hong *et al.,* 2004).

CONCLUSIONS

In various cells like RAW cells, rat peritoneal macrophages and animal studies, many flavonoids were found to have good anti-inflammatory activity and also seemed to have negligible adverse effect on human systems. The immunomodulatory effects of the flavonoids are consolidated in Table 12.1. Immunomodulation of flavonoids is an area under wide research scrutiny yielding many broad spectrum as well as specific inhibitors.

Table 12.1. Summary of the immunomodulatory effects of the different flavonoids in macrophage

Chemical compound	Species	Biological activity
Apigenin	*Adinandra nitida*	Inhibition of (IL-1β and TNF-α), IL-2 secretion, COX-2, iNOS, NF-κB, leukotrienes and phospholipase A2, Prostaglandins E2
Kaempferol	Onions, apples, grapes, leeks, Citrus fruits and red wine	Inhibition of cytokines(IL-1β and TNF-α), NO, COX-2, iNOS, Protein kinase C and NF-κB
Resveratrol	Red grapes	Inhibition of cytokines (IL-1β and TNF-α), iNOS, PKC, COX, AKT, (NF)-κB. Decrease the production of ROS and prostaglandins E2, inhibit p38 MAPK phosphorylation, 5-lipoxygenase
Catechin	Henna, green tea	Decrease the production of ROS, Repression of NO production and TNF-α secretion, Increased IL-2 secretion
Hydroxystilbene resveratrol		Decrease the production of ROS, NO inhibitor
Epicatechin	Cocoa, tea, grapes	Decrease the production of ROS, Increased IL-2 secretion, NO inhibitor
Quercetin	Onions, apples, and tea	Decrease the production of ROS, Inhibits NO, iNOS, and NF-κB, COX-2, TNF-α and Protein Kinase C. Increased IL-2 secretion. Attenuation of Prostaglandin E2 production, blocked the phosphorylation of ERK, JNK, p38.
Rutin	*Artemisia scoparia* thumb, buckwheat	Inhibits osteoclast formation, Inhibits cytokines TNF-α, NF-κB. Decrease the production of ROS.
Luteolin	*Salvia tomentosa*	Increased IL-2 secretion, NF-κB inhibitor.
Naringenin	Orange, citrus fruits	Repression NO production and TNF-α secretion, iNOS, NF-κB inhibitor.
Fisetin	Strawberries	Repression NO production and TNF-α secretion, Inhibition of IL-2 secretion
Chrysin	*Passiflora caerulea*	Increased IL-2 secretion, COX and iNOS inhibitor

Table 12.1. *Contd.*

Chemical compound	Species	Biological activity
Galangin	*Passiflora* caerulea	Increased IL-2 secretion
Epigallocatechin	*Alpinia galanga*	Repression NO production and TNF-α secretion, Inhibition of IL-2 secretion
Epigallocatechin gallate	Green tea leaves	Increased IL-2 secretion
Genistein	Green tea leaves	COX-2, iNOS, NO, NF-κB inhibitor
Diazein	Soyabean	iNOS, NF-κB inhibitor
Silymarin	*Silybum marianum*	Inhibition of iNOS, NO, TNF-α, Inhibits PGE2 and IL-1β
Pelargonidin	Strawberries	iNOS, NO, NF-κB inhibitor
Curcumin	*Curcuma longa* Linn	Inhibiton of NF-κB, AP-1, iNOS,COX-1, COX-2, interleukin-8, 5-lipooxygenase, Phospholipase A2, Leukotriens B4, Leukotriens C4, Reduces the production of TNF-α and (IL-1β). Enhancement of secretion of 6-keto PG F1α
Wogonin	*Scutellaria baicalensis*	Inhibition of inducible prostaglandins E2, iNOS, NO
Baicalin	*Scutellaria baicalensis*	Inhibits iNOS, NO
Baicalein	*Scutellaria baicalensis*	Inhibition of iNOS, NO, Lipooxygenase, leukotriene C4 biosynthesis

ACKNOWLEDGEMENTS

One of the authors PVK acknowledges Rameshwardaji Birla Smarak Kosh for the financial assistance. The authors also gratefully acknowledge the computational and bioinformatics facility provided by the Alagappa University Bioinformatics Infrastructure Facility (funded by Department of Biotechnology, Government of India; Grant No. BT/BI/25/001/2006).

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13

Herbal Immunomodulators

CHAUHAN SINGH NAGENDRA[1,*], SHARMA VIKAS AND DIXIT V.K.[1]

ABSTRACT

Herbs have shown potential of being used as immuno-modulators especially the drugs used in alternative system of medicine, have been documented to possess immunomodulatory properties. Many drugs used in Ayurvedic system of medicine for Rasayana therapy have been investigated for their effect on immune system. The plants employed as Rasayan, the active phyto-constituents as well the mechanism involved in the pharmacological findings have been discussed with a view to appreciate immunomodulation of these plants.

Key words : Ayurveda, immunomodulating, Indian medicine, herbal, pharmacological, rasayana

INTRODUCTION

Immunology acquires an important place in modern biology, particularly in medical science. Immunology also plays an important role in the process of diagnosing diseases. The immune response is involved in the etiology as well as in the pathophysiological mechanism in the process of diseases. Immunology is one of the most developing areas of biomedical research. It also opens the doors of great hopes and major advances in the prevention and treatment in wide range of disorders. Today diseases like arthritis ulcerative colitis, asthma, allergy, parasitic and infectious diseases are primarily considered as immunological disorders. Immune mechanism is also involved in variety of other diseases such as cancer, diabeties mellitus,

1. Natural Product Research Laboratories, Department of Pharmaceutical Sciences, Dr. H.S. Gour. University, Sagar (M.P. 470 003) India.

* *Corresponding author* : E-mail : chauhan.nagendra@gmail.com

myocardial diseases, cirrhosis and atherosclerosis (Herausgegben *et al.*, 1984; Samter, 1971).

Immunomodulation is a process which can alter the immune system of an organism by interfering with its function, if it results in an enhancement of immune reaction is *immunostimulation* and primarily implies stimulation of non-specific system *i.e.* stimulation of the function and efficiency of granulocytes, macrophages, complement, certain T-lymphocytes and different effectors substances. Immunosuppression implies mainly to reduce resistance against infection, stress and may be because of environmental or chemotherapeutic factors (Patwardhan *et al.*, 1990).

Immunomodulation is the regulation and maintenance of immunity either by enhancing or the dampening of the immune response. Modulation in immune response may involve induction, expression or amplification of immune response. Immunomodulation may be specific *i.e.* limited to a given antigen or non-specific *i.e.* general effect on immune response. Levels of immunomodulation may be affected by modulation in antigen reorganization, lymphoid cell interaction, balance of helper and suppressor cell function, proliferation of responding cell population, differentiation of effectors cells, synthesis of immune products (lymphokines, specific antibodies) and accessory effector system (macrophage, mast cell and compliment activity).

The use of medicinal plant products as possible prophylactic/therapeutic measure for the modulation of immune response have become a subject of scientific investigations. Many Indian medicinal plants have been screened by various research workers in the search of novel immunomodulatory compounds. Lazaraw (1947), Lindquist and Teuscher (1985), Wagner (1987), Wagner and Prokesh (1985) and Labadie *et al.* (1989) have reviewed the natural products which have been a reputation in ethanomedicinal practice and which gives an advance picture in the field of immunomodulation. The rejuvenation therapy is define as that attains longevity, memory, intellect, freedom from diseases, youth, and excellence of luster, true sense-organ and respect, brilliance and vak-siddhi (*i.e.* what he says comes true). This rejuvenation therapy is known as Rasayana therapy. The materials used in the therapy are termed as "Rasayana". These rasayana may be inducer of enzymes and hormones, which the body needs for adaptations and survival during normal health, stress and in diseases (Sharma & Das, 1996). Rejuvenation or revitalization therapy suggests that the body fluids can be replenished renewed by proper medications. It is possible to achieve not only vitality and vigor but also greater resistance to diseases, longevity, memory, intelligence, body strength, personal beauty and sense perceptions (Ray & Gupta, 1965).

This concept of rasayana is well known to Ayurvedic physicians that the delicate cellular machinery of the body suffers from trauma (stress)

resulting in wear and tear of different body structures and detoriation of the functional capacity of human being. This procedure of revitalization and rejuvenation were adopted to increase the power of resistance to disease (increased immunity) (Sharma, 1981). Modulation of immune response to alleviate the diseases has been of interest for many years and the concept of Rasayana in Ayurveda is based on related principles. Rasayana drugs in Ayurveda are known to prevent aging, increase longevity and offer resistance to disease by augmenting the immune system. (Reg *et al.,* 1999).

PLANTS HAVING IMMUNOMODULATORY POTENTIAL

A list of various Immunomodulating plants with their family, plant part and name of active extract is given in Table 13.1. Chemical constituents responsible for immunomodulators and their chemical structures are given in Table 13.2 and Fig 13.1. Studies on single *immunomodulator* herbs are presented here.

Andrographis paniculata

Andrographis paniculata (Burm.f) Nees; family: Acanthaceae, also known commonly as "kalmegh", is a well known medicinal plant of Ayurveda and has been used for centuries in Asia. About 26 different polyherbal formulations of this plant are mentioned in Ayurveda as a popular remedy for the treatment of various disorders. *A. paniculata* is an annual shrub which grows abundantly in India and cultivated extensively in China and Thailand. The aerial parts of the plant (leaves and stems) are used to extract the active phytochemicals (Kumar *et al.,* 2004). *A. paniculata* ethanolic extract and andrographolide were found to enhance IgG antibody levels against *S. typhimurium* and also showed a remarkable increase in the production of IFN-γ following stimulation with the bacterial lysate, indicating an induction of Salmonella-specific cell-mediated response/ immune response (Xu *et al.,* 2007). It also show significant enhancement of haemagglutinating antibody and delayed type hypersensitivity in a mice treated with sheep red blood cells as compared to untreated animals. There was a significant enhancement [not induction] of non-specific or innate immune response as measured by an increase in macrophage migration indices, phagocytosis of 14 C labeled *E. coli*, and increased incorporation of 3H-thymidine in PHA [phytohaemagglutinin]-stimulated splenocyte supernatants. It also showed non-specific immune response of mice administered with andrographolide by the intraperitoneal route higher than when the route of administration was the oral route (Puri *et al.,* 1993). Dichloromethane fraction of the methanolic extract retains the three active diterpene compounds, *i.e.* [1] andrographolide, [2] 14-deoxyandrographolide and [3] 14-deoxy-11, 12-didehydroandrographolide that contributing for both the anticancer and immunostimulatory activities. Dichloromethane fraction significantly inhibits the proliferation of HT-29 (colon cancer) cells and augments the proliferation of human peripheral blood lymphocytes

Table 13.1. Following is a list of various immunomodulating plants with their family, plant part and name of active extract

S. No.	Plant name	Family	Plant part(s)	Extract	Activity	Reference(s)
1.	*Argyreia speciosa* Sweet	Convolvulaceae	Roots	Ethanolic	Immunostimulant	Gokhale *et al.*, 2003
2.	*Aloe vera* Linn	Aloaceae	Leaves	Aqueous	Immunostimulant	Pugh *et al.*, 2001
3.	*Albizzia lebbeck* Linn.	Mimosaceae	Bark	Aqueous and butanolic	Immunostimulant	Barua *et al.*, 2000
4.	*Aerva lanata* Juss	Amaranthaceae		Pet. ether	Immunomodulatory	Nevin & Vijayammal, 2005
5.	*Baliospermum montanum* (Willd.) Müll. Arg.	Euphorbiaceae	Roots	Ethanolic	Immunostimulant	Wadekar *et al.*, 2008
6.	*Biophytum sensitivum* (L) DC	Oxalidaceae	Aerial part	Alcoholic	Immunomodulatory	Guruvayoorappan & Kuttan, 2007
7.	*Boerhaavia diffusa* Linn	Nyctaaginaceae	Roots	Aqueous and ethanolic	Antilymphopro-liferative	Mugantiwar *et al.*, 1999
8.	*Boswellia serrata* Roxb	Burseraceae	Bark	Oleo gum resin	Immunomodulating	Sharma *et al.*, 1996
9.	*Buchanania lanzan* Spreng.	Anacardiaceae	Seeds	Ethanolic	Immunostimulant	Puri *et al.*, 2000
10.	*Capparis zeylanica* Linn	Capparidaceae	Leaves	Ethanolic and water	Immunomodulatory	Ghule *et al.*, 2006
11.	*Cedrus deodara* (Roxb.) Loud.	Pinaceae	Wood	Volitile oil	Immunomodulatory	Shinde *et al.*, 1999
12.	*Centella asiatica* Linn	Apiaceae	Whole herb	Methanolic, aqueous	Immunomodulatory activities	Jayathirtha & Mishra, 2004; Patil *et al.*, 1998
13.	*Carica papaya* Linn	Caricaceae	Seeds	Hexane and aqueous extract	Lymphocyte stimulation	Mojica-Henshaw1 *et al.*, 2003
14.	*Chlorophytum borivilianum* Sant. & F	Liliaceae	Roots	Ethanolic	Immunomodulatory	Thakur *et al.*, 2006
15.	*Cissampelos pareira* Linn	Menispermaceae	Roots	Methanol	Immunostimulant	Bafna & Mishra, 2005

Table 13.1. *Contd.*

S. No.	Plant name	Family	Plant part	Extract	Activity	Reference(s)
16.	*Commiphora mukul* Engl	Burseraceae	Gum	Ethyl acetate	Immunostimulant	Manjula *et al.*, 2006
17.	*Curculigo orchioides.* Gaertn	Amaryllidaceae	Rhizomes	Methanol	Immunomodulatory	Lakshmi *et al.*, 2003
18.	*Cryptolepis buchanani* Roem. & Schult	Asclepiadaceae	Roots	Ethanolic	Immunostimulant	Kaul *et al.*, 2003
19.	*Eclipta alba* (Linn.) Hassk	Asteraceae	Whole herbs	Methanol	Immunomodulatory	Jayathirtha & Mishra, 2004
20.	*Euryale ferox* Salisb	Nymphacaceae	Seeds	Ethanolic	Immunostimulation	Puri *et al.*, 2000
21.	*Euphorbia tinucalli* Linn	Euphorbiaceae	Latex	Ethy acetate	Immunosuppressive	Bani *et al.*, 2005
22.	*Ficus benghalensis* Linn	Moraceae	Aerial Root	Methanolic	Immunostimulation	Gabhe *et al.*, 2006
23.	*Heracleum nepalense* D. Don	Apiaceae	Roots	Methanolic	Immunostimulation	Dash *et al.*, 2006
24.	*Hippophae rhamnoides* Linn	Elaeagnaceae	Leaves and fruits	Alcoholic	Cytoprotective and antioxidant	Geetha *et al.*, 2002
25.	*Lawsonia alba* Lam	Lytheraceae	Leaves	Napthoquinone	Antiviral and antitumoral	Kulkarni & Karande, 1998
26.	*Luffa cylindrical* Linn	Cucurbitaceae	Seeds	Methanolic	Immunostimulatory effect	Khajuria *et al.*, 2007
27.	*Magnifera indica* Linn	Anacardiaceae	Stem bark	Alcoholic	Immunostimulant	Makare *et al.*, 2001
28.	*Nyctanthes arbro-tristis* Linn	Oleaceae	Whole plant	Ethanolic	Immunostimulant	Puri *et al.*, 1994
29.	*Psoralea corylifolia* Linn	Fabaceae	Seeds	Ethyl alcohol	Antitumour	Latha *et al.*, 2000
30.	*Phoneix dactylifera* Linn	Arecoceae	Fruit	Ethanolic	Immunostimulant	Puri *et al.*, 2000

Table 13.1. *Contd.*

S. No.	Plant name	Family	Plant part	Extract	Activity	Reference(s)
31.	*Premna tomentosa* Willd	Verbenaceae	Leaves	Methanolic	Immunomodulatory and cytoprotective	Pandima *et al.*, 2003, 2004
32.	*Piper longum* Linn	Piperaceae	Fruits	Alcoholic	Immunomodulatory and antitumor	Sunila & Kuttan *et al.*, 2004
33.	*Prunus amygdalus* Batsch	Rosaceae	Seed	Ethanolic	Immunostimulant	Puri *et al.*, 2000
34.	*Punica granatum* Linn	Punicaceae	Fruit	Aqueous	Immunostimulant	Gracious *et al.*, 2001
35.	Pistacia integerrima J. L. Stewart ex Brandis	Anacardiaceae	Leaf	Aqueous and methanolic	Immunomodulatory and adaptogenic	Joshi & Mishra, 2008
36.	*Semecarpus anacardium* Linn.	Anacardiaceae	Nut	Milk	Immunomodulatory, anti-inflammatory	Ramprasath *et al.*, 2006
37.	*Sphaeranthus indicus*	Compositae	Flower heads	Methanolic	Immunostimulant	Bafna & Mishra, 2004
38.	*Sphaeranthus indicus* Linn	Compositae	Flower	Petroleum ether	Immunostimulant	Bafna & Mishra., 2007
39.	*Selaginella involvens* (Swartz) Spring	Selaginellaceae	Whole plant	Aqueous	Immunostimulant and antioxidant	Gayathri *et al.*, 2005
40.	*Rubia cordifolia* Linn.	Rubiaceae	Root	Ethanolic	Immunomodulatory	Joharapurkar *et al.*, 2003
41.	*Taxus wallichiana* Zucc	Taxaceae	Needles	Methanolic	Immunomodulatory and cytotoxic	Chattopadhyay *et al.*, 2006
42.	*Tephrosia purpurea* Linn.	Leguminosae	Aerial parts	Ethanolic	Immunostimulant	Damre *et al.*, 2003
43.	*Trigonella foenum graecum* Linn	Leguminosae	Whole plant	Aqueous	Immunostimulatory effect	Hafeez *et al.*, 2003
44.	*Tinospora sinensis* (Lour.) Merrill	Menispermaceae	Stems	Water and ethanolic	Immunostimulatory effect	Manjrekar *et al.*, 2000
45.	*Tridax procumbens* Linn	Compositae	Aerial parts	Aqueous	Immunostimulatory effect	Tiwari *et al.*, 2004
46.	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome	Ethanolic	Immunostimulant	Puri *et al.*, 2000

Table 13.2. Chemical constituent(s) responsible for immunomodulators

Biological source	Chemical constituent(s)	Mechanism of action	Reference(s)
Aristolochia clemalites Linn. Aristolochiaceae	Aristolochic acid	Enhanced phagocytosis	Wagner, 1986
Azadiracta indica A Juss. Meliaceae	Catechins	Enhanced phagocytosis	Wagner and Proksch, 1985
Azadirachta indica A Juss. Meliaceae	Volatile oil	Activate macrophage	Sairam *et al.*, 1997
Azadirachta indica A Juss. Meliaceae	Fixed oil	Immunostimulation	Upadhyay *et al.*, 1992
Azadirachta indica A Juss. Meliaceae	Glycoprotein	Cytotoxicity	Chakraborty *et al.*, 2008
Abrus precatorious Linn. Fabaceae/Leguminosae	Lectin	Macrophage stimulation	Tripathi & Maiti, 2003
Aloe barbadensis Mill. Liliaceae	Acemannan, aloeride	Induced nitric oxide production, increase interleukin level and TNF factor	Ramamoorthy *et al.*, 1996; Pugh *et al.*, 2001
Aloe vera Mill. Liliaceae	Dihydrocoumarin derivatives, acemannan	Immunomodulation, anticomplement activity	Zhang *et al.*, 2006; Lee *et al.*, 1997
Abrus precatorius L. Leguminosae	Abrus agglutinin	Immunomodulatory and anti-tumor	Ghosh *et al.*, 2007
Andrographis paniculata Nees	Diterpenes acanthaceae	Cell differentiation	Mastuda *et al.*, 1994
Apium graveolens L. Apiaceae	Quercetin, rutin	Immunomodulatory activities	Cherng *et al.*, 2007
Astragalus membranaceus Fisch Leguminosae	Daucosterol	Immunoregulatory activity	Lee *et al.*, 2007
Asparagus racemosus Willd. Liliaceae	Shatavarin-IV and immunoside	Immunomodulating	Gautam *et al.*, 2004
Aegle marmelos (Linn.) Corr. Rutaceae	Propelargonidin	Anticomplement activity	Abeysekera & De silva, 1996
Allium sativum Linn Amaryllidaceae	14-kDa glycoprotein	Immunomodulatory activity	Ghazanfaria *et al.*, 2002
Artemesia annua Linn Asteraceae	Artemesin, dihydroartemesin and arteether	Immunomodulating	Tawfik *et al.*, 1990
Berberis aristata DC. Berberidaceae	Berberine	Enhance phagocytosis	Mastumoto *et al.*, 1996
Boswellia serrata Roxb. Ex Colebr.	BOS 2000, boswellic acid burseraceae	Immunostimulatory	Khajuria *et al.*, 2008; Sharma *et al.*, 1996

Table 13.2. *Contd.*

Biological source	Chemical constituent(s)	Mechanism of action	Reference(s)
Brassica oleracea Linn. Brassicaceae	Sulforaphane	Immunomodulation	Thejass & Kuttan, 2007
Boswellia carterii Flueck. Burseraceae	Triterpenoids	Immunostimulant activity	Badria *et al.*, 2003
Cajanus indicus Spreng. Leguminosae	CI-1	Immunopotentiation	Datta *et al.*, 1999
Coriandrum sativum L Umbelliferae	Quercetin, rutin	Immunomodulatory activities	Cherng *et al.*, 2007
Curculigo orchioides Gaetrn. Hypoxidaceae	Curculigoside	Increase phagocytosis	Kubo *et al.*, 1983
Cephaelis ipecacuanha (Brotero), A. Richard. Rubiaceae	Emetine	Increase phagocytosis	Wagner, 1986
Catharanthus roseus Linn. G. Donn Apocynaceae	Vincristine	Induce antibody production	Wagner, 1986
Claviceps purpueria (Fr.) Tul Convolvulaceae	Ergot alkaloids	Immunomodulatory	Fiserova *et al.*, 1997
Camptotheca acuminate Decne Nyssaceae	Camptothecin, tenulin	Induce interferon, production of antibodies	Wagner, 1986
Canavailia ensiformins (L.) DC.Leguminosae-Papilionoideae	Concavolin A	Stimulate lymphocytes	Wagner *et al.*, 1985
Curcuma longa Linn. Zingiberaceae	Curcumin	Inhibits cell proliferation	Abu-Rizq *et al.*, 2008
Cedrus deodara (Roxb.) Loud Pinaceae	Wood oil	Inhibit neutrophil adhesion	Shinde *et al.*, 1990
Crocus sativus Linn. Iridaceae	Proteoglycan	Induced nitric oxide production	Escribano *et al.*, 1999
Datura quercifolia Kunth. Solanaceae	1b, 5a, 12a-trihydroxy-6a, 7a, 24a, 25a-diepoxy-20S, 22R with-2-enolide (lactones)	Stimulate antibody production	Bhat *et al.*, 2005
Dysoxylum binectaniferum Roxb. Hook. F. Ex Bedd. Meliaceae	(+)-cis-5, 7-Dihydroxy-2-methyl-8-[4, (3-hydroxy-1-methyl) piperid	Immunomodulatory and anti-inflammatory	Naik *et al.*, 1988
Daucus carota L. Apiaceae	Quercetin, rutin	Immunomodulatory activities	Cherng *et al.*, 2007

Table 13.2. *Contd.*

Biological source	Chemical constituent(s)	Mechanism of action	Reference(s)
Desmodium gangeticum (L.) DC. Fabaceae	Aminoglucosyl glycerolipid, glycosphingolipid	Increae nitric oxide level	Mishra *et al.*, 2005
Foeniculum vulgare Mil Umbelliferae	Quercetin, rutin	Immunomodulatory activities	Cherng *et al.*, 2007
Ipomoea batatas (l.) Lam. Var. Cannabina Hallier F. Convolvulaceae	(1 → 6)-alpha-D-glucan	Immunostimulating	Zhao *et al.*, 2005
Lawsonia inermis Linn. Lythraceae	*p*-coumaric acid lawsone, apigenin luteolin, and 2-methoxy-3-methyl-1, 4-naphthoquinone, cosmosiin and apiin	Immunostimulant and antioxidant	Mikhaeil *et al.*, 2004
Linum usitatissimum L. Linaceae	Cyclonopeptide A	Immunosupression	Morita *et al.*, 1997
Luffa cylindrica (L.) M. J. Roem. Cucurbitaceae	Sapogenins 1 and 2	Immunostimulant	Khajuria *et al.*, 2007
Magnifera indica L. Anacardiaceae	Allergens	Immunomodulation	Edwards *et al.*, 1995
Ocimum sanctum Linn Labiatae	Fixed oil	Immunomodulatory	Mediratta *et al.*, 2002
Plumbago zeylanicum L. Plumbaginaceae	Plumbagin	Stimulate phagocytosis	Wagner *et al.*, 1985
Picrorhiza kurroa Royle Ex Benth Scrophulariaceae	Picroside I, II, kutkoside, vanillic acid, Apocynin; Iridoid glycoside	Anticomplement activity, Immunostimulant	Wagner *et al.*, 1985; Puri *et al.*, 1992
Trigonella foenum-graecum Linn. Leguminosae-Papilionoideae	Galactomannan	Increase phagocytosis	Ramesh *et al.*, 2002
Plumbago zeylanica	Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone)	Improve macrophage function	Abdul & Ramchender, 1995
Plantago ovata Forsk. Plantaginaceae	Mucopolysaccharides	Increase macrophage activation	Rezaeipoor *et al.*, 2000
Petroselinum crispum Umbelliferae	Quercetin, rutin	Immunomodulatory activities	Cherng *et al.*, 2007

Table 13.2. *Contd.*

Biological source	Chemical constituent(s)	Mechanism of action	Reference(s)
Stevia rebaudiana Bertoni (Asteraceae)	Stevioside	Immunostimulant	Sehar *et al.*, 2008
Tinospora cordifolia (Willd.) Hook f. &. Thoms Menispermaceae	(1, 4)-alpha-D-glucan, cardifolioside A and B	Activation of macrophage, immunostimulating	Nair *et al.*, 2006; Maurya *et al.*, 1996
Withania somnifera (L) Dunn Solanaceae	Sitoindosides IX and X	Immunostimulating	Ghosal *et al.*, 1989
Viscum album L. Loranthaceae	Viscumin	Stimulate lymphocytes	Wagner *et al.*, 1985

(HPBLs) at low concentrations. Andrographolide showed anticancer activity in various cancer cells representing different types of human cancers. Whereas all three molecules showed enhanced proliferation and interleukin-2 (IL-2) induction in HPBLs (Kumar *et al.,* 2004). One of the diterpene lactones andrograpanin isolated from *A. paniculata* exhibited anti-inflammatory property in lipopolysaccharide-induced macrophage cells through down-regulating the p38 MAPKs signaling pathways (Lie *et al.,* 2008).

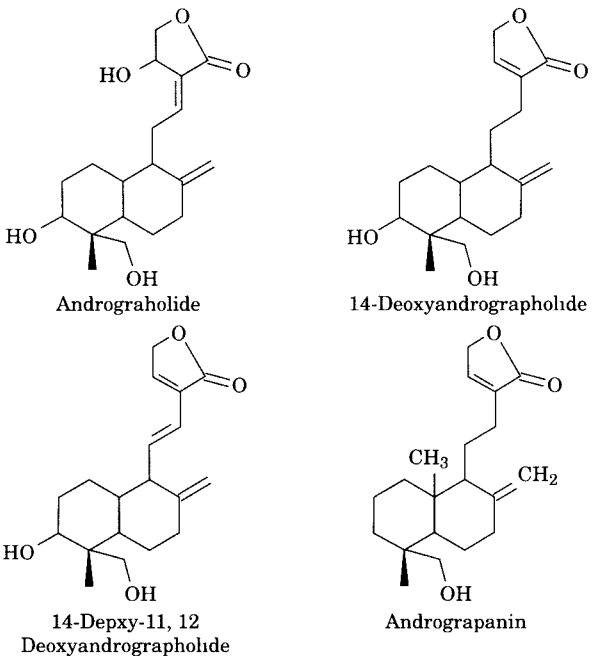


Fig 13.1. Chemical structures responsible for Immunomodulatory activity

Allium sativum

Garlic (*Allium sativum* L; family: Amaryllidaceae) is a general spicy flavoring agent used since olden times. Its bulb is carminative, aphrodisiac and stimulant (Chopra *et al.,* 1996). *A. sativum* contain diallyl sulphide, diallyl disulphide and allyl methyl sulphide sulphur containing compounds may potentiate stem-cell proliferation and differentiation and probably stimulate humoral immune responses. Sulphur containing compounds may interfere with the function of the gamma-glutamyl cycle, as well as act as inhibitors of some of the enzymes with a SH-group at their active site. Recently these compounds were found to inhibit the tumour with cell metastasis in experimental animals, which may be partially due to the immunostimulation of stem cells (Kuttan, 2000). Allicin is one of the active principles in garlic and immunostimulatory activity of allicin may be mediated through

upregulation of secretory molecules in macrophages that activate the cellular functions of macrophages to kill tumor cells and to produce various molecules such as NO, H_2O_2, TNF-α, IL-1 and IL-6 (Kang *et al.,* 2001). Garlic extract has chemopreventive potential against cycophosmamide induced chromosomal mutations in Swiss albino mice (Shukla & Taneja, 2002). DAS could inhibit the NAT activity (N-acetylation of AF) and gene expression (mRNA NAT) on three kinds of human colon cancer cell lines such as colo 205, colo 320 DM and colo 320 HSR (Chunga *et al.,* 2004). DADS, an oil-soluble organosulfur compound in processed garlic, inhibit the proliferation of human colon, lung, and skin cancer cells (Sundaram & Milner, 1996a), thus suggested that DADS induces apoptosis of human colon tumor cells by increasing intracellular calcium concentration (Sundaram & Milner, 1996b). Both garlic aqueous and ethanolic extract significantly modulate lymphocyte proliferation, triggered by this potent T-cell mitogen, depending on the type and dilutions of extracts and concentrations of Con A. Allicin, the main biologically active substance of freshly crushed garlic, modulates cytokine and chemokine secretion from intestinal epithelial cell lines (Lang *et al.,* 2004).

Aloe vera

Aloe vera Linn; family: Aloaceae has been very widely used in cosmetics, health foods and traditional medicines. Various pharmacological activities are reported in aloe together with anti-inflammatory, anti-oxidative, anti-aged, anti-cancer and immunomodulatory. The aqueous and chloroform extracts of the *A. vera* gel contain compounds with a potential to reduce carrageenan induced-edema hence show potent anti-inflammatory activity (Vfizquez *et al.*, 1996) Isolated polysaccharides from the gel act as immunopotentiators and promote phagocytosis (Egger *et al.,* 1996; Shida *et al.,* 1985). Lectin-like proteins may be involved in the anti-inflammatory action of *A. vera* gel (Grindlay & Reynolds, 1986). *Aloe* contain a polysaccharide that may have immunological adjuvant activity (Thart *et al.,* 1989). It is also reputed to have anti-inflammatory properties. Compounds isolated from the inner gel, such as salicylates, magnesium lactate, bradykinin, thromboxane inhibitors, sterols and a beta linked acetyl mannan (acemannan) have been proposed as an active anti-inflammatory components (Lee, 2001; Talmadge, 2004; Vazquez, 1996). Anti-inflammatory activity of the inner leaf gel component of *Aloe barbadensis* was proved using *in vitro* assay and was designed to determine the effect of the inner gel on bacterial-induced pro-inflammatory cytokine production, namely TNF-α and IL-1β, from peripheral blood leukocytes stimulated with *Shigella flexneri* or LPS (Habeeb, 2007).

Asparagus racemosus

Asparagus racemosus Willd family Liliaceae, commonly known as Shatavari has significant medicinal properties and forms the essential component of

established ayurvedic drugs. It is used for the treatment of diarrhoea, dysentery, rheumatism and nervous breakdown, and as an aphrodisiac (Nadkarni, 1976). Ethanolic extract of *A. racemosus* significantly inhibited ochratoxin A-induced suppression of chemotactic activity and production of inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-alpha by macrophages (Dhuley, 1997). *A. racemosus* aqueous root extract was evaluated in experimental animals immunized with diphtheria, tetanus, pertussis (DTP) vaccine. *A. racemosus* aqueous extract exhibited three important characteristics in this experimental setup—immunostimulation, immunoprotection and adjuvant activity. Presence of steroidal saponins in plant may be responsible for immunostimulatory activity (Gautam *et al.,* 2004). *A. racemosus* also show protection towards cyclophosphamide induced myelo- and immunoprotection as evident by significant increase in white blood cell counts and hemagglutinating and hemolytic antibody titers (Diwanay *et al.,* 2004).

Azadirachta indica

Neem (*Azadirachta indica* A Juss; family: Meliaceae) is perhaps the most useful traditional medicinal plant in India. Each part of the neem tree has some medicinal property and is thus commercially exploitable. Neem has been extensively used in ayurveda, unani and homoeopathic medicine and has become a cynosure of modern medicine. The Sanskrit name of the neem tree is 'Arishtha' meaning 'reliever of sickness'. The aqueous extract of leaf increases humoral and cell-mediated responses. Administration of aqueous extract of leaf 100 mg/kg after three weeks of oral administration causes higher IgM and IgG levels along with increased antibody titre (Biswas *et al.,* 2002). *A. indica* leaf extract-activated NK- and NK-T-cells in mice which may regulate tumor cell cytotoxicity by enhancing the secretion of different cytotoxic cytokines (Haquea & Baral, 2006). The aqueous extract of neem bark possesses anticomplement activity, acting both on the alternative as well as the classical pathway of complement activation in human serum (Vander *et al.,* 1987). Neem oil selectively activates the cell-mediated immune (CMI) mechanisms by enhanced response to subsequent mitogenic or antigenic challenge thus acts as a non-specific immuno-stimulant (Upadhyay *et al.,* 1992). Aqueous extract of the stem bark showed dose-dependent strong anticomplementary effects in the classical complement pathway assay, increase in the production of migration inhibition factor by lymphocytes and a decrease in the chemiluminescence of polymorphonuclear leukocytes (Vander *et al.,* 1987).

Boerhaavia diffusa

Boerhaavia diffusa Linn (family Nyctaginaceae) is an abundant creeping weed found all over India. In the Indian traditional system of medicine, *B. diffusa* roots have been widely used for the treatment of dyspepsia,

jaundice, enlargement of spleen, abdominal pain, and as an anti-stress agent (Chopra *et al.,* 1996). Oral administration of the alkaloid fraction of *B. diffusa* significantly inhibited sheep red blood cells (SRBC)-induced delayed type hypersensitivity (DTH) reaction in Swiss albino mice (Mungantiwar *et al.,* 1999). Crude ethanolic extract of *B. diffusa* roots has antiproliferative (Mehrotra *et al.,* 2001) and immunosuppressive potential as it inhibited human NK-cell cytotoxicity *in vitro*, production of NO in mouse macrophage cells, IL-2 and TNF-α in human PBMCs (Mehrotra *et al.,* 2002). The leaf extracts contains bioflavonoid (eupalitin-3-O-h-D-galactopyranoside) purified from ethanolic extract is the active principle for its having cell type specific immunosuppresive activity as shown by suppressive against lymphocyte and monocyte/macrophage lineage cells but not neutrophils and NK-cell (Pandey *et al.*, 2005).

Boswellia serrata

Boswellia serrata Roxb. ex Colebr. Family: Burseraceae contain gum, its lipophilic portion termed "frankincense," is a traditional Ayurvedic remedy. It has been used in Asia and Africa as a medical therapy for at least 3500 years and has been used to treat a wide variety of ailments, including respiratory problems, diarrhoea, constipation, flatulence, central nervous system disorders, rheumatism, liver disease, wound healing, fat reduction, and fevers. It has also been used as a mental tonic, taste enhancer, and even as an aphrodisiac (Ammon, 2006; Poeckel & Werz, 2006). The crude methanolic extract and the pure compound [(6aR, 6bS, 8aR, 11R, 12S, 12aR, 14bS)-1, 2, 4a, 5, 6, 6a, 7, 8, 8a, 9, 10, 11, 12, 12a-tetradecahydro-4, 4, 6a, 6b, 8a, 11, 12, 12a, 14b, non-amethyl picene-3, 14 (4H, 6bH, 14aH, 14bH)-dione (12-ursene 2- diketone)] were analyzed for immunomodulatory effect by inhibiting effect on TNFα, IL-1β and IL-6 release. The mechanism involved in this process that all three cytokines mediated through the JNK pathway, which leads to the decrease of c-jun and thus regulates AP-1 expression and subsequently brings about down regulation of inflammatory mediators (Gayathri *et al.,* 2007). Boswellic acids the active principle constituent from *B. serrata* possess anticomplementary activity due to inhibition of C3-convertase of the classical complement pathway (Kapil & Moza, 1992).

Curcuma longa

Curcuma longa Linn; family: Zingiberaceae are the most commercially cultivated spice crops of India for the production of turmeric, and are indigenous to southern Asia. Rhizome of *C. longa* has been used in Indian systems of medicine as an antiseptic, carminative, stomachic, appetizer and tonic. The ethyl acetate extract of *C. longa* contain diferuloylmethane which is most potent in inhibiting TNF-α induced expression of ICAM-1, VCAM-1 and E-selectin on human umbilical vein endothelial cells as it

significantly blocks the cytokine induced transcript levels for the leukocyte adhesion molecules (Gupta & Ghosh, 1999). The aqueous extract of *C. longa* promotes induction of adhesion of peripheral neutrophils to human umbilical vein endothelial cells by inducing the expression of ICAM-1 and E-selectin on endothelial cells and also it increases the steady state transcript levels of ICAM-1, VCAM-1, and E-selectin (Madan *et al.,* 2001). Curcumin (diferuloylmethane), a major curcumanoid inhibits the mitogen/ antigen induced lymphocyte proliferation, development of cell-mediated cytotoxicity, and the production of cytokines. The inhibition of the development of these responses results at least in part from suppression of the activation of transcription factor NF-κB by curcumin (Gao *et al.,* 2004).

Emblica officinalis

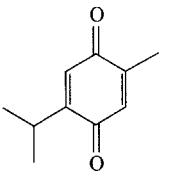
Fruits of *Emblica officinalis* Gaertn; family: Euphorbiaceae commonly known as "amla" or the Indian gooseberry, member of a small genus *Emblica*, claimed to have anti-fungal, anti-bacterial, anti-diabetic, anti-clastogenic and hepatoprotective properties besides having significant anti-oxidant, adaptogenic and anti-tumor activities Amla fruit has also been demonstrated to possess cytoprotective properties in acute cadmium toxicity. Immunomodulatory properties of fruits of *E. officinalis* were evaluated in adjuvant induced arthritic (AIA) rat model. Extract show significant anti-inflammatory activity in AIA animals. There was a significant reduction in swelling and redness of inflamed areas in treated animals than in untreated controls. It also significantly decreases the induction of nitric oxide synthase (NOS) (Ganju *et al.,* 2003). The fruits extracts *E. officinalis* showed the immunomodulating activity by relieving immunosuppressive effects of Cr on lymphocyte proliferation, restored the IL-2 and γ-IFN production considerably and along with it also inhibited significantly both lipopolysaccharide and concanavalin-A-stimulated lymphocyte proliferation. Amla significantly inhibited Cr-induced free radical production and restored the anti-oxidant status back to control level. It also inhibited apoptosis and DNA fragmentation induced by Cr (Ram *et al.,* 2002).

Nigella sativa

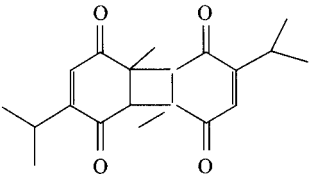
Nigella sativa Linn; family: Ranunculaceae is a herbaceous plant growing in the Mediterranean countries and Western Asia. The plant has been considered for millennia as one of the greatest healing herbs and has long been used for strengthening the immune system (Obeida *et al.,* 2006). Melanin extracted from *N. sativa* has a direct modulatory effect on cytokine production by monocytes, PBMC and THP-1 cell line. RT-PCR results showed that melanin up-regulated the expression of TNF-α and IL-6 mRNA in monocytes and total peripheral blood mononuclear cells (PBMC) in a dose-dependent manner. The levels of both cytokines were significantly

elevated following treatment with HM. Vascular endothelial growth factor (VEGF) expression was also found to change after melanin treatment. In contrast to its action on TNF-α and IL-6, HM inhibited the secretion of basal VEGF by monocytes and PBMC (Obeida *et al.,* 2006). *N. sativa* seeds were found to be immunopotentiating by increasing T4: T8 ratio as well as natural killer cell activity (Elkadi & Kandil, 1986). The immunomodulatory effect of whole *N. sativa* and fractionated proteins was monitored in mixed lymphocyte cultures and cells activated with poke weed mitogen (PWM) from different donors. Fractionation of *N. sativa* proteins by using DEAE Sephadex A50 ion exchange chromatography, coupled with the effect of these proteins was also observed on the production of inflammatory cytokines (IL-1β. IL-4, IL-8 and TNF-α) by using non-activated, PWM-activated and allogeneic peripheral blood mononuclear cells (PBMC). The total extracts of *N. sativa* or its fractionated proteins could modulate cytokines production (Haq *et al.,* 1999). The ethanolic extract of *N. sativa* had a effective cytotoxic effect as well as a potentiating effect on the cellular immune response (Swamy & Tan, 2000). *N. sativa* oil showed significant immunopotentiating effect by increasing the phagocytic activity and phagocytic index of peritoneal macrophages and lymphocyte count in peripheral blood compared with untreated diabetic hamsters in hamsters (Fararh *et al.,* 2004). The whole and soluble fractions of *N. sativa* seeds on *in vitro* effect investigated on human peripheral blood mononuclear cells (PBMC) response to different mitogens, the components did not show any significant stimulatory effect on the PBMC responses to the T-cell mitogens phytohemagglutinin (PHA), or concanavalin-A (Con A). By contrast, the components expressed stimulatory effect on the PBMC response to pooled allogeneic cells (Haq *et al.,* 1995). The stimulatory effects of *N. sativa* on the cellular immunity are dependent on the nature of the immune (*e.g.* ConA versus allogenic) response. *N. sativa* oil on proliferation of T-cells, its ethyl-acetate column chromatographic fraction and water fraction enhanced the proliferative response to ConA, but again not to the B-cell mitogen LPS. These results indicate that certain constitutions of *N. sativa* oil possess potent potentiating effects on the cellular (T-cell-mediated) immunity, while other constituents possess suppressor effects on B-cell-mediated (humoral) immunity. *N. sativa* seed is a complex substance of more than 100 compounds, some of which have not yet been identified or studied. A combination of fatty acids, volatile oils and trace elements are believed to contribute to its effectiveness. The original research articles published so far have shown the potential immunomodulatory and immunotherapeutic potentials of *N. sativa* seed active ingredients, in particular thymoquinone. The immunotherapeutic efficacy of thymoquinone is linked to its antitoxic, anti-histaminic and anti-inflammatory properties. These effects with its immunomodulatory properties can explain the anti-microbial and anticancer properties of *N. sativa* oil or thymoquinone (Salem, 2005).

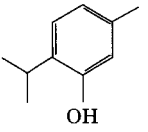
CHEMICAL STRUCTURES OF IMMUNOACTIVE LEADS FROM PLANTS



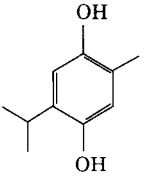
Thymoquinone (TQ)



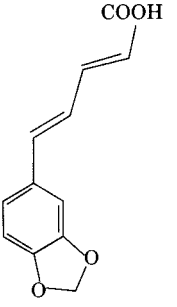
Dithymoquinone (DTQ)



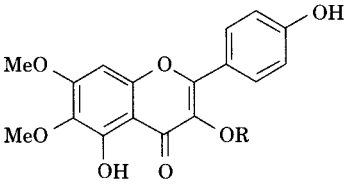
Thymol (THY)



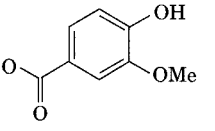
Thymohydroquinone (THQ)



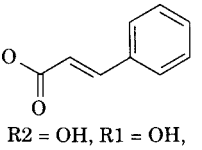
Piperinic Acid



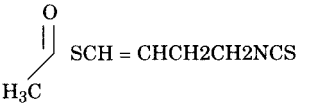
Eupalitin-2-O-h-dgalactopyranoside
R1 = OH, R3 = OH, R2



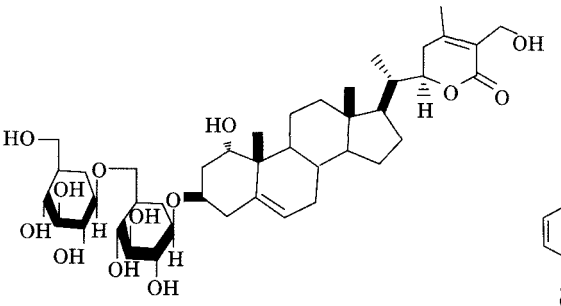
Kutkoside



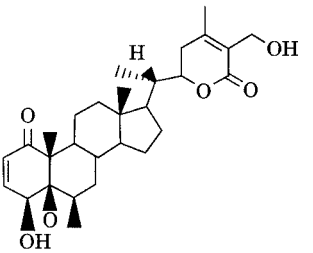
R2 = OH, R1 = OH,
R3 =
Picroside I



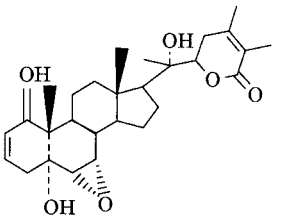
Sulforaphane



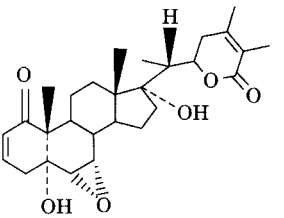
Withanoside-VI



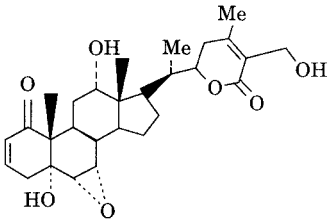
Withaferin-A



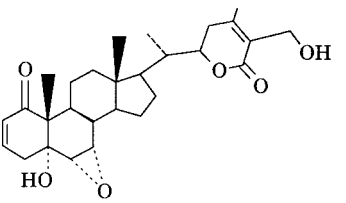
Withanolide-A



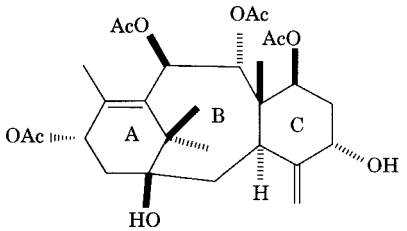
Withanone



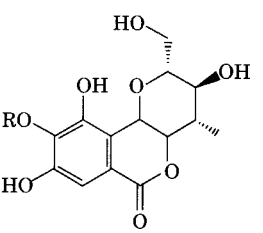
Withastramonlide



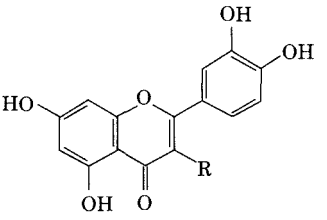
12-Deoxywithastramonlide



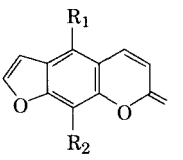
taxoid 1 (1-hydroxy-2-deacetoxy-
5-decinnamoyl-taxinine)



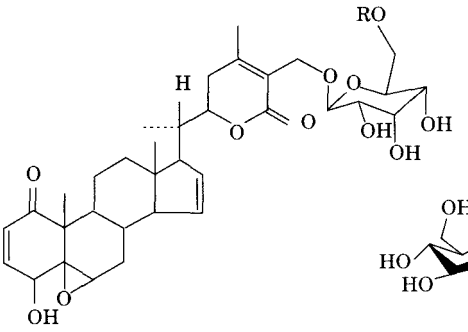
R = Me (1) bergenin
R = H (2) Norbergenin



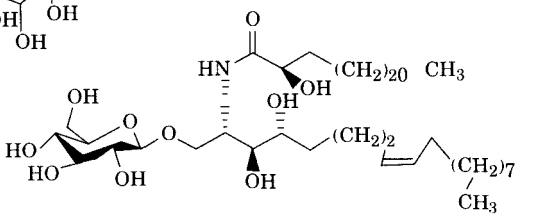
R	Type
Quercetin OH	1
Rutin O-rutinoside	3



	R1	R2	Type
Bergapten	OCH3	H	3
Isopımpinellin	OCH3	OCH3	2
Xantjptpxoin	H	OCH3	3

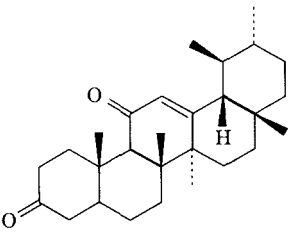


1 : R = H
2 : R = PALMITOYL

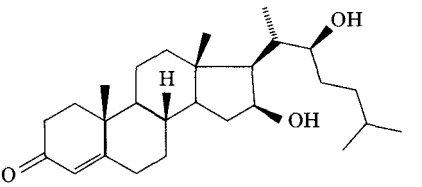


Glycosphıngolipid

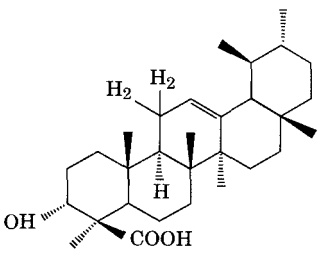
Sitoındosides IX and X



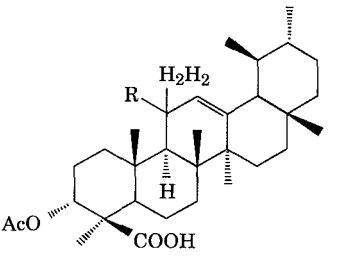
(6aR. 6bS, 8aR, 11R, 128, 14bS)-1,2, 4a, 5, 6, 6a 7, 8, 8a, 9, 10, 11, 12, 12a-tetradecajudrp-4, 4, 6a, 6b, 8a, 11, 12, 12a, 14b, nonamethyl pıcene-3, 14 (4H, 6bH, 14aH, 14bH) dione (12-ursene 2-diketone)



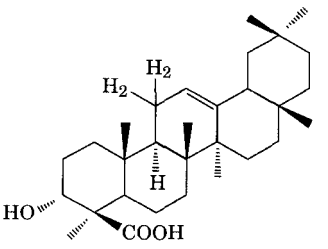
8S, 10R, 13S. 17R -Hydroxy-17-(S)-2-Hydroxy-1, 5-dimethylhexyl) 10, 13-dimethy 1-1, 2, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17-tetradechydro-cyclopenta [a] Phenanthren-3-one (Gggulsterol)



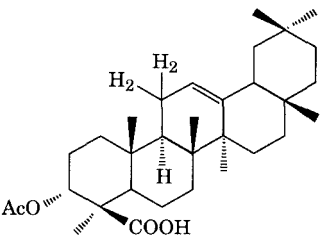
(a)



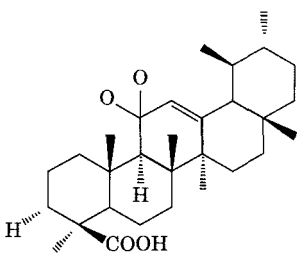
(b)



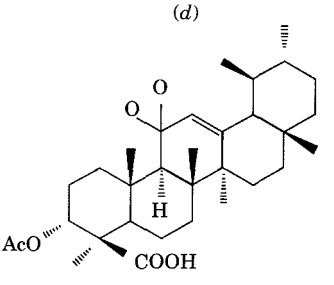
(c)



(d)

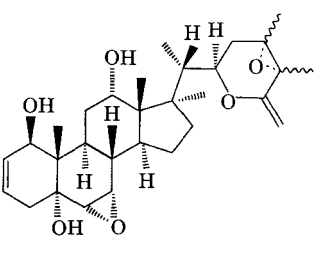


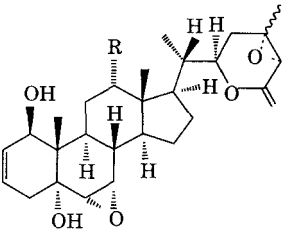
(e)



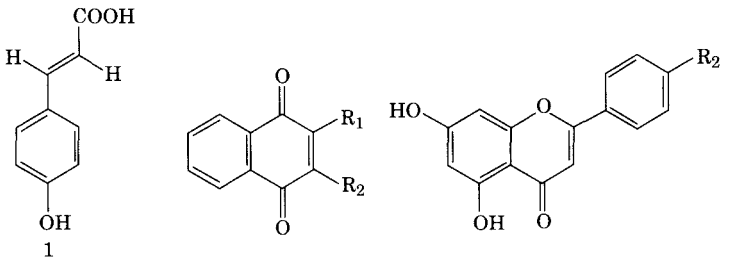
(f)

(a) β-boswellic acid, (b) aceyl-β-boswellic acid, (c) α-boswellic acid, (d) Aceyla-α-boswellic acid, (e) 11-keto-β-boswellic acid and (f) acetyl 11-keto-β-boswellic acid

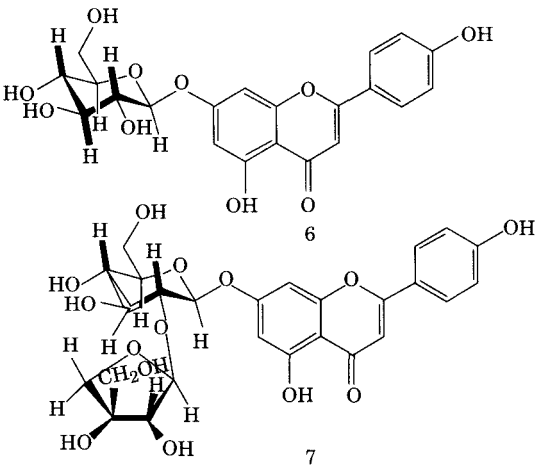




Datura Lactones



	R1	R2		R1	R2
2.	OH	H	3.	OH	H
5.	OCH3	CH3	4.	OH	OH



1. *P*-coumaric acid, 2. lawsone, 3. apijenin, 4. luteolin, 5. 2-methoxy-3-methyl-1, 4-naphthoguinone, 6. cososiin, 7. apiin

Ocimum sanctum

Ocimum sanctum Linn; family: Labiatae normally known as 'Holy basil' has been broadly used in the Ayurvedic system of medicine and claimed to be valuable against a wide variety of diseases (Singh *et al.*, 1996). Immunostimulant activity of *O. sanctum* was evaluated in albino rats against the antigenic challenge of *Salmonella typhosa* and sheep erythrocytes. (Godhwani *et al.*, 1988). Herbal preparation Immu–21 (Indian Herbs, Saharanpur, India) containing *O. sanctum* as major constituent increased the microbicidal activities of neutrophil and increased antibody titre in rats (Chatterjee, 1994). Extract also enhanced cell mediated immunity and lymphoproliferation in poultry infected with IBD virus (Sadekar *et al.*, 1998). *O. sanctum* seed oil (OSSO) appears to influence both humoral and

cell-mediated immunological parameters in naïve non-stressed, as well as stressed animals. As pretreating the rats with OSSO produced a significant increase in the anti-SRBC antibody titre and a decrease in the antigen induced histamine release from the peritoneal mast cells of the sensitized non-stressed animals. In the case of cellular immunity parameters, *i.e.* foot pad thickness and percentage leucocyte migration inhibition were significantly reduced after OSSO treatment. RS produced a reduction of antibody titre as well as footpad thickness and percentage leucocyte migration inhibition. Pretreating the animals with OSSO effectively blocked the immunosuppressive effect of RS on both humoral and cell-mediated immune responses and these immunomodulatory properties could be mediated via GABAergic pathways (Mediratta *et al.*, 2002). Crude aqueous extract of *O. sanctum* (leaf) having biologically active principles that's treatment reduced the total bacterial count and increased neutrophil and lymphocyte counts with enhanced phagocytic activity and phagocytic index. Similarly, the lysosomal enzymes contents of the milk polymorphonuclear cells (PMNs) were also enhanced significantly in animals treated with the extract, showed that it had antibacterial and immunomodulatory actions in nature (Mukherjee *et al.*, 2005).

Picrorhiza kurroa

Picrorhiza kurroa Royle ex Benth; family: Scrophulariaceae is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhoea, and scorpion sting. Other traditional uses include dyspepsia, bilious fever, chronic dysentery, and scorpion sting. The roots and rhizomes are the part of the plant used medicinally. Ethanolic extract of leaf of *Picrorhiza kurroa* was found to stimulate the cell-mediated and humoral components of the immune system as well as phagocytosis in experimental animals (Sharma *et al.*, 1994). Immunostimulatory activity of biopolymeric fraction RLJ-NE-205 from *P. kurroa* proved by as its significantly increased the phagocytic function of macrophages. Simultaneously, the proliferation of Con-A and LPS stimulated spleenic lymphocytes, the CD4/CD8 population in spleen, HA titre, PFC assay, the DTH reaction and cytokines levels (IL-4 and IFN-γ). This suggests that the fraction RLJ-NE-205 may enhance non-specific, humoral and cellular immunity in mouse model (Gupta *et al.*, 2006).

Piper longum

Traditionally (*Piper longum* Linn; family: Piperaceae) has been used since time immemorial for its anti-inflammatory and other properties. The *Piper* species are traditionally employed in the local folk medicinal preparations. Extract of *P. longum* and piperine was found to increase the circulating antibody titre and antibody forming cells indicating its stimulatory effect

on humoral arm of immune system. Administration of this drug could also significantly inhibit the growth of solid tumor induced by Dalton's lymphoma ascites (DLA) cells and Ehrlich ascites carcinoma (EAC) cells. Immunomodulatory activity of *P. longum* and piperine may be due to the combined action of humoral and cell-mediated immune responses (Sunila & Kuttan, 2004). Immunomodulation by piperine may be clearly attributed to its multi faceted activities such as anti-oxidative, anti-apoptotic and restorative ability against cell proliferative mitogenic response, splenic B- and T-cell population and cytokine release (Pathak & Khandelwal, 2007). Immunoregulatory potential of *P. longum* and piperinic acid, one of its active constituent, in Balb/C mice (*in vivo*) and human PBMCs (*in vitro*) models was also observed. Piperinic acid moderated the proinflammatory mediators and cytokines. At doses of 10, 20, 40 and 80 mg/kg p.o. PL showed a dose dependent decrease of lymphocytes ($CD4^+$ and $CD8^+$ T-cells) and cytokine levels in sensitized Balb/c mice with a marked inhibition at 40 mg/kg. At an *in vitro* dose of 20 μg/mL of PL and 5 μg/mL of piperinic acid, there was a significant inhibition of mitogen induced human PBMC proliferation, mRNA transcripts of IL-2 (ConA) and TNFα, IL-1β and iNOS (LPS) respectively under stimulated conditions in time dependent (6 h, 12 h and 24 h respectively) expression studies. In parallel, induced nitric oxide production was also reduced by stimulated macrophages (Devan *et al.*, 2007).

Trigonella foenum-graecum

Fenugreek, *Trigonella foenum graecum* L.; family Leguminoceae is one such plant whose seeds and leaves are used not only as food but also as an ingredient in traditional medicine. *T. foenum graecum* has appreciable immunostimulatory effect as it increases both cellular and humoral immunity. *T. foenum graecum* extract elicited a significant increase in DTH response, haemagglutination titre, phagocytic index, phagocytic capacity of macrophages and PFC. Plant extract also show stimulatory response in lymphoproliferation assay (Hafeez *et al.*, 2003). *T. foenum graecum* seeds show immunomodulating effect as resulted in restoration of humoral responses in deltamethrin treated (immunosuppresed) animals as shown by a significant ($p<0.01$) increase in plaque-forming cell (PFC) response as well as QHS (quantitative haemolysis assay) in deltamethrin-treated animals. The antioxidant property of fenugreek seeds might be contributing to modulatory action resulting in its protective effect in immunosuppresed mice (Rehman *et al.*, 2004). Fenugreek galactomannan rich fraction show activation of phagocytosis and proliferation of cells in cell line HB4C5 and it also show inhibition of IgM secretion (Ramesh *et al.*, 2002).

Tinospora cordifolia

Tinospora cordifolia Miers (Menispermaceae), commonly called Amrita, Gurcha or Jetwatika, is a large glabrous deciduous climbing shrub. It is

mentioned in Ayurvedic literature as a constituent of several compound preparations used in general debility, dyspepsia, fever and urinary diseases. The active principles of *T. cordifolia* a traditional Indian plant were found to possess anticomplementary and immunomodulatory activities. Syringin and cordiol inhibited the *in vitro* immunohaemolysis of antibody-coated sheep erythrocytes by guinea pig serum. Macrophage activation was reported for cordioside, cordiofolioside A and cordiol and this activation was more pronounced with increasing incubation times (Kapil & Sharma, 1997). The immunostimulating properties of a polysaccharide and isolated polysaccharide (1, 4)-α-D-glucan, is due to the β-glycosidic linkages, degree of branching and solution conformation (Nair *et al.,* 2004, 2006). Plants showed immunomodulatory activity by inhibit cyclophosphamide-induced anemia (Manjrekar *et al.,* 2000). Immunostimulatory property *T. cordifolia* stem methanolic extract was shown as it increases in total white blood cells and bone marrow cells. It also improved the number of α-esterase-positive cells; stimulate the humoral immunity by increase in antibody-producing cells and circulating antibody titre and also stimulate the phagocytic activity (Mathew & Kuttan, 1999).

Tylophora indica

Tylophara indica Merr; family: Asclepiadaceae is a branching climber or shrub that grows up to 1.5 meter found in the eastern and the southern regions of India. The leaves are ovate-oblong to elliptic-oblong, 3–10 cm long and 1.5–7 cm wide (Kirtikar & Basu, 1991). *T. indica* alkaloids inhibit cellular immune responses like contact sensitivity to dinitro-flurobenzene and delayed hypersensitivity to sheep red blood cells but not have any humoral response (Ganguly & Sainis, 2001). Alkaloids have a concentration dependent biphasic effect on Con A induced mitogenesis. The alkaloid mixture was found to inhibit proliferation of splenocytes at higher concentrations and augment the same at lower concentrations. At lower concentrations they augment Con A induced lymphoproliferation by enhancing IL-2 production. Inhibition of proliferation at higher concentrations of alkaloid is due to inhibition of IL-2 production and activation of macrophages, which have a cytostatic effect (Ganguly *et al.,* 2001).

Withania somnifera

Ashwagandha, *Withania somnifera* (L. Dunal) (Solanaceae), is an Ayurvedic medicinal plant which is popular as a home remedy for several diseases and human requirements. It is mentioned in Vedas as a herbal tonic and health food. It is an official drug and is mentioned in the Indian Pharmacopoeia. *Withania* extract administration was found to raise the haemoglobin level, RBCs and decrease serum cholesterol, ESR etc. (Ziaudidin *et al.,* 1996). Aqueous fraction of *W. somnifera* root at graded

doses of 25, 50, 100 and 200 mg/kg p.o. caused significant potential of modulator the population of T-cell and Th1 cytokin in chronically stressed mice (Khan *et al.,* 2006). Withaferin-A has been found to inhibit growth of Ehrlich ascites carcinoma in mice within 24 h followed by complete disappearance of tumor cells after 2–4 days of treatment by activating immune responses (Sbohat *et al.,* 1970). WS increased significantly not only chemotakis but also IL-1 and TNF-α production in macrophage (Dhuley, 1997). *Withania* extract administration was found to raise the haemoglobin level, RBCs and decrease serum cholesterol, ESR etc. (Ziaudidin *et al.,* 1996). Administration of *W. somnifera* could decrease the lecopenia, enhanced the bone marrow cellularity and the ratio of normochromatic to polychromatic erythrocytes in mice treated with nonlethal dose of gamma radiation (Kuttan, 1996). Administration of *Withania* could also improve the total WBC count, bone marrow cellularity as well as esterase positive cells in mice treated with cyclophosphamide (Davis & Kuttan, 1998). *Withania* extract of root increase the total WBC count, bone marrow cellularity, and α-esterase positive cell number. There is enhancement in the circulating antibody titre and the number of plaque forming cells (PFC) in the spleen when extract along with the antigen (SRBC) is given. It inhibited delayed type hypersenstivity reaction and also showed an enhancement in phagocytic activity of peritoneal macrophages in mice (Davis & Kuttan, 2000). *W. somnifera* resulted in protection towards CP-induced myelo- and immunoprotection as evident by significant increase in white cell counts and hemagglutinating and hemolytic antibody titres (Diwaney *et al.,* 2004). *W. somnifera* treatment restored the number and function of these cells as well as immunoglobulin level in benzo(a)pyrene induced cancer animals and paclitaxel treated animals. *W. somnifera*, the natural antioxidant, can counteract the side effect of the synthetic anticancer drug as well as improve the clinical efficacy. *W. somnifera* exerts a more beneficial effect than paclitaxel alone and it can be used as a potential chemotherapeutic agent along with paclitaxel in the treatment of experimental lung cancer (Senthilnathan *et al.,* 2006). *W. somnifera* stimulates macrophage-derived NO production, and is able to up-regulate iNOS expression through NF-nB transactivation in murine macrophages. This could increase the cytotoxic/cytostatic effect of macrophages and thus may provide a mechanistic basis for the immunostimulating properties of *W. somnifera* (Iuvone *et al.,* 2003). Withaferin A, a steroidal lactone showed a marked tumour inhibition when tested *in vitro* against cells derived from human nasopharyngeal carcinoma (Sbohat *et al.,* 1970) and experimental mouse tumour (Devi *et al.,* 1995; Sharda *et al.,* 1996). Withanolide D, another steroidal lactone occurring in the leaves of *W. somnifera* also showed antitumour activity against Sarcoma-180 and Ehrlisch ascites cells (EAC) (Das *et al.,* 1985). The root extract of *W. somnifera* were able to increase the proliferation of T- and B-lymphocytes favoring Th1 immunity as evidenced by enhanced secretion

of IFN-γ, IL-2 and IgG2a. Concurrently, AGB also enhanced macrophage activation, by augmenting the production of IL-12, TNF-α and NO (Malik *et al.*, 2007).

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14

Immunomodulatory Activity of Botanicals

KAUL A.[1,*], KHAJURIA A., SINGH S., SINGH G.D.,
CHANDAN B.K. AND GUPTA V.K.

ABSTRACT

Immunomodulation is basically a process which can alter the immune system of an organism by interfering with its functions. The inference results in either immunostimulation, an enhancement of immune reactions or immunesuppression imply mainly to reduce resistance against infection, stress which may be because of environmental or chemotherapeutic factors. Bioactive natural products provide excellent raw material for the discovery and development of novel immunomodulatory compounds. A good number of bioactive natural products used as medicinal plants have stood the test of time, particularly for the treatment of allergic metabolic and degenerative diseases associated with aging. These bioactive natural products are believed to promote positive health and maintain organic resistance against infections by reestablishing body equilibrium and conditioning the body tissues. A large variety of natural bioactive plants mentioned in ayurveda for their immunomodulating, adaptogenic and rejuvenating properties have been under study. The current approach is to know about the medicinal plants that are biologically active and could potentially be of help in the development of modern and new immunomodulating agents. As in the modern day life, extensive exposure to industry based pollutants/zenobiotics has resulted into emergence of a variety of immune deficiencies or hypersensitivity situations, where immunology can play an important role.

Key words : Immunomodulation, immunostimulation, immunosuppressive, homeostasis, arthritis, ulcerative-colitis, asthma, allergy, vitiligo, atherosclerosis

1. Division of Pharmacology, Indian Institute of Integrative Medicine (CSIR), Canal Road Jammu-180 001, India.

* *Corresponding author* : E-mail : dranpurna@gmail.com

INTRODUCTION

Historical period documents medicine and botany hand-in-hand in service of mankind. Bioactive medicinal plants have been and are still in use by the practitioners of the traditional system of medicine. According to WHO survey of 1993, 90% of patients are still treated by using bioactive medicinal plants/plant extracts in Bangladesh, 85% in Burma, 80% in India, 75% in Nepal, 65% in Sri Lanka and 60% in Indonesia and Pakistan respectively. In India medicinal plants endowed with leads from Ayurveda, Siddha and Unani have been used with success in the management of various diseased conditions. Claims made about these drugs are basically their safety besides being effective and economical (Siddique, 1993).

Ayurveda, the Indian system of medicine deals with plant drugs, the main stress being on strengthening body's own defense system. The concept of "Rasayana" was put forth in ayurveda, meaning a group of plants with potential to treat body's main defense system. This documented use of herbal medicinal plants and the concept of immune system came in India with Ayurveda.

Plants/plant extracts are being used in Europe for rejuvenating therapy and treatment of chronic disorders. These have been shown to possess immunostimulatory activity (Wagner *et al.*, 1983). Chopra *et al.* (1958) have described plants with immunostimulatory activity in India and China. Plants with above activity have also been documented by Dua *et al.* (1989) and Nadkarni (1954).

Herbals are known to bring general improvement in the homeostasis. Plants have been reported to have regulatory effects on immunity, endocrine, neural and cardiovascular systems (Patwardhan *et al.*, 1990). Certain bioactive herbals have been suggested to act through modulation of these systems and are effective against some auto-immune diseases (Haruki, 1992). On the contrary they have actually been contributing to the modern medicine, *e.g.* morphine, quinine, reserpine, atropine, vincristine, and vinblastine (Patwardhan *et al.*, 1990).

Study of the defense system of the body is in vogue and has evoked great interest recently. The balance has shifted to a greater extent from simple treatment of symptoms to the basic understanding of immunology of disease states.

This study has been designated as science of immunology. Immunology is not only the most rapidly developing area of biomedical research but also possess great promise of being the major advance in the prevention and treatment of wide range of chronic disease (William & Barbara, 1984). Arthritis, ulcerative-colitis, asthma, allergy, vitiligo and many more parasitic and infectious diseases are now primarily considered to be immunological disorders. Disturbance of immune mechanisms have been

reported to be involved in a variety of other metabolic diseases such as diabetes mellitus, cancer, atherosclerosis (Samter, 1971) myocardial disorders and cirrhosis.

Modulation of immune response as a basis of therapy has been of interest since many years (Gulableanvorba, 1940). First scientific report was put forth by Lazarev in 1947, who demonstrated the capacity of diabazol to increase non-specific resistance. This compound possessed adaptogenic activity which was supported by Anosov *et al.* (1956) and Lazarev (1958). Development of a series of synthetic compounds followed the impact being on to utilize these to balance immune system. Synthetic drugs named as Cyclophosphamide (alkylating agents), 6-mercaptopurine (pureantagonists), mathotrekate (folic acid antagonists), 5-fluorouracil (pyrimidine antagonists) and adramycin (Sanots & Owens, 1964; Turk & Poulter, 1972; Mansour & Nelson, 1977; Speirs *et al.,* 1979) are some of the examples.

Designing of any immunomodulator requires on the onset and in-depth understanding of various components of immune system. An attempt has been made here to give a bird's eye view of some important components of this complex system. Immune response includes both antigen specific and antigen non-specific processes.

Antigen Specific Immune Response (Protective Host Defense Mechanism)

The basic requisite of the immune system of the body is to recognize and destroy the "Non Self" which includes protection against various infections maintaining homeostasis and immune surveillance (Bellanti, 1978).

Components involved in the immune response can be classified under two major components *i.e.* molecular or humoral and the cellular factors. Antigen specific immune response are represented through T-lymphocytes (T-cells) capable of activating B-lymphocytes which are then triggered by antigens to become plasma cells, secreting antibodies. Immunoglobulins IgM, IgG, IgA, IgD, IgE and their subclasses are also produced by B-cells. T cells play a significant role in the interaction of B-cells with macrophages. Cellular immunity is functionally related to the immune activity of cells, particularly the T-cells, whereas the humoral immunity emphasizes the production of B-cells or antibodies.

During the immune response to a particular antigen the production of antibodies by B-cells is governed by following possible mechanisms; (a) humoral immunity, (b) a temporary loss of reactive B-cells, (c) T-suppressor action directly on B-cell differentiation and (d) indirect T-suppressor action on T-helper cells (Sell, 1980). The mechanism of action of specific antibodies inhibit the production of antibody of the same specificity by feed back control system (Smith *et al.*, 1968).

Antibodies are generally operative against bacteria or bacterial products, whereas cellular reactivity works against viral and other micro-organisms thus showing protective effect against infections thereby enhancing the immunity. Hence immune reactions of both humoral and cellular types play an important role in the defense of the host against infections.

Immune Memory

In order to develop an immune response characterized by antibody synthesis, the participation of two types of lymphocytes (T- & B-Cells) is necessary. Other types of lymphocytes and their metabolic products *i.e.* lymphokines or interleukins (ILS) and natural killer cells (NK) function as messengers.

The messengers are carried to other lymphocytes, phagocytes or non-immune cells to produce immune responses. Macrophages both inactive and active remain essentially the same (Jerne, 1973). To begin an immune response immunologically competent lymphocytes with receptors must make contact with antigen. Starting from the response to antigen till the final product antibody is synthesized we can observe an amazing specificity. This specificity is revealed by the capacity of antibody to react against a particular determinant of an antigenic molecule. The antibody to a particular antigen is so exquisitely specific that even minor alterations in the determinant molecule or the portion responsible for its antigenic reactivity markedly alter the response.

Non-Specific Immune Response

Prominent function of non-specific immune response is through activation of complement cascade which potentiates the macrophages in the process of ingesting foreign material (antigen) without the usual aid of immunoglobulin or antibody synthesis. Macrophages and neutrophils engulf almost any thing. Polymorphonuclear leucocytes (PMNL), mononuclear phagocytes and macrophages are part of above mentioned non-specific immune system. These components function by the production of reactive oxygen species (ROS) by activated PMNL. C3 component of complement is found to mobilize leucocytes *in vitro* and *in vivo* promoting leucocytosis factor (Rother, 1972; Gherbrehiwet *et al.*, 1978).

Immune Regulation-Cell Interaction

Interaction between different components of immune system leads to consecutive efforts to prevent the diseased conditions. In totality the functional interactions between an antigen non-specific and antigen specific (humoral & cellular) components of immune system are manifold, intensive and intimate. For instance, macrophages and lymphocytes interact through the phenomenon of antigen dependent action.

Macrophage processed antigen is presented to T-lymphocytes. In this process cell to cell contact takes place, macrophage and lymphocytes interacting in the process of immune response share histocompatibility linked gene products. The macrophage seem to select discrete regions of the antigen molecule for recognition by sensitized T-cells (Erb & Feldman, 1975; Rola-pleszczynski, 1982; Sirois & Rola-Pleszczynski, 1982). Other cells involved in this response are activated monocytes and macrophages producing monokines that interact with lymphocytes.

The involved mediator cells *e.g.* mast cells, basophils, eosinophils and platelets, release soluble mediators such as chemotactic factors, platelet activating factors, heparin, prostaglandins and leukotrines which in turn are responsible for inflammatory manifestation following an immune reaction (Fernandez *et al.*, 1978; Goldstein, 1980; Detmers & Wright, 1988; Ward & Marks, 1989; Brown, 1991; Summers, 1991). Phagocytes are also activated by leukocytes through this process to perform phagocytosis. It is very clear that any immune response is brought about through functional interactions of the different components of the immune system. Antigen non-specific and antigen specific processes are closely and interdependently linked in a regulatory network of the immune response. Besides this direct cell to cell contact, macrophages and lymphocytes influence individual functions indirectly also.

Such interaction are mediated through secretions of cytokines *i.e.* monokines (IL-1) and lymphokines that provoke specific activities resulting in lymphocyte proliferation. lymphokines produced by T-lymphocytes designated as IL-2 activate macrophages. The above described activation of macrophages leads to enhance phagocytosis, microbial killing through secretory activities due to adherence of foreign molecules to macrophageal surface. Lymphokines such as IL-5 are known to have released chemotactic effects on eosinophils, basophils and neutrophils at the site of inflammation.

Activated T-lymphocytes also produce IL-4, a lymphokine that stimulates B-cells to produce antibodies. In cases of allergic or inflammatory reaction the antibody (IgE) produced would trigger mast cells to release inflammatory mediators including histamine, prostaglandin D-2 (PGD2) and leukotrine C-4 (LTC4). Together with basophils mast cells play a key role in immediate hypersensitivity and other allergic manifestations. The picture given above of the functional interactions between cellular and humoral immunofactors is far from complete. Still, it illustrates however the interdependency of regulatory and effector cells, their molecular messengers and the mediators in the antigen specific and the antigen non-specific immune response. Interaction of various immune components and relevant non-immune cells results in correction of immune system (Labadie, 1993).

Immunomodulation

Immunomodulation is basically a process which can alter the immune system of an organism by interfering with its functions. This interference results in either immunostimulation, an enhancement of immune reactions or immunosuppression which imply mainly to reduce resistance against infections stress that may be because of environmental or chemotherapeutic factors (Patwardhan *et al.*, 1990).

Immunostimulation and Immunosuppression both are needed to be tackled depending on the type of immunological disturbance. Hence immunostimulating and immunosuppressing agents have their own standing. Recently search for better moieties with the activities is becoming the field of major interest. Research focused on the development of immunomodulators is directed towards activities that can be expressed in terms of stimulation or inhibition of immunofactors and their integrated function (Labadie, 1993).

Apart from specific stimulative or suppressive activity certain agents have been shown to process activity to normalize or modulate the pathophysiological processes in the underline immune response and hence the term immunomodulation or immunomodulatory agents are now used (Wagner, 1983). This activity varies with dose level and most of the immunosuppressants show immunostimulation at low doses (Wagner *et al.*, 1988). Most of the chemical agents which are fairly known to have effect on immune system are immunosuppressants and cytotoxic agents (Muftuogy *et al.*, 1984). Azathioprine and cyclophosphamide have been extensively studied as standard immunosuppressors. Azathioprine inhibits DNA synthesis and has antinflammatory activity by virtue of its effects on IFN and monocyte production although the immunopharmacology of this non-specific agents is not well understood even today.

Cyclophosphamide's importance and safety is not well established (Gordwin *et al.*, 1974). The other group of drugs described and worked in immunopharmacology is immunostimulant mostly derived from natural resources. Levamisole, the earlier anti-parasitic drug now has been identified as immunomodulator and has been studied in a number of diseases including rheumatoid arthritis where it reduced rheumatoid arthritis factor titres (Vischer, 1978). As described above immunomodulation represents an important therapeutic approach in the treatment of various diseases. Immunomodulators so for available have been categorized on the basis of their origin as under.

Biological Origin

1. ***Products of Immune System***

   Thymic hormones

   Thymosine

Thymopoietine
FTS
Lymphokines
Lymphokines non-purified
Interferon
Interleukine-2
Others Tuftsine
Bacterial preparations
Bacillus Calmette Guerin (BCG)
Brucella abortus
Corynebacterium parvum
Pseudomonas aeruginosa
Bordetella pertussis
Nocardia

2. ***Chemically Identified Bacterial Extracts of***
   M. smegmatis
   K. pneumoniae
   M. tuberculosis
3. ***Chemically Identified Compounds from Fungi***
   Krestin
   Glucan
   Lentinan
   Cyclomunine
   Bestatin
4. ***Natural Products or their Analogs***
   Vitamin A and its derivatives
   Vitamin E
   Lysolecithenes
   Lynesterol
   Synthetic Compounds
   Sulfur-containing compounds
   Levamisole
   Diethyldithiocarbamate
   Cimetidine

5. ***Compounds with A Nucleotide***

   Poly A: U, Poly I: C

   Isoprinosine

   NTP 15392

6. ***Other Miscellaneous Compounds***

   Azimexon

   Therafectine

   Tilorone

   Indomethacine

With the discovery of numerous immunomodulators and a good deal of information about their mechanisms of action, great hopes have arisen envisaging that in the future, we shall be able to modulate the complex immune system into desired direction (Fenichel & Chirigos, 1984).

Natural Product Resources and their Selection

Synthetic compounds although very effective in controlling different components of immune system have inherent severe side effects. However, almost limitless resources are provided by the plethora of potential medicinal plants, for the treatment of a variety of disorders including disturbed immune system. It has now become possible to analyze that extracts from reputed plants can prove to be beneficial immunologically with availability of various *in vitro* and *in vivo* testing models. This motivation has to be persuade to search for new and better drugs from plant resources (Labadie, 1993).

Immunopharmacological studies have been carried out in the recent past on large number of plants. The most notable amongst them are enumerated below:

Acanthopanax obovatus Hoo (Arallaceae)

A. obovatus is a Chinese traditional herb being used as a tonic. Polysacchride isolates AOPS from this plant has shown immunomodulatory activity. Administration of this fraction resulted in increase in the weight of the spleen and the number of cells. This isolate augments the phagocytosis of peritoneal macrophages both in normal and immune suppressed mice (Wang *et al.,* 1991). In a haemagglutinnin assay AOPS increased the production of specific antibodies and antagonized the suppressive effect of cyclophosphamide in all the parameters under study. Enhancement in the degree of *in vitro* spleen cell mediated red blood cells (SRBC) hemolysis was observed (Wang *et al.,* 1991) on administration of AOPS.

Aconitum heterophyllum Wall (Ranunculaceae)

A. heterophyllum has been used in folklore medicine in giardiasis and as an antidiarrhoel (Singh & Chaturvedi, 1981, 1982). Ethanolic extract (95%) of bark has been reported to stimulate phagocytosis and inhibit the humoral immune response against SRBC in mice (Atal *et al.*, 1986). It has shown 35–50% inhibitory activity against *S. lutea* and a large variety of organisms tested (Pandya *et al.*, 1990).

Abutilon indicum L. (Malvaceae)

Dried powdered material of *A. indicum* has been reported to increase the humoral antibody response to *Salmonella typhimurium* antigen in rabbits. Survival time of rabbits against virulent *Staphylococcus aureus* challenge increases on administration of *A. indicum* (Dixit *et al.*, 1978).

Allium cepa L. (Liliaceae)

Handa *et al.* (1983) have shown the bronchodilatory effect of the chloroform fraction of the cold 95% ethanolic extract of *Allium cepa* and quercetin.

Arctostaphylos uva-ursi Spreng (Ericaceae)

Methanolic extract (50%) (U-ext) from *A. uva-ursi* leaf given orally (100 mg/kg) showed an inhibitory effect on the swelling induced by picryl chloride (PC-CD). The inhibitory effect was enhanced when u-ext and predinisolone was used in combination (Kubo *et al.*, 1990). Arbfutin isolated from the leaves of *A. uva-ursi* plus indomethacin showed inhibitory effect on the swelling induced by PC-CD and SRBC stronger than that of indomethacin alone (Mastuda *et. al.*, 1991).

Aristolochia clematitis L. (Aristolochiaceae)

Aristolochic acid isolated from *A. clematitis* has been shown to enhance the phagocytic function of leucocytes and peritoneal macrophages (Kluthe *et al.*, 1982). It has been reported to prevent the prednisolone induced reduction of rosette forming cells and check chloramphenicol and tetracycline induced reduction of phagocytosis (Lemperle, 1972). It has been reported to be carcinogenic (Mengs *et al.*, 1982, 1983) for which reason the drug containing this compound have been withdrawn from the market.

Arnica montana L. (Compositae)

A. montana is widely used externally in traditional medicine in Europe and North America because of its anti-inflammatory and anti-microbial activity. Immunostimulant properties of crude polysaccharide from the herb have been described (Wagner *et al.*, 1985). One of the two homologus

polysacchrides isolated from *A. montona* cell culture showed a pronounced enhancement of phagocytosis *in vitro*. Arabino-3, 6-galactin protein displayed a strong anti-complementary effect and stimulated macrophages (Puhlmann *et al.,* 1991) to excrete the tumour necrosis factor (TNF-α).

Astragalus mongholicus Bunge (Fabaceae)

Glucoarabinan obtained from *A. mongholicus* (Chen *et al.*, 1981) has been demonstrated to stimulate phagocytosis, T lymphocytes and increase plasma cell counts.

Asparagus racemosus Wild (Liliaceae)

A. racemosus protected rats against coecal-ligation induced sepsis and mice against *E. coli* peritonitis. Plant extract administration reduced the mortality rate due to *S. aurcus* sepsis in neutropenic and hemispleenectomized mice. This plant extract reduced *C. albicans* induced sepsis and mortality. It is reported to have immunorestorative effect against the myelosuppression induced by single or multiple doses of cyclophosphamide (Dahanukar *et al.,* 1989).

Azadirachta indica Juss (Meliaceae)

Various preparation of *A. indica*, an evergreen tropical tree are used in Ayurveda and other systems of traditional medicine for the treatment of inflammatory disorders (Vander *et al.,* 1991a; Bhargava *et al.,* 1970).

Fujiwara *et al.* (1982, 1984a, 1984b) have described polysacchrides isolated from the bark of this plant to possess anti-tumour interferon inducing and anti-inflammatory activities. Aqueous extract of the bark has been shown to increase the production of migration inhibitory factors (MIF) (Vander *et al.,* 1986). It inhibits complement activation by both classical pathway (CP) and alternative pathway (AP). Luminal dependent chemiluminescence activated human polymorphonuclear leucocytes was also inhibited by this plant extracts (Vander *et al.,* 1987, 1989).

Isolate (NB-11) from this plant has also been shown to enhance the induction of delayed type hypersensitivity (DTH) response and antibody formation in mice (Vander Nat, 1990). Phenolic compounds contribute to the *in vitro* anti-inflammatory activity of its preparations (Vander *et al.,* 1991a, 1991b).

Boerhaavia diffusa Linn. (Nyctajinaceae)

Roots of *B. diffusa* are documented in literature to possess diuretic properties and are useful in jaundice, ascities, internal inflammations and gonorrhea. It is very good expectorant and is used for antistress activity (Sharma *et al.,* 1995).

The drug is reported to be useful for the eyes, dropsical swellings, heart diseases, blood purifier and maintains balance between vata, kapha. Its usefulness during enlargement of spleen and dyspepsia is also documented. It is in combating of scorpion string (Kiritikar & Basu, 1987). The plant shows hepatoprotective, anti-inflammatory and cardiovascular activity. The leaves possess antitumour, appetizer, alexiteric activities, used in rheumatism and muscular pain, blood purifier and hasten delivery.

Boswellia serrata Roxb. (Burseraceae)

The alcoholic extract of salai guggal (AESG), the oleogum resin of *Boswellia serrata*, has been shown to possess prominent anti-inflammatory and anti-arthritic activities (Singh & Atal, 1986).

Reduction of humoral antibody and development of DTH reaction to SRBC by AESG and Boswellic acids has been reported by Sharma *et al.* (1988a). It inhibits migration of leucocytes into the pleural cavity and reduced the volume pleural exudates. Antichemotactic activity has been demonstrated by its inhibitory effect on arthus reaction (Sharma *et al.*, 1988b) and synthesis of catalysed mediator (Ammon *et al.*, 1991). Salai guggal and boswellic acid have been shown to inhibit the proliferation of murine spleenocytes and thymocytes in response to mitogens (Sharma *et al.*, 1989a).

Cryptolepis buchanani Roem and Chult. (Asclepiadaceae)

C. buchanani holds a very prestigious position in Ayurveda. It is distributed throughout India, preferably in hot deciduous forests. The plant is used in traditional medicine as decoction given to children to cure them of rickets. It is also given to women in combination with *Euphorbia mycrophylla* Hyne, when lactation is deficient or fails. Different parts of this plant are used in sores, ascites, dropsy, anasarca, cholera, dysentery, body ache and snake bite. Roots of *C. buchanani* is used as a blood purifier. Ethanolic extract of roots and stem shows hypotensive and as central nervous system depressant and antiamphetaminic (Bhakuni *et al.*, 1969). Paste given internally in abdominal pain (Joshi *et al.*, 1980).

Ethanolic extract of arial parts of plant shows diuretic activity (Dhawan *et al.*, 1977). Pounded roots are given to women to increase milk secretion (Bhav, 1969). Root bark is used in rheumatic pains (Mudgal & Pal, 1980). Stem constituents are alkaloids and triterpines, leaves constituents are *a*- and *B*-amyrin (Asolkar *et al.*, 1992) and cryptolepine—the methyl-quinolanol alkaloid of *C. sanguinolenta*.

The alcoholic extract of the root shows the presence of sterols, reducing sugars and traces of glycosides exhibited antiplatelet effects *in vitro* in human, rabbits and rats. In rats, it exhibited ADP—aggregation *in vitro*

with delayed on set and prolonged action *in vitro* cryptolepine disaggregated platelets aggregated by ADP, AA and thrombin. In addition, it exhibited an indirect fibrinolytic action in the rat possibly by causing the release of plasminogen activators from the vascular endothelium (Oyeken *et al.,* 1988).

Carthamus tinctorius L. (Compositae)

A water soluble polysaccharide isolated from blossoms of C. *tinctorius* has been shown to induce antibody synthesis in mice following intraparitoneal injection (Caldes *et al.,* 1981).

Carthamus ipecacuanha (Brot.) Rich. (Rubiaceae)

Emetine an alkaloid isolated from *C. ipecacuanha* as an amoebicide and expectorant only has been demonstrated to possess antiviral activity (Grollman, 1968). Increased phagocytosis suggested an immunostimulatory action of emetine has been demonstrated by Wagner *et al.* (1985a).

Cynanchum caudatum (Asclepiadaceae)

Cynacoside, a glycoside isolated from the roots of *C. caudatum* is also an immunostimulant as it has been shown to increase phagocytosis and cellular immunity in mice (Zenyaku, 1980).

Curcuma longa L. (Zingiberaceae)

C. longa has been reported to possess anti-bacterial (Basu, 1971) anti-inflammatory, anti-arthritic (Arora *et al.,* 1971; Srimal *et al.,* 1971; Chandra & Gupta, 1972; Yegnanarayan *et al.,* 1976) and anti-asthmatic properties (Jain *et al.*, 1979). Curcumin, the active ingredient in *C. longa* has shown anti-arthritic (Deodhar *et al.,* 1980), cytotoxic and anti-tumour activities (Soudamini & Kuttan, 1988). Curcumin has shown to inhibit mitogen induced proliferation of mouse spleenocytes (Sharma *et al.,* 1989a).

Cryptolepis sanguinolenta (Asclepiadaceae)

The alcoholic extract of the root shows the presence of sterols, reducing sugars and traces of glycosides exhibited antiplatelet effects *in vitro* in human, rabbits and rats. In rats, it exhibited ADP-aggregation *in vitro* with delayed on set and prolonged action *in vitro* cryptolepine disaggregated platelets aggregated by ADP. Anti-diarrhoeal activity of *C. sanguinolenta* was studied by Alexandra *et al.* (1994).

Cryptolepine is the main alkaloid of *C. sanguinolenta* (Lindl.) Schlechter, a plant frequently used in West Africa, Ethanol and aqueous extract of roots were effective for 65 strains of *Campylobacter jejuni,* 41 strains of *Campylobacter coli* isolated from sporadic cases of gastroenteritis in Portugal and 86 strains of *Vibrio cholerae*, its activity was claimed to be

equal to ampicillin. The results suggest that these roots could be a therapeutic alternative for bacterial etiologic diarrhoea in West Africa. Studies were conducted on inhibition of carrageenan induced oedema by Cryptolepine (Boakyeyiadom, 1979; Bamgbose *et al.*, 1981; Paulo *et al.*, 1992; Alexandra *et al.*, 1994). The roots of *C. sanguinolenta* acts as anti-hepatitis and bronchodilatory (Boyed *et al.*, 1983; Cimanga *et al.*, 1991; Gomes *et al.*, 1993).

Epimedium davidii Franch (Berberidaceae)

A novel flavonoid compound baohuoside-1 (3, 5, 7-trihydroxy-4-methoxyl-8-prenylflavone-3-O-a-L-rhamnopyranoside) isolated from the plant (Li & Liu, 1988) has been shown to produce significant suppressive effect on neutrophil chemotaxis, mitogen-induced lymphocytes transformation, mixed lymphocyte culture, NK-cell cytotoxicity and IL-2 production (Li *et al.*, 1991). These inhibitory effects suggests anti-inflammatory/ immunosuppressive potential of baohuoside-1.

Eucommia ulmoides Oliver (Eucommiaceae)

An acid polysaccharide 'eucomman' isolated from the dried bark of *E. ulmoides* has shown significant potentietion of reticuloendothelial system using carbon clearance test in mice (Tomada *et al.*, 1990).

Eupatorium cannabinum L. (Asteraceae)

E. cannabinum is currently used as an ingredient of immunostimulatory drug preparation. Of the two homogeneous polysacchrides (PI and PII) isolated from *E. cannabinum* and *E. perfoliatum*, PI has been reported to differentiate to enhance the microphagocytosis chemiluminescence by a much larger margin than PII (Vollamar *et al.*, 1986).

Gynostemma pentaphyllum (Cucurbitaceae)

Total saponins of *G. pentaphylla* markedly acted against the immunity inhibition due to cyclophosphamide administration in experimental animals. Saponins have shown to have immunomodulatory action in healthy mice (Chonguan *et al.*, 1990).

Hemidesmus indicus Linn. (Asclepiadaceae)

The roots of this plant are used as a substitute for sarsaparilla. It is considered to be demulcent alternative, diaphoretic, diuretic and tonic. Root is bitter and are used in loss of appetite, fever, skin diseases, leucorrhoea, syphilis, rheumatism, scorpion sting and snake bite. It is also used as aphrodisiac, antipyretic, cure for leprosy, leucoderma, itching, good for the diseases of the brain, liver and kidney. The plant is considered useful in fever (Jain, 1965). It is useful in leucoderma, paralysis and epileptic fits in

children (Kiritikar & Basu, 1987). The aqueous extract caused a slight increase in the urinary flow in rats. Rise in B.P. was observed with alcoholic extract in rats.

The petroleum ether, chloroform and alcoholic extracts of *H. indicus* roots showed antibacterial activity. Essential oil obtained from the plant exhibited marked antibacterial activity against *B. proteus, P. aeruginosa, S. pyogenes* and *E. coli* at a concentration of 0.2% (Prasad *et al.,* 1983). The aqueous, ethanolic extract of *H. indicus* showed antiviral activity (100%) against ranikhet disease virus (RDV) (Babbar *et al.,* 1982). Ethyl acetate and saponin from *H. indicus* was found to have anti-inflammatory activity against formaline induced oedema (Dutta *et al.,* 1982). The survey revealed that twenty one plant species are used by tribals of Kerala for treating cancer symptoms (Mathew *et al.,* 1992). *H. indicus* popularly known as Indian Sarsaparilla finds extensive application in the Indian system of medicine as blood purifier and anti-rheumatic agents (Banejit *et al.,* 1992). Two new coumarino-lignoids *i.e.* Hemidesmin-1 and Hemidesmin-2 have been isolated from the root of *H. indicus* (Das *et al.,* 1992). A 95% ethanolic extract of *H. indicus* has been reported to suppress the cell mediated (CMI) and humoral components of immunity (Atal *et al.,* 1986). Mice, infected with *M. leprae*, when treated with *H. indicus* showed a delay in multiplication of organisms in the mouse foot pads while supporting the anti-leprotic action of an extract on Ananatamul (*H. indicus*) (Gupta, 1981).

Holarrhena antidysenterica Linn. (Apocynaceae)

It is found throughout India, and is safe cheap reliable drug for the treatment of diarrhoea, spleenic disorders, cholera, menorhagia and dog bites (Jain & Tarafder, 1970). The bark is used as anti-helminthic, in skin diseases, fever, piles, leprosy, kapha, thirst and dysentery. Leaves of *H. antidysenterica* are used as tonics, aphrodisiac, muscular pain, in chronic bronchitis and for cure of boils and ulcers. It is useful in regulating the menstruation. Seeds are used for fatigue, as hepatoprotective, used in leprosy, halluicinations and astringent to the bowels. Seed oil of *H. antidysenterica* showed antifungal action (Deshmuk & Jain, 1981; Kiritikar & Basu, 1987; Chopra, 1933). The therapeutic utility of kurchi in acute and chronic amoebic dysentery has been known for a long time (Chopra *et al.,* 1927, 1933).

H. antidysenterica showed promising activity against experimental amoebiasis in rats and hamster (Basu & Jayaswal, 1968; Dutta & Iyer, 1968). It lowers the B.P. in dogs. Singh and Singh (1972) studied the antiviral activity of bark extract (50% ethanolic) against potato virus X (PVX). The fruit extract (50% ethanolic) showed anti-prtozoal effect against *E. histolytica* stain STA, *Trypanosoma evansi.* Dhar *et al.* (1968) has demonstrated anticancer effect against human epidermoid carcinoma of

the nasopharynx in tissue culture and hypoglycemic activity in rats. *H. antidysenterica* (95% ethanolic extract) has been found to enhance the phagocytic functions of reticulo-endothelial system (RES) and suppress the humoral component of the immune system (Atal *et al.*, 1986).

Ichnocarpus frutescens Linn. (Apocynaceae)

It is found in all parts of India. Roots are used as a demulcent, alternative tonic, diuretic, diaphoretic and as a substitute for Indian Sasaparilla. Leaves and stems are used in fever, roots used in skin diseases, useful in night blindness, bleeding of gums, ulcerated tongue, sores, enlargement of spleen, convulsions, haematuria, dysentery cough, dog bite, in snake bite and atrophy (Jain & Tarafder, 1970; Meheshwari *et al.*, 1980). The ethanolic extract (50%) of *I. frutescens* (Whole plant) showed anti-viral activity against ranikhet disease virus but was inactive against Vaccinia virus. It had anti-bacterial, anti-fungal, anti-protozoal, anti-helminthic, hypoglycaemic and anti-cancer activity (Satyavati *et al.*, 1987). Alkaloids and flavinoids were found in roots (Kapoor *et al.*, 1969). It has no effect on respiration and blood pressure in cats and dogs (Dhar *et al.*, 1968).

Malva verticellata L. (Malvaceae)

A novel acidic polysaccharide designated as MVS-VI isolated from seeds of *M. verticellata* has been shown (Gonda *et al.*, 1990) to possess significant potentiating activity on reticuloendothelial system using carbon clearance test and anti-complementary activity.

Ocimum gratissimum L. (Labiatae)

A 95% ethanolic extract of *O. gratissimum* leaves have been reported (Atal *et al.*, 1986) to improve the phagocytic function of reticulo-endothelial system without affecting the humoral or cell mediated immune responses in mice.

Ocimum sanctum L. (Labiatae)

Godhwani *et al.* (1988) have reported the immunostimulant properties of 50% methanol extract and aqueous suspensions of *O. sanctum*. Both methanol extract and aqueous suspension of *O. sanctum* leaves have been found to enhance the humoral responses to SRBC and typhoid 'H' antigens and to increase the count of Erosetting lumphocytes.

Panax ginseng Meyer (Araliaceae)

Panax ginseng has been classified as an adaptogenic or antistress drug plant (Brekham & Dardymov, 1969). In combination with 6-MFA (an interferon inducing antiviral substance of viral origin), has been found to

significantly enhance antibody titre against SRBC. Cell mediated immunity Semliki Forest Viries antigen, natural killer cell activity in mice and production of interferon by an interferon inducer 6-MFA has been observed to be effected. This plant augments natural killer cells and antibody dependent cytotoxic activities and increase the protective effect against above described antigen as compared to 6-MFA along (Singh *et al.,* 1983).

Polysacchrides from ginseng root were found to markedly stimulate phagocytosis and production of antibody (Wang *et al.,* 1980). They caused an increase of serum complement content in guinea pigs, raised serum IgG level in mice and increased B-lymphocytes to T-lymphocyte cell ratio. The polysaccharide fractions from hot water extract of *P. ginseng* have been found to have immunological, antitumour and hypoglycemic activity (Moon *et al.,* 1983; Konno *et al.,* 1984).

Administration of ginseng polysaccharides (400–800 g/kg) for 10 days markedly increased the number of plaque forming cells (PFC) and specific rosette forming cells in tumour bearing mice immunized with SRBC (Qian *et al.,* 1987). The two polysaccharides have the similar effect on immune functions causing increase in weight of spleen, enhanced phagocytosis, promoting the production of serum specific antibody hemolysin and IgG level in mice (Li *et al.,* 1989). Enhanced DTH of foot pad induced by SRBC is also controlled by above mentioned isolate.

Picrorhiza kurroa Royle ex. Benth. (Scrophulariaceae)

Root extracts of the small Himalayan herb *Picorhiza kurroa* are used therapeutically in traditional medicine of almost of all Asian countries to treat many conditions of illness including inflammatory disorders (Nadkarni, 1954; Dey, 1980; Jayaweera, 1982). *P. kurroa* extract is being used in the treatment of bronchial asthma (Rajaram *et al.,* 1976), infectious hepatitis (Mittal *et al.,* 1978), and joint pain (Langer *et al.,* 1981; Yegnanarayan *et al.,* 1982) has been documented. 95% ethanolic extract of roots and leaves have been shown to enhance the cell mediated and humoral immune responses to sheep erythrocytes and phagocytic function of reticuloendothelial system (Atal *et al.,* 1986; Sharma *et al.,* 1988b). *P. kurroa* was also found effective in vitiligo patients (Bedi *et al.,* 1989).

A glycoside fraction from *P. kurroa* roots has been shown to augment the bronchodilatory effect of isoprenaline adrenaline, rendering guinea pigs less sensitive to histamine. It reduces histamine contents of lung tissues and inhibit immunological release of histamine and show reacting substance of anaphylaxis (SRS-A) from chopped lungs (Mahajani & Kulkarni, 1977). Iridoid glycosides *Picroside–I* and *kutkoside* have been found to stimulate the cell mediated and humoral components of immune system, Improve the phagocytic function of RES and abrogate the suppressive effects of cyclophosphamide and betamethasone (Sharma, 1991).

Plumeria acutifolia Linn. R. Br. (Apocynaceae)

It is an evergreen partly deciduous tree, cultivated as an ornamental plant throughout India. Root bark has been used as purgative, in gonorrhea and venereal sores. Stem bark is given in diarrhoea. Latex of this plant is employed as a rubefacient in rheumatism. Jain and Tarafder (1970) described its use in indigestion and cholera. Flowers are potent contraceptives (Tiwari *et al.,* 1982). Chak and Patnaik (1972) demonstrated local anesthetic activity of aqueous extract of *P. acutifolia* in rabbits, guinea pigs, cats and dogs. Antibacterial/antifungal activity has been reported by Bhatnagar *et al.* (1961). Root bark cures tumours and rheumatic pains. Plasters made of the bark are said to be useful in dispersing hard tumours (Kiritikar & Basu, 1988). Plumeride isolated from *P. acutifolia* has been reported to possess anti-fungal (Jewers *et al.,* 1975) and immunostimulatory effect in mice, on cell mediated and humoral responses. Triterpenes from leaves of *P. acutifolia* have shown strong anti-tumour activity. Bark extract possess stimulant action and is powerful antitherpeutic (Fugimoto *et al.,* 1988).

Rehmannia glutinosa libosch var. *hueichingensis* (Scrophulariaceae)

On immunological investigations two new phenethyl alcohol glycosides designated as Jionoside A1 and B1 have been shown to possess immunosuppressant activity suppressing haemolytic plaque forming cells (HPFC) in mice (Sasaki *et al.,* 1989).

Sida spp. (Malvaceae)

Dixit *et al.* (1978) have reported *S. cordifolia* L., *S. rhombifolia* L. and *S. veronicaefolia* Lamk to increase humoral antibody response to *S. typhimurium* 'O' antigen and to increase the survival time of rabbits against virulent *Staphylococcus aureus* challenge. However, no direct antibacterial activity against *S. aureus* or specific change in immunoglobulin pattern on treated animals has been recorded.

Sphaeranthus indicus L. (Compositae)

A new sesquiterpene glycoside, spaeranthonolide isolated from the flowers of *S. indicus* has been shown to produce 40% increase in the antibody secreted cells (Shekhani *et al.,* 1990). It has been used for glandular swelling, bronchitis, jaundice and nerves depression (Nadkarni, 1976).

Strobilanthes hyneanus Nees (Acanthaceae)

The aqueous and 95% ethanolic extract of stem have been shown to possess analgesic, anti-inflammatory and immuno-suppressive activity. In mice treated with these extracts, significant suppression of antibody formation

against sheep red blood cells was noticed comparable to betamethasone (Shanker *et al.,* 1987). It affects neurologic disorders oedema, healing of ulcers (Agnivesa, 1970).

Swertia chirata Ham. (Gentianaceae)

It is used as tonic, anti-helminthic, anti-diarrhoeal. It is laxative, used in fever gout, ulcers and acts as anti-inflammatory (Chowdhury *et al.,* 1995), cures burning sensation, pain in body, asthma, bronchitis and is good in vomiting. It is used as an antiworm (Kiritikar *et al.,* 1987). Hexane fraction of *S. chirata* induces fall in blood sugar after single oral administration in albino rats. A multiple dose of *S. chirata* has significantly lowered the blood sugar while showing an increase in plasma IRI along with a significant rise in liver glycogen. Swerchirin may have application in control of diabetes mellitus (Satayavati *et al.,* 1987).

Taraxacum platycarpum (Compositae)

A polysaccharide fraction from *T. platycarpum* has been shown to possess a potent immunopotentiating activity with a antitumour activity (Jeong *et al.,* 1991). The fraction having small amount of protein inhibited the growth of solid tumour and increased the peritoneal exudate cells and immunoorgan weight in normal mice and hypersensitivity in tumour bearing mice.

Tinospora cordifolia Miers (Menispermaceae)

The ethanolic extract of *T. cordiofolia* has been reported to enhance the cell mediated immunity and non-specific resistance in mice (Atal *et al.,* 1986). It is associated with early wound healing in patients with perforated peritonitis and local sepsis (Arya *et al.,* 1989).

Dahanukar *et al.* (1989) have reported *T. cordifolia* to protect rats against coecal ligation induced abdominal sepsis and mice against *E. coli* peritonitis. The plant induced leucocytosis with predominant neutrophilia associated with stimulation of phagocytic and bactericidal capacity of neutrophils and macrophages. Rege and associates (1989) have reported the restorative effect of aqueous extract of *T. cordifolia.*

Tripterygium wilfordii Hook. F. (Celastraceae)

Wilfortrine and euonine isolated from *T. wilfordii* (Deng *et al.,* 1987) showed marked depressant effects on humoral and cell mediated immunity. Wilfortrine exhibited depressant effect on graft vs host reaction and marked suppressant effects on DTH reaction in mice. Wilfortrine and euonine significantly decreased the clearance rate of charcoal particles and weights of spleen and thymus (Zheng *et al.,* 1989).

Tylophora indica Burm F. Merr. (Asclepiadaceae)

The ethanolic extract (95%) of *Tylophora* leaves has been shown to possess immunosuppressive activity (Atal *et al.,* 1986). At 100 mg/kg P.O. dossage, it significantly inhibited the expression of SRBC induced DTH reaction and humoral antibody synthesis, reduced the carbon clearance rate and prolonged the graft rejection time. Sharma *et al.* (1989) have shown tylophorine to significantly reduce the proliferative responsiveness of spleenocytes to PHA, CON A, and LPS at a concentration of 125 ug/mL. It has been used against bronchial asthma (Nadkarni, 1954) and rheumatism (Chopra *et al.,* 1958) and anti-asthmatic effect (Shivpuri, 1973, 1975; Gore *et al.,* 1980).

Uncaria tomentosa DC Prod. (Rubiaceae)

Pure oxindole isolated from *U. tomentosa* has been reported to increase phagocytosis suggesting its immunostimulatory activity (Wagner *et al.,* 1985b).

Viscum album L. (Loranthaceae)

Immunomodulating properties have been attributed to polysaccharide fraction. It has a biphasic effect on mononuclear phagocytic system and an adjuvant effect on humoral immune response (Bloksma *et al.,* 1982). It has no effect on human granulocyte phagocytosis *in vitro* (Wagner & Jorden, 1988).

Woodfordia fruticosa Kurtz. (Lythraceae)

A macerate of flowers of *Woodfordia fruticosa* has been shown to inhibit both the alternative and classical pathways of human complement system, and zymosen induced chemiluminescence of human PMNL and proliferation of T-cells (Labadie *et al.,* 1989).

Zexmenia brevifolia Gray (Compositae)

Zexbrevins A and B, the two germ acranolides isolated from *Zexmenia brevifolia* have been shown to display a pronounced activity as immuno adjuvants (Vivar *et al.*, 1970). Administration of zexbrevin prior to or along with antigen (SRBC, BSA or mouse albumin) has been observed to produce a pronounced increase in the number of specific rosette like cells in the mouse spleen. It has been shown to have no mitogenic activity.

Literature cited so far put forth the claim that natural products represent prominent and promising sources of molecules with interesting immunomodulating properties. This line of research although is a programmed way is relatively young, but fast growing, advancement in

the field of immunopharmacology, plant products have been reviewed by Lindequist and Teuscher (1985); Wagner and Proksh (1985); Wagner (1987) and Labadie (1989).

CONCLUSIONS

Leads derived from traditional medicine through scientific studies appear to be influential for the discovery of immunomodulatory compounds and novel mechanisms of action. Such compounds may serve innovative to drug development, and can be of great impact on drug therapy as a whole. The brief review of the immunopharmacological work done on medicinal plants in the past brings out potential of these herbals for effectively modulating the immune expression of an individual and opens a great avenue for researchers.

Although terms depicting immunostimulation and immunosuppression are not exactly described in the ancient literature but the therapeutic efficacy described points more or less towards their use as immunomodulators. Recently many plants have been screened on the basis of their use as antibacterial, anti-inflammatory, anti-viral, anti-stress, anti-fungal and antitumour activities. These plants/extracts or isolates are good candidates for modern immunological test screening.

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The Potentials of Immunomodulatory Substances of Natural Origin in Contemporary Medical Practice

CHUKWUEMEKA SYLVESTER NWORU[1,*] AND PETER ACHUNIKE AKAH[1]

ABSTRACT

Stress and pressure of modern society are taking a toll on the immune system. This has left many with weakened immunity and more susceptible to infection and disease. The rampaging effect of high profile immune-destructive diseases such as acquired immunodeficiency syndrome (AIDS), the concern about bioterrorism and biological warfares, the emergence of new and more virulent viruses have all contributed in the increase in the need to maintain or rebuild a healthy immune defence. These facts have led researchers to minerals, plants and fungi in search of natural substances with health-supporting properties. Compounds that are capable of interacting with the immune system to up-regulate or down-regulate specific aspects of the host response are classified as immunomodulators. Those substances which appear to stimulate the human immune response are being developed for the treatment of cancer, immunodeficiency diseases, or for generalised immunosuppression following drug treatment; for combination therapy with antibiotics; and as adjuvants for vaccines while compounds that suppress immune reactions are also potentially useful in the management of autoimmune diseases, host-graft rejection and other conditions of deleterious hyper-immune reactions. In this chapter, we will take an overview of the research efforts in identifying and harnessing the potentials of plant-derived compounds with promising immunomodulatory properties and their application in medical practice.

1. Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001, Enugu State, Nigeria.

* *Corresponding author* : E-mail : csnworu@yahoo.com

Key words : Adjuvants, immunomodulation, immunorestoration, nutraceuticals, phytoimmunomodulators, dietary supplements

INTRODUCTION

The immune system is a highly specialized defense system that identifies, remembers, attacks and destroys disease-causing invaders or infected cells. The human immune system is a sophisticated network of organs, blood cells and specialized tissues that protect the body from foreign substances such as bacteria, viruses, fungi and parasites. Essentially, the immune system is the body's means of surveillance, intended to protect the body from disease by searching and destroying any health-damaging agents. The system also defends against invaders from within the body, which can be very life-threatening.

There are two aspects of immune protection-the innate response and the adaptive response (Abbas & Litchman, 2004). Innate immunity is present at birth and provides the first barrier against microorganisms. The skin, mucus secretions and the acidity of the stomach are examples of innate immunity and act as barriers, shielding vulnerable tissues from unwanted germs. These mucosal surfaces contain types of immunoglobulins (primarily IgA), which function as antibodies to help determine which non-self organisms are pathogenic and which are innocuous (Janeway *et al.*, 2005). Phagocytes may also release pyrogens to fight off infection. Should foreign substance eventually enters the body, the immune system destroys the matter by phagocytosis, a process which eats and digests the unfamiliar matter. The cells involved in phagocytosis include granulocytes and macrophages.

Adaptive immunity is the second barrier against infection (Janeway *et al.*, 2005). It is acquired later in life, for example, after an immunization or after fighting off an infection. The adaptive immune system retains a memory of all the invaders it has faced (measles and chicken pox are common examples). However, some viruses (*e.g.* cold virus) disguise themselves to escape dictation and must be fought off time and again by the immune system.

The innate and adaptive mechanisms could be modified by substances to either enhance or suppress their ability to resist invasion by pathogens (William, 2001). Both immunostimulation and immunosuppression are clinically relevant and search for substances, and leads with these activities is becoming a field of major interest all over the world (Naved *et al.*, 2005). There has been a growing interest in identifying and characterising natural compounds with immunomodulatory activities after some researchers suggested their possible clinical use (Atal *et al.*, 1985; Atal *et al.*, 1986; Wang *et al.*, 1991; Sharma *et al.*, 1994; Lee *et al.*, 1995). The high profile

immune-destructive diseases such as AIDS and the concern about bioterrorism are leading consumers to seek natural ways to boost their immune systems. In an era of chronic and emerging new viruses, with very high virulence, immunomodulating substances from natural sources could play a significant role in human disease prevention and treatment (Dixon, 2001).

Those compounds which appear to stimulate the human immune response are being sought for the treatment of cancer, immunodeficiency diseases, or for generalised immunosuppression following drug treatment; for combination therapy with antibiotics; and as adjuncts for vaccines. Those compounds that suppress immune reactions are potentially useful in the remedy of autoimmune (an abnormal immune response against self-antigens) or certain gastro-intestinal tract diseases (*e.g.* Crohns) (Badger, 1983).

Whether immunomodulators enhance or suppress immune responses will depend on a number of factors such as dosage, route of administration, and timing and frequency of administration (Tzianabos, 2000).

Immunostimulation

The immune response can be modified to enhance the humoral or cell-mediated immune response against antigens. Immunostimulation reinforces the immune system. The mechanisms of potentiation of immune responses may include one or all of the following situations: an increase in the rate; a heightened intensity or level; an extension of normal immune response; and the induction of immune response to an otherwise non-immunogenic substance. Many humoral and cellular immune factors are subject to stimulation.

Cellular Factors

Cell mediated processes which could be stimulated include, but not limited to: polymorphonuclear leukocytes activation; peritoneal macrophage activation; natural killer cell activation; activation of helper T-cells; activation of killer T-cells; inhibition of suppressor T-cell activity; activation of cytotoxic macrophages; delayed-type hypersensitivity reaction and mitogenicity. Activation of these processes will invariably lead to the stimulation of the cellular immune responses mediated by them.

Humoral Factors

Humoral factors activation which will result in immunopotentiation include: increased production of antibodies, opsonin, colony-stimulating factor (CSF), lymphocyte-activating factor (interleukin-1), interferon, tumor necrosis

factor (TNF). Increased complement C3 production and C3-splitting activity, inhibition of the release of prostaglandin and other immunosuppressive factors.

Immunosuppression

The term immunosuppression refers to impairment of any component of the immune system resulting in decreased immune function (Descotes *et al.*, 2000). Clinically, immunosuppression manifest as myelosuppression (*e.g.* pancytopenia, leukopenia, lymphopenia, and other blood dyscrasias); decreased immune system organ weights and histology (*e.g.* hypocellularity of immune system tissues such as the thymus, spleen, lymph nodes, or bone marrow); decreased serum globulin levels; increased incidence of infections; and increased incidence of tumors (Basketter *et al.*, 1995; Dean *et al.*, 1998; Richter-Reichhelm & Schulte, 1998).

It is also necessary to distinguish between adverse (unintended) immunosuppressive effects and pharmacodynamic (intended) effects. For example, many antitumor drugs are toxic to rapidly dividing cells; therefore, immunosuppression due to bone marrow toxicity would be considered an adverse effect during the treatment of a solid tumor, but not in the treatment of a haematologic malignancy. For drugs intended to be used for prevention of transplant rejection (*e.g.* cyclosporine), immunosuppression is the intended pharmacodynamic effect (Kwak *et al.*, 2000; Colville-Nash & Gilroy, 2001). Methods of detecting immunosuppression in standard toxicology studies have been described and these includes determination of serum biochemical markers such as globulin levels, hematology (including differential), gross pathology findings, immune system-related organ weights, and histologic examination of immune system-related tissues (Kuper *et al.*, 2000).

Clinically, some drugs (including natural products), acting through one or some of the mechanisms outlined above have been used to successfully achieve immunostimulation and immunosuppressive states in patients. The major drawback which has limited the clinical application of immunomodulatory substances is non-specific immune system effects and toxicity. There are situations where the use of an immunostimulant drug results in non-specific and uncontrolled immunopotentiation leading to over-reactivity of immune responses.

MEDICAL APPLICATION OF IMMUNOMODULATORS OF NATURAL SOURCES

Herbal immunomodulatory substances have a great deal of areas of application in contemporary medical practice.

Nutraceuticals and Supplements

The use of natural immunomodulators as adjuncts in dietary supplements and beverages has more than any other application popularized this group of medicinal products. There is hardly any dietary supplement without an ingredient with a claim on "boosting the immune system". The most popular plant products which are household name among the consuming public include Ginseng, *Echinacea, Gingko biloba*, Noni (*Morinda citrifolia*), Ashwagandha root (*Withania somnifera*) and so many others. These products have consistently demonstrated ability to cause an increase in non-specific resistance to infection and a general improvement in immune status. Immunocompromised subjects have also benefited from the immune-boosting properties of these products. These claims are also well supported by literature and research which has shown that extracts and phyto-constituents from these plants actually have effects on the components of the immune system which could lead to immunopotentiation.

Adjuncts in Cancer Chemotherapy

Immunomodulators of plant sources could also be beneficial to tumor patients where they are either used alone for their inherent anti-tumor effects or in combination with other chemotherapeutic regime. Many natural products with immunomodulatory effects which have shown good potentials as anti-tumor agents include *Allium sativum* (Kandil *et al.*, 1987; Amagase *et al.*, 2001), *Rhaponticum carthamoides* (Wagner, 1995), *Tinospora cordifolia,* (Chen & Chen, 2004; Winston, 2004), *Gynostemma pentaphylla* (Blumart & Jialiu, 1999), *Codonopsis pilosula* (You-ping, 1998), *Calendula officinalis* (Jimenez-Medina *et al.*, 2006) and *Eleutherococcus senticosis* (Lee *et al.*, 2004). In human studies, Eleuthero has been successfully used to treat bone marrow suppression caused by chemotherapy or radiation (Halstead & Hood, 1984). These herbal products are safer with little or no side effects when compared with the limiting toxicities of conventional anti-tumor agents. Herbal immunomodulators could also be used in leukemia and multiple myeloma, abnormal, where cancerous immune cells crowd out the normal stem cells of the bone marrow. These abnormal cells reduce the number of B cells and lead to hypogammaglobulinemia or secondary immune deficiency (Roitt & Delves, 2001; Janeway *et al.*, 2005).

Treatment of Immunosuppressive Disorders

Immunostimulant natural products are also very beneficial to immuno-compromised patients. The use of immunomodulators, especially those of natural origin in tackling the menace of HIV holds a lot of promise in preventing or treating opportunistic infections (Yamaguchi, 1992).

An immune deficiency disease occurs when one or more cells within the immune system do not operate properly, or the system is absent

altogether. In primary immune deficiency, the abnormalities of the immune system develop from an inborn defect in the cells. Cells that are readily affected include T-cells, B-cells, phagocytic cells or the complement system (Janeway *et al.*, 2005). Most primary immune deficiencies are inherited diseases; examples include X-linked agammaglobulinemia (XLA) and severe combined immunodeficiency disease (SCID) which appear to run in families. Other primary immune deficiencies, such as common variable immunodeficiency (CVID), appear less obviously inherited, but the causes of the defects are unknown and genetic factors cannot be ruled out.

Secondary immune deficiencies occur when damage is caused by environmental factors. Radiation, chemotherapy, burns and infections contribute to the many causes of secondary immune deficiencies (Goldsby *et al.*, 2000; Roitt *et al.*, 2002). Acquired Immune Deficiency Syndrome (AIDS) is a secondary immune deficiency caused by the Human Immunodeficiency Virus (HIV) that destroys T4 cells. Diets lacking sufficient protein are associated with impaired cell-mediated immunity, complement activity, phagocytes function, immunoglobin A (IgA), antibody concentrations, and cytokine production. Deficiency of single nutrients such as zinc; selenium; iron; copper; vitamins A, C, E, and B_6; and folic acid (vitamin B_9) also reduces immune responses (Chandra, 1997).

Recently, in our laboratory, we demonstrated the immunorestorative properties of Kolaviron, a mixture of three related biflavonoids of *Garcinia kola* in immunocompetent and immunocompromised animal models (Nworu *et al.*, 2007, 2008). There are also many other phyto-immunomodulators which have been found very useful in immunodeficiency conditions.

Adjuncts in Antibiotics Therapy

It is known that strong immune response in the face of an infection will augment the rate of bacterial clearance by antibiotics. It is therefore reasonable to use immune-boosting adjuvants in combination during antibiotics therapy especially in patients with low immune profile. In this regard, potent phyto-immunomodulators could readily be used for reasons of tolerability, acceptability and cost-effectiveness.

Remedy of Autoimmune Disorders

Sometimes the immune system's recognition apparatus breaks down, and the body begins to manufacture antibodies and T-cells directed against the body's own constituents-cells, cell components, or specific organs. Autoimmune reactions contribute to many enigmatic diseases. For instance, autoantibodies to red blood cells can cause anaemia, autoantibodies to pancreas cells contribute to Type 1 diabetes, and autoantibodies to nerve and muscle cells are found in patients with myasthenia gravis. Autoantibody known as rheumatoid factor is common in persons with rheumatoid

arthritis. Persons with systemic lupus erythematosus (SLE), whose symptoms encompass many systems, have autoantibody that affects the immune system at several levels. The causes of autoimmune disease are not well understood, but several factors are likely to be involved. These may include viruses and environmental factors such as exposure to sunlight, certain chemicals, and some drugs, all of which may damage or alter body cells so that they are no longer recognizable as self (Goldsby *et al.*, 2000; Roitt *et al.*, 2002). Many types of therapies are being used to combat autoimmune diseases. These include corticosteroids, immunosuppressive drugs developed as anticancer agents, radiation of the lymph nodes, and plasmapheresis, a sort of "blood washing" that removes diseased cells and harmful molecules from the circulation (Goldsby *et al.*, 2000; Roitt *et al.*, 2002).

Herbal immunosuppressive therapies formulated with plant materials which have been shown in research and folklore practices to possess suppressive effects on the various components of the immune system could be used in autoimmune conditions. Licorice rhizome (*Glycyrrhiza glabra, Glycyrrhiza uralensis*) is an immune amphoteric and can be useful for autoimmune disorders (Lupus, Scleroderma, Crohn's disease, rheumatoid arthritis) as well as immune deficiency conditions (*e.g.* cancer and HIV) and ulcers (Murray & Pizzorno, 1990). Research has also shown that Holy Basil (*Ocimum sanctum*) reduces excess immune responses (Ghosal *et al.*, 1989) and is therefore a good candidate for development as remedy for autoimmune diseases.

Graft Versus Host Diseases

Transplantation of tissues and organs can cause life-threatening reactions in recipients. Graft rejection reaction is immunologic, it shows specificity and memory. It has been shown to be mediated by lymphocytes. The antigens that serve as the principal targets of rejection are proteins encoded in the major histocompatibility complex (MHC). The mainstay of preventing and treating the rejection of organ transplant is immunosuppression, designed mainly to inhibit T-cell activation and effector function (Abbas & Lichtmann, 2004; Janeway *et al.*, 2005). Herbal immunosuppressants and other immunosuppressive therapies of natural origin could be very beneficial to organ-transplant patients.

Adjuvants in Vaccines Development

The formulation of vaccines with immune potentiators (also termed adjuvants) is an attractive approach for obtaining robust and long-lasting immune responses to these vaccines. Adjuvants have diverse mechanisms of action and should be selected based on the route of vaccine administration and the type of immune response (antibody, cell-mediated, or mucosal

immunity) desired. Adjuvant mechanisms of action include: (1) increasing the biological or immunological half-life of vaccine antigens; (2) improving antigen delivery and presentation; and (3) inducing the production of immunomodulatory cytokines (Vogel, 1998).

Many synthetic compounds have been explored for possible use as vaccines adjuvants, but toxicity-related issues have limited the number of such compounds licensed for use in vaccine development. Immunostimulatory compounds of plant-sources have also demonstrated good potentials as vaccines adjuvants and some have even been patented (Gautam *et al.*, 2004; Vega, 2005; Khajuria *et al.*, 2007). The saponin adjuvants of *Quillaia saponeria* and *Quillaia brasiliensis* are an examples of successes recorded in the use of phyto-adjuvants in vaccine development (Fleck *et al.*, 2006).

IMMUNOMODULATORY SUBSTANCES OF NATURAL ORIGIN

Several classes of compounds have been shown to modulate the immune system. Low molecular weight compounds such as alkylamides, phenolic compounds, alkaloids, quinones, saponins, sequiterpenes, di- and triterpenoids and high molecular weight compounds such as proteins, peptides, polysaccharides, glycolipids (lectins), lipopolysaccharides, glycoproteins, and glucans have all been classified as molecules that have potent effects on the immune system (Labadie, 1993; Wagner *et al.*, 1999). The idea of boosting immunity has led many researchers and consumers to vitamins, minerals, mushrooms and herbal products that may strengthen or modulate the immune system naturally (Tzianabos, 2000).

Vitamins and Minerals

Vitamins and minerals are necessary in maintaining a healthy immune system. Within the body, vitamins and minerals act as enzymes and coenzymes for every function of the body. Research in Vitamin A continues to show that the nutrient may have immunomodulatory effects. One study comparing patients with common variable immunodeficiency (CVI) with healthy controls demonstrated that the majority of CVI patients had low vitamin A levels, and that supplementation of vitamin A in these patients resulted in increased immune function (Aukrust *et al.*, 2000).

Vitamin B

Vitamins B group act as co-enzymes that help the body metabolize food into energy, synthesize antibodies for the immune system and provide daily support. Perhaps one of the most important nutrients for supporting immune function is vitamin B6, which is needed to make antibodies and slow tumor growth (Vanderhaeghe and Bouic, 1999). Studies have shown

that vitamin B6 supplementation can influence tumor growth and disease processes (Tryfiates, 1986; Matsubara *et al.*, 2003). Vitamin B6 should be taken in conjunction with the other B vitamins to maximize the vitamins' synergies.

Vitamin C

Vitamin C ascorbic acid has been shown to improve immune function in humans (Delafuente *et al.*, 1986; Banic, 1982). Human volunteers who ingested 2–3 g ascorbate daily for several weeks exhibited enhanced neutrophil motility to chemotactic stimulus and stimulation of lymphocyte transformation (Anderson *et al.*, 1980). Neutrophil motility and lymphocyte transformation were also stimulated by 1 g intravenous ascorbic acid in six healthy volunteers. Alterations in these activities were related to serum ascorbic acid levels. A study found that individuals with low to inadequate NK-cell and B-cell activity could boost NK-cell activity tenfold and improve B-cell function by consuming vitamin C at levels of 60 mg/kg of body weight (Gunnar & Aristo, 1997).

Vitamin D

Vitamin D has been shown to either prevent or markedly suppress autoimmune disease by stimulating TGFβ-1 and IL-4 production, which can suppress inflammatory T-cell activity (Deluca & Cantorna, 2001; Griffin *et al.*, 2003).

Vitamin E

Vitamin E, it is an antioxidant with pronounced effect on the thymus and on white blood cells, which are prone to oxidative stress and viral illnesses such as AIDS and hepatitis. Vitamin E supplementation has also been said to improve cell-mediated immunity, enhance digestion in cells and increase resistance to infection (Vanderhaeghe, 2001).

Selenium

Low levels of selenium can make the body susceptible to cancer, viruses and free radical damage. As part of the antioxidant enzyme glutathione peroxidase, selenium may protect white blood cells from free radical damage and also appears to increase antibody production, as well as accelerate production of leukocytes (Hawkes *et al.*, 2001; de Jong *et al.*, 2001).

Zinc

Many scientific investigations have proved that zinc have the most specific effect on the immune system. Its effects are across the board, impacting defense systems from skin barrier to gene regulation in infection-fighting

lymphocytes. According to researchers, zinc plays a unique role in T-cell mediated response and, when combined with thymic hormones, it forms a biologically active thymic hormone molecule (Dardenne *et al.*, 1985). Supplementation with zinc increases the ability of macrophages to digest invaders and enhances the ability of the immune system to eliminate bacteria (Duchateau *et al.*, 1981; Fraker *et al.*, 1986).

Mushrooms

The value of medicinal mushrooms to human health is increasingly gaining acceptance as researchers provide information on the vast number of bioactive compounds found within these fascinating fungi. Mushrooms grow wild in many parts of the world and also are commercially cultivated. It is low in calories and carbohydrates; high in vegetable proteins and essential amino acids; a source of some fibre; and rich in some important vitamins and minerals, including B vitamins, iron, potassium, selenium and zinc (Mattila *et al.*, 2001). Researchers have found that mushrooms can directly stimulate the lymphocytes, neutrophils and secondary immune responses (immunoglobulins IgE, IgA, IgG) of the immune system. This stimulus can increase production of immune defenders such as cytokines and macrophages, which play important roles in recognizing and removing foreign antigens, as well as releasing chemical mediators including interleukin-1 (Adach *et al.*, 1995; Ohio *et al.*, 1996).

Some substances with immunomodulatory activities have been isolated from different species of mushroom. These include β-glucans, lentins, polysaccharides, polysaccharide-peptide complexes, triterpenoids, nucleosides and other secondary metabolites (Lui *et al.*, 1998; Eo *et al.*, 1999; Zhu *et al.*, 1999; Ooi & Lui, 2000). Many of these bioactive substances, through their stimulatory effects on the immune system, are showing powerful antitumour, antimutagenic and anticancer activity (Borchers *et al.*, 1999).

Of the hundreds of known mushroom varieties, several have been studied for their ability to enhance the human immune system and fight infections. Some well-known medicinal mushrooms which are beneficial to the immune system are presented in Table 15.1.

Table 15.1. Some mushrooms with identified immunomodulatory activities

Name of mushroom	Common name	Reference(s)
Cordyceps sinensis	Cordyceps (Caterpillar fungus)	Bo & Bau, 1980; Chang, 1981; Liu *et al.*, 1992; Ying *et al.*, 1987
Ganoderma lucidum	Reishi	Chang, 1981; Lin *et al.*, 2006; Dharmananda, 1988, 1986; Liu, 1993; Miyazaki & Nishijima, 1981; Willard, 1990

Table 15.1. *Contd.*

Name of mushroom	Common name	Reference(s)
Trametes versicolor	Kawaratake (Turkey Tail)	Arora, 1986; Ebina, 1987
Auricularia auricular	Kikurage	Arora, 1986; Bo & Bau, 1980; Ying *et al.*, 1987
Agaricus blazeii	Himematsutake	Arora, 1986; Bo & Bau, 1980; Ying *et al.*, 1987
Trametes versicolor	Kawaratake (Turkey Tail)	Arora, 1986; Ebina, 1987
Auricularia auricular	Kikurage	Arora, 1986; Bo & Bau, 1980
Agaricus blazeii	Himematsutake	Arora, 1986; Bo & Bau, 1980; Ying *et al.*, 1987
Lentinula edodes	Shitake (Snake butter)	Liu *et al.*, 1992
Grifola frondosa	Maitake	Arora, 1986; Adachi *et al.*, 1994; Weill, 1990
Hericium erinaceus	Lion's Mane Hericium	Yang & Jong, 1989
Grifola umbellata	Choreimaitake (zhu ling)	Arora, 1986; Suzuki, 1990
Flamulina velutipes	Enokitake (Velvet Foot)	Arora, 1986; Zhou *et al.*, 1989
Tremella fuciformis	White fungus	Arora, 1986; Ying *et al.*, 1987
Poria cocos	Bukuryo (Hoelen)	Narui *et al.*, 1980

Probiotics

Probiotics have been defined as live microorganisms that (when ingested) have a beneficial effect in the prevention and treatment of specific medical conditions. These microorganisms are believed to exert biological effects through a phenomenon known as colonization resistance, whereby the indigenous anaerobic flora limits the concentration of potentially harmful (mostly aerobic) germs in the digestive tract. *Lactobacillus* is one member of a large family of beneficial bacteria populating the human body, predominantly in the gastrointestinal system. Lactobacillus and other probiotics are crucial for proper gastrointestinal function as well as immune recognition and response in the GI tract. *Lactobacillus GG* is an isolate of the lactobacillus species that has been found to have excellent effects on the immune system. In one human study, three weeks of lactobacillus supplementation increased white blood cells' ability to attack bacteria by 19% and natural killer cells' ability to kill cancer cells by more than double (Sheih *et al.*, 2001). *Lactobacillus rhamnosus* has also been shown clinically to be very beneficial to the immune system.

Generally, probiotics help create a barrier effect in the intestinal tract and crowd out pathogenic organisms (*e.g.* Salmonella and other enterococci). This activity helps strengthen the body's first line defense, thereby preventing allergens and pathogens from getting into the system. Probiotics also compete for nutrients with pathogens, thereby inhibiting their growth. Some probiotics is also known to secrete anti-microbial substances such as

bacteriocins. These are like natural antibiotics which eliminates pathogenic bacteria (Erickson, 2000; Gill & Rutherford, 2001). Probiotics are now used to fortify yogurts as immune boosting agents.

Phyto-Immunomodulators

Plants have been widely studied as potential sources of immunomodulatory substances (Yamaguchi, 1992; Wagner *et al.*, 1999). Recent researches are now focused on harnessing natural products especially from plant sources which has demonstrated potent immunomodulatory effects for clinical use. These efforts have proved quite successful as there are a number of phytotherapeutic products commercially available as immune-boosting preparations and as dietary supplements. Some of these plants which have been demonstrated by research to possess immunomodulatory activities are shown in Table 15.2.

Table 15.2. Immunomodulatory activities of some medicinal plants

Plant species	Family	Immuno-activity	Reference(s)
Echinacea purpurea L.	Asteraceae	2, 4	Coeugniet & Elek, 1987; Wang *et al.*, 2004
Echinacea angustifolia L.	Asteraceae	2	Bauer, 1996; Hobbs, 1994
Eupatorium perfoliatum L.	Asteraceae	2	Coeugniet & Elek, 1987; Wagner *et al.*, 1999
Eleutherococcus senticosus (Rupr. & Maxim.)	Araliaceae	2	Lee *et al.*, 2004
Morinda citrifolia (Noni) L.	Rubiaceae	19	Hirazumi *et al.*, 1999
Allium sativum L. (Garlic)	Alliaceae	3	Keiss *et al.*, 2003; Sahu *et al.*, 2007
Sabal serrulata (Michx.) Nutt. ex Schult. & Schult. f.	Arecaceae	2	Koch *et al.*, 2001
Calendula officinalis L.	Asteraceae	2	Barbour *et al.*, 2004
Pfaffia paniculata (Mart.)	Amaranthaceae	6	Pinello *et al.*, 2006;
Gynostemma pentaphylla (Thunb.)	Cucurbitaceae	19	Zhang *et al.*, 1990; Blumart & Jialiu, 1999
Trichopus zeylanicus J. seed	Dioscoreaceae	19	Singh *et al.*, 2001; Patwardhan, 2005
Baptisia tinctoria L.	Fabaceae	2	Bodinet *et al.*, 2002
Glycyrrhiza glabra L.	Fabaceae	18	Schepetkin & Quinn, 2005
Withania somnifera L.	Solanaceae	18	Bhattacharya *et al.*, 1987; Agarwal *et al.*, 1999; Furmanowa *et al.*, 2001
Arnica montana L.	Asteraceae	2	Schepetkin & Quinn, 2005; Bellavite *et al.*, 2006
Achyrocline satureioides L.	Asteraceae	2, 4, 15	Ruffa *et al.*, 2002

Table 15.2. *Contd.*

Plant species	Family	Immuno-activity	Reference(s)
Carthamus tinctorius L.	Asteraceae	2	Ando *et al.*, 2002
Chamomilla recutita L.	Asteraceae	2	Wagner *et al.*, 1985
Althaea officinalis L.	Malvaceae	2	Bakuridze *et al.*, 1993; Kayser *et al.*, 2003.
Plantago major L.	Plantaginaceae	2	Gomez-Flores *et al.*, 2000; Samuelsen, 2000
Eupatorium cannabinum L.	Compositae	2	Kintzios, 2006
Angelica acutilo (Siebold & Zucc	Umbelliferae	5, 8, 15	Chang, 2002; Kim *et al.*, 2006
Aloe vera L.	Liliaceae	12, 15, 16	Winters, 1993; Marshall *et al.*, 1993
Viscum album	Viscaceae	3, 7	Coeugniet & Elek, 1987 L. (Mistletoe)
Azadirachta indica, A. Juss.	Meliaceae	12, 15, 16	Upadhyay *et al.*, 1992; Baral *et al.*, 2005; Mandal-Ghosh *et al.*, 2007
Picrorhiza kurroa Royle ex Benth.	Scrophulariaceae	14, 15, 16	Gupta *et al.*, 2006; Simons *et al.*, 1990
(*Curcuma longa*) L.	Zingiberaceae	13	Gautam *et al.*, 2007
Astragalus mongholicus, Bunge	Leguminosae	4	Mao *et al.*, 2005; Tang *et al.*, 2003
Artemisia princes Pampan	Compositae	13	Yamada *et al.*, 1985
Asparagus racemosus (Willd.)	Liliaceae	16	Gautam *et al.*, 2004
Panax ginseng, C.A. Mey.	Araliaceae	13	Lim *et al.*, 2004; Wang *et al.*, 2004
Garcinia kola Heckel	Clusiaceae/ Guttiferae	11, 13, 16	Nworu *et al.*, 2007, 2008
Mangifera indica L.	Anacardiaceae	9, 10, 12	Makare *et al.*, 2001
Amaranthus spinosus L.	Amaranthaceae	1	Lin *et al.*, 2005
Brassica oleracea	Brassicaceae	6, 17	Wataru *et al.*, 2002
Theobroma cocoa L.	Sterculiaceae	8	Ono *et al.*, 2003

Legends to Table 15.2: stimulation of B-cell proliferation-1; stimulation of phagocytic activity of granulocyte-2; stimulation of natural killer (NK) cell cytotoxic activity-3; stimulation of phagocytic activity of macrophages-4; stimulation of mitogenic activity-5; activation antitumor macrophages-6; activation of lytic effector cell-tumor cell conjugates-7; stimulation of interferon production-8; stimulation of IgG antibody response-9; stimulation of IgM antibody response-10; inhibition of delayed type hypersensitivity-11; induction of delayed-type hypersensitivity in mice footpad-12; anticomplementary activity-13; anticomplementary activity with the classical pathway selectivity-14; anticomplementary activity in classical and alternative pathways-15; adjuvant activity on specific antibody production-16; induction of cytotoxic macrophages-17; immune amphoteric-18; general and non-specific immunostimulation-19.

Immunomodulatory substances of herbal origin are encouraged for reasons of safety, acceptability, and could also show efficacy comparable or even better that the synthetic immunomodulators.

CONCLUSIONS

It is obvious from the discussions that immunomodulatory substances of natural origin are of great importance in health care. They have wide application ranging from their use in dietary supplements to its application as adjuvants and delivery agents in vaccines development. Unlike synthetic immunomodulators, natural immunomodulators could be harnessed and used routinely in short and chronic immunotherapies because of their lower toxicities, acceptability and availability.

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Study of Immunomodulatory Activity of *Sphaeranthus indicus*

VIKANI K.V.[1], LAHIRI S.K.[1], SANTANI D.D.[2], KAPADIA N.S.[1] AND SHAH M.B.[1,*]

ABSTRACT

The immunomodulatory effect of the whole plant Sphaeranthus indicus (Linn.) was evaluated by studying its effect on phagocytic activity (carbon clearance assay), E. coli induced peritonitis, humoral and cell mediated immune response as well as effect over antibody forming spleen cells. Hydroalcoholic extract and its ethyl acetate soluble and insoluble fractions were screened primarily. Of the extracts tested, ethyl acetate (sesquiterpene rich) fraction possessed potent immunomodulatory activity. The same extract was also found to withhold considerable antioxidant activity as evident by its DPPH, superoxide, hydroxyl, radical scavenging activity, erythrocyte membrane stabilizing and lipid peroxidation assays. The results ensure it as a potent Rasayana drug.

Key words : Antioxidant, eudesmenolide, Gorakhmundi, HPTLC, immunomodulation, *Sphaeranthus indicus*

INTRODUCTION

Sphaeranthus indicus (Linn.), Gorakhmundi (Family: Compositae) is a herb widely distributed throughout India, in tropical Himalaya ascending to 5000 feet and southward to Srilanka, Singapore, Africa and Australia. The herb is reputed general tonic, alterative, aphrodisiac and it is highly valued in obesity, indigestion and bronchitis etc (Kirtikar & Basu, 1975). A literature survey on the phytopharmacological aspects of this species led

1. Department of Pharmacognosy, L.M. College of Pharmacy, Ahmedabad, Gujarat, India.
2. Department of Pharmacology, L.M. College of Pharmacy, Ahmedabad, Gujarat, India.
* *Corresponding author* : E-mail : mbshah2007@rediffmail.com

us to determine the possible immunomodulatory and antioxidant effects of the plant.

MATERIALS AND METHODS

Animals

Swiss albino mice of either sex weighing 25–30 g were used in this study. The distribution of animal in groups, the sequence of trials and the treatment allotted to each group were randomized. The animals were given standard pellet diet and water *ad libitum* under standard conditions of 12 h light and dark period, humidity and temperature. The study was permitted by the institutional animal ethical committee.

Plant Material and Extraction

Fresh flowering plants of the *S. indicus* were collected in the month of November, 2004. The authenticity of the plant was established and the voucher specimen (No. 139) was deposited in Department of Pharmacognosy and Phytochemistry, L. M. College of Pharmacy Ahmedabad. The plant material was dried under shade and powdered to 60# mesh. 30 g of powdered plant material after removing volatile oil through steam distillation was extracted with 50% aqueous ethanol (3 × 500 mL) exhaustively. The hydro alcoholic extract (Ext A, 11.23%) after striping off alcohol was fractioned using ethyl acetate (4 × 200 mL) to get ethyl acetate soluble fraction (Ext B, 1.21%) and ethyl acetate insoluble fraction (Ext C, 9.97%).

Immunomodulatory Studies

The method was executed as reported earlier (Gonda *et al.,* 1990). The clearance of carbon particles from blood was determined at 650 nm and phagocytic index (PI) was calculated as slope of time by concentration curve of test divided by that of control. Group I was control receiving distilled water, group II (Ext A), III (Ext B) and IV (Ext C) were given extracts respectively (300 mg/kg body weight, p.o.) for 5 days. PI between 1.0–1.2, was considered active, 1.3–1.5 was considered moderately active and >1.5 was considered strongly active. The active fraction was further studied for its immunomodulatory and antioxidant potential.

Evaluation of Immunoprophylactic Effect

The method described earlier (De *et al.,* 1998) was adapted. The control group received 1.0% Na CMC solution as vehicle; while animals in the treatment group were given the test extract (Ext B, 300 mg/kg, *p.o.*) in 1.0% Na CMC daily for 15 days (n = 8). On 15^{th} day, 3 h after the last dose of Ext B, *E. coli* (2.5×10^8 cells suspended in normal saline) were injected intraperitoneally in both groups of mice. Apart from percentage mortality of animals within 24 h, bacteremia was determined at 1, 6 and 12 h of *E. coli*

post challenge by enumeration of colony forming units (cfu) on Mac Conkey's agar medium.

Humoral Antibody (HA) Titre and Delayed Type Hypersensitivity (DTH) Response

The method described elsewhere (Puri *et al.*, 1993) was employed with grouping and dosing as above done for a week, followed by 0.1 mL of 20% of fresh sheep red blood cells (SRBC) immunization. Antibody levels were determined by haemagglutination technique and the reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

On 11^{th} day, the thickness of the right hind footpad was measured using Vernier caliper. The mice were then rechallenged (Puri *et al.*, 1993) by injection of 20 µl of 1% SRBCs in right hind footpad. Foot thickness was again measured after 24 h of this challenge. The difference between the pre and post challenge foot thickness expressed in mm was taken as a measure of delayed type hypersensitivity (DTH).

Plaque Forming Cell (PFC) Assay

The assay was done according to the technique of Jerne and Nordin (1963). As per grouping and dosing done in HA titre assay, the spleen cells were separated in RPMI-1640 medium washed with the same and suspended in the same to a concentration of 1×10^6 cells/mL on 8^{th} day. Agarose plates were prepared as reported earlier and plaques in presence of guinea pig (complement source) were expressed as count per 10^5 spleen cells.

Antioxidant Activity

The active fraction was evaluated for antioxidant potential.

Antiradical Activity (Free Radical Scavenging Activity) by DPPH Method

Antiradical activity was measured (Navarro, 1993) by observing decrease in absorbance at 516 nm of a methanolic solution of colored DPPH (1, 1-diphenyl-2-picryl hydrazyl, a stable free radical) brought out by the sample. A stock solution of DPPH (4.3 mg/3.3 mL methanol) was prepared such that, 75 µl of it in 3 ml methanol gave an initial absorbance of 0.9. Decrease in absorbance in the presence of Ext B at different concentration (20–70 µg) was measured at 516 nm up to 2 min at an interval of 30 sec. Ascorbic acid was used as a reference standard and 75 µl of stock solution of DPPH in 3 mL methanol as a control. The activity was expressed as an effective concentration at 50% (EC_{50}).

Superoxide Radical Scavenging Activity

Superoxide anion radical scavenging assay was performed by monitoring the reduction of nitroblue tetrazolium (NBT) to a blue colored formazan as reported elsewhere (Beauchamp & Fridovich, 1971), which was measured at 590 nm at regular interval of 30 sec up to 2.5 min and terminally at 4 min. The EC_{50} from percent Inhibition of superoxide radical scavenging activity was calculated.

Hydroxyl Radical ($HO^{\cdot}$) Scavenging Activity

The formaldehyde formed during the oxidation of dimethylsulphoxide (DMSO) by Fe^{+3}-ascorbic acid was used to detect hydroxyl radicals (Rekka *et al.,* 1989). The reaction mixture with different concentrations of test sample (10, 100 and 1000 µg/mL) dissolved in phosphate buffer or 100 µl ascorbic acid (2 mm) was incubated for 30 min at 37°C. The reaction was stopped by adding 125 µl of trichloroacetic acid (17.5% w/v) and the formaldehyde formed, followed by another reaction for 10 min at 50°C, was determined by measuring the absorbance of reaction mixture at 412 nm (Nash, 1953).

Erythrocyte Membrane Stabilizing Activity

The assay was carried out according to the procedure described by Navarro *et al.* (1993), wherein the hemolysis of RBC was induced with superoxide radical by a riboflavin-light-NBT system. The percent protection provided by test (300 µg/mL) was calculated as described previously. Hydrocortisone was taken as a reference standard and control was prepared without test solution.

Lipid Peroxidation

As reported earlier (Rekka *et al.,* 1989; Liu *et al.,* 2002) in presence of hydroxyl radical and deoxyribose, lipids from liver homogenate of wistar rats, fragmentize to malonyl dialdehyde (MDA) that binds to 2-thio-barbituric acid (TBA) to form pink MDA-TBA chromogen was estimated at 532 nm. Test (6, 8, 10, 12, 14 and 16 µg/mL) and α-tocopherol taken as a reference standard showing antioxidant activity were expected to interfere with the above process by scavenging hydroxyl radical, evident from malondialdehyde content was calculated.

Statistical Analysis

Statistical significance of results expressed as the mean ± SEM was determined by one way analysis of variance (ANOVA factor) followed by Tuckey's t test and the level of significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Immunomodulatory Activity Carbon Clearance Assay

Carbon clearance assay reveals effect of phagocytosis in *in vivo* assay, where enhanced phagocytic activity of macrophages was evaluated by the rate of the elimination of the exogenously administered antigen such as colloidal carbon particles. In the present study the stimulation rate was significantly higher for Ext B (5.24) as compared to Ext A (1.77) and Ext C (1.33). Results thus reflect a marked increase in the rate of phagocytosis. Rise in the carbon clearance rate indicates stimulation of reticuloendothelial system and activity of macrophages (Ponkshe, 2002). In this, *in vivo* assay macrophages probably secrete a number of cytokines, which in turn stimulates other immunocytes. This may give the host, the defense ability to counter the infectious stress. Phagocytic index and rate of clearances are shown in the Table 16.1.

Table 16.1. Phagocytic index of the Ext A, Ext B and Ext C in carbon clearance assay

Drug	Slope of control (K)	Slope of treated (K_1)	Phagocytic index (K_1/K)
Ext A	–0.00062	–0.0011	1.774194
Ext B	–0.00062	–0.00337	5.435484
Ext C	–0.00062	–0.00082	1.322581

Evaluation of Immunoprophylactic Effect

In this assay, after challenging animals with fatal dose of *E. coli* i.p. (2.5×10^8 cells suspending in normal saline), significant difference was found in bacteraemia induced in control and Ext B treated groups (Table 16.2). Also percentage clearance of bacteria in treatment group ($82.56 \pm 5.82\%$ reduction of cfu) was found quite higher than the control group ($16.34 \pm 3.37\%$ reduction of cfu). The result was supported by the percentage mortality observed in the control and Ext B treated groups in 24 h which was 100% and 37.5% respectively. All (n = 8) animals of control group died within 15 h while three out of eight animals of treatment group died at 17, 18.5, 23 h and remaining showed survival without any symptoms of peritonitis. Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by opsonization of the parasites with the antibody and complement C3b, leading to more rapid clearance of parasites from blood (Sagle, 2004).

Table 16.2. Percentage clearance of bacteria in immunoprophylactic assay

Group	Colonies at 1 h	Colonies at 6 h	% Clearance 12 h	Colonies at 6 h	% Clearance 12 h
Control (n = 8)	355.25 ± 17.8	330.62 ± 18.72	7.15 ± 0.77	290.12 ± 23.25	19.04 ± 3.30
Treated (n = 8)	97.5 ± 7.70	36.87 ± 4.84	59.85 ± 7.35	15.25 ± 4.08	82.56 ± 5.82

Haemagglutinating Antibody (HA) Titre

Increase in the humoral antibody titre was evident on the 3rd day post immunization in treated mice in comparison with untreated mice (n = 8). Anti-SRBC hemagglutination antibody titre with the use of the Ext B treated mice was found to be 1:512 as compared to control mice 1:128. In the test for humoral immunity there is a significant ($F = 4.425$, $p<0.05$) enhancement of antibody titre in treatment group of mice (Fig 16.1). The potential of humoral immune response may be mediated by T-lymphocytes. Presensitization with sub-optimal doses of antigen is known to generate helper T-cells for humoral responses and all the memory calls for DT to SRBC (Sainis, 1983) hence its effect on DTH was evaluated. The cell-mediated immune response was measured by hypersensitivity test. Footpad thickness was measured after 24 h of challenge with SRBC. After primary immunization control and Ext B treated animals (n = 8) showed significant increase ($F = 42.65$, $P\leq0.001$) in the cell mediated immune (CMI) response (0.080 ± 0.0054), when compared to that of control animals (0.039 ± 0.0019) (Fig 16.2). Increase in paw volume in case of SRBC immunized mice indicates an immunopotentiating effect of CMI. It appears that the Ext B of the plant could have stimulated T-lymphocyte activity. This result is also supported by the *in vitro* plaque forming assay.

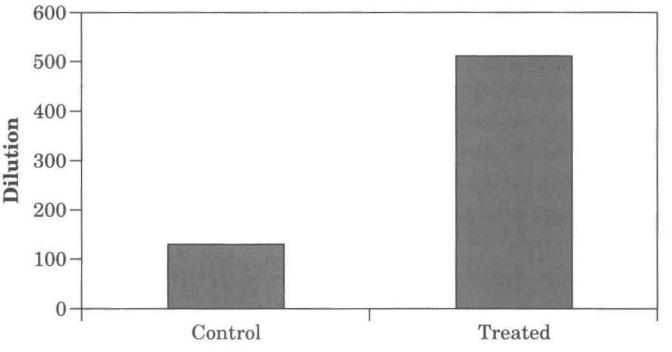

Fig 16.1. Effect of Ext B on haemaggultination titre value

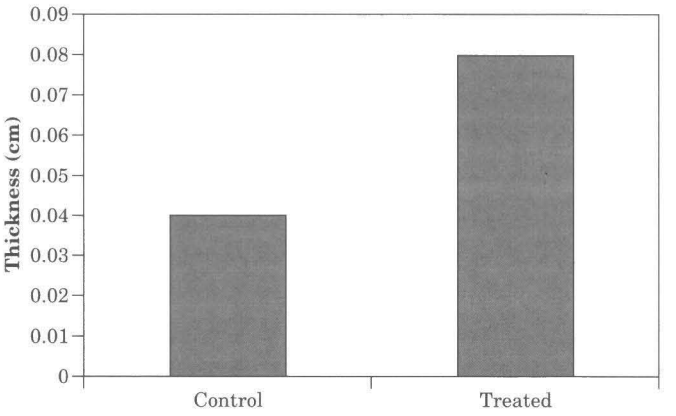

Fig 16.2. Effect of Ext B on DTH Response

Plaque Forming Cell (PFC) Assay

Increase in the humoral antibody level in the antibody forming cells was found to be increasing (Fig 16.3) after immunization in treated mice (201 ± 3.43 colonies, $p \leq 0.001$) in comparison with untreated mice (100.75 ± 2.63 colonies). Thus, nearly two-fold increase in the IgM antibody plaque formation was also observed in the spleen cells of the Ext B treated mice as compared to control animals.

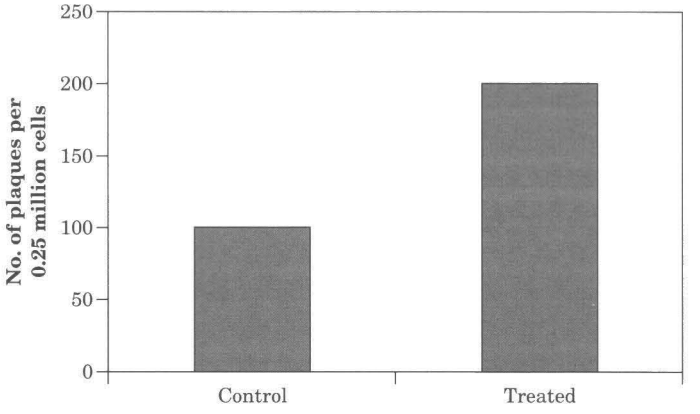

Fig 16.3. Effect of Ext B over PFC

Antioxidant Activity

Antiradical Activity

Ext B showed a concentration dependent antiradical activity by inhibiting DPPH radical, with the IC_{50} value of 30.73. Whereas, ascorbic acid showed at IC_{50} value of 10.05 µg/mL (Table 16.3).

Table 16.3. Antiradical activity of Ext B of *S. indicus* observed with DPPH

Sample	Concentration (μg/mL)	% Inhibition	IC_{50} (μg/mL)
Ext B	10	10.5 ± 0.13	30.73
	20	37.43 ± 0.61	
	30	53.84 ± 1.24	
	40	61.71 ± 1.94	
	50	80.52 ± 2.13	
Ascorbic acid	2.5	16.72 ± 3.1	10.05
	5	23.54 ± 4.1	
	7.5	32.67 ± 4.3	
	10	47.06 ± 3.9	
	12.5	66.9 ± 2.7	

Superoxide Radical Scavenging Activity

Ext B also showed decrease in the formazan formation revealing scavenging of the superoxide radicals in concentration dependent manner, with IC_{50} value of 70.51 μg/mL (Table 16.4).

Table 16.4. Super oxide scavenging activity of Ext B of *S. indicus* observed with riboflavin-light-NBT system

Sample	Concentration (μg/mL)	% Inhibition at different time (seconds)				IC_{50} (μg/mL)
		60	90	120	150	
Ext B	20	11.28	19.25	22.8	20.08	70.51
	40	24	30.58	32.36	33.8	
	60	29.25	39.41	41.26	44.16	
	80	31.25	50.61	53.12	55.26	

Hydroxyl Radical ($HO^{\cdot}$) Scavenging Assay

Ext B effectively scavenged the hydroxyl radical (HO^{-}) as evident from reduced amount of formaldehyde formation (IC_{50} value of 76.65 μg/mL) (Table 16.5).

Table 16.5. Inhibition of formaldehyde production by Ext B of *S. indicus* induced by DMSO

Sample	Concentration (μg/mL)	% Inhibition	IC_{50} (μg/mL)
Ext B	40	26.17	76.65
	60	37.76	
	80	52.27	
	100	65.20	
	120	79.83	

Erythrocyte Membrane Stabilization Assay

Ext B inhibited the hemolysis of the erythrocytes against thermal stress, osmotic stress as well as oxidative stress induced by riboflavin-light-NBT system. Ext B showed considerable protection to the membrane of the erythrocytes with EC_{50} value of 338.4 µg/mL (Table 16.6).

Table 16.6. Erythrocyte membrane stabilization of Ext B of *S. indicus* measured in terms of % Inhibition of hemolysis induced by riboflavin-light- NBT system

Sample	Concentration (µg/mL)	% Inhibition	IC_{50} (µg/mL)
Ext B	100	10.23 ± 2.35	338.44
	200	15.62 ± 3.51	
	300	49.12 ± 4.36	
	400	61.45 ± 5.21	
	500	78.51 ± 5.89	
Hydrocortizone	**100**		**36.76**

Lipid Peroxidation

Lipid peroxidation induced by the hydroxyl radicals were also reduced by the Ext B with EC_{50} 79.86 µg/mL, which was even better than standard used α-tocopherol (Table 16.7).

Table 16.7. Inhibition of lipid peroxidation by Ext B of *S. indicus* induced by iron-TBA system in mice liver homogenate

Sample	Concentration (µg/mL)	% Inhibition	IC_{50} (µg/mL)
Ext B	20	15.41	79.86
	40	29.15	
	80	47.82	
	120	74.11	
α-tocopherol			**136.02**

Herbs have been the main source of the medicines through out human history. That they are still widely used today is not a throwback to the dark ages, but an indication that herbs are a growing part of modern medicines. Researchers today examine folk and traditional uses of plants to find new drugs for cancer, AIDS, and many incurable diseases (Steven, 1996). Ayurveda, the science of life, is an integral part of our proud heritage. Ayurveda says that to restore health we must understand the exact quality, nature and structure of disease, disorder, or imbalance. The body has its own intelligence to create balance, realized by four main classifications of management of disease in Ayurveda, namely shodhana (cleansing), shaman

(palliation), rasayana (rejuvenation) and satvajaya (mental hygiene). Beside other three, rasayana is the group of non-toxic herbal drugs, which are used to the general health in normal and diseased conditions by the stimulation of the immune system (Praveen *et al.*, 1999). Observations of the present study indicated that Ext B is significantly active in different immunomodulatory modules of specific and nonspecific immune system. This prompted us to proceed with the investigation of its antioxidant potential.

Free radicals are involved in the normal physiology of the living organisms. They act as the messengers for signal transduction and also affect gene expression. There are several proteins and biomolecules in the living organisms, which act as free radical scavengers. Besides these biomolecules, several dietary components containing vitamins, polyphenols and flavones also play a significant role in this matter. The role of free radical oxidative stress in various disease conditions has been well established. Superoxide radical, hydroxyl radical, peroxy radical and singlet oxygen are some of the important reactive oxygen species that cause damage to the biological systems (Tripati, 1999). The active fraction Ext B was also found to be potent antioxidant. The Ext B reported to contain 7-hydroxy eudesmolide (0.066%) (Shah, 2008) may also play some vital role in the underlined activities.

Thus it can be said that the whole plant is a potent immunostimulator with excellent antioxidant activity affirming it as a Rasayana drug.

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