STUDY ON TUMOR REGRESSION BY PRESENTING LIPOSOME-ENCAPSULATED TUMOR-ASSOCIATED ANTIGENS (TAA) TO THE IMMUNE SYSTEM

ABSTRACT

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SUBMITTED IN PARTIAL FULFILMENT OF REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

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ABSTRACT

Immunotherapy has been a fast-evading area of cancer research because some clinical responses have been demonstrated against malignant tumors. This therapy has an advantage of specific lysis of malignant cells without destroying the normal cells. One approach is specific active immunotherapy with antigenic tumor cells (rendered non-tumorigenic by, e.g., irradiation) or with extracted tumor-associated antigens (TAA), the objective being to stimulate the host's immune response against the tumor. Antigens on tumor cells are usually also found on normal cells. The expression of such molecules on tumor cells, however, differs from the expression on normal cells. For instance, they are present at high levels on tumor cells and in trace amounts on normal cells, they are usually distributed over the cell membrane, or they are expressed at an inappropriate phase of ontogenesis, as in case of fetal antigens on tumor cells in adults. Little evidence is available on the existence of real tumor-specific antigens in man. Therefore, antigens on tumor cells are usually called tumor-associated antigens (TAA).

The main obstacle in specific active immunotherapy is the generally weak immunogenicity of TAA. Therefore a major challenge in tumor immunology is to develop methods that augment the immune response to TAA. Different methods have been explored in order to achieve a potent immune response against tumor and are as follows:

1. Instead of whole tumor cells, preparations containing TAA of tumor cells (TAA extract) have been explored for active immunization. Potential advantages of the use of TAA extracts over whole tumor cells include the immunization of contamination with potentially oncogenic nuclear materials from tumor cells as well as the opportunity of chemical characterization, sterilization, and refinement of the antigen preparation.
2. Tumor cells have been modified to enhance their immunogenicity. This has been accomplished in various ways, for example, by enzymatic unmasking of TAA with enzymes such as neurominidase, by additional immunogenic cell surface determinants, or by rigidification of cell membranes with lipids. All these make the antigen more immunogenic.
3. Another approach is to employ immunological adjuvants. Recently less toxic immunostimulants such as cytokine and analogs of bacterial products are under investigation. Beside these, extensive efforts are currently being made to augment anti-tumor immunity by using genetically modified autologous cancer cells as

vaccines and also the cloning of genes encoding TAAs which have significantly improved the prospect for cancer immunotherapy.

4. Several investigators have focused on the delivery of TAA-derived proteins/peptides or TAA genes to professional antigen presenting cells, to elicit immune responses capable of eradicating tumor cells.

The recent isolation and biochemical characterization of certain TAA enabled the study of immune reactions against highly purified TAA. Encouraging results were reported in cancer patients for specific active immunotherapy with crude as well as highly purified TAA preparations. Considering the antigenic heterogeneity of human tumors it is expected that tumor vaccine should contain multiple TAA. Ideally, vaccines should be prepared from a mixture of pure antigens.

The TAA responsible for mediating tumor rejection are integral membrane molecules or molecules associated with the outer cell surface. Their immunogenicity is closely related to their presentation form.

Liposomes have been found to elicit the immune response to a variety of antigens and therefore proposed as biodegradable vehicles for the presentation of antigens to the immune system. Liposomes are lipid vesicles made up of concentric bimolecular leaflets of phospholipids separated by aqueous spaces (0.1 – 1.0 μm). They are versatile presentation vehicles, as antigen can be included in the aqueous or lipid phase of liposome, and their size, surface charge, and other properties can be controlled.

The work embodied in this thesis is an attempt to develop a safe and effective liposomal-TAA formulation that could mount a strong rejection response against the host's tumor. Liposome has successively been used as a potential carrier in drug delivery and drug targeting with a number of cancer therapies. However, its application in tumor immunotherapy as carrier in yet to be proved.

Diethylnitrosamine (DEN), a potent hepatocarcinogen was used for tumor induction in mice. The carcinogen administered by i.v. route for a period of about three

months at weakly intervals. Cancer induction studied by monitoring the marker enzymes activities, i.e. γ-glutamyl transpeptidase (GGT) and acetylcholine esterase (AChE). Histology and electrophoretic studies of surface membrane glycoproteins in liver were also carried out in support of cellular transformation. Tumor-associated antigen (TAA) was extracted from the liver cells of DEN-treated animals using 1-butanol, which extract exclusively membrane surface glycoproteins. Mice having complete DEN treatment were immunized with TAA-extract after encapsulating it into liposomes, which served as carrier for the presentation of TAA to the immune system. Antibody response elicited by liposomal-TAA formulation monitored using ELISA, whereas the cellular response followed by cell proliferation assay using BrdU labeling kit. The assay is a cellular immunoassay, which uses a mouse monoclonal antibody directed against BrdU based on ELISA principle. Effect of immunization on tumor regression was studied. For the regression studies the same parameters employed in induction studies were used.

In the present investigation it was found that a strong immune response against host's tumor could be achieved by administering TAA encapsulated in liposomes. Liposome encapsulated TAA was found to be more potent immunogen compared to TAA alone. The elicited immune response produced satisfactory results. The main points emerging from the work embodied in the thesis are:

⇒ Chronic exposure of DEN to Swiss albino mice induced cellular transformations in the liver, as substantiated by the pronounced alterations in the activities of the marker enzymes such as γ-glutamyl transpeptidase and acetylcholine esterase.

⇒ DEN treatment resulted in a distinct change in the morphology of the hepatocytes such as the variations in the cell shape and size, appearance of more densely stained nuclei and multinucleated cells as elucidated in the histological studies.

⇒ Cell surface membrane glycoproteins in liver exhibited differential expression upon DEN exposure. A glycoprotein of approximately 68 kDa was over expressed while some between 20 kDa to 29 kDa molecular weight found under expressed as compared to their normal counterparts as revealed by SDS-PAGE electrophoretic study.

⇒ Liposomes prepared by the dry film method exhibited highly reproducible entrapment efficiency. Further, the immunogenicity of entrapped TAA remained unaltered.

⇒ DEN-exposed mice upon immunization with liposomal-TAA formulation elicited humoral immune response against TAA as substantiated by the presence of significantly high circulating antibody concentration in immune serum.

⇒ Cell proliferation assay *in vitro* of lymphocytes, obtained from immunized animals, clearly indicate the induction of cell mediated immune response in DEN-exposed mice upon immunization.

⇒ Reversal of the marker enzyme activities, in the liver of DEN-exposed and immunized mice, towards normal level is likely due to the effect of immunization on tumor regression.

⇒ Attainment of normal morphology and cell shape of hepatocytes upon immunization in DEN-exposed mice supports tumor regression.

From the above observations, it is evident that this particular approach could be useful to elicit immune response against tumor-associated antigens. It is further suggested that vaccine based on liposomal antigen formulation in particular with non-fractional cell extracts as a source of tumor antigen may be equally effective to induce immune responses to that of the cell based modified tumor vaccine.

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Dedicated With Love
To My Parents

NORTH-EASTERN HILL UNIVERSITY

Shillong, India

I, **Imliwati Longkumer**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University or Institute.

This is being submitted to the North-Eastern Hill University for the degree of Doctor Of Philosophy in Biochemistry.

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1 INTRODUCTION

1. INTRODUCTION

A cell, of any origin has a definite regulatory mechanism for its existence. In animals, a balance is generally maintained between cell renewal and cell death in most organs and tissues. The various types of mature cells in the body have a given life span; as these cells die, new cells are generated by the proliferation and differentiation of various types of stem cells. Under normal circumstances, the production of new cells is so regulated that the numbers of any particular type of cell remain constant. Occasionally, though, cells arise that are no longer responsive to normal growth-control mechanisms. These cells gives rise to clones of cells that can expand to a considerable size producing a tumor, or neoplasm.

A tumor, which is not capable of inducing growth and does not envade the healthy surrounding tissue extensively, is benign. A tumor that continues to grow and becomes progressively invasive is malignant; the term cancer refers specifically to a malignant tumor. In addition to uncontrolled growth, malignant tumors exhibit metastasis; in this process, small clusters of cancerous cells dislodge from a tumor, invade the blood or lymphatic vessels, and are carried to other tissues, where they continue to proliferate. In this way a primary tumor at one site can give rise to a secondary tumor at another site.

Malignant tumors are classified according to the embryonic origin of the tissue from which the tumor is derived. Carcinomas are tumors arising from endodermal or ectodermal tissues such as skin or the epithelial lining of internal organs and glands. Sarcomas, which arise less frequently, are derived from mesodermal connective tissues such as bone, fat and cartilage. The leukemias and lymphomas are malignant tumors of haematopoietic cells of the bone marrow. Leukemias proliferate as single cells, whereas lymphomas tend to grow as tumor masses. It is important to realize that cancer is not a single disease with a single cause and a single type of treatment. There are more than 200 different kinds of cancer, each with its own

name and treatment. The major forms of treatment of cancer are chemotherapy or radiotherapy. Therefore, it is highly desirable to identify methods by which selective killing of cancer cells is increased while preventing the side effects (65).

1.1 CANCER THERAPY

1.1.1 RADIOTHERAPY OF CANCER

Radiotherapy plays an important role in the treatment of cancer. Cancer cells are more sensitive to radiotherapy than normal cells and will, therefore, be destroyed at a greater rate. Radiotherapy stops the growth of cells by interfering with their normal function. The normal cells in the area being treated will also be affected but, unlike cancer cells, they are able to repair themselves very quickly. Irradiation is popular because it does not require any cutting or incision and therefore has no immediate mechanical effects on the function of organs. It does, however, damage the living cells. It causes mutation that is also a cause for the development of cancer. Radiotherapy has a stronger damaging effect on cancer cells than it has on normal cells, particularly if its administration has been calibrated at a favorable energy level. It is therefore regarded as an effective anti-cancer treatment.

Radiotherapy is generally applied in combination with surgery and chemotherapy; its use as the sole agent for treatment is rather rare. The object of its combination with other modalities is to supplement the effects of the other therapies.

The following are the options in the administration of radiotherapy that depend on the course of treatment and location of the malignancy:

1. Irradiation of the external body,

2. Irradiation through a device inserted into a body cavity or

3. Irradiation of the tumor in which there is direct contact between the radiation source and the cancer cells

Ionizing radiation - similar to x-rays - can penetrate tissue, and alter the part of the cell, which regulates its growth and reproduction. Uncancerous cells can recover from this damage, while cancer cells cannot. There are two types of radiotherapy - delivered from outside the body by a machine, and using radioactive implants placed inside the body. Researchers are working to increase the effectiveness of radiotherapy by targeting the beam of energy more precisely, and making the cancer cells more sensitive to it.

The principal use of radiotherapy is to tackle solid tumours found in just one location, for example skin, brain, breast or uterine cancers. Sometimes doctors will use the treatment to shrink a tumour so that a subsequent operation will be more effective. In some cases, for example in invasive bladder cancer, radiotherapy is considered as the first option, as an alternative to surgery that would have permanent effects on the lifestyle of the patient. But although radiotherapy alone can cure many cancers, in other cases the radiotherapy is given after surgery over the surrounding area to "mop up" any remaining cells which have spread from the original cancer site. If there is a suspicion or firm evidence that cells could have spread further afield, then chemotherapy may be the preferred option. Although the treatment itself is painless at the time, the cumulative effect of many sessions does produce side effects. The radiation can produce a sunburn-like effect on the skin as it passes through. The extent of this depends on the intensity and number of treatments. There can be hair loss in the area being treated, which is usually temporary. The treatment can also leave the patient feeling fatigued and generally lethargic. Ionizing radiation produces changes within the genetic structure of the body's cells, and there is a small risk that an increased radiation dose leads to changes in healthy cells, which can cause cancer. However, the dose is lessening steadily as modern radiotherapy equipment targets tumours more precisely. The risks of medical radiation exposure are

miniscule when compared to the risks to the patient's health of not having the treatment. Old-fashioned radiotherapy equipment tends to give the patient a slightly higher dose. Therefore, the main efforts are now focus on delivering a more powerful radiotherapy beam accurately to smaller and smaller targets. Another field of research is looking at heating cells in a specific area to make them more sensitive to radiotherapy (36)

A great change has occurred in both medical and patient attitudes to radiotherapy. Originally regarded as a palliative treatment for inoperable or incurable cancers, it is now seen as a partner and partial replacement for surgery in the curative treatment of certain cancers such as those in skin, larynx, and cervix. Advances in both technology and technique have meant that radiotherapy can be accurately applied to the region of the cancer with minimal damage to surrounding tissue, especially the skin. How radiation kills cancer cells selectively is not fully understood but all dividing cells are particularly sensitive to radiation damage and consequently rapidly proliferating tumour cells are especially vulnerable. One of the drawbacks of radiation treatment is that therapeutic doses may also kill dividing cells in normal tissues.

Two main techniques are used for the delivery of radiation, which is given either as an external beam or as short-range radiation from an implanted radioactive source. External beam radiation usually involves megavoltage produced by linear accelerator as photons or electrons or from cobalt sources in the form of relative low energy X-rays or gamma rays. The latter are often used to treat relatively superficial lesions such as basal cell carcinoma or recurrences within the skin. High-energy radiation can be used to treat deeply located lesions such as prostatic carcinomas without delivering an excessive dose to adjacent normal tissues. The total dose of radiation is usually delivered in several fractions to maximize the tumor killing while sparing normal tissues, the exact method of fractionation differing from institution to institution.

Interstitial (implant) irradiation gives a high local dose to the tumor and usually employs sources such as radium, iridium, or caesium used in the form of needles or wires implanted in the tumor. This technique is widely used in the treatment of head and neck cancers to deliver a high tumor dose without irradiation to sensitive organs such as the lens of the eye or the spinal cord. A combination of interstitial and external radiation may be used to treat the potentially malignant peritumoural field as well as the site of the primary carcinoma. Insertion of the radioactive sources may be carried out under general anesthesia. As an alternative, tubes to contain the radioactive sources may be inserted under anesthesia and, after measurements of the implant characteristics, loading of appropriate doses of isotopes can be carried out subsequently (afterloading). This technique of afterloading has been used with caesium sources to treat cancers of the body of the uterus.

1.1.2. CHEMOTHERAPY OF CANCER

Chemotherapy is a relatively new method of treating cancer (78). The tradition of treating cancer with surgery had been in existences for over a century, and radiotherapy had been used for atleast a quarter of a century before chemotherapy made its appearance in the middle of the Second World War. Malignant disease, such as leukemia, which was generalized when it first presented, or tumors, which had become disseminated, was incurable up to that time. Chemotherapy gave a first promise of cure.

Chemotherapeutic drugs act directly on tumor cells whereas other drugs be must be activated by metabolic processes, either in the tumor cells or in the organs such as in the liver. Some important drugs designed for cancer treatment are listed below.

Anti-cancer drugs

Classification	Drugs
Alkalating agents	Mechlorethamine
	Busulphan

	Cholambucil
	cyclophosphamide
	Melphalan
	Thiotepa
Antimetabolite	Methotrexate
	6-Mercaptopurine
	Thioguanine
	5-Fluorouracil
	Cytosine arabinoside
	5-Azacytidine
Plant alkaloids	Vinsblastin
	Vincristine
	VP-16
Antibiotics	Actinomycin D
	Doxorubicin
	Bleomycin
	Daunorubicin
	Mithramycin
	Mitomycin C
Nitroureas	Carmustin
	Lomustine
	Semustin
	Streptozotocin
Enzymes	L-Asparaginase
Random synthetics	*Cis*-Platinum diammine dichloride
	Dacarbazine
	Dibromomannitol
	Hexamethylmelamine
	Hydroxyurea
	Mitotane
	Procarbazine

These drugs can be divided into those that are active on dividing cells and affect a very particular phase of cell cycle (phase specific drugs), and those that affect all or most of the phases of the cell cycle. The phase and cycle specific drugs are listed as follows: -

Phase and cycle specific drugs

Phase specificity	**Acts on**	**Drugs**
S phase specific	DNA synthesis	Methotrexate

		Cytosine arabinoside Hydroxyurea
Relatively S phase specific	DNA, RNA, and protein synthesis	5-Fluorouracil 6-Mercaptopurine
Cycle specific	DNA at all phases of cycle	Nitrogen Mustrad Nitrosourea Cyclophosphamide

Whatever the mode of action of the chemotherapeutic agent, a very important finding is that it destroys malignant cells according to first order kinetics; in other words the same proportion of cells is killed for each dose of the agent

Chemotherapeutic drugs kill cancer cells. Different cancer cells respond to different drugs. Sometimes a combination of as many as eight different drugs is used in combination to get the best effect. Chemotherapy is often associated with debilitating side effects, but many types of modern chemotherapy cause only mild problems. Because chemotherapy drugs are usually injected into the blood, they travel around the body and can attack cancer cells regardless of where they find them. For this reason, doctors will use them when they think there might be cancer cells in more than one part of the body.

Radiotherapy, which uses radiation to destroy cancer cells, can only be given to small areas of the body or it will cause damage to too many healthy cells. Often, after an operation to remove cancer, chemotherapy will be given to "mop up" any remaining cells. Some cancers, such as leukemia, need chemotherapy because they involve cells, which are found throughout

the body. Chemotherapy can be given to shrink a tumour to make it easier for the surgeon to remove. It can also ease the symptoms of patients whose cancer is not curable.

Chemotherapy, in its traditional sense, is a chemical, which is poisonous to cancer cells and kills them. This is called a cytotoxic chemical: - One very early chemotherapy was produced from mustard gas, which was used as chemical weaponry during the First World War. However, anything, which is poisonous to cancer cells, may also be poisonous to the body's healthy cells, which it needs to survive. The trick with chemotherapy is to find a chemical which kills as many cancer cells as possible, and as few healthy cells. Doctors have been getting increasingly successful at developing such chemicals, by spotting the differences between the cancer cells and neighboring normal cells, and exploiting them. The principle difference between many cancer cells and normal cells is the speed at which they reproduce, or divide. Cancers tend to be dividing and growing faster than other cells in the body, which is why lumps or tumours sometimes appear. Other cancer cells may become more or less active in response to natural chemicals called hormones produced by the body. Some chemotherapy harnesses this reaction to control the growth of the cancer cells. Cancer cells are not attacked by the body's own immune defense system because the immune system does not recognize them as foreign. Some chemotherapies try to program the immune system to see the cancer cells as foreign so they can be attacked and destroyed. Normally chemotherapy is delivered by injection into a blood vein. In many cases a saline drip will be set up to dilute the drug as it enters the body. This stops to harming the vein because it is so concentrated. Sometimes, a concentrated dose of chemotherapy is needed on a particular part of the body, and side effects can be lessened by injecting it directly onto the cancerous area. For example, for some bladder cancers, the drug can be pumped into the bladder so it works directly on the tissue involved. How long chemotherapy courses last varies between different cancer types, with some being given intensively over a fortnight, normally in hospital, and some over a period of months. What side-effects can be expected? Because some chemotherapy targets fast-growing or fast-dividing

cells, it is more likely to harm similar cells in the body. These include the cells in the hair follicles, which are why cancer treatment is often associated with hair loss, although hair does regrow once treatment has ended. Other fast-dividing cells can be found in the stomach and bowel lining, which leads to nausea and diarrhea. There are, however, drugs which help control this, and timing meals to avoid having a full stomach when the drugs take effect can also help in some cases. Other types of normal cell that can suffer are the blood cells. Red cells are important to carry oxygen to keep other cells alive. Other blood cells help stave off infection. As a result, chemotherapy patients may be more prone to infections, and find them harder to fight off.

Convention chemotherapeutic or radiotherapeutic regimens often fail to cure patients because they do not kill all malignant cells. One reason is that the dose needed to iradicate cancer is so large that patient's own tissues would be severely damaged. Active specific immunotherapy with antigenic tumor cells, (rendered non tumorigenic by e.g. irradiation) or extracted tumor associated antigens is another approach for improved systemic cancer therapy

1.1.3. IMMUNOTHERAPY OF CANCER

The concept of the immune system being involved in the development of cancer and of manipulating it as a part of cancer therapy dates back almost 100 years. For immune surveillance against cancer to be a plausible theory, cancer cell must express antigens recognizable as foreign and accessible to the immune system, which must in turn be able to mount a response against cells bearing such antigens. Immunotherapy is treatment by immunological means. The aim of treatment in cancer is to eliminate the tumor without harming the host.

Tumor immunity can be enhanced by providing co-stimulatory signal necessary for activation of Cytotoxic-T Lymphocytes precursors (CTL-Ps). When mouse CTL-Ps are incubated with melanoma cells in vitro, they do not proliferate and differentiate into effector CTLs, but when melanoma cells are transfected with gene encoding the B7 ligand, then the CTL-Ps differentiate into effector CTLs (6). The identification of novel B7-family members, the modulation of CD40 to reverse tolerance to tumor-associated antigens and the use of CX40 to enhance antitumor response of CD4+ T cells have all contributed to the development of more powerful immunomodulatory cancer therapies (55). In human chronic myeloid leukemia (CML) model, tumor cells incubated in Granulocyte Macrophages-Colony Stimulating factors (GM-CSF), IL-4 and TNF differentiated into mature dentritic Cells capable of stimulating an autologous anti-CML CTL response (30). Some malignant cells in patients with acute myelogenous leukemia, upon cytokine administration (i.e. GM-CSF or IL-4), undergo differentiation into APCs that contain high levels of co-stimulatory and MHC molecules. These cells are ideal Antigen Presenting Cells as presumably the entire repertoire of tumor antigens is presented on them (23, 15).

The dentritic cells of mouse when cultured in GM-CSF and incubated with tumor fragments have been shown to activate both T-helper (T_H) cells and CTLs specific for the tumor antigens. When these mice were subsequently challenged with live tumor cells, they displayed tumor immunity. In one approach, tumor cells were transfected with GM-CSF gene (26). These engineered tumor cells, when reinfused back into the patient, secrete GM-CSF, enhancing the differentiation and activation of host antigen-presenting cells, especially dendritic cells. As these dentritic cells accumulate around the tumor cells, the GM-CSF secreted by the tumor cells will enhance the presentation of tumor antigens to T_H and CTLs cells by the dendritic cells. More recently, it has been found that dendritic cells (DC) phagocytose apoptotic influenza-infected monocytes and cross-present influenza antigen to CD8+ T cells, generating a specific CTL response (60).

Several adjuvants, such as attenuated strain of Mycobacterium bovis called bacillus Calmette-Guerin (BCG) and Corynebacterium parvuum, are used to boost tumor immunity. These adjuvants activate macrophages increasing their expression of various cytokines, class II MHC molecules, and the B7 co-stimulatory molecule. Thus, the macrophages activates T_H cells, resulting in generalized increases in both humoral and cell-mediated responses

A variety of experimental and clinical approaches have been developed to use recombinant cytokines, either singly or in combination, to augment the immune response against cancer. Various cytokines used in cancer immunotherapy are interferon α,β and γ, IL-1, IL-2, IL-4, IL-5, IL-12, and IL-15, GM-CSF and TNF (82,63,65,67). Purified recombinant preparations of the interferon, IFN-α, IFN-β and IFN-γ are available, each of which has shown some promise in the treatment of human cancer. To date, most of the clinical trials have involved several mechanisms. All three types of interferon have been shown to increase class I MHC expression on tumor cells; IFN-γ have also been shown to increase class II MHC expression on macrophages. Given the evidence for decreased levels of class I MHC molecules on malignant tumors, the interferons may act by restoring MHC expression, thereby increasing CTL activity against tumors. Interferons have been shown to inhibit cell division of both normal and malignantly transformed cells in vitro. IFN-γ increases the activity of T_C cells, macrophages, and NK cells, all of which play a role in the immune response to tumor (6).

Tumor necrosis factors, TNF-α and TNF-β, have been shown to exhibit direct antitumor activity, killing some tumor cells and reducing the rate of proliferation of others while sparing normal cells. In the presence of TNF-α or TNF-β a tumor undergoes visible hemorrhagic necrosis and tumor regression. TNF-α has also been shown to inhibit tumor-induced vascularization (angiogenesis) by damaging the vascular endothelial cells in the vicinity of tumor, thereby decreasing the flow of blood and oxygen that is necessary for progressive tumor

growth. Injection of TNF- α directly into the tumor has led to complete tumor regression in some patients, but not in others. TNF-α therapy has several limitations: the short half-life of TNF-α necessitates frequent injections; and its adverse side effects include fever, chills, blood-pressure changes, and decreased counts of white blood cells. (65).

Monoclonal antibodies have been used in various ways as experimental immunotherapeutic agents for cancer (42). Anti-idiotype monoclonal antibodies have been used with some success in treating human B-cell lymphomas and T-cell leukemias. Monoclonal antibodies also have been used to prepare tumor-specific immunotoxin (82). These agents consist of the inhibitor chain of a toxin (e.g. diphtheria toxin) linked to an antibody against a tumor-specific or tumor-associated antigen. *In vitro* studies have demonstrated that those "magic bullets" can kill tumor cells without harming normal cells.

Another cancer immunotherapy involves using monoclonal antibodies to bridge activated T cells directly to a tumor. In this approach two different monoclonal antibodies are produced: one specific for a tumor-cell membrane molecule and other specific for the CD3 membrane molecule of the TCR complex. A hybrid monoclonal antibody, or heteroconjugate, is then prepared with specificity for the tumor antigen and for CD3. *In vitro* experiments with these hetero-conjugates have revealed that they are able to cross-link and activate T cells directly on the surface of the tumor cell. A variety of tumors express significantly increased levels of growth-factor receptors suggests that treatment with monoclonal antibodies against receptors might inhibit tumor-cell activity. Monoclonal antibodies to the EGF receptors; to the p97 (transferrin) receptor, and to the IL-2 receptor have been produced. In the present study specific active immune response was induced against tumor antigen in mice bearing tumor. The tumor was initiated by chronic exposure of chemical carcinogen to experimental animals.

1.2 CHEMICAL CARCINOGENS

Substances capable of producing neoplasms are classified as carcinogens. Carcinogens are reactive intermediates, as electrophilic reactants or radical cations, that can interact with cellular macromolecule (130). They include a highly diverse collection of chemical substances, both organic and inorganic chemicals, solid-state materials, hormones, and immunosuppressants (83). Chemical carcinogens can be classified into two major categories- genotoxic and epigenetic.

Genotoxic carcinogens are identified by the biochemical demonstration of DNA damage or by the demonstration of genotoxic effects in short-term tests. Most likely, alteration in DNA is the key event in the initiation of carcinogenicity by these compounds. Genotoxic carcinogens are occasionally effective after a single exposure and frequently carcinogenic at subtoxic doses. They act in a cumulative manner together with other DNA-reactive carcinogens having the same organotropism. They usually produce neoplasms in more than one target organ and have a short latent period. Examples of genotoxic carcinogens are ethyleneimine, dimethylnitrosamine, and diethylnitrosamine, vinyl chloride and nickel (129).

The second broad category, designated as epigenetic agents, comprises those chemicals for which no evidence exists of direct interaction with genetic material, but which produce another biological effect that could be the basis for their individual carcinogenicity. Possible mechanisms for epigenetic effects include chronic tissue injury, hormonal imbalance, immunologic effects, or promotional activity on cells that are either genetically abnormal or have been independently altered by genotoxic carcinogens (129). This category contains cytotoxic agents, solid-state carcinogens, hormones, immunosuppressants, and promoters. Examples of epigenetic carcinogens include nitrolotriacetic acid, asbestos, phorbol esters, Estradiol, Azothioprine.

The production of cancer in humans and animals by chemical carcinogens is thought to be the end result of a complex series of individual reactions that are subject to and controlled by a number of modifying factors. The reactions can be group into two sequences. In the first sequence, the normal cell is converted to a neoplastic cell, and in the second sequence, the neoplastic cell develops into a overt neoplasm. In the neoplastic conversion different enzyme systems can function in the detoxification and elimination of xenobiotics (128). Many carcinogens, however, undergo enzymatic activation to a reactive ultimate carcinogen. A small number of carcinogens, mostly industrial intermediates and chemotherapeutic drugs, are reactive in their parent form and therefore do not require activation. Carcinogens that form reaction reactive species undergo covalent reactions with a variety of cellular macromolecules, including DNA (48). If the cell replicates while DNA damage is persistent, permanent alterations in the genome can be produced in several possible ways: the mispairing of bases leading to point mutations; errors in replication yielding frame-shift mutations; transpositions resulting in codon rearrangement; and combinations of these alterations in sequential steps. Codon rearrangement may involve sequences known as oncogenes, which are emerging as critical gene sequences for transformation (127). In the case of interactions with the mitotic apparatus, chromosomal mutations and aneuploidy could result. All these alterations generate a permanently abnormal cell with an altered genotype and distinct phenotypes.

Thus, the abnormal cell formed may be held in check by tissue homeostatic factors or, if the conditions of exposure or abnormalities generated in the cells permit, they may undergo limited proliferation to form "preneoplastic" lesions. Now, cells with the requisite abnormalities may have the capacity to proliferate beyond tissue constraints to form neoplasms. Neoplasms can undergo qualitative changes in their phenotypic properties, possibly including transition from benign to malignant behavior (41). This change probably reflects the selection during growth of a population with a genotype coding that produces advantageous phenotypic

properties. New genotypes could arise in neoplasms through errors in DNA replication (133) or alterations in chromosome constitution.

1.2.1 CARCINOGENICITY OF DIETHYLNITROSAMINE

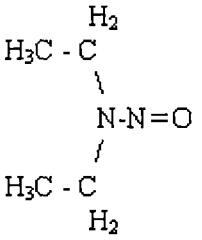
Diethylnitrosamine (DEN) is a synthetic deriviative of nitrosamine. It is an established and potent hepatocarcinogen. The carcinogenic activity of this compound was shown by Schmahl and Preussaman (109) in 1960. The LD50 for DEN is 216 mg/kg when administered by intraperitoneal route injection in rat,. The lowest published toxic dose is 100 mg/kg body weight when administered intraperitoneally (25). The acute toxic effects from exposure to DEN are similar to those produced by dimethylnitrosamine with serious destruction of liver tissue as the most important result (77). There is no correlation between acute toxic effects and carcinogenic potential for nitrosamines. This is demonstrated by the fact that even though DEN has only about one-sixth the acute toxicity of dimethylnitrosamine (27), if administered continuously to rats, it is probably a more active liver carcinogen (28).

DEN has been shown to be carcinogenic to the mouse, the rat, the hamster, the guinea-pig, the rabbit, the dog, the pig, the monkey, and to aquarium fish. The agent induces tumors primarily in the nasal cavity, trachea, esophagus, and liver. It causes cancer after different modes of exposure, which include ingestion, inhalation, and skin painting. It is carcinogenic in single doses and following prenatal exposure. In lifetime feeding studies with rats in which daily doses between 1 and 10 mg/kg body weight were administered, tumor yields approaching 100% have been obtained (56).

In a dose-response study conducted using rats DEN was administered in drinking water and the daily exposure was between 0.075 and 14.2 mg/kg body weight in 9 groups of animals. The total dose, until death occurred, was between 64 and 965 mg/kg body weight. The tumor

induction time was between 68 and 840 days. All daily doses higher than 0.15 mg/kg body weight gave a tumor incidence of 100%. When a dose of 0.15 mg/kg body weight per day was administered, a tumor yield of 90% was obtained. At 0.075 mg/kg body weight per day, 20 rats survived for more than 600 days and 11 of the 20 animals had tumors of the liver, esophagus, or the nasal cavity. All 4 of the animals that lived longer than 940 days at this dose level had tumors (56).

1.2.2 STRUCTURE AND PHYSICAL PROPERTIES OF DEN

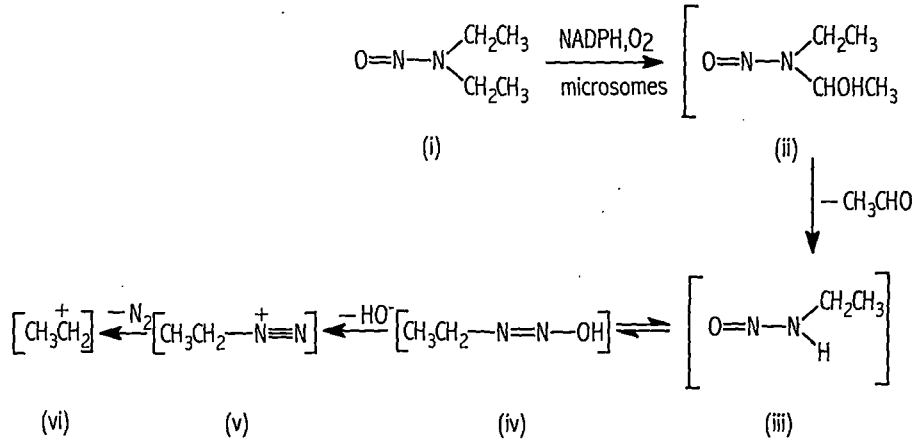


mol wt:	102.1
physical appearance:	a yellow, volatile liquid
boiling point:	177°C (760 mm Hg) 64-65°C (17 mm Hg)
density:	0.9422 (20/4°C)
refractive index:	1.4386 (20°C)
solubility:	about 10% in water; soluble in organic solvents and in lipids.

1.2.3 METABOLIC ACTIVATION OF DEN

Diethylnitrosamine must be metabolically activated to reactive intermediates to interact with cellular macromolecules and initiate carcinogenesis. The metabolic activity occurs in the microsomes of liver and in presence of O_2 and NADPH forms the intermediate monodiethylnitrosamine upon oxidative deethylation (29). Diethylnitrosamine-ethylase is the probable activating enzyme, with acetaldehyde as the metabolic product. Mirzahi and Emmelot

(89,88) were the first to study the properties of DEN-ethylase. The metabolic activation of DEN follows the pattern of DMN- monodealkylation followed by the liberation of a reactive ethonium ion from the remainder of the molecule as demonstrated by Dutton and Heath (29). The α-hydroxylation hypothesis is currently accepted mechanism of metabolic activation of DMN.



Mechanism of metabolic activation of diethylnitrosamine

Hydroxylation of DEN (29), at the α-carbon is believed to be the critical, rate-limiting step. The putative α-hydroxylated diethylnitrosamine (ii) is extremely unstable and yields, upon hydrolysis, acetaldehyde and monoethylnitrosamine (iii). The overall reaction is N-deethylation. Monoethylnitrosamine (iii) is also highly unstable and readily undergoes non-enzymatic, spontaneous rearrangement or breakdown to a "ethylating intermediate", which, is assumed to be carbonium ion (vi), although the possibility that the ethylating intermediate may be monoethylnitrosamine (iii) itself, or its tautomeric form, ethyldiazonium hydroxide (iv) or diazoalkene (vii) has been suggested at various times.

Thus, the liberation of the reactive ethonium ion which, is an electrophile ethylates with the nucleophilic centers in vivo of DNA, RNA and protein. The ethylation of rat liver DNA by DEN has been studied by Scherer *et al* (107). The potential adduct found include O^2- and O^4-ethylthymidine, and O^6- methylguanine.

1.3 TUMOR ASSOCIATED ANTIGENS (TAA)

Antigens of tumors induced by chemical carcinogens exhibit little or no immunologic cross-reactivity; each tumor exhibits a unique antigenic specificity. Thus, cells of a given tumor, arising from a single transformed cell, all share common antigens, but different tumors, even if induced by the same carcinogen, are antigenically distinct from one another. This absence of cross reactivity is probably due to the random mutations induced by the chemical or physical carcinogens, leading to a large array of different antigens. (10)

Tumor-specific transplantation antigens (TSTAs) or simply tumor-specific antigens (TSAs), are antigens found on the surface of malignant cells and consist of structures that are unique to the cancerous cells and are not present on their normal counterparts. The existence of these antigens comes from the study of transplanted tumors in inbred animals. Some of these tumors express antigens that elicit T cell-mediated immune rejection responses in syngeneic hosts. These antigens can be specific for individual tumors or common to a class of tumors (115).

Antigens on tumor cells are usually also found on normal cells. The expression of such molecule on tumor cells, however differs from the expression on normal cells. For instance, they are present on high levels on tumor cells and in trace amount on normal cells, they are usually distributed over the cell membrane or they are expressed in an appropriate phase of

ontogenesis. Little evidence is available on the existence of real tumor-specific antigens in man. Therefore, antigens on tumor cells are usually tumor-associated antigens (TAA).

TAA represent a heterogeneous group of macromolecular structures (glycoproteins, glycolipids) in or on tumor cells (69) and can serve on effective target for active immunotherapy against tumor. In this context, the preparations containing extracted TAA of tumor cells (TAA extract) and purified TAA have been explored for active immunization. Encouraging results have been found.

Extraction of viable tumor cells with low concentrations of butanol has become increasingly popular for obtaining TAA. With this procedure mainly peripheral membrane components are released, thus avoiding extraction of cytoplasmic or integral membrane components (68). Butanol extraction does not exert cytolytic effects as viability and proliferation capacity of the tumor cells is preserved.

1.4 TAA BASED IMMUNOTHERAPY

One approach in immunotherapy is specific active immunotherapy with antigenic tumor cells which have been rendered non-tumorigenic or extracted tumor-associated antigens (TAA). The objective being to stimulate the host immune response against the tumor (80,19). Antigens on tumor cells are usually also found on normal cells. The expression of such molecules on tumor cells, however, differs from the expression on normal cells, they are usually distributed over the cell membrane, or they are express at an inappropriate phase of oncogenesis, as in the case of fetal antigens on tumor cells in adults.

One of the main problems in specific active immunotherapy is the generally weak immunogenicity of TAA (67). Therefore, a major challenge in tumor immunology is to develop

methods that augment the immune response to TAA. Different methods have been explored. Tumor cells have been modified to enhance their immunogenicity. This has been accomplished in various ways, for example, by enzymatic unmasking of TAA with enzymes such as neurominidase (111), by additional immunogenic cell surface determinants (50), or by rigidification of cell membranes with lipids (113). Immunity against these more immunogenic tumor cells usually cross-reacts with the original tumor cells. Thus, this approach may lead to success. A second approach is to employ immunostimulants as immunological adjuvants. *Bacillus Calmette-Guerin* (BCG) and *Corynebacterium parvum* mixed with irradiated tumor cells have been especially studied in the past (53). More recently better defined and probably less toxic immunostimulants, such as cytokines and analogs of bacterial products, are under investigation (75,76,81). A somewhat different approach involves the reduction of suppressor T cells. This kind of treatment is based on observations that tumor-bearing animals may have T cells that can specifically suppress the immunological response to TAA (95). Most of the attention is focus upon cyclophosphamide to achieve this goal.

Instead of whole tumor cells, preparation containing extracted TAA of tumor cells (TAA extract) have recently been explored for active immunization (40,62). Immunization with purified TAA may be more effective than whole tumor cells. In the latter case, the relevant TAA may be present at a low density and consequently swamped by other determinants. The recently isolation and characterization of certain TAA enable the study of immune reactions against highly purified TAA with encouraging results in cancer patients (99,51).

1.5 USE OF LIPOSOMES IN TUMOR IMMUNOTHERAPY

The TAA responsible for mediating tumor rejection are integral membrane molecules or molecule associated with the tumor cell surface. Their immunogenecity is closely related to their presentation form. Liposomes have been shown to potentiate the immune response of a

variety of antigens. Liposomes are artificially prepared spheres, consisting of concentric phospholipids bilayers separated by aqueous compartments. Liposomes have been first described by Alec D. Bangham (13). They are formed when phospholipids are confronted with water. The phospholipid molecules prefer to find a conformation in which their hydrophobic fatty acid chains are prevented from contact with water. For that reason, phospholipid are formed in which the relatively hydrophilic head groups are made up of both of the outer parts of each bilayer, whereas the hydrophobic fatty acids groups are located directly opposite each other as the inner part of the bilayer.

Part of the aqueous solution, together with hydrophilic molecules dissolved in it will be encapsulated during the formation of the liposomes, whereas lipophilic bilayers molecules may be associated with the phospholipid bilayers. Amphiphatic molecules, as the phospholipids themselves, attempt to find a conformation, with their hydrophilic parts extended in the aqueous compartments and their hydrophobic parts inserted in the bilayers. Liposomes may differ in their dimensions, composition (different phospholipids and cholesterol contents), charge (resulting from the charges of the composing phospholipids), and structure (Uni- or multilamellar liposomes consisting of only one or more phospholipid bilayer surrounding one aqueous compartment).

The *in vivo* distribution of liposomes are biodegradable and may be composed of non-toxic and immunologically inert phospholipids, they have been suggested as promising carrier for haptens and antigens. Immunopotentiation has been established both for antigens exposed on liposomal outer surfaces and for antigens encapsulated within the liposomes. Interest centered on the possible applicability of liposomes as an adjuvant for the preparation of vaccines and for the preparation of tumor antigens to the immune system (122)

The immunopotentiating ability of liposomes has been first shown by Allison and Gregoriades in 1974. They have reported the adjuvant effect of liposomes in eliciting anti-diphtheria toxoid immune response (5). Subsequently various workers confirmed the immunoadjuvanicity of liposomes in case of proteins (123) peptides (73), lipids (8) and carbohydrates (108).

Thus, Liposomes may be an effective tool to obtain enhanced immune reactions against TAA by acting as an adjuvant, because effective immunotherapy employing TAA depends upon reconstitution into an environment that enhances immunogenicity. Direct interaction between liposome-associated antigens and lymphocytes is well documented for the *in vivo* stimulation of T-cells (126). A likely prerequisite for direct stimulation of lymphocytes by liposome-associated antigens is the presentation of the antigens on the outermost layer of liposomes. However, the antigen might also be incorporated into the interior of the liposomes and consequently, not directly available for interaction with immunocompetent cells. Thus the immune response evoked by liposomal antigens can also be the result of their targeting to the antigen presenting cells.

Various technical problems have impeded the development of liposomes as viable clinical tools for the delivery of cytotoxic antineoplastic agents to tumor e.g., the poor entrapment efficiency of drugs, increase uptake by liver and spleen macrophages and hepatocytes and the disintegration into blood. However, encouraging progress have been made in overcoming some of these problems. Recently, a method to produce liposomes capable of achieving high entrapment efficiency was developed and has been successfully used for entrapment of toxin, radiomodulators and radiosensitizer (1,110). The method is highly reproducible and the biological activity of the entrapped material was found to remain unaltered. In many experimental situations the immunogenicity of the antigen-containing liposomes was still relatively low. Therefore, immunostimulants were applied as adjuvants to

further potentiate the immune response. Lipoidal adjuvants that activate macrophages have been studied extensively. Lipid A and lipophilic derivatives of MDP, in particular have shown to be effective (18). Some new lipophilic MDP derivatives and the sodium phthalyated derivative of lipopolysaccharide (SPLPS) have been found potent adjuvants in stimulation of immune responses (2,3,105,112,121). There is no doubt that antibody response can be induced by TAA containing liposomes. However, the potential of the antibodies to mediate tumor regression is still a matter of controversy. On the other hand, antibodies may kill tumor cells in vitro by complement dependent lysis or by antibody mediated cell-dependent mechanisms. Clinical responses in some patients treated with monoclonal antibodies have been observed (85.58). On the other hand, the presence of antibodies on tumor cells is regarded as unfavorable as they can block the immune response in vivo or can form antigen-antibody complexes that can induce suppressor cells (94). Generally, it is believe that in most case cell-mediated immune response rather than humoral response is of prime importance in tumor rejection. Therefore, in order to induce both types of immune responses in host against TAA, incorporation of macrophage activators such as MDP, INF-r, and IL-2 or B-cell mitogen such as SPLPS in the liposomal TAA formulation seems realistic.

Little information is available on the mechanism behind the potentiation of antitumor immune responses after incorporation of TAA into liposomes. In general, antigen presenting cells (APCs) present TAA in association with the proper MHC molecules to helper cells. This activates T-cells to the production of nonspecific T cell factors, the lymphokines. These factors participate in the induction of cytotoxic macrophages, natural killer (NK) cells, cytotoxic T cells, and Ig synthesis by B cells. It is not known to what extend these effectors mechanisms are stimulated by liposomal TAA and whether these mechanisms can be manipulated by the liposomal characteristics. The immunopotentiation of TAA by liposomes might be explained by a better targeting of TAA to antigen presenting cells and consequently, improved stimulation of one or more tumoricidal effector mechanisms.

Rodent and human macrophages activated by various immunomodulators acquire the ability to destroy neoplastic cells in vivo while leaving non-neoplastic cells unharmed. Macrophages are also an important component of both the afferent and efferent arms of the immune system and can also be important in the defense against neoplasm (39). Liposomes provide a particularly convenient non-toxic carrier for the delivery of biologically active materials to mononuclear phagocytes in vivo (40). Following i.v. administration, liposomes are taken up by the reticuloendothelial (RE) cells in the liver and spleen by circulating monocytes (96). By exploiting this localization pattern, it is possible to target liposome-encapsulated materials to macrophages *in vivo*. Preferential delivery of the liposome-encapsulated substance can be achieved in vivo because mainly phagocytic cells are exposed to the liposome-entrapped antigens. Once phagocytosed, biological active materials are release into the cytoplasm of the phagocyte, thereby avoiding the problem of dilution, serum protein binding, and rapid clearance, and minimizing the elicitation of undesirable side effects. This natural localization pattern also allows efficient targeting of liposomes and their contents to various macrophage components in the body. Therefore, the systemic activation of macrophages by liposome-encapsulated TAA and supplemented by immunostimulator can provide a successful approach to the eradication of established cancer metastases.

The present study uses tumor bearing animal model for TAA vaccination in an attempt to devise an effective formulation using liposomes that would elicit both humoral and cellular responses against tumor. The following objectives were conceived for the investigation embedded in this thesis.

1.Induction of Tumor in mice by diethlynitrosamine (DEN)

2.Isolation of Tumor-Associated Antigens

3.Encapsulation of TAA into Liposomes

4.Stimulation of Immune Reaction against Liposomal-TAA

5.Study The Effect Of Immune Responses and Assessment Of Tumor Regression.

2 Materials And Methods

2.1 CHEMICALS

All the chemicals used were of analytical grade. The various chemicals were obtained from different sources as listed below: -

Sigma Chemicals: N-Nitrosodiethylamine (DEN), [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB), Acetylcholine chloride, Ammonium persulphate, Bovine serum albumin (BSA), Bromophenol blue, Cholesterol, Citrate, Coomassie Brilliant Blue G-250 & R-250, Diaminobenzidine(DAB), Dicetyl Phosphate (DCP), Dithiothreitol (DTT), DL- α-Phosphatidylcholine, DL- α-Phosphatidylcholine, Dipalmitoyl (DPPC), Ethylenediaminetetraacetic acid (EDTA), Fetal calf serum, Fruend's Adjuvant (complete and incomplete), Glycine, Glycylglycine, L-γ-glutamyl-p-nitroanilide, L-arginine, L-asparagine monohydrate, L-glutamine, N,N,N'N'- Tetramethylethylenediamine (TEMED), N'N'-bis-methylene-acrylamide, O-phenylenediamine hydrochloric (OPD), Protein A peroxidase, Tris base, 2-mercaptoetanol, Trizma Base (Tris[hydroxymethyl]aminomethane), Trypan Blue, Sodium azide, Trypsin, Acrylamide, Tween-20,

Boehringer Mannheim: 5-Bromo-2'-deoxy-urudine Labeling and Detection Kit III, Cell proliferation kit I (MTT), Penicillin-Streptomycin.

Qualigens: Butanol, Chloroform ($CHCl_3$), D.P.X., di-sodium hydrogen ortho-phosphate (Na_2HPO_4), Eosin, Formaldehyde solution (HCHO), Glacial acetic acid (CH_3COOH), Glycerol, glycerol, Haematoxylin, Methanol (CH_3OH), Orthophosphoric acid (H_3PO_4), Sodium dihydrogen ortho-phosphate ($NaH_2PO_4.2H_2O$), Sodium hydroxide flakes (NaOH), Sucrose, Trichloroacetic acid (TCA), Xylene,

Hi Media: Dulbecco's Modified Eagle Medium Base (DMEM), Potassium hydrogen phosphate (monobasic) KH_2PO_4, Potassium hydrogen phosphate (dibasic) K_2HPO_4.

Merck: Ammonium sulphate $(NH_4)_2SO_4$, Citric acid-1-hydrate($C_6H_8O_7.H_2O$), Hydrochloric acid (HCl), Paraffin Wax with ceresin, Sodium bicarbonate($NaHCO_3$), Sodium carbonate, Sodium hydrogen carbonate.

BDH: Sodium chloride (NaCl),

Bengal Chemicals & Pharmaceuticals Ltd.: Ethanol (C_2H_5OH) 90%.

CDH: Picric acid.

Miscellaneous: Nitrogen gas ($N_2 \uparrow$).

2.2 INSTRUMENTS AND APPARATUS

Aspirator and Rotary vacuum evaporator: EYELA A-3S and type N-1 respectively.

Centrifuge: (i) Remi C 24, (ii) Beckman Optima™ TL Ultracentrifuge and (iii) Spinwin model MC - 01.

Electrophoresis Unit: Bio-Rad model Mini-PROTEAN II cell.

ELISA: Polystyrene microtitre plate (Corning NY), Multiskan MS India Serial RS-232 C.

Homogenizer: Remi Motors type RQ - 127 A, H.P 1/8, R.P.M. 8000.

Microscope: (i) Carl Zeiss JENA 30 - G0603 and (ii) Leitz Dialux 20.

Microtomy: Weswox Rotary Microtome.

Protein blotting: PhastSystem™ Semi dry Transfer Kit Pharmacia Biotech.

Spectrophotometer: Hitachi Model U-2001 UV/Vis Spectrophotometer.

Weighing balance: (I) Mettler Toledo model AB54, (ii) Sartorius type 1409 & 2405.

Pippetes: Gilson pippetes (France).

2.3 ANIMALS

Adult Swiss albino mice (Balb/c), bred by a random breeding method from the outbred colony maintained at the department were used for the entire course of study. The animals were housed at (20 ± 2°C), with 12 h light and 12 h darkness. Water and pellet dry feed was given at *ad libitum*.

2.4 INDUCTION OF TUMOR

Diethylnitrosamine (DEN), a potent hepatocarcinogen (109) was used for the induction of cancer in mice (101). Stock solution was prepared in glass double distilled water to deliver a dose of 25 mg DEN per Kg body weight.

Healthy 6-8 weeks old mice were administered aqueous preparation of DEN (25 mg/kg body weight) through *i.v.* route up to three months at weekly intervals. The following studies were carried out to monitor cancer induction in mice upon DEN exposure.

(i) Body mass index (BMI).

(ii) Tumour marker enzyme activities (*viz.* GGT and AChE).

(iii) Histological examination of liver tissue.

(iv) Differential expression of protein(s) in hepatocytes.

2.5 TISSUE PREPARATION FOR ENZYMATIC ASSAYS

After complete DEN treatment mice were killed by cervical dislocation and the liver was quickly excised and removed, rinsed in chilled normal saline (0.9% NaCl), blotted dry and weighed. A 50% homogenate was prepared in chilled 0.32M sucrose solution. The homogenate was solubilized in 0.5% Triton X-100 (1:1) and then centrifuged at 20,000 × *g* for 30 min at 4°C. The resulting supernatant was used for the enzyme assays as described below. The total protein content in the supernatant was estimated using Bradford method [16].

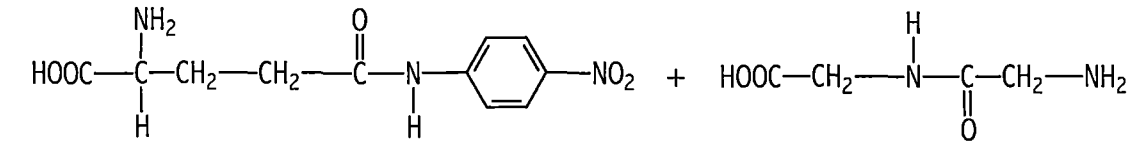
2.6 γ-GLUTAMYL TRANSPEPTIDASE ACTIVITY ASSAY

γ-Glutamyl transpeptidase activity was assayed according to the method as described by Meister *et al.*, (7) with slight modifications.

Assay principle

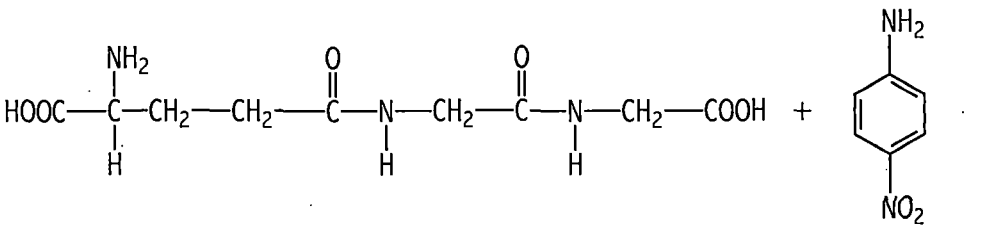
In presence of the substrate L-γ-glutamyl-p-nitroanilide and glycylglycine as the acceptor, GGT catalyzes the transfer of the L-γ-glutamyl moiety to glycylglycine forming L-γ-glutamyl glycylglycine and releasing p-nitroaniline, which absorbs at 410 nm. The activity of the enzyme was measured by following the increase in absorbance at 410 nm spectrophotometrically.

Reaction mechanism



L-γ -GLUTAMYL-p-NITROANILIDE + GLYCYLGLYCINE

GGT



L-γ -GLUTAMYL-GLYCYLGLYCINE + p-NITROANILINE ($A_{410\,nm}$)

Reagents

Buffer: 0.1 M Tris HCl, pH 8.0 at 25°C.

Glycylglycine: 0.1 M, pH 8.0, prepared in H_2O and pH adjusted with 2 N NaOH.

L-γ-glutamyl-p-nitroanilide: 2.5 mM, pH 8.0, prepared by dissolving in 20 ml of 0.5 N HCl followed by addition of 30 ml of H_2O, pH was adjusted with Trizma Base and the final volume was made up to 60 ml with H_2O.

Procedure

The following additions were made to a spectrophotometer quartz cuvette (1 ml): – 920 µl Tris HCl buffer, 20 µl L-γ -glutamyl-p-nitroanilide and 10 µl glycylglycine. The solution mixture was brought to 37°C in a thermostatted cuvette holder of the spectrophotometer. Reaction was initiated by adding 50 µl of the enzyme preparation and the rate of release of p-nitroaniline was recorded at 410 nm (ϵ =8800 $M^{-1}cm^{-1}$). The specific activity was expressed as units per mg protein.

Calculation

If the change in extinction is ΔE per minute, then the activity in International Units is:

GGT activity = (ΔE x 1000 x 1) /(8800 x 0.05)

ΔE = Extinction change per minute

1000 = Factor to obtain moles µmoles

1 = Total volume of reaction mixture (ml)

0.05 = Volume of enzyme (ml)

8800 = molar extinction coefficient of chromophore at 410 nm (litres $mol^{-1}cm^{-1}$).

Enzyme specific activity is the number of enzyme units (U) per milligram of protein. Therefore, specific activity is = ΔE x 2.27 liter moles $min^{-1}ml^{-1}$ x 1000 / Conc. of protein mg/ml

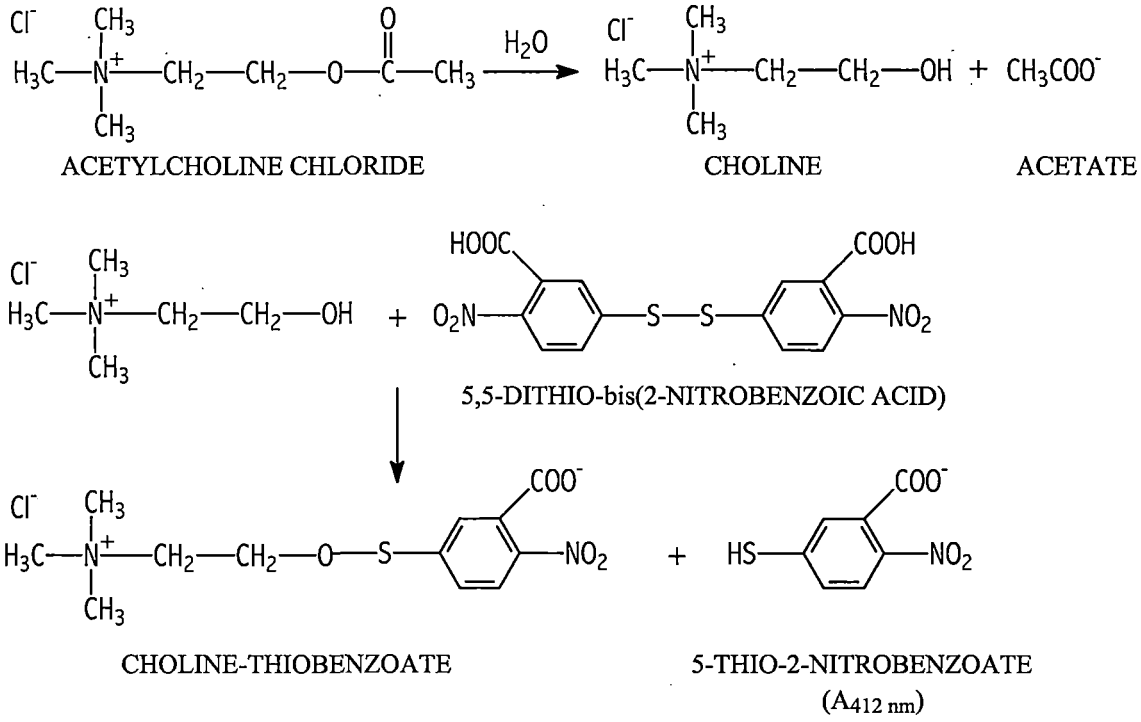
2.7 ACETYLCHOLINE ESTERASE ACTIVITY ASSAY

Acetylcholine esterase activity was assayed according to the methods of Ellman *et al.* (31), and Plummer & Wright (132) with slight modifications.

Assay principle

Acetylcholine chloride, the ester of choline and acetic acid, was used as the substrate in this assay system. AChE hydrolyses acetylcholine chloride to choline and acetate. Choline reacts with an oxidizing agent, 5,5'- dithiobis-(2-nitrobenzoic acid) (DTNB), which splits into two products, one of which i.e. 5-thio-2-nitrobenzoate, absorbs at 412 nm. The activity of the enzyme was then measured by following the increase in absorbance at 412 nm spectrophotometrically.

Reaction mechanism



Buffer:	Sodium phosphate 0.1 M, pH 8.0
DTNB:	10 mM, freshly prepared in buffer containing $NaHCO_3$.
Acetylcholine chloride:	158.5 mM, freshly prepared in buffer.

Procedure

The following additions were made to a spectrophotometer quartz cuvette: –

50 µl of the enzyme preparation was added to 3.0 ml of sodium phosphate buffer and incubated at room temperature for 5 min. Then, 10 µl of DTNB was added and the reaction was initiated by the addition of 20 µl of acetylcholine chloride. The rate of release of 5-thio-2-nitrobenzoate was recorded at 412 nm (ϵ=1.36x10^4 litres mol^{-1}cm^{-1}).

was expressed as units per mg protein. Protein concentration was determined by Bradford's method of protein estimation, using BSA as standard.

Calculation

If the change in extinction is ΔE per minute, then the activity in International Units is:

AChE activity = (ΔE x 1000 x 3.17) /(1.36 x 10^4 x 0.05)

ΔE = Extinction change per minute

1000 = Factor to obtain moles μmoles

3.17 = Total volume of reaction mixture (ml)

0.05 = Volume of enzyme (ml)

1.36 x 10^4 = molar extinction coefficient of chromophore at 412 nm (litres $mol^{-1}cm^{-1}$).

AChE activity = ΔE x 4.66 liter moles $min^{-1}ml^{-1}$

Enzyme specific activity is the number of enzyme units (U) per milligram of protein.

Therefore, specific activity is = ΔE x 4.66 liter moles $min^{-1}ml^{-1}$ x 1000 / Conc. of protein mg/ml

2.8 PROTEIN ESTIMATION

Total protein content of enzyme preparation was determined by the method of Bradford (16), using BSA as standard.

Reagents

Reagent A – Coomassie Brilliant Blue G-250 (0.2 g in 100 ml of 95% ethanol).

Reagent B – Phosphoric acid, 85%.

Procedure

Stock solution was prepared by mixing 50 ml of Reagent A and 100 ml of Reagent B, this was thoroughly mixed and stored in a dark bottle at 4°C. The working solution was prepared by diluting 15 ml of the stock solution to 100 ml, with distilled water, and this was filtered through Whatman filter Paper No. 1 and the resultant filtrate was used immediately for the estimation. The final concentration of the reagents was 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% ethanol (v/v) and 8.5% phosphoric acid (v/v). A diluted protein sample (0.1 ml) was added to 5 ml of the working solution, thoroughly mixed in a

cyclomixer, followed by incubation at room temperature (25°C) for 10 min. The intensity of colour developed was measured at 595 nm. Protein concentrations were determined using a computed standard curve prepared by different concentrations of BSA (0.01 – 0.1 mg/ml).

2.9 MICROTOMY

The histological sections of the liver tissue of the normal and DEN treated animals were prepared following the method as described by Ratcliffe (102). The procedure involved the following steps: -

Fixation

The fixative used was Bouin's fluid, which had the following composition:

Picric acid (saturated aqueous solution)	75 ml
Formalin (40% aqueous)	25 ml
Acetic acid (glacial)	5 ml

Freshly excised liver tissue was immediately placed in about 6 ml of freshly prepared Bouin's fluid and cut to approximately 0.4 cm and fixed for 24 h in the same fixative. Excess fixative was washed overnight in running tap water.

Dehydration

Dehydration was carried out in a graded series of ethanol, placing the tissue serially in 30%, 50%, 70%, 90% and absolute alcohol; leaving for about 2 h in each grade and, about 3 h in absolute grade with 3 changes.

Clearing

This step involved placing the tissue in xylene for 2-6 h till the tissue became translucent.

Wax infiltration and casting

Wax infiltration impregnates and surrounds the tissue providing support and prevents the collapse and distortion of the tissue during sectioning. Wax infiltration was done by the xylene method: -

(1) From absolute alcohol ⟶ xylene (1 change)	2 h
(2) xylene: wax (50: 50) ⟶ in oven (58°C-60°C, 1 change)	1 h
(3) Molten wax ⟶ in oven (3 changes)	1 h each

The tissue was transferred from the last wax change and embedded in fresh molten wax and allowed to cool gradually. Care was taken not to overheat the casting blocks.

Section cutting (microtomy) and mounting

The embedded tissue was fixed to a specimen holder (wooden cube), which fitted on the microtome, and sectioned with a sharp knife at 7 μm. The sections were handled with a moist paintbrush.

Mounting

Glass slides were uniformly smeared with undiluted egg albumen and the sections were mounted on the slides. A drop or two of water was added on each slide, and gently passed over a hot plate in order to flatten the mounted sections. The slides were dried overnight at 37°C.

Staining

Haematoxylin and eosin procedure was used for staining. Haematoxylin stains the nuclei dark blue and eosin stains the cytoplasm pink. This step involved several sub-steps: -

(1) The slide were de-waxed in xylene	5-10 min.
(2) Absolute ethanol	2 min.
(3) 90% ethanol	1 min.
(4) 70% ethanol	1 min.
(5) Haematoxylin	10-15 min.

(6) Excess stain was removed by acid alcohol mixture (100 ml of 70% ethanol, 0.5 ml concentrated HCl) followed by washing in tap water.

(7) The slides were dipped in 70% ethanol for few seconds.

(8) Eosin (70% ethanol)	5-10 min.
(9) 70% ethanol	1 min.
(10) 90% ethanol	10-15 sec.
(11) Absolute ethanol	2-3 min. x 2

(12) Cleared in fresh xylene.

(13) Mounted in 1-2 drops of D.P.X. and covered with cover slips. Care was taken not to allow the slides to dry up at any stage.

Observation

Finally, the prepared slides were observed and studied under a phase contrast microscope, Leitz Dialux 20.

2.10 TAA EXTRACTION

Selective extraction by membrane surface glycoprotein from liver tissues of DEN exposed and age-matched normal mice was carried out using 1-butanol as described by Liao *et al.* (72).

Reagents

(i) 3% 1-butanol in PBS (10 mM pH 7.4)

(ii) 0.02% ETDA

(iii) 0.25% Trypsin-EDTA solution

Procedure

Liver tissue excised from DEN-exposed and age-matched normal mice was washed in normal saline and minced over fine wire screen. It was washed in 0.02% EDTA followed by gentle dispersion in 0.25% trypsin EDTA solution. Minced tissue was washed again in PBS (10 mM, pH 7.4) and then incubated for 7 min. with 3% 1-butanol diluted in PBS at 22°C with occasional agitation. Finally, the mixture was centrifuged at 100,000 x g for 1 h at 4 °C. The supernatant obtained was concentrated and was used as TAA extract to resolve membrane glycoproteins using SDS-PAGE and for induction of immune responses in DEN exposed mice.

2.11 SDS-PAGE GEL ELECTROPHORESIS

SDS-PAGE analysis was carried out to resolve hepatocyte cell surface membrane glycoproteins. The gel slab was casted according to Laemmli (66) under reducing condition. Low and high molecular weight reference standards from Sigma served as markers.

Reagents

A. Acrylamide/bis (30%T, 2.67%C)

87.6 g acrylamide (29.2 g/100 ml)

2.4 g N'N'-bis-methylene-acrylamide (0.8 g/100ml)

Made to 300 ml with deionized water, filtered and stored at 4°C in the dark.

B. 1.5 M Tris-HCl, pH 8.8

27.23 g Tris base (18.15 g/100 ml)

80 ml double distilled water.

The pH was adjusted to 8.8 with 6 N HCl. Volume was made up to 150 ml with double distilled water and stored at 4°C.

C. 0.5 M Tris-HCl, pH 6.8

6 gTris base

60 ml double distilled water

The pH was adjusted to 6.8 with 6 N HCl and the volume was made up to 100 ml with double distilled water and stored at 4°C.

D. 10% SDS

10 g SDS was dissolved in 90 ml water with gentle stirring and volume made up to 100 ml with dd H_2O.

E. Sample buffer

dd H_2O	3.8 ml
0.5 M Tris –HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
2-mercaptoethanol	0.4 ml
1% (w/v) bromophenol blue	0.4 ml
TOTAL VOLUME	8.0 ml

F. 5 x electrode (Running) buffer, pH 8.3

Tris base	9 g	(15 g/l)
Glycine	43.2 g	(72 g/l)
SDS	3 g	(5 g/l)

Volume made up to 600 ml with dd H_2O

Separating gel Preparation (for 10% gel)

H_2O	4.0 ml
1.5M Tris-HCl, pH 8.8	2.5 ml
10% (w/v) SDS stock	100 µl
Acrylamide/bis (30% stock)	3.3 ml
10% Ammonium persulphate	100 µl
TEMED	4 µl
TOTAL MONOMER	10 ml

Stacking Gel preparation- 4.0% gel, 0.125M Tris, pH 6.8

H_2O	6.1 ml
0.5M Tris-HCl, pH 6.8	2.5 ml
10% (w/v) SDS stock	100 µl
Acrylamide/bis (30% stock)	1.33 ml
10% Ammonium persulphate	50 µl
TEMED	10 µl
TOTAL MONOMER	10 ml

Running condition

The power condition during the run was maintained at 200 volts with constant voltage setting.

Staining with Coomassie brilliant blue

The gel was stained for 30 minutes with 0.1% Coomassie brilliant blue R-250 in fixative (40% MeOH, 10% HOAc).

Destaining

The gel was stained with several changes of 40% MeOH 10% HOAc till the background was removed.

2.12 LIPOSOMES PREPARATION & ENCAPSULATION OF TAA

Dry film method

Liposomes were prepared by dry film method originally described by Bangham *et al.* (12). Phospholipids, DPPC : Chol : DCP were taken in a molar ratio of 1.0 : 0.9 : 0.25 and dissolved in a mixture of chloroform and methanol (ratio 2:1 v/v). The solvent was then evaporated under nitrogen stream to make a lipid film. The film was dried under vacuum at 40°C for 1 h. The lipid film was then suspended in 1.0 ml of aqueous TAA extract (1 mg/ml glycoprotein in 10 mM tris-buffered saline pH 7.4) and kept for 5 min. at 40°C in a water bath. The mixture was thoroughly vortexed for 10-15 min. till all the lipid film turned into a turbid lipid viscous. It was then centrifuged for 30 min. at 14000 rpm in a refrigerated centrifuge at 4°C. The supernatant was carefully aspirated and the pellet was washed for 2-3 times with buffer and supernatants were pooled together. Protein content in the supernatant was measured by Bradford method (16) and thus glycoprotein entrapped into liposomes was calculated by subtracting free from the total (Table 2).

2.13 IMMUNIZATION

For immunization the TAA extract was made into three formulations:-

(i) TAA-alone in saline,

(ii) TAA-emulsified in Fruend's adjuvant and

(iii) TAA-encapsulated into liposome.

DEN-exposed mice described above were divided into three groups each of 8-10 animals. First two groups were immunized by giving three intramuscular injections of 10 μg equivalent of TAA extract alone and liposome encapsulated at fortnightly intervals. Whereas, the third group was immunized by single intramuscular injection of same antigen dose emulsified with CFA (ratio 1:1, v/v). A booster was given one month after the last immunization in all groups. For the third group the booster was given with the antigen emulsified with incomplete Fruend's adjuvant.

Blood was collected by retro-orbital bleeding at different time points and the pooled sera stored at -20^{o}C before antibody titration.

2.14 ENZYME-LINK IMMUNOSORBANT ASSAY (ELISA)

The presence of circulating antibodies against TAA in the test sera were determined using ELISA describe by Catty. D, *et al.* (21).

Buffers and Solutions

Coating buffer - carbonate/bicarbonate 0.05M pH 9.6

Diluting/incubation buffer (PBS)-Tween) - PBS, 0.1M, pH 7.4 containing 0.05% (v/v) Tween-20.

Washing solution (Saline-Tween) - 0.9% (w/v) saline with 0.05% (v/v) Tween-20.

BSA coating solution – 100 μg/mg in coating buffer.

Substrate buffer – citrate buffer, 0.15M, pH 5.0

Substrate (OPD) solution – freshly prepared.

(i) O-Phenylenediamine hydrochloric (OPD) was dissolved at a concentration of 0.4 mg/ml of substrate buffer.

(ii) 5 μl of undiluted H_2O_2 [(or 0.5 ml of 30% (v/v) was added and mixed.

Enzyme conjugate – Protein A-peroxidase conjugate (HRP) diluted 1:1000 in PBS-T

Stopping solution – HCl 2.5M.

Procedure

1. *Coating plates with antigen*

   The TAA extract at 1 μg/ml in coating buffer was pipette to each wells of a microtitre plate. The plate was covered and incubated overnight (16 h) at 4°C.

2. *Washing plates*

   The plate was washed with washing solution (Saline-Tween) by shaking out vigorously over a sink and banged the inverted plate several times onto a filter paper. This step was repeated 4-5 times.

3. *Blocking*

   The plate was then blocked by applying 100 μl of BSA solution at 100 μg/ml to each well. The plate was covered and incubated overnight (16 h) at 4°C. The BSA solution was then ejected and the plate was washed as above.

4. *Applying primary antibodies*

   100 μl of the serum was applied to each well of the plate and the plate was incubated for 2 h at 37°C, then washed as above.

5. *Applying the conjugate*

   The diluted Protein-A peroxidase conjugate was applied at 100 μl to each well and incubated for 2 h at 37°C, then washed as above.

6. *Applying the substrate*

   Finally, 100 μl of the substrate solution was applied to each well. The plate was covered and incubated in the dark at 37°C for 10 min.

7. *Stopping the reaction*

   When the colour was optimally developed, the reaction was stopped by adding 25 μl of the stopping solution to each well.

8. *Reading the plate*

   The plate was read on an ELISA reader with filter setting at 492 nm for OPD. Results are expressed as the mean absorbance of triplicate wells after subtracting the background values.

2.15 IMMUNOBLOTTING

Specificity of the antibodies against the TAA was checked by western-blot analysis (44,59). The test antigens were electrophoresed by SDS-PAGE separation on PhastGel gradient 10-15. The gel was then blotted onto nitrocellulose membrane (NC) using the PhastTransfer Semi-dry Transfer Kit as described in the Users Manual (Pharmacia Biotech PhastSystem^{TM})

Reagents

1. *Transfer buffer:* 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol.
2. *Blocking buffer.* 20 mM Tris, 500 mM NaCl and 3% gelatin.
3. *Enzyme conjugate:* Protein-A peroxidase diluted in blocking buffer at 1/1000 of the commercial stock.
4. *Substrate solution:* 6 mg of Diaminobenzidine (DAB,3,3'4,4'-tetraaminobiphenyl) dissolved in 10 ml of blocking buffer, filtered through Whatman no.1 filter paper and 10 µl of 30% H_2O_2 added.

Procedure

After the transfer -

(i) The NC membrane was blocked for 30 min. in blocking buffer.

(ii) The NC membrane was next incubated with the test serum (diluted in the blocking buffer) 60 min. at room temperature.

(iii) Rinsed in distilled water.

(iv) Washed twice in the buffer for 10 min.

(v) Incubated with Protein A peroxidase conjugate for 60 min. at room temperature.

(vi) Rinsed in distilled water.

(vii) Washed as in step (iv).

(viii) The substrate solution, 3,3'-Diaminobenzidine (DAB) was added and allowed to develop for 15 min.

(ix) The reaction was stopped with distilled water.

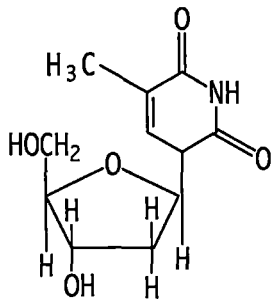
2.16 CELL PROLIFERATION ASSAY USING 5-BROMO-2'-DEOXY-URIDINE, (BrdU)

For *in vitro* lymphocytes cell proliferation assays, the mice were killed by cervical dislocation to harvest spleens on third day after administering the booster injection.

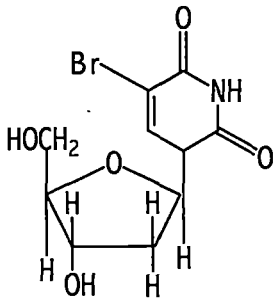
This assay was performed as described initially by Gratzner (47) and by subsequent workers (124,46,84,86) .BrdU an analogue of thymidine was used for this assay.

Molecular structure of thymidine and BrdU .

Thymidine
(5-methyluracil-2'-deoxyribose)



5-Bromo-2'-deoxyribose
(5-Bromouracil-2'-deoxyribose)



Assay principle

The assay is a cellular immunoassay, which uses a mouse monoclonal antibody directed against BrdU based on the cell ELISA principle. Cells cultured in a microtiter plate are incubated with BrdU. The cells are fixed with 0.5 M ethanol/HCl, and are then incubated with nucleases to partially digest the DNA. Incorporated BrdU is detected with the monoclonal anti-BrdU-POD, Fab fragments, and the bound conjugate is visualized with the soluble chromogenic substrate ABTS and measured spectrophotometrically at 405 nm using an ELISA reader.

Reagents

The 5-Bromo-2'-deoxy-Uridine Labeling and Detection Kit III (Boehringer Mannheim) was commercially obtained. The supplied kit contained the following: -

1. BrdU labeling reagent, (1000x). 1 ml containing 10mM BrdU stock solution (1000x), in phosphate buffered saline (PBS), pH 7.4; sterile.
2. Washing buffer concentrate (10x), 125 ml PBS, 10x concentrated.
3. Incubation buffer, 125 ml containing 66 mM Tris buffer, 0.66 mM $MgCl_2$, and 1 mM 2-mercaptoethanol.
4. Nucleases, stabilized and lyophilized.
5. Anti-BrdU-POD, Fab fragments, one glass vial containing anti-BrdU (monoclonal antibody, Fab fragments from mouse) conjugated with peroxidase (25 U), stabilized, lyophilized.
6. Substrate buffer, 125 ml ABTS-substrate buffers (sodium perborate and citric acid/phosphate buffer).
7. ABTS-substrate, 2,2'-azino-di-[3-ethylbenzthiazolin-sulfonat (6)]. One glass vial containing ABTS-substrate powder for 125 ml substrate buffer.
9. Substrate enhancer, one glass vial containing 125 mg substrate enhancer.

Preparation of working solutions

Solution I. BrdU labeling solution. The vial of the BrdU labeling reagent was diluted with sterile culture medium in the proportion of 1:90, (resulting concentration: 110 μM BrdU). Freshly prepared.

Solution II. Washing buffer. Washing buffer concentrate (10x) was diluted with redist.water in the proportion of 1:10.This washing buffer (solution II), was used to (a) prepare the anti-BrdU-POD, working solution and (b) wash cells after incubation with anti-BrdU-POD.

For other washing steps culture medium containing 10% FCS (fetal calf serum) was used.

Solution III. Incubation buffer. Ready to use, and it is used for diluting the nucleases.

Solution IV. Nucleases. Stock solution was diluted with incubation buffer in the proportion of 1:100.

Solution V. Anti-BrdU-POD, Fab fragments. The whole content of the supplied vial was dissolved in 1.25 ml redist. water (final concentration: 20 U/ml).

Solution Va. Anti-BrdU-POD, Fab fragments. Freshly prepared by diluting the supplied stock solution with washing buffer (Solution II) in the proportion of 1:100, supplemented with 10 mg/ml BSA.

Solution VI. Peroxidase substrate. A vial of supplied ABTS powder was dissolved in substrate buffer, stirred at room temperature till a clear solution was obtained.

Other reagents

1. *Culture medium:* DMEM was sterilized by autoclaving and the following were added: - 10% heat inactivated FCS, 2 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine monohydrate and streptomycin.
2. *Fixative*: Ethanol p.a. (70%) in HCl (final concentration 0.5 M) i.e. for one microtiter plate, 7 ml ethanol p.a., 100%, was diluted with 2.33 ml redist. water and 670 µl of HCl p.a., 25% was added. This was stored at -20°C and used pre-cooled.
3. *Washing medium:* Culture medium containing heat inactivated 10% FCS.

Procedure

Lymphocytes obtained from the harvested spleen of immunized and normal mice (age-matched) were suspended in the culture medium. The cell viability test was done by Trypan blue method using Neubauer hemocytometer as described in sigma bulletin.

The lymphocytes were taken at 100 µl/well in 96-well microtiter plates. The cell suspension was labeled with 10 µl of freshly prepared working BrdU labeling solution, added to each well and incubated for 6 h at 37°C. During this incubation period, BrdU incorporates into freshly synthesized DNA. The cell suspension was centrifuged at 300 × *g*, using a rotor device for centrifugation of MTP, for 10 min. at room temperature. The labeling medium was carefully discarded and the adherent cells were washed twice with the wash medium (250 µl × 2). The cell suspension was air dried in an incubator at 60°C for 2 h. The air dried cell suspension was next fixed with 250 µl of the pre-cooled (-20°C) fixative and incubated for 30 min. at –20°C. The fixative was discarded and the wells were washed with wash medium (250 µl × 3). The cells were then incubated with 100 µl of nucleases per well at 37°C for 30 min. to partially digest the cellular DNA and thus, exposing the incorporated BrdU to immunodetection. The nucleases were discarded and washed with wash medium (250 µl × 3), the wash medium of the last wash was carefully removed and 100 µl of peroxidase-conjugated Anti-BrdU antibody (Anti-BrdU-POD) was added to each well and incubated for 30 min. at 37°C. Anti-BrdU-POD located the BrdU label in the DNA and bound to it. The unbound antibody conjugate was removed and the cells were washed with washing buffer (Soln. II) (250 µl × 3). The washing buffer of the last wash was removed carefully and 100 µl of the peroxidase substrate, 2,2'-azino-di-[3-

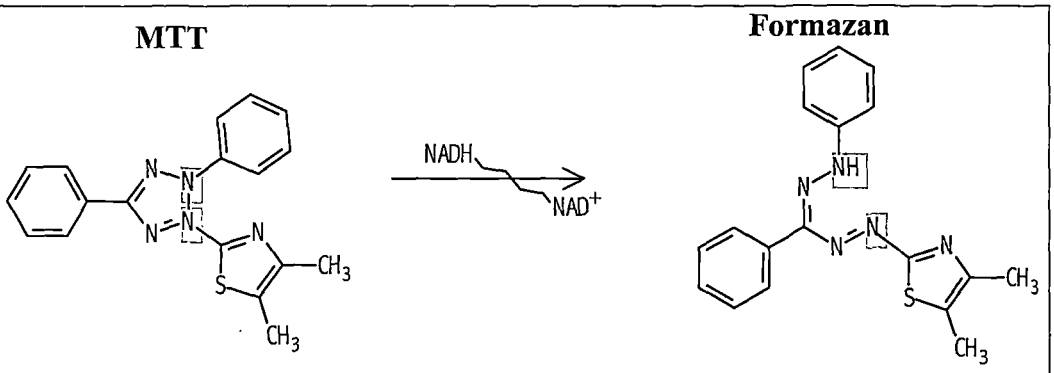
ethylbenzthiazolin-sulfonat (6)] (ABTS) was added to each well. Absorbance was read using an ELISA reader at 405 nm. The absorbance of the sample determined was directly correlated to the level of BrdU incorporated into cellular DNA. Results are expressed as the mean of triplicate wells.

2.17 METABOLIC ACTIVITY ASSAY USING THE TETRAZOLIUM SALT MTT

MTT assay was performed to determine the metabolic activity of immune lymphocytes using the Cell Proliferation Kit I (MTT). The non-radioactive, calorimetric assay system using MTT was first described by Mosmann, T. *et al.* (92) and improved in subsequent years by several other investigators (117,27,43,49,125,98). The assay is designed for the spectrophotometric quantification of cell growth and viability without the use of radioactive isotope.

Test principle

The assay is based on the tetrazolium salt MTT: (3-[4,5-dimethylthiazol-2-yl]-2,5-diphyltetrazolium bromide) by the viable cells. MTT is cleaved to formazan by the "succinate -tetrazolium reductase" system, which belong to the mitochondrial respiratory chain and is active only in viable cells.



Molecular structure of MTT and its corresponding reaction products.

MTT (yellow in colour) undergoes reduction in presence of NADH and NADPH as cofactors to form formazan crystals, which is purple in colour. The resulting colored solution is measured at 440 nm in an ELISA reader.

Reagents

Culture medium

DMEM was sterilized by autoclaving and the following were added: - 10% heat inactivated FCS, 2 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine monohydrate and streptomycin.

The supplied kit contained the followings: -

1. *MTT labeling reagent, (1x, ready to use)*

   5 vials containing 5 ml MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) labeling reagent (1x), 5 mg/ml, in phosphate buffered saline (PBS), non-sterile, ready to use.

2. *Solubilization solution, (1x, ready to use)*

   3 bottles solubilization solution (90 ml per bottle), 10% SDS in 0.01M HCl.

Procedure

Lymphocytes obtained from immune and normal mice suspended in culture medium were seeded ($1x10^4$/100µl/wells) in 96 wells, flat bottom polystyrene microtiter plates. An aliquot of 10 µl of yellow MTT solution (final concentration 0.5 mg/ml) was added to each wells and the plate incubated for approximately 4 hrs at 37°C. After this incubation period, purple formazan salt crystals were formed. These salt crystals are insoluble in aqueous solution, but were solubilized by adding 100 µl of solubilization solution (10% SDS in 0.01 M HCl) followed by overnight incubation in humidified atmosphere (37°C, 6.5% CO_2). The solubilized formazan product was quantified spectrophotometrically at 550 nm. using an ELISA reader.

2.18 STATISTICAL ANALYSIS

The result values are recorded as means ± SEM. The data were analysed by Student's t-test; difference at $P < 0.05$ were considered as statistically significant.

3 Results

The results of the experiments conducted are organized under two subheadings:-

3.1 TUMOR INDUCTION STUDIES

3.2 TUMOR REGRESSION STUDIES

3.1 TUMOR INDUCTION STUDIES

3.1.1 Body Mass Index

The body weight of each mouse was recorded at weekly intervals during the entire period of DEN administration. The change in body mass index is shown in Figure 1. Treated animals showed a gradual decrease in the body weight during the treatment course as compared to the age-matched normal control mice. Overall, loss in body weight of DEN-exposed mice was found to be −19 g/100g b.w., that was statistically highly significant. (Table. 1)

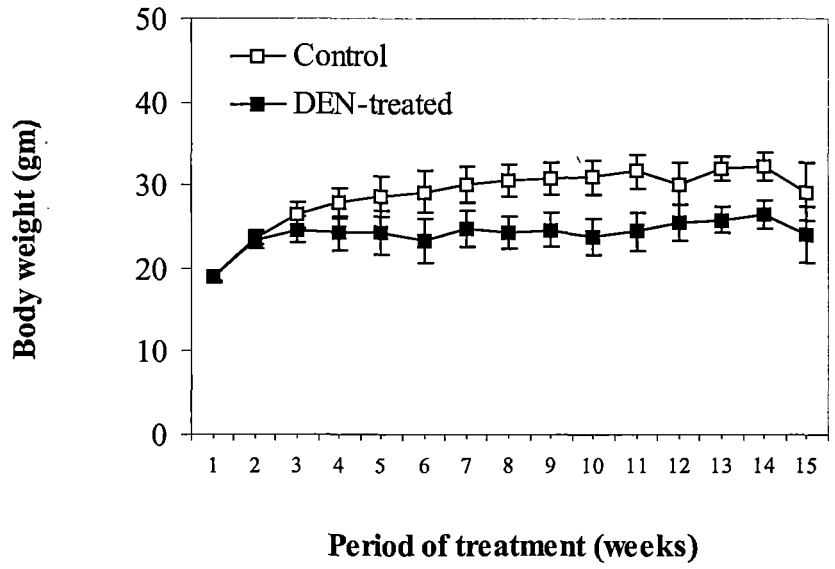


Figure 1 Body weight of Control and DEN-treated mice recorded during the course of DEN administration at weekly interval. Data from 20-25 mice in each case (means ± SD).

Majority of the animals survived during the complete DEN-administration course, however, signs of lethargy and sluggishness were observed in treated animals.

3.1.2 Effect of DEN on GGT Activity

Animals upon DEN administration exhibited alteration in GGT activity both in serum as well as in liver. In DEN-treated animals enzyme serum was monitored at an alternate week and was compared with the age-matched normal control mice (Figure 2). Significant change was found only after 12 weeks onward treatment. Control group of animals showed an average GGT activity of 0.00645 U/mg protein while the treated animals showed an activity of 0.00925 U/mg protein. Upon comparison, the difference in enzyme activity was found to be statistically significant ($P < 0.001$) as shown in Table 1.

In liver the GGT activity was monitored after 12 weeks of DEN treatment. Enzyme

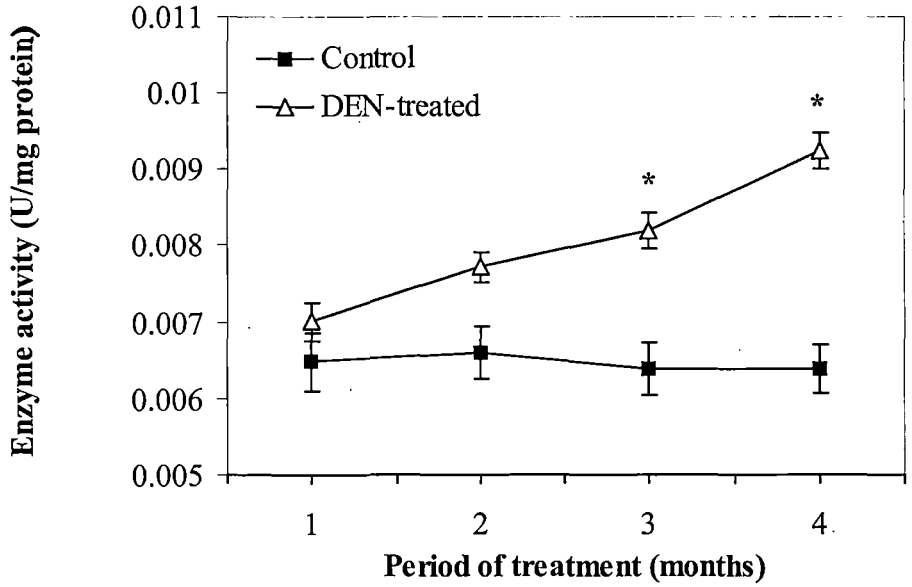


Figure 2 γ-glutamyltranspeptidase specific activity in the serum of Control and DEN-treated mice during DEN administration (25 mg/kg b.wt.) up to 12 weeks. Age-matched normal mice served as Control.
* $P < 0.001$, n = 25.

activity increased by 6 folds in comparison to the age-matched normal control mice (Figure

3). The average GGT activity in control was found to be 0.00415 U/mg protein while that of DEN-treated was 0.0270 U/mg protein and was statistically highly significantly ($P < 0.001$)

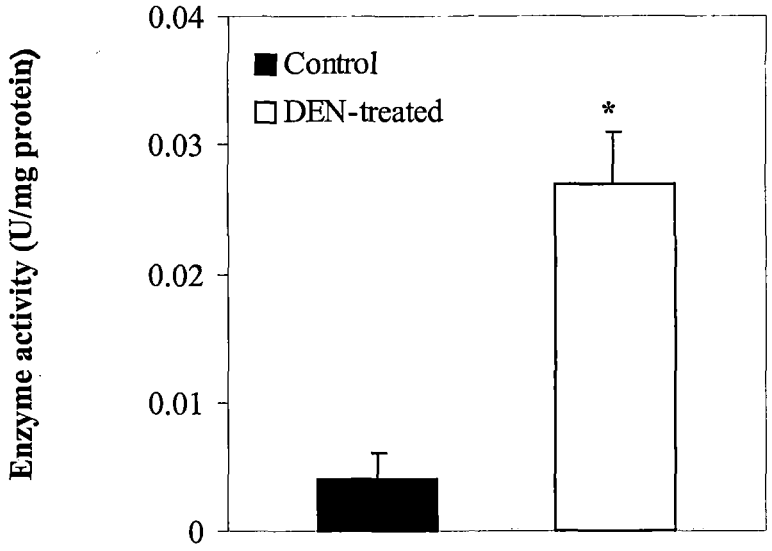


Figure 3 Liver γ-glutamyltranspeptidase specific activity in the supernatant fraction of Control and DEN-treated mice after 12 weeks of DEN administration (25 mg/kg b.wt.). Age-matched normal mice served as Control.
* $P < 0.001$, n = 25.

3.1.3 Effect of DEN on AChE activity

The AChE activity in serum as well as in liver of DEN-treated animals was also monitored and found elevated when compared with age–matched normal control mice (Figure 3 & 4). The enzyme activity raised significantly from 3rd month of treatment. In serum the level was elevated from 0.00595 ± 0.00021 to 0.0086 ± 0.00032 U/mg protein in comparison to control (Table-1). However, in liver the elevation in enzyme activity was more pronounced than it was observed in serum. It increased by more than 2 folds in comparison to the control (Figure 4). The average AChE activity in the liver of age-matched control mice was found to be 0.0072 U/mg protein while that of DEN–treated was 0.0158 U/mg protein (Table 1).

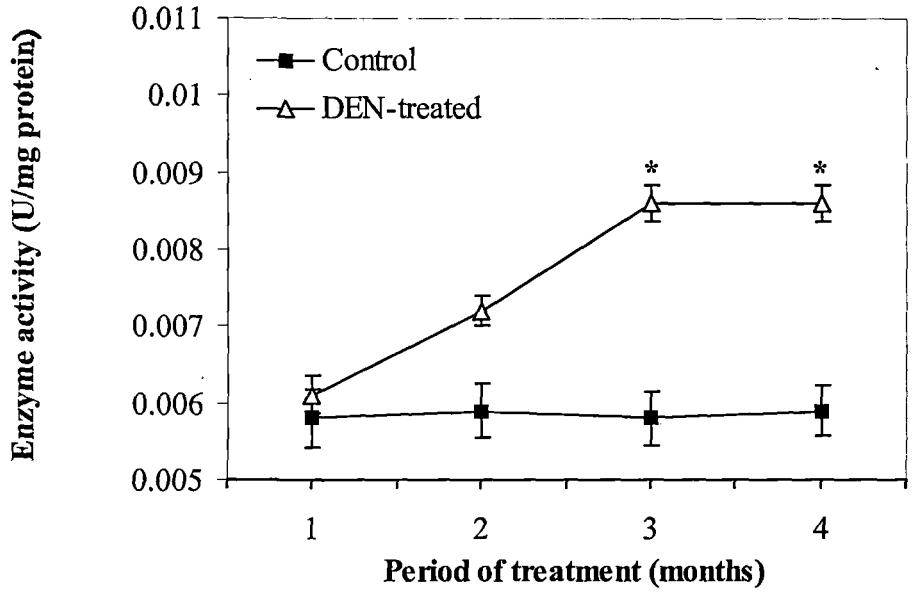


Figure 4 Acetylcholine esterase specific activity in the serum of Control and DEN-treated mice upon DEN administration (25 mg/kg b.wt.) upto 12 weeks. Age-matched normal mice served as Control.
* $P < 0.001$, n = 25.

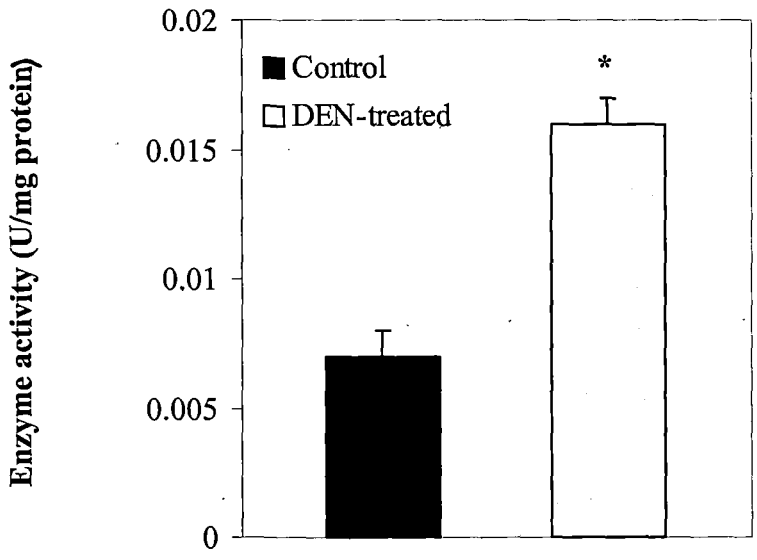


Figure 5 Liver acetylcholine esterase specific activity in the supernatant fraction of Control and DEN-treated mice after 12 weeks of DEN administration (25 mg/kg b.wt.). Age-matched normal mice served as Control.
* $P < 0.001$, n = 25.

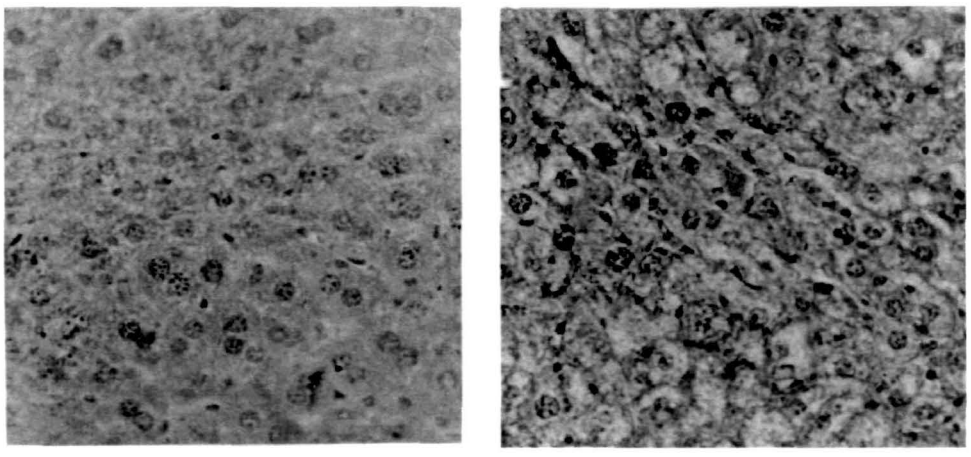
Table 1 Effect of DEN on body weight, GGT and AChE activity.

Mice group	Total change in b.wt (g/100g b.wt)	GGT activity (U/mg protein) means ± SD		AChE activity (U/mg protein) means ± SD	
		Serum	Liver	Serum	Liver
Control		0.00689 ± 0.0002	0.00415 ± 0.0015	0.00595 ± 0.0002	0.00720 ± 0.0011
DEN-treated	-19.21	0.00924 ± 0.0002*	0.02700 ± 0.0030*	0.00860 ± 0.0003*	0.01580 ± 0.0012*

* $P < 0.001$; n = 25 in each case.

3.1.4 Histological Analysis

The histological analysis of liver tissue of DEN treated and normal mice showed marked differences. As seen, in the DEN-exposed liver there is a loss of regular arrangements of cells, increased density staining and less binucleated cells as compared to their normal counterpart which has smooth, well defined, symmetrical, mono and bi-nucleated cells (Figure 6).



A B

Figure 6. Liver section showing details of hepatic cells. Normal (A) and DEN exposed (B) mice. Haematoxyline and eosin counterstain. **M** X 250.

3.1.5 SDS-PAGE Analysis

The total protein content in TAA-extract obtained from age-matched normal and DEN-exposed mice was estimated by Bradford's method. Equal amount of the TAA was loaded on 10% acrylamide gel to resolve membrane surface glycoproteins. No difference in protein profile of DEN exposed mice was observed when compared with the normal mice liver extract. However, differential expression in several proteins were observed, such as a glycoprotein of approximately 68 kDa was found over expressed and few glycoprotein of molecular weight between 29 kDa and 20 kDa were under expressed in the DEN-exposed mice. The results are shown in Figure 7.

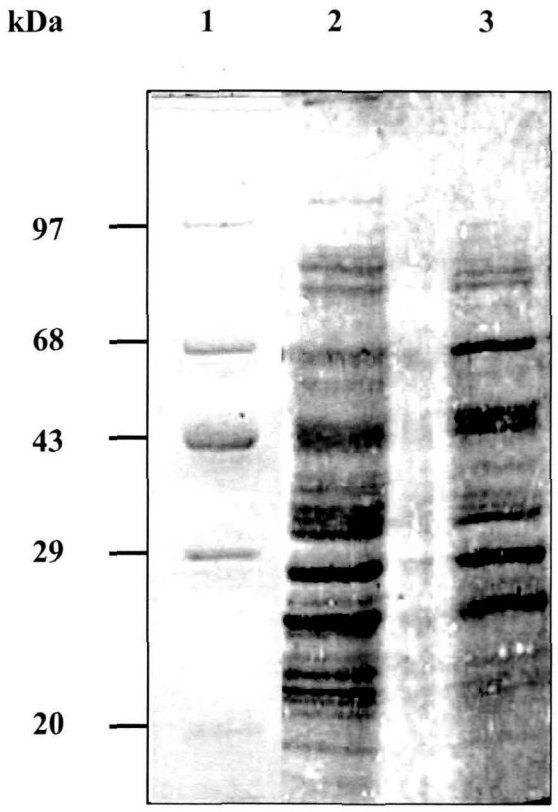


Figure 7. 1-Butanol extract of membrane glycoproteins from Control and Treated mice resolved by SDS-PAGE (10%). Tris-glycine gel stained with Coomassie Brilliant Blue. Lanes: (1) Molecular size standards (Pharmacia); (2) Control, (3) DEN-treated.

Thus, the loss of body weight, alterations in tumor marker enzymes, morphological changes in the hepatocytes and the differential expression of the protein indicated that DEN exposure leads to hepatocellular transformation and cancer induction in mice. Therefore, the next objective in the experiment was to stimulate the host immune reaction against TAA encapsulated into liposome in tumor bearing mice and to assess the effect on tumor regression.

3.2 TUMOR REGRESSION STUDIES

Immune responses against TAA were induced in DEN-exposed mice by using liposome as vehicle to deliver the antigen to the immune system. Presence of circulatory antibodies against TAA and the induction of cellular responses in immunized mice were monitored using ELISA and in vitro cell proliferation assay of lymphocytes respectively. The tumor regression studies in these animals were carried out by monitoring the same parameters used for the induction of carcinogenesis..

3.2.1 Entrapment of TAA into Liposome

Generally, liposome prepared by Dry film method yields low entrapment effenciecy. However, an advantage of Dry film method is that it does not involve any organic solvent, detergent or sonication that may cause denaturation and alter the immunogenicity of TAA. The method used for TAA entrapment has been found simple and highly reproducible.

Table 2 Lipid composition and percent entrapment of TAA into liposome

Liposomal phospholipids (mg)	Molar Ratio	TAA (μg)	% Entrapment means ± SD
DPPC : Chol : DCP (5.0 : 2.5 : 1.0)	1.0 : 0.9 : 0.25	1000	30.71 ± 2.20

Lipid composition of liposomal membrane and the entrapment efficiency of TAA into liposomes are shown in Table-2. An average of 30.71 % entrapment was achieved.

3.2.2 Antibody response

The anti-TAA antibody concentration in blood serum was determined using ELISA. Mice immunized with TAA in saline served as positive control to compare the antibody responses elicited by liposomal-TAA formulation. The antibody level was determined at two different time points i.e. on day 3 and day 15 after administering the booster injections. As

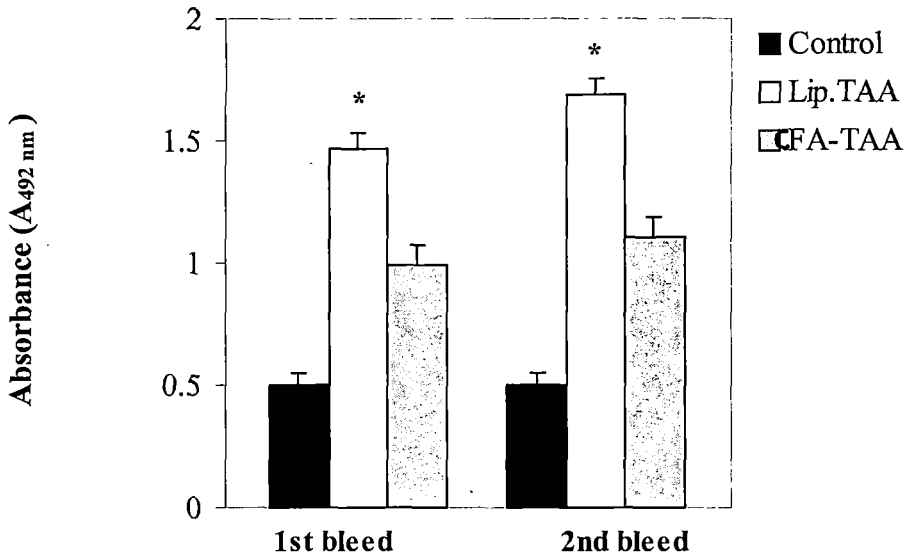


Figure 8 Humoral immune response against tumor-associated antigens in DEN-exposed mice. Antibody titer was measured separately in pooled immune sera of different group of animals collected at different time points. The means ± SD of the antibody in triplicate wells as determined by ELISA in 1:10 diluted serum sample.
* $P < 0.001$ against Control.

shown in figure 8, immunization by liposomal TAA formulation elicited significantly high ($P < 0.001$) antibody level in comparison to the control. Antibody response elicited by liposomal-TAA formulation was substantially higher but statistically not significant in comparison to Fruend's adjuvant (Figure 8 & Table 3).

3.2.3 Anti-TAA antibody specificity

Specificity of the antibody to the over expressed glycoprotein (approximately 68 kDa mol.wt.) in DEN-exposed mice was checked by western immunoblot analysis as discussed in method and material section. Cross reactivity with the above specific glycoprotein was observed along with other major proteins transferred on nitrocellulose (Figure 9).

Table 3 Humoral immune response against tumor-associated antigens.

Mice group	Absorbance (A_{492nm}) Mean ± SD	
	1st bleed	2nd bleed
Control	0.4976 ± 0.0550	0.498 ± 0.055
Lip.-TAA	1.4694 ± 0.0653*	1.69 ± 0.0650*
CFA.-TAA	0.9950 ± 0.0813	1.105 ± 0.0813

* $P < 0.001$ against control.

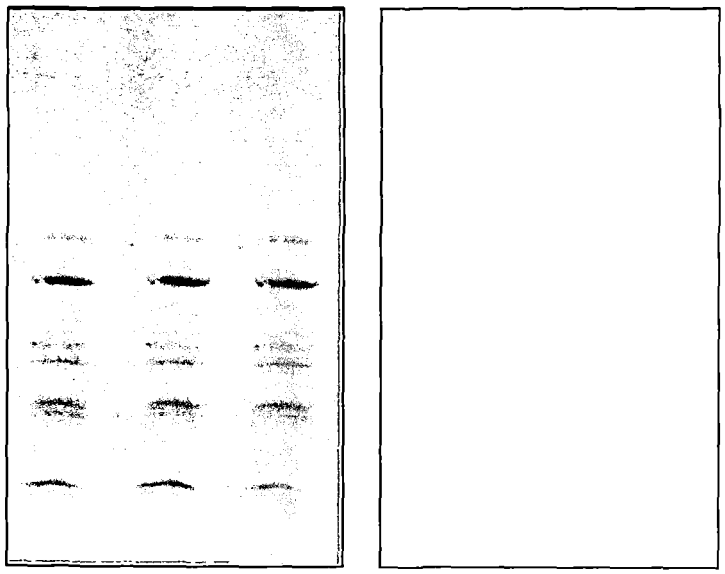


Figure 9. Immunobloting after SDS-PAGE separation on Phast Gel gradient 10-15. The transfer to a NC membrane was performed for 20 minutes at 20°C. The blot was reacted with mice antisera directed against TAA, incubated with Protein A-peroxidase conjugate and the immune complex was visualized by DAB. Left: the gel stained with Phast gel Blue R. Right: immunoblot of TAA.

3.2.4 Cellular response

Induction of cellular immune response is usually measured by delayed type hypersensitivity reaction or lymphocyte proliferation. We monitored it by BrdU incorporation

into the DNA of replicating cells. The incorporation of BrdU in place of tritiated thymidine was monitored as a parameter for DNA synthesis. BrdU incorporates into freshly synthesized DNA. After fixation of cells, a peroxidase labled antibody (monoclonal) to BrdU was added that binds to BrdU and the enzyme activity was measured spectrophotometrically after adding substrate for peroxidase. BrdU incorporation by different populations of lymphocytes is shown in Figure 10. It was observed that for every 10 folds increase in cell number, there was an increase of 0.16 - 0.34 in OD. This experiment was carried out to know the increase in OD when the lymphocytes are increased by 10 fold, which may help to find out the number of cell division cycle during in vitro culture of proliferating cells.

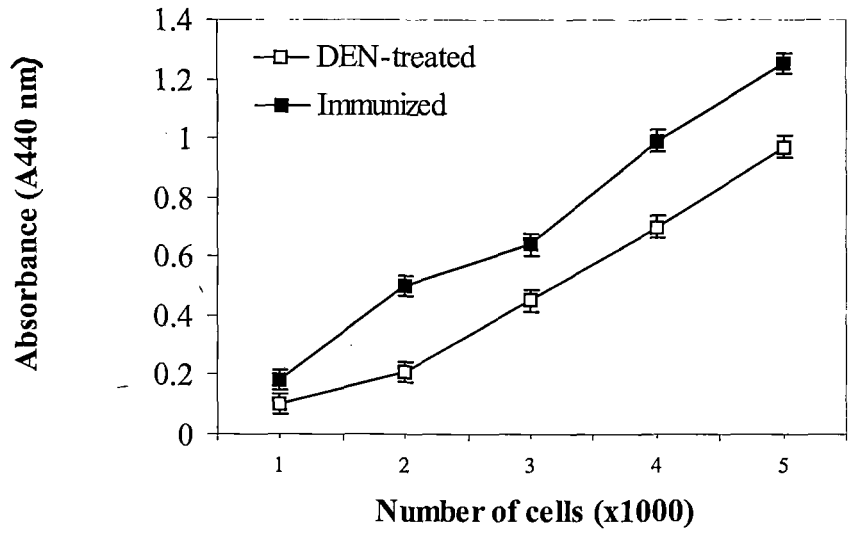


Figure 10 BrdU incorporation into DNA of lymphocytes of DEN-treated and immunized mice. Immunized mice were sacrificed on 3 day after booster injection. Lymphocytes from spleen were harvested and were reciprocally diluted in DMEM containing 10% heat inactivated FCS (fetal calf serum), 2 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine-monohydrate and assayed immediately for BrdU incorporation. Each point shows the mean SD of 3 replicates from five mice each.

In the other set of experiment, where the cell proliferation was monitored at different time intervals, it was observed that incorporation of BrdU was more significant for cells

cultured for 6 and 12 hours, than those cultured for 24 h as is evident from the increase in absorbance (Figure 11). Non-immunized lymphocytes (obtained from age-matched normal mice) when assayed for BrdU incorporation after culturing for different time points did not show any increase in the absorbance (Figure 11).

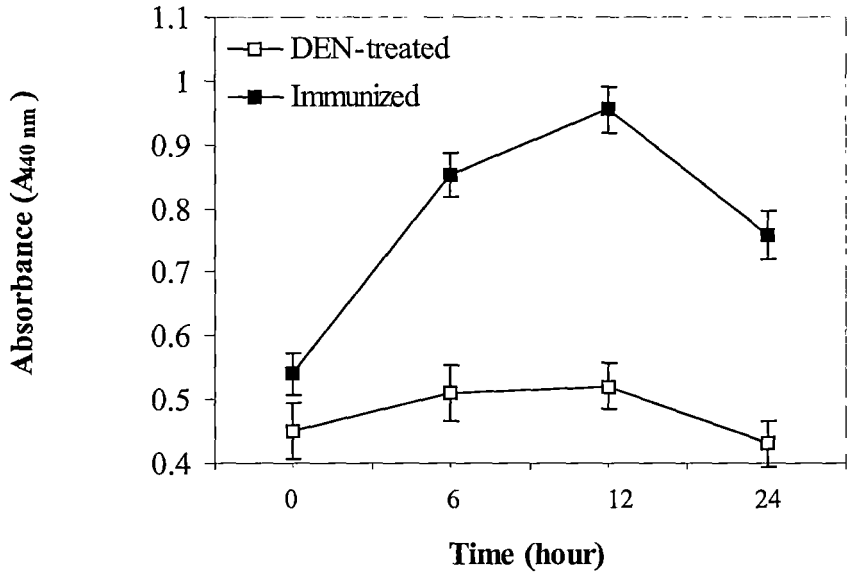


Figure 11 BrdU incorporation into DNA of proliferating lymphocytes. RBC deprived lymphocytes ($3x10^5$/100 µl of culture medium) were seeded into wells of ELISA plate and cultured *in vitro*. BrdU was performed at zero point and after 6,12 & 24 h culture. Each point shows the mean ± SD of 3 replicates from five mice each.

3.2.5 Metabolic Assay

Since the proliferating cells are metabolically more active than non proliferating (resting) cells, therefore, the metabolic activity of lymphocytes upon immunization was monitored using MTT proliferating Kit as described in material and method section. The results given in Figure 12 show the metabolic activity of viable immune and resting lymphocytes. The immune lymphocytes were found to be metabolically more active than the resting one as is evident from the observed increase in absorbance (Figure 12, table 4).

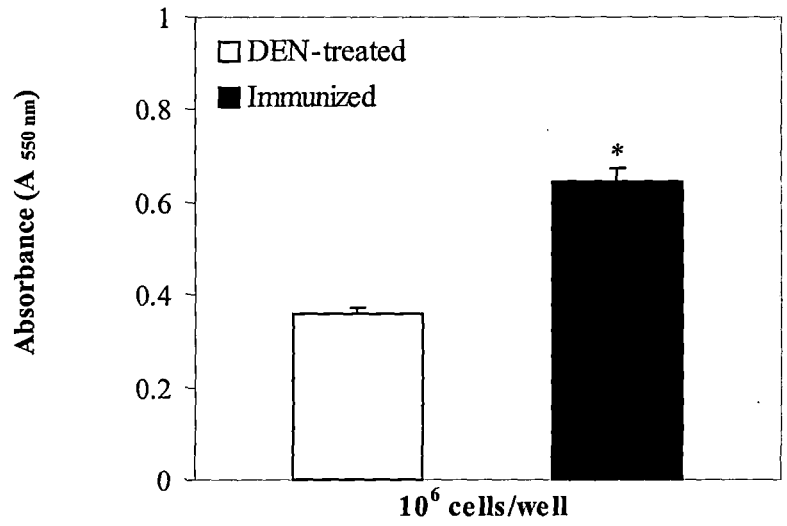


Figure 12 Absorbance measurement as a function of metabolic activity of viable cells. Lymphocytes (10^6/well) were taken from DEN-treated and immunized mice. Metabolic activity was measured using the MTT Cell proliferation Kit. Each point shows the mean ± SD of 3 replicates from five mice each.
* $P < 0.0001$

Table 4 Metabolic activity in viable and resting lymphocytes.

Mice group	Metabolic activity (A_{550nm}) Mean ± SD
DEN-treated	0.3606 ± 0.0101
Immunized	0.6465 ± 0.0265*

* $P < 0.0001$.

3.2.6 Histological Analysis following immunization

Histological study was also taken up for monitoring the regression process. The histological section of liver tissue of immunized mice exhibited a significant change in the morphology when compared with DEN-exposed liver tissue (Figure 13). The hepatocytes of immunized mice found returning towards normal morphology and cell shape. The number of multinucleated cells were also less in comparison to DEN-treated.

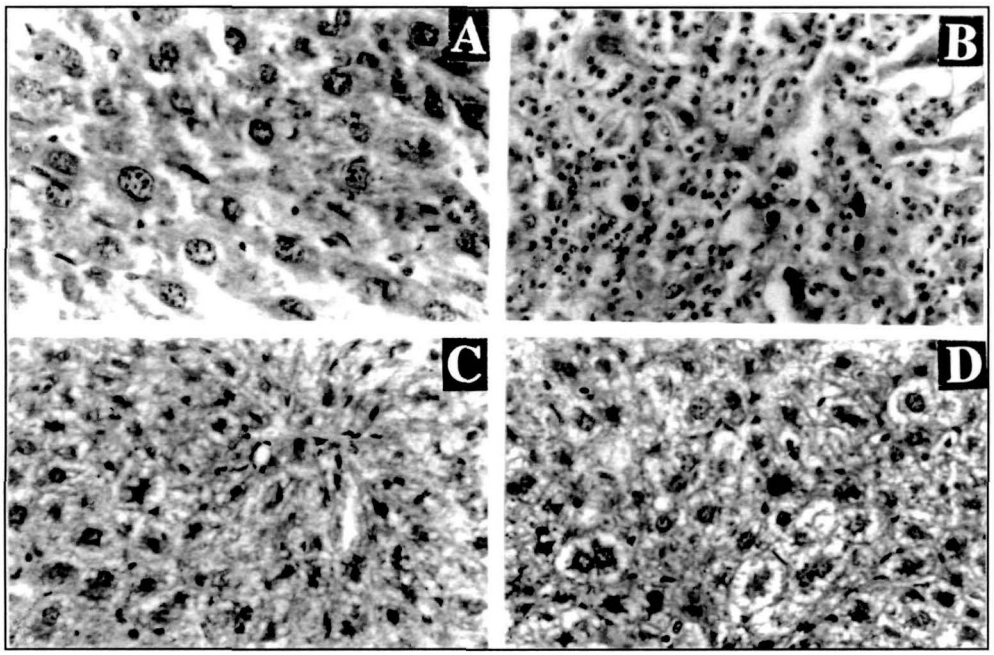


Figure 13. Liver sections of mice showing hepatic cells. Normal (A), DEN exposed (B) and Immunized: 40 day (C) & 60 day (D), Haematoxyline and eosin counterstain. M X250.

3.2.7 Marker Enzymes activities following Immunization

The activities of the marker enzymes in mice after immunization showed a trend declining towards normal value (Figure 14 & 15). A significant decrease both in GGT and AChE observed upon immunization when compared with DEN exposed mice and was found statistically significant ($P < 0.001$). The values of marker enzymes activities of age-matched normal control, DEN-exposed and immunized mice are given in Table 5.

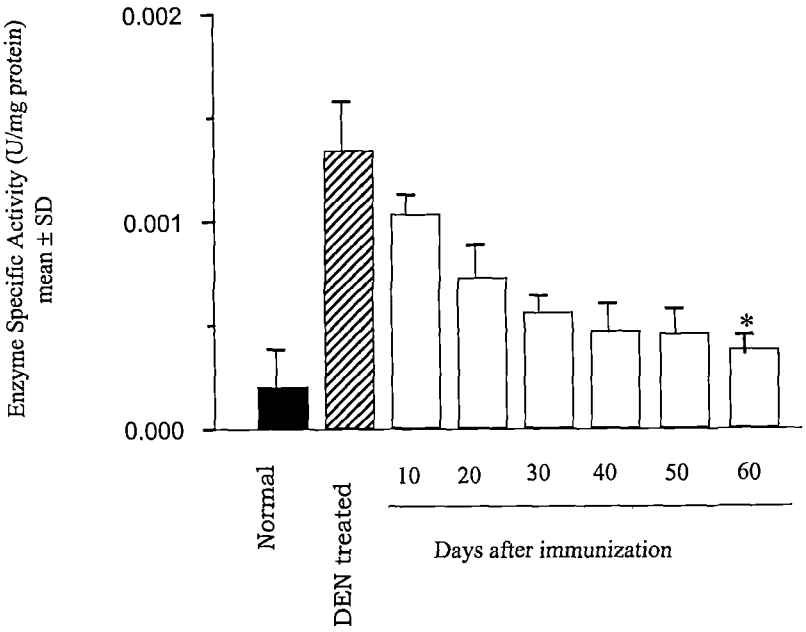


Figure 14 Liver GGT Specific Activity in the supernatant fraction of Normal, DEN treated and Immunized mice.
* $P < 0.001$ against DEN treated, n = 17.

Table 5. Gamma glutamyltranspeptidases specific activity in normal, DEN treated and immunized

Mice group	Days after immunization.	Specific Activity (U/mg protein) Mean ± SD
Normal	-	0.0002077 ± 0.000179
DEN treated	-	0.0013501 ± 0.000073
Immunized	10	0.001038 ± 0.0000784
	20	0.000736 ± 0.0001475
	30	0.000566 ± 0.0000577
	40	0.000473 ± 0.0001551
	50	0.000460 ± 0.0001345
	60	0.000383 ± 0.000070*

*$P < 0.001$ against DEN treated, n = 17.

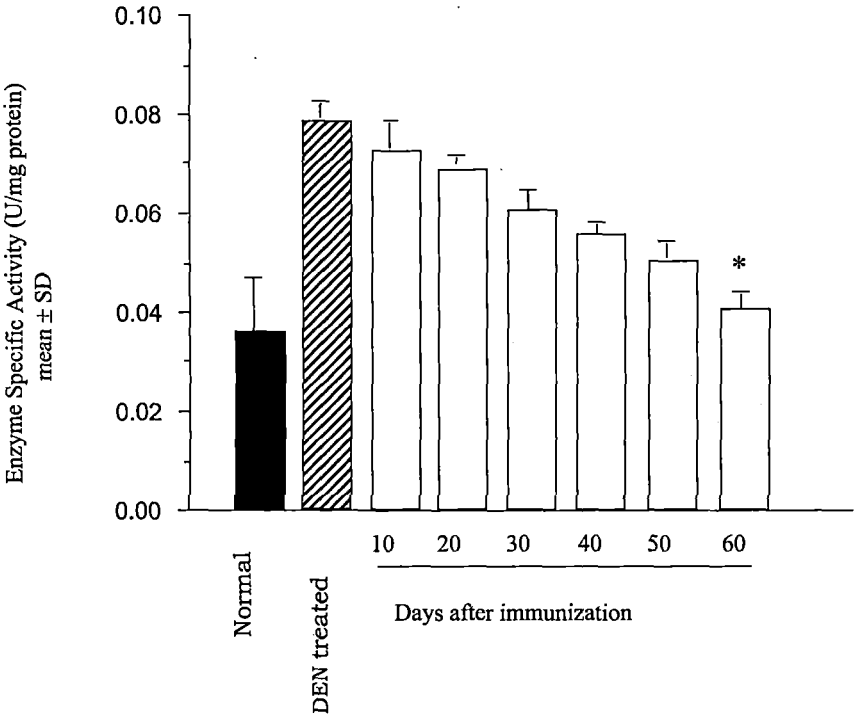


Figure 15. Liver Acetylcholine esterase Specific Activity in the supernatant fraction of Normal, DEN-treated and Immunized mice after DEN administration.
* $P < 0.001$ against DEN treated, n = 17.

Table 6 Acetylcholine esterase specific activity in Normal, DEN treated and immunized

Mice group	Days after immunization.	Specific Activity (U/mg protein) Mean ± SD
Normal	-	0.036 ± 0.011
DEN treated	-	0.079 ± 0.011
Immunized	10	0.073 ± 0.005
	20	0.069 ± 0.001
	30	0.061 ± 0.004
	40	0.056 ± 0.002
	50	0.051 ± 0.007
	60	0.041 ± 0.003*

*$P < 0.001$ against DEN treated, n = 17.

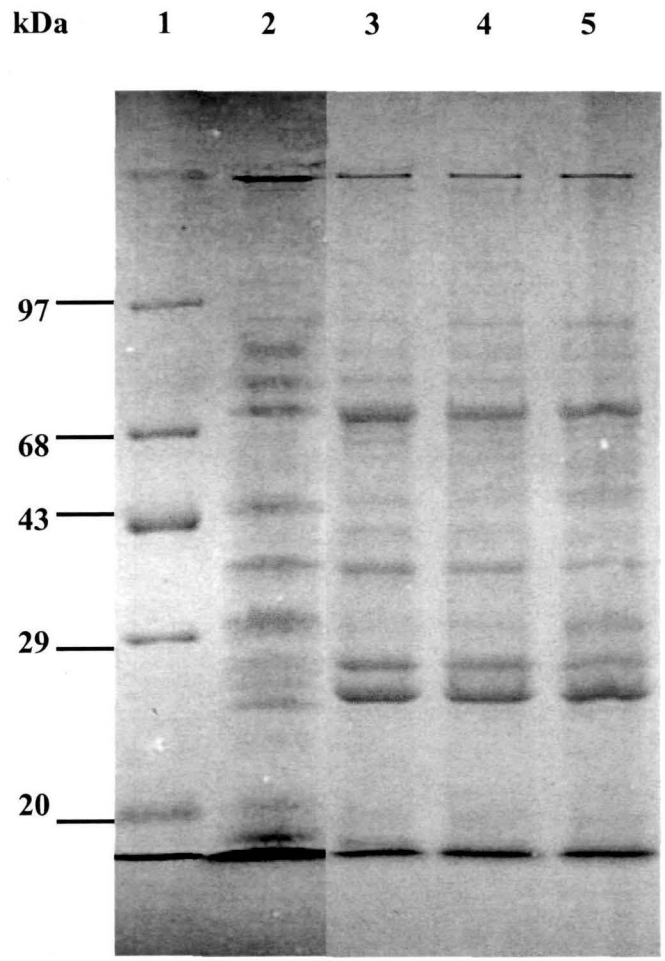


Figure. 16 1-Butanol extract of mouse liver membrane glycoproteins resolved by SDS-PAGE (10%). Tris-glycine gel stained with Coomassie Brilliant Blue. Lanes: (1) Molecular weight markers; (2) Control, (3) DEN-treated, (4) 30 day, (5) 60 day after booster injection (DEN exposed and immunized).

3.2.8 SDS-PAGE ANALYSIS

Equal amount of protein (TAA-extract) was loaded on each well along with mol.wt markers and resolved on 10 % acrylamide gel. No difference in protein profile was observed visually in the immunized (lane 4 & 5) when compared to that of DEN-treated (Figure-16).

4 Discussion

Carcinogenesis being a multi-step process involves multiple alterations in the structure and functions of the normal cells. Consequently, any intervention to the process of carcinogenesis may also utilize the same molecular mechanism to check further development of cancer and possibly, reverse the process. Immunotherapy is one such intervention. The present investigation, therefore, was aimed to study the effects of immunization against TAA on tumor regression in mice bearing tumor. The work embedded in this thesis comprises the study on tumor induction and the effect of immunization on tumor regression.

Diethylnitrosamine (DEN), an established hepatocarcinogen (109) was used to induce carcinogenesis in experimental mice. It has been reported that DEN is itself a carcinogen which, does not require promoters for its activation. DEN undergoes metabolic activation in the liver through enzymatic oxidation and the ultimate carcinogen form is an ethyl carbonium ion, that ethylates with the nucleophilic centers of DNA, RNA and proteins. The hepatocytes were initiated by an intravenous administration of DEN at a dose of 20 mg kg^{-1} body weight at weekly intervals for a period of two months. This dose causes necrosis and a regenerative hyperplasia extending to numerous initiated hepatocytes. Several procedures have been described, which selectively and rapidly stimulate the growth of the carcinogen altered cells into foci islands of hepatocytes following initial treatment with a carcinogen, which resulted in an increased cancer incidence (97, 114).

The body mass index of each animal during the entire period of DEN-treatment was recorded at regular intervals and compared with age-matched normal control mice (Figure 1). Significant decrease was observed in the body weight of

animals upon DEN exposure. This is likely due to the effect of carcinogen on the physiology and metabolic activities, since these animals recovered their body weight up to a significant extent when the treatment is over. Majority of the animals survived, however, signs of lethargy and sluggishness were noticed during the treatment.

Because of its metabolic capacity, the liver is the target for many carcinogens. During liver carcinogenesis, several distinct hepatocellular lesions precede the development of carcinomas (116). The first lesions to appear is the altered focus, and it can be demonstrated in routine histologic tissue sections when sufficiently developed. However, more sensitive techniques permit reliable and objective identification at earlier stages by detecting abnormal characteristics of altered foci. For instance, in rat liver, abnormalities in the activities of enzymes such as γ-glutamyl transpeptidase, glucose-6-phosphatase, and adenosine triphosphatase have previously been used as markers for initiation of carcinogenesis (32, 90, 33, 131). Another finding that may have application in carcinogen screening is that GGT-positive foci is accompanied by an elavation of serum GGT (103). This change could be monitored in animals to determine the most appropriate time to terminate the induction study in animals.

The activities of the marker enzyme γ-glutamyl transpeptidase both in serum as well as liver tissue underwent a marked elevation following DEN treatment (Figure 2 & 3). High concentration of GGT has been reported in the liver, bile ducts, and the kidney (93, 38). However, the GGT activity in liver is negligible in adult mice, and the activation of GGT activity is achieved only in case of spontaneous or experimentally induced hepatocarcinogenesis (24, 14, 39). A strikingly high and rapid elevation of liver and serum GGT has also been reported to occur after treatment with a hepatocarcinogen (12,13). In fact it has been reported that after a chronic treatment and until the development of hepatocellular carcinoma (HCC), a 20 to 60 fold increase in liver GGT can be achieved which is comparable to the activity measured

in fetal rat liver (17, 20). The level of GGT, however, seems to be independent of the malignancy of the tumor, as all transplantable hepatomas investigated showed a high GGT activity, regardless of the degree of differentiation and the rate of proliferation (14). Therefore, the reappearance of GGT in treated animals, indicates the reactivation of the enzyme on cancerization of hepatocytes, and this reappearance is often referred to as a 'carcino-embryonal' feature (14). This high GGT level is a characteristic of carcinogenic transformation and not rapid proliferation of hepatocytes (24). These observation, thus indicate that the hepatocytes had undergone cellular transformation, and it was further substantiated by other parameters studied.

Alteration in plasma membrane is a known feature characteristic of cellular transformation. Acetylcholine esterase (AChE), an oligomeric enzyme, is predominantly membrane bound and its activity in the soluble fraction of liver has been used as a parameter for studying membrane changes (Sharan et al 1995). Any cellular transformation brought upon under the influence of the administered DEN could therefore, be monitored by assaying AChE activity. Since, AChE rapidly hydrolyses the acetylcholine to acetate and choline, and so it efficiently terminates the chemical impulse, thereby setting the basis for rapid, repetitive responses and enabling the re-uptake (and recycling) of choline (4). AChE activity was found elevated significantly, both in the serum and supernatant fraction of liver following DEN administration (Figure 4 &5). The hyper-activation of the AChE activity in DEN-exposed mice also signifies hepatocellular transformation.

Cancer is a disorder of cells, and so for diagnosis and assessing of tumors, microscopic examination of tissue is a reliable method for routine use. Histological differentiation is concerned with alterations in the structure of the tissue, i.e. the relationship of cells to each other and to their underlying stroma. Histology by microtomy technique was carried out to see the morphological alterations in liver cells

upon DEN-exposure. The microphotographs of DEN-exposed liver cells exhibited significant alterations in the morphology (Figure 6). The liver of the treated animals seemed to have undergone a lot of alterations with the following characteristics:-

(a) That there was a loss of the normal regular arrangement of cells. Cells were disorganized and disoriented in the DEN treated as compared to the normal control animal which have smoother, well aligned columnoid, distinct and defined cell arrangement.

(b) That the variations in the cell shape and size were also observed indicating loss of normal characteristics which is known as dedifferentiation or anaplasia. The cell size of DEN treated were smaller with distorted and ill-defined cell shape as compared to the normal hepatocytes which had a definite shape and their size were almost regular.

(c) That the DEN treated hepatocytes also showed an increase in density of staining which reflected an increase in total DNA. This is due to the condensation of chromatin, which indicates an increase in mitotic activity.

These morphological changes in hepatocytes, therefore, indicate that the liver cells of DEN treated mice were in an active state of cell division and thus, suggests a state of carcinogenesis in liver.

Several methods have been reported for the extraction of membrane surface proteins/glycoproteins. In the present investigation, butanol-extraction method (71) was employed. This method extracts exclusively the membrane surface glycoproteins. It is believed that this organic solvent competes effectively for the polar side chains of the protein with alcohol and displaces the lipid, thus causing the dissociation of the lipoprotein complex (91).

The TAA-extracts obtained from DEN-treated and age matched normal control mice were subjected to resolution on SDS-PAGE. No difference in protein

profile was observed. However, the butanol extract of DEN-treated mice showed a significant over-expression and under-expression of several surface membrane glycoproteins (Figure 7), such as a glycoprotein of mol. wt. approximately 68 kDa found over-expressed and few glycoproteins of mol. wt. between 29 kDa and 20 kDa were under-expressed. The butanol extraction method, exclusively extracts surface membrane glycoproteins and therefore, the above observation indicates that DEN inflicted major alterations in cell surface membrane glycoproteins as is evident from the different intensity of the protein bands in the SDS-PAGE gel. These alterations could be involved in causing distortion of cell membranes upon DEN treatment.

The loss in body weight, the elevation of the marker enzyme activities, the distinctive features of hepatocytes depicted in histology and the differential expression of membrane surface glycoproteins are the direct evidences of hepatocellular transformation and induction of carcinogenesis in mice upon DEN treatment.

Animals after having complete DEN treatment were immunized against TAA. Three formulations i.e. TAA alone in saline, Liposomal–TAA and TAA emulsified with CFA were used for induction of immune response. Induction of immune response was followed monitoring both the humoral as well as CMI responses. Mice immunized with TAA alone served as positive control. The regression studies in these animals were carried out after administering the booster injection and by monitoring the same parameters used for the induction of carcinogenesis followed it.

Due to low immunogenicity TAA alone could not mount a strong rejection response against the host's tumor. Therefore, in order to potentiate the immune responses, liposomes were used as a carrier and as an adjuvant for the presentation of TAA to the immune system. The liposome encapsulated TAA formulation used is

self-contained without further addition of other adjuvant materials or carrier molecules.

Dry film method was used to encapsulate TAA into liposomes. At present there are several methods of preparing liposomes, each having their own advantages and disadvantages (87). One of the major disadvantages of these methods is that the protein to be entrapped into liposomes requires exposure to the organic solvent or detergent, which may lead to denaturation. Dry film method is simple and highly reproducible which involves extremely mild conditions so that the immunogenicity of the entrapped material remains unchanged. Although, the entrapment efficiency produced by dry film method has been significantly low in comparison to the other conventional methods, but found highly suitable for entrapment of TAA. The liposomes, thus produced appeared to be relatively more stable than those produced by other methods such as reverse phase evaporation, detergent dialysis, alcohol or ether injection methods etc. More significantly, the immunoreactivity of the entrapped TAA was preserved, which is much more important for this study. Low entrapment efficiency by this method is reported in literature.

The phospholipid composition of liposomal membrane is a determining factor in the humoral immunogenicity of haptenated liposomes. Liposome made up of DPPC, induce a much higher antibody response than prepared from egg PC, which is mainly because of their phase transition temperature. Membrane fluidity can be increased or decreased by the addition of cholesterol. An intermediate fluidity of the bilayer is required for the induction of an optimal humoral response to haptenated liposome (1). Thus the lipid compositions presently used in the liposome preparation are likely to be more appropriate for induction of immune response.

The liposomal-TAA formulation elicited significantly high antibody response in comparison to TAA-alone and was equivalent to the response elicited by TAA

emulsified with CFA, the most potent adjuvant known so far (Figure 8). Encapsulation or incorporation of antigens into liposomes markedly enhances the immunogenicity of the antigen. It has been shown that viral glycoproteins encapsulated into liposomes has resulted in enhancement of the humoral response as seen in the rise of serum antibody levels which is several folds higher than that elicited by free antigen alone (63).

Liposome-associated antigens elicit IgG response in addition to the preceding IgM response and generate the immunological memory, and thus behave as real T cell dependent (TD) antigens. Protein antigens also elicit an immune response when given in its free form. However, the response is very poor in comparison to liposomal antigen. It has also been demonstrated that antigens either encapsulated into liposomes or exposed on their outer surfaces both induce significantly high antibody response (122).

Macrophages play an important role in the processing and presentation of liposome-associated antigens. Liposomes are effective in targeting the antigens to macrophages, consecutively allowing these cells to process and present the associated antigens to T cells. The importance of macrophage-mediated presentation of liposomal-TAA to the immune system has been investigated and a correlation found between the response (IL-2 production) of antigen-specific T cells and the uptake and processing of the liposomal antigen by macrophages in vitro (106).

Thus, the immune response evoked by liposomal-TAA formulation is likely to be the result of targeting to antigen presenting cells (APC). Following administration liposomes are taken up by the reticuloendothelial system. They are ingested by macrophages, that consecutively digest the phospholipid bilayers using lysosomal phospholipases, and it is probable that liposome-mediated targeting of antigens to macrophages forms part of the mechanism of the immuno-adjuvant activity of

liposomes (27,28). Our findings therefor, are in agreement with these observations. Further, in studying the efficacy of liposomal-TAA formulation over TAA emulsified with CFA, it was observed that the formulation is in equivalence to the CFA-TAA formulation (Figure 8).

The antibody specificity to the over expressed high molecular weight glycoprotein in the DEN treated mice was checked by western immunoblot analysis. The immune sera contained antibody to the above specific glycoprotein molecule along with other major proteins transferred on nitrocellulose (Figure 9). These findings support that a good immune induction could be achieved with liposome entrapped antigens and therefore, this particular approach could be useful to elicit immune response to a self-existing bio-molecules. Antibodies may kill tumor cells in vitro by complement dependent lysis or by mediating cell-dependent mechanisms. Clinical responses in some patients treated with monoclonal antibodies have been observed (57, 85). However, the presence of antibodies on tumor cells are not favorable as they can block the immune response *in vivo* (11) or can form antigen-antibody complexes (94) that can induce suppressor cells. Generally, it is believed that in most cases cell-mediated responses rather than humoral responses are of prime importance in tumor rejections.

When the antigen presentation is in the appropriate MHC context it would result in T cell activation. More precisely, soluble antigen is acquired and processed by professional antigen-presentation cells (APCs) and presented in the context of major histocompatibility complex class II molecules to tumor-reactive CD4+ T - helper (Th) lymphocytes. The resulting activated CD4+ T cells provide 'help' to antigen-specific CD8+ T cells in the form of cytokine(s). These CD8+ T cells have presumably been directly activated by target cells presenting endogenously synthesized antigenic peptide in the context of their MHC class I molecules. It was therefore, studied the induction of cellular responses in mice immunized with

liposomal-TAA formulation. Cellular induction of immune responses usually measured by delayed-type hypersensitivity reaction or lymphocyte proliferation (96. 101). In the present study the lymphocyte proliferation monitored by quantitating BrdU incorporation into the DNA of replicating cells. Since, the assay is based on a microtiter plate format and the color development is measured spectrophotometrically, it was desirable to know the change in ratio of absorbance in the reciprocally diluted lymphocytes in order to find out the number of cell division cycle during *in vitro* culture. BrdU incorporation for different populations of lymphocytes is shown in Figure 10. It observed that for every 10-fold increase in cell numbers, there was an increase by 0.16- 0.34 in the absorbance. Based on these observations, same numbers of the normal and immune lymphocytes were cultured for 0, 6,12 and 24 hrs respectively and assayed for BrdU incorporation. The immune lymphocytes showed significant rise in OD at 6 and 12 hrs which, clearly indicates that the cells had undergone proliferation, whereas, the normal (resting) lymphocytes did not show any significant change in absorbance (Figure 11). However, a significant cell death occurred both in the resting as well as rapidly dividing cells when cultured for more than 12 hr. Therefore, the decrease in absorbance at 24 hrs time point may be due to cells death, since the BrdU incorporation occurs only in viable cells.

The change in OD observed after 12 hr culture (Figure 11) signifies a ten folds increase in cell numbers when compared with the reciprocally diluted cells (Figure 10). It, thus appears that the immune lymphocyte had undergone about three cell division cycle during culture. This observation is in agreement with the fact that the lymphocytes upon antigenic stimulation undergo 3 to 4 cell division cycles in every 24 hrs up to 4 or 5 days *in vivo* system.

Since the proliferating cells are metabolically more active than non-proliferating (resting) cells. Therefore, the metabolic activities of both the cells were studied using cell proliferation kit (MTT). The tetrazolium salt (MTT) has been used

to develop a quantitative calorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of the activation of the cells. This method is therefore used to measure cytotoxicity, proliferation, or activation of metabolically active cells. The results can be read on a multi-well scanning spectrophotometer (ELISA reader) and show a high degree of precision. MTT is cleaved to formazan by the "succinate-tetrazolium reductase" system that belongs to the mitochondrial respiratory chain and is active only in viable cells. The purple formazan salt crystals formed dissolved by adding solubilization solution and the solubilized formazan product was spectrophotometrically quantified. Immune lymphocytes were found metabolically more active than the normal resting lymphocytes as was evident from increase in the absorbance measured at 550 nm. (Figure 12). The reduction of MTT to a formazan product appears to be carried out by all the cell types. These include mitogen stimulated T and B cells myeloma, T lymphoma and macrophage-like tumor cell lines, as well as various IL-2 dependent T cell lines (92). Thus the observation (Figure 12) further support that the lymphocytes of DEN-exposed mice were sensitized by the liposomal antigens *in vivo*, and they became metabolically more active during cell proliferation.

These observations, therefore, suggest that vaccine based on liposomal-antigens formulation in particular with unfractionated cell extracts as a source of tumor antigen may be equally effective to induce immune responses to that of other cell-base modified tumor vaccines reported recently (1, 134). Vaccination with tumor cell extracts circumvents the need for identifying specific tumor antigens and hence extends the use of active immunotherapy to the vast majority of cancers, in which specific tumor antigens have not been identified.

After having established that liposomal-TAA formulation can generate both humoral as well as CMI responses in DEN-exposed mice, regression studies were

carried out. The parameters monitored for regression were those employed for induction studies.

During immunization, animals did not show any decrease in the body weight as it was observed during DEN treatment. However, majority of animals had more or less constant body weight in comparison to control in which it increased linearly. Immunization it self did not cause any casualty.

The regression studies by assays of marker enzymes in mice upon immunization showed a trend declining towards normal value. A significant decrease both in GGT and AChE level was observed in comparison to the DEN-exposed mice (Table 5). However, the normal value could not be achieved for the marker enzymes, this might be due to the various factors which induce suppression mechanism as has been reported earlier (94). Both of the marker enzyme activities exhibited a common trend in all immunized animals. Decreased tumor incidence and decreased tumor growth rate were seen in animals injected with viable tumor cells after immunization with liposomal-glycolipid (9,54).

The treatment of the tumor with anti-neoplastic drugs, radiation or immunotherapy, all includes the basic mechanism of programmed cell death or apoptosis. Without apoptosis, there is practically no tumor regression, of any kind (61). The immunological mechanism is probably the main effector mechanism for tumor regression with its trigger being apoptosis. Therefore, histological study was also taken up for monitoring the tumor regression process. The microphotographs, which were taken at the same magnification (M X40), exhibited notable difference in the morphology of hepatocytes (Figure 13). The distinctive features that observed in DEN-exposed liver cells were lost and the cells attained more towards the normal features in the immunized animals. However, the level of expression of membrane surface glycoprotein remain unchanged. The SDS-PAGE profile of the butanol liver extract obtained from immunized animals showed a similar pattern of the protein

bands as that observed in DEN treated animals (Figure 16). Since the results are primitive and preliminary, it is difficult to correlate this particular observation with the regression of carcinogenesis.

The experimental results, thus obtained from the regression studies, suggest a partial recovery of chemically induced carcinogenesis upon immunization. The return of marker enzymes activities toward the normal value, improvement over the body weight of the animals and the histological examination of the liver cells following immunization support a positive effect on tumor regression with liposomal-TAA formulation.

Cancer vaccines have become the major clinical interest of tumor immunologists. While many of the vaccines currently under investigation are designed to generate CD8+ T cell responses, it is clear from studies of basic immunology that CD4+ T cells are a crucial component of potent and long-lasting immunity. They provide help for generating CTL and IgG responses, are important in maintaining immune memory and can directly kill target cells via lysis or cytokine secretion. Thus, there is growing interest in defining tumor-associated antigens recognized by helper T cells, so that these may be incorporated into new, and potentially more effective, cancer vaccines. Although there is no existing cloning methodology for discovering antigens restricted by MHC class II that can match the efficiency of the genetic methods successfully used to identify antigens that are restricted by MHC class I, a number of different approaches are currently being explored. Perhaps the most straightforward of these is to screen candidate antigens for recognition by CD4+ T cells. Thus, CD4+ T cells specific for an autologous tumor, that are raised from cancer patients (potentially following vaccination), can be tested for recognition of tumor-associated antigens that are lineage-specific, over expressed or commonly mutated. Following this approach, CD4+ T cells from a melanoma patient that were restricted by HLA-DR4 and that recognized a melanocyte lineage-

specific antigen were found to be specific for non-mutated tyrosinase epitopes (120, 119).

There are good evidences that tumor-specific cytotoxic T cells are responsible for destruction of tumor *in vivo*. Although helper T cells participate in the induction and regulation of cytotoxic T cells, the destruction of tumor cell is achieved by the CD8+ cytotoxic T lymphocytes (CTL) with specificity for the antigens on the surface of the tumor cell. This preference has been bolstered by numerous adaptive transfer studies in which CD8+ T cell lines and CD8+ clones specific for tumor antigens that have been stimulated *in vitro* can mediate antitumor immunity when transferred back into tumor-bearing hosts (104). Furthermore, recent reports suggest that immunization using either adjuvant or dendritic cells with pure tumor peptides can result in productive antitumor immunity that is restricted by MHC class I (35, 79).

While most of the focus is on CD8+ cytotoxic T lymphocyte responses, recent evidence indicates that CD4+ T cells are an equally critical component of the antitumor immune response. They provide help for generating CTL and IgG responses, are important in maintaining immune memory and can directly kill target cells via lysis or cytokine secretion such as tumor necrosis factors (TNF) and INF-12. The critical role for CD4+ T cells in induced antitumor immunity has been consistently demonstrated in vaccine/challenge experiments employing antibody-mediated depletion of CD4+ T cells or using CD4-knockout mice. Abrogation of antitumor immunity in CD4-knockout mice or mice depleted of CD4+ T cells has been demonstrated in cases of cell-based vaccines, recombinant viral vaccines and recombinant bacterial vaccines (34, 22, 52, 51, 50).

The ability of adaptively transferred tumor-specific CD4+ T cells to mediate antitumor immunity also suggests that they may mediate other effector pathways. Recent evidence in cell-based vaccine models indicates that CD4+ T cells can

mediate a number of antitumor effector pathways independent of CTLs. In particular, there is direct evidence for both Th1 and Th2 effector pathways that, respectively, either activate macrophages to produce reactive oxygen intermediates or activate eosinophils; these are critical effector pathways. In these studies, activated eosinophils and macrophages were found in the tumor-challenge site of vaccinated animals. These activated macrophages and eosinophils were dependent on the presence of CD4+ T cells in the animals but not on CD8+ T cells. Thus, independently CD8+ T cells and CD4+ T cells that are specific for tumor antigens were critical in orchestrating activated macrophages to produce nitric oxide and superoxides and were critical in recruiting and activating eosinophils; the CD4+ T cells, macrophages and eosinophils mediated the complete antitumor response. In these studies, as with the studies of adoptive transfer of CD4+ T cells in the FBL-3 murine leukemia tumor model, the tumors themselves were negative for MHC class II. The most plausible model to account for this is that tumor antigens released at the challenge site are ingested, processed and presented to CD4+ T cells by macrophages within the tumor bed; primed, tumor-specific CD4+ T cells secrete lymphokines in response to antigen presentation by these macrophages. The results, taken together, suggest a model in which CD4+ T cells orchestrate multiple effector arms of antitumor immunity including CTL activation, macrophage activation and eosinophil activation. While the products of activated macrophages (i.e. reactive oxygen intermediates) and activated eosinophils are themselves not antigen specific, antigen specificity is nonetheless mediated geographically by the localized activation of CD4+ T cells by macrophages that are positive for MHC class II.

In the present study, the initial laboratory investigation support that a good immune induction could be achieved with liposomal formulation and therefore, this particular approach could be useful to elicit immune responses against tumor-associated antigens. Our observations, further suggest that vaccine based on liposomal antigen formulation in particular with non-fractional cell extracts as a source of tumor

antigen may be equally effective to induce immune responses to that of the cell based modified tumor vaccine. Vaccination with tumor cell extracts circumvents the need for identifying specific tumour antigens and hence extends the use of active immunotherapy to the vast majority of cancers, in which specific tumor antigens have not been identified.

5 Conclusion

Based on the results obtained from the experiments carried out, it may be concluded that:

1. Chronic exposure of DEN to Swiss albino mice induced cellular transformations in the liver, as substantiated by the pronounced alterations in the activities of the marker enzymes such as γ-glutamyl transpeptidase and acetylcholine esterase.

2. DEN treatment resulted in a distinct change in the morphology of the hepatocytes such as the variations in the cell shape and size, appearance of more densely stained nuclei and multinucleated cells as elucidated in the histological studies.

3. Cell surface membrane glycoproteins in liver exhibited differential expression upon DEN exposure. A glycoprotein of approximately 68 kDa was over expressed while some between 20 kDa to 29 kDa molecular weight found under expressed as compared to their normal counterparts as revealed by SDS-PAGE electrophoretic study.

4. Liposomes prepared by the dry film method exhibited highly reproducible entrapment efficiency. Further, the immunogenicity of entrapped TAA remained unaltered.

5. DEN-exposed mice upon immunization with liposomal-TAA formulation elicited humoral immune response against TAA as substantiated by the presence of significantly high circulating antibody concentration in immune serum.

6. Cell proliferation assay *in vitro* of lymphocytes, obtained from immunized animals, clearly indicate the induction of cell mediated immune response in DEN-exposed mice upon immunization.

7. Reversal of the marker enzyme activities, in the liver of DEN-exposed and immunized mice, towards normal level is likely due to the effect of immunization on tumor regression.

8. Attainment of normal morphology and cell shape of hepatocytes upon immunization in DEN-exposed mice also support tumor regression.

These findings therefore, support that a good immune induction could be achieved with liposome encapsulated antigens and therefore, this particular approach could be useful to elicit immune response to the self-existing bio-molecules.

APPENDIX ABBREVIATIONS

ABTS	2,2í-azino-di-[3-ethylbenzthiazolin-sulfonat]
AChE	Acetylcholine esterase
BrdU	5-Bromo-2í-deoxyuridine
BSA	Bovine serum albumin
CFA-TAA	Complete Freund's Adjuvant Tumor-associated antigen
$CHCl_3$	Chloroform
CH_3OH	Methanol
Chol.	Cholesterol
D.P.X.	Diesterene Plasticizer Xylene
DCP	Dicethyl choline phosphate
DMEM	Dulbecco's Modified Eagle Medium
DMPC	Dimyristoyl phosphatidyl choline
DPPC	Dipalmitoyl phosphatidyl choline
DTNB	5,5í-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
ELISA	Enzyme-linked immunoadsorbent assay
FCS	Fetal calf serum
g	Gravitational force
GGT	Gamma glutamyl transpeptidase
h	Hour
HMW	High molecular weight
kDa	Kilo Dalton
Lip TAA	Liposomal Tumor-associated antigen
mA	Milliampere
MTP	Microtitre plate
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid)
DEN	Diethylnitrosamine
NDMA	N-Nitrosodimethylamine
$(NH_4)_2SO_4$	Ammonium sulphate
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidyl choline
POD	Peroxidase
SDS	Sodium dodecyl sulphate
TAA	Tumor-associated antigen
TCA	Trichloro acetic acid
TNF	Tumour necrotic factor
Tris	Tris (hydroxymethyl)-aminomethane
Triton X-100	(t-Octylphenoxypolyethoxyethanol)
U	Enzyme specific activity
V	Volt
Vh	Volt hour

REFERENCES

1. Alam, A., Bhuri, S. R. K., Mavila, A. K. and Singh, V. (1992). Design of liposome to improve encapsulation efficiency of gelonin and its effect on immunoreactivity and ribosome inactivating property. *Mole Cell Biochemistry* **112:** 97.
2. Alam, A., Capoor, A. K., and Rao, L. V. (1991). Evaluation of adjuvanticity of promising new synthetic MDP analogues. *Immunology Letters.* **27**: 53.
3. Alam, A., Singh, O. and Talwar, G. P. (1989). Stability of an antifertility vaccine consisting of gonadotropin subunits linked to tetanus toxoid. *Vaccine,*. **7**: 129.
4. Alfred, M. (1991). 'Acetlycholine esterase: the structure'. TIBS-Oct., pp. 355-356.
5. Allison, A. C., and Gregoriades, G. (1974). Liposomes as Immunological adjuvants. *Nature* (London). **252**, 252.
6. Allison, J. P., Hurwitz, A. A. and Leach, D. R. (1995). Manipulation of costimulatory signals to enhance antitumor T-cell responses. *Curr. Opin. Immunol.,* **7**: 682.
7. Alton Meister, Suresh S. Tate, Owen W. Griffith, γ-Glutamyl Transpeptidases (1981) *Methods in Enzymology*, vol. **77** pp 237-251.
8. Alving, C. R. (1977).In: The Antigens (Ed. M. Sela), Academic Press, New York, Vol.**4**, pl.
9. Ansel, S. and Blangy, D. (1984). In vivo induction of tumor-specific immunity by glycolipid extracts of SV40-transformed cells. *Int. J. Cancer.* **34**: 555.
10. Baldwin, R. W., Harris, J. R., and Price, M. R. (1973). Fractionation of plasma membrane associated tumor-specific antigen from an aminoazo dye-induced rat hepatoma. *Int. J. Cancer* **11**: 385.
11. Baldwin, R. W., Price, M. R., and Robins, R. A. (1972). Blocking of lymphocytes-mediated cytotoxicity for rat hepatoma cells by tumor-specific antigen antibody complexes, Nature (London) *New Biol.,* **238**: 158.
12. Bangham, A. D., Standish, M. M., and Ulatson, J. C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.,* **13**: 238.
13. Bangham, A. D., Standish, M. M., and Watkins, J. C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.,* **13**: 238-52.
14. Boelsterli, U. (1979) 'Gamma-glutamyl transpeptidase (GGT)- an early marker for hepatocarcinogens in rats'. TIPS- Oct., 1979 pp. 47-49.
15. Boyer, M. W., Waller, E. K., Bray, R. A., Unangst, T., Johnson, T. S., Phillips C., Jurickova, I., Winton, E. F. and Yeager, A. M. (2000). Cytokine upregulation of the antigen presenting function of acute myeloid leukemia cells. *Leukemia,* **14**: 412-418.
16. Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilysing the principle of protein dye-binding. *Anal. Biochem.* **72**: 248-254.
17. Braun, J. P., Rico, A. G., Bernard, P., and Burgat-Sacaze, V. (1977). Gamma-glutamyltransferase. Human pathology and animal biology. *Ann. Biol. Clin.,* (Paris) **35**, 433-457.
18. Brynestad, K., Babbitt, B., Huang, L. and Rouse, B.T. (1990). Influence of peptide acylation, liposome incorporation, and synthetic immunomodulators on the immunogenicity of a 123 peptide of glycoprotein D of herpes simplex virus: implication for subunit vaccines. *J Virol*, **64**: 680.
19. Bystryn, J. C. (1990). Tumor vaccines. *Cancer metast. Rev.* **9**: 81.
20. Cameron, R., Kellen, J., Kolin, A., Malkin, A., and Farber, E. (1978). Gamma-glutamyltransferase in putative pre-malignant liver cell populations during hepatocarcinogenesis. *Cancer Res.,* **38**: 823-829.**D15**
21. Catty, D., and Raykundalia, C. In Antibodies: a practical approach, Catty, D., Ed.; Vol. 2, PRACTICAL APPROACH SERIES, Oxford, (1989); pp.97-154.
22. Cavallo, F., Giovarelli, M., Gulino, A., Vacca, A., Stoppacciaro, A., Modesti, A., Forni, G. (1992). Role of neutrophils and CD4+ T lymphocytes in the primary and memory response to nonimmunogenic murine mammary adenocarcinoma made immunogenic by IL-2 gene. *J. Immunol.,* **149**: 3627-3635.
23. Charbonnier, A., Gangler, B., Sainty, D., Lafage-Pochitaloff, M., and Olive, D. (1999). Human acute myeloblastic leukemia cells differentiate *in vitro* into mature dendritic cells and induce the differentiation of cytotoxic T cells against autologous leukemias. *Eur .J . Immunol.*, **8**: 2567-2578.
24. Cheng, S., Nassar, K., and Levy, D. (1978). Gamma-glutamyl transpeptidases activity in normal, regenerating and malignant hepatocytes. FEBS Lett. **85**, 310-312.
25. Christensen, H. E., Fairchild, E. J., Eds., Carroll, B. S., Lewis, R. J., Project Coordinators. (1996 Edition). *Registry of Toxic Effects of Chemical Substances.* U.S.Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, U.S. Government Printing Office, Washington, D.C. (1976).
26. Cournoyer, D., and Caskey, C. T. (1993). Gene therapy of the immune system. *Annu. Rev. Immunol.* **11**: 297.
27. Denizot, F., and Lang, R. (1986). Rapid colorimetric assay cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability.*J. Immunol. Methods,* **89**: 271-277.
28. Druckey, D., Preussmann, R., Ivankovic, S., and Schmahl, D., (1967). Organotrope Carcinogene Wirkany bei 65 Verschiedenen N-Nitroso-ver-bindungen and BD-Ratten, *Z. Krebsforsch,* **69** (103): 103-201.
29. Dutton, A. H.and Heath, D. F. (1958). *Nature.* (London) **178**, 644.
30. Eibl, B., Ebner, S., Duba, C., Bock, G., Romani, N., Erdel, M., Gachter, A., Niederweiser, D., and Schuler, G. (1997). Dendritic cells generated from blood precursors of chronic myelogenous leukemia

patients carry the philadelphia translocation and can induce a CML- specific primary cytotoxic T-cell response. *Genes Chromosomes Cancer,* **20**: 215-223.
31. Ellman, G.L., Courtney, D.K., Andres, V. and Featherstone, R.M. (1961) *Biochem. Pharmacol.* **7**, 88-95.
32. Emmelot, P., Scherer, E.(1980). The first revelant cell stage in rat liver carcinogenesis. A quantitation approach. *Biochim. Biophys. Acta.*, **605**: 247-304.
33. Farber, E. (1980). The sequential analysis of liver cancer induction. *Biochim.Biophys. Acta.*, **605**: 145-66.
34. Fearon, E. R., Pardoll, D. M., Itaya, T., Golumbek, P., Levitsky, H. I., Simons, J. W., Karasuyama, H., Vogelstein, B., Frost, P. (1990). Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell*, **60**: 397-403.
35. Feltkamp, M. C., Smits, H. L., Vierboom, M. P., Minnaar, R. P., de Jongh, B. M., Drijfhout, J. W., Ter Schegget, J., Melief, C. J., Kast, W. M. (1993). Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol.* , **23**: 2242-2249.
36. Fentiman, I. S. (1985). The local treatment of cancer. In *Introduction to the cellular & molecular biology of cancer* 2nd Edition, edited by L. M. Franks and N.M. Teich, Oxford University Press, pp. 434-450.
37. Fiala, S., and Fiala, E. S. (1973). Activation by chemical carcinogens of gamma-glutamyl transpeptidase in rat liver. *J. Natl. Cancer Inst.* **51**, 151-158.
38. Fiala, S.,Reuber, M.D (1970). Gann, 61, 275.
39. Fidler, I. J. (1988). Targeting of immunomodulators to mononuclear phagocytes for therapy of cancer. *Adv. Drug. Deliv. Rev.* **2**: 69.
40. Fidler, I. J. (1994). Therapy of cancer metastasis by systemic activation of macrophages. *Adv. in Pharmacol.* **30**: 271.
41. Foulds, L., (1969). *Neoplastic Development I*; Academic: New York.
42. George, J. T., Spooner, R. A., and Epenetos, A. A. (1994). Applications of monoclonal antibodies in clinical oncology. *Immunol.Today* **15**: 559.
43. Gerlier, D., and Thomasset, N. (1986). Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods,* **94**: 57-63.
44. Gershoni, J. M., Palade, G. E. (1983). Protein blotting: Principles and applications. *Anal. Biochem.,* **131** (1983) 1-15.
45. Golumbek, P. T., Lazenby, A. J., Levitsky, H. I., Jaffee, L. M., Karasuyama, H., Baker, M., Pardoll, D. M. (1991). Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science*, **254**: 713-716.
46. Gonchoroff, N. J. *et al.* (1985). A monoclonal reactive with 5-bromo-2-deoxy-2-uridine (BrdU) that does not require DNA denaturation.*Cytometry* **6**: 506-505.
47. Gratzner, H. G. (1982). Monoclonal antibodies to 5-bromo-and 5-iododeoxyurdine: A new reagent for detection of DNA replication. *Science,* **218**: 474-475.
48. Grover, P. L., Ed. *Chemical Carcinogens and DNA*; CRC: Boca Raton, F. L., (1979), Vol. **1-2.**
49. Hansen, M. B., Nielson, S. E., and Berg, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods,* **119**: 203.
50. Heicappell, R., Schirrmacher, V., Von Hoegen, P., Ahlert, T. and Appelhans, B. (1986). Precvention of metastastic spread by postoperative immunotherapy with virally modified autologous tumor cells. I.Parameters for optimal therapeutic effects. *Int. J. Cancer.* **37**: 569.
51. Hellstrom, K. E. and Hellstrom, I. (1989). Oncogene-associated tumor antigens as targets for immunotherapy. *FASEB J.*, **3**: 1715.
52. Hock, H., Dorsch, M., Diamantstein, T., Blankenstein, T. (1991). Interleukin 7 induces CD4+ T cell-dependent tumor rejection. *J. Exp. Med.*, **174**: 1291-1298.
53. Hoover, H. C. and Hanna, M. G. (1989). Active immunotherapy in colorectal cancer. *Semin. Surg. Oncol.* **5**: 436.
54. Huet, C. (1980). Tumor rejection induced by tumor cell glycolipids. *Biol. Cell.,* **39**: 269.
55. Hurwitz, A. A., Kwon, E. D., Van Elsas, A. (2000). Costimulatory wars: the tumor menace. *Current opinion in Immunology* 2000, **12**(5): 589-596.
56. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. (1971), Vol. **1**, International Agency for Research on Cancer, Lyon, France, World Health Organization, 107-124.
57. Irei, R. F. and Morton, D. L. (1986). Regression of cutaneous metastasis melanoma by intralesional injection with human monoclonal antibody to gangglioside GD2, *Proc. Natl. Acad. Sci.* U.S.A., **83**: 8694.
58. Irei, R. F. and Mortton, D. L. (1986). Regression of cutaneous metastases malanoma by intralesional injection with human monoclonal antibody to ganglioside GD2. *Proc. Natl. Acad. Sci.* U.S.A., **83**: 8694.
59. Jacobson, G. (1988). Semi-dry electroblotting from PhastGel Media. In Electrophoresis '88, C. Schafer-Nielsen (Editor), VCH Verlagsgesellschaft, W. Germany, 418-423.

60. Jenne, L., Arrighi, J. F., Jonuleit, H., Saurat, J. H., Hauser, C. (2000). Dentritic cells containing apoptotic melanoma cells prime human CD8+ T cells for efficient tumor cell lysis. *Cancer Res.*, **60**: 4446-4452.
61. Kaiser H. E., Bodey, B. Jr, Siegel S. E, Groger, A. M., Bodey, B. (2000). Spontaneous neoplastic regression: the significance of apoptosis. *In vivo* **14**(16): 773-88.
62. Kleinerman, E. S., Maeda, M., and Jaffe, N. (1993). Liposome-encapsulated muramyltripeptide: a new biological response modifier for treatment of Osteosarcoma. *Canc. Treat. Res.*, **62**: 101.
63. Knuth, A., Jager, D., Jager, E. (2000). Cancer immunotherapy in clinical oncology. *Cancer Chemother. Pharmacol.*, 46 Suppl: S4 6-51.
64. Kradin, R. L., *et al.* (1984). Tumor infiltrating lymphocytes and interleukin-2 treatment of advanced cancer. Lancet. (March 18): 577.
65. Kuby, J. Cancer and immune system. In *Immunology,* (1997), 3rd Ed. pp. 573-596.
66. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680.
67. Law, L.W. (1984). Characteristics of tumor specific antigens.*Cancer Surv.* **4**: 1-19.
68. LeGrue, S. J. (1985). Noncytolytic extraction of cell surface antigens using butanol. *Cancer Metast. Rev.* **4**: 209.
69. LeGrue, S. J., Kahan, B. D., and Pellis, N. R. (1980). Extraction of a murine tumor-specific transplantation antigen with 1-Butanol. I. Partial purification by isoelectric focusing. *J. Natl. Cancer Inst.* 65: 191.
70. Levitsky, H. I., Lazenby, A., Hayashi, R. J., Pardoll, D. M. (1994). In vivo priming of two distinct antitumor effector populations: the role of MHC class I expression. *J. Exp. Med.* ,**179**: 1215-1224.
71. Liao, S. K., Smith, W. J., and Kwong, C. P. (1984). Selective extraction by 1-butanol of surface glycoprotein antigens from human melanoma cells. *Cancer Immunol. Immunother.*, **17**: 95-99.
72. Liao, S., Smith, J. W., and Kwong, P. C. (1984). Selective extraction by 1-butanol of surface glycoprotein antigens from human melanoma cells. *Cancer Immunol. Immunother.*, **17**: 95.
73. Lifshitz, R., Gitler, C., and Mozes, E. (1981). Liposomes as immunological adjuvants in eliciting antibodies specific to the synthetic polypeptide poly (LTyr, LGlu)-poly (DLAla)-(LLys) with high frequency of site-associated idiotypic determinants. *Eur. J. Immunol.* **11**: 398.
74. Lin, K. Y., Guarnieri, F. G., Staveley-O'Carroll, K., Levitsky, H. I., August, J. T., Pardoll, D. M., Wu, T. C.(1996). Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res.*, **56**: 21-26.
75. Livingstone, P.O., Jones, M., DeLeo, A. B., Oettgen, H. F., and Old, L.J. (1985). The serologic response to Meth A sarcoma vaccines after cyclophosphamide treatment is additionally increase by various adjuvants. *J. Immunol.* **135**, 1505.
76. Maas, R. A., Dullens, H. F. J., De Jong, W. H., and Den Otter, W. (1989). Immunotherapy of mice with a large burden of disseminated lymphoma with low-dose interleukin 2. *Cancer Res.* **49**: 7037.
77. Magee, P.N., and Barnes, J. M., (1967). Carcinogenic Nitroso Compounds. *Advances in Cancer Research* **10**: 163-247 .
78. Malpas, J. S. (1985). Chemotherapy. In *Introduction to the cellular & molecular biology of cancer* 2nd Edition, edited by L. M. Franks and N.M. Teich, Oxford University Press, pp. 451-467.
79. Mandelboim, O., Vadai, E., Fridkin, M., Katz-Hillel, A., Feldman, M., Berke, G., Eisenbach, L. (1995). Regression of established murine carcinoma metastases following vaccination with tumour-associated antigen peptides. *Nat Med.* , **1**: 1179-1183.
80. Marx, J. L. (1989). Cancer Vaccines show promise at last. *Science.* **245**: 813.
81. McCune, C. and Marquis, D. M. (1990). Interleukin 1 as an adjuvant for active specific immunotherapy in a murine tumor model. *Cancer Res.* **50**: 1212.
82. Meazza, R., Lollini, P. L., Nanni, P., De Giovanni, C., Gaggero, A., Comes, A., Cilli, M., Di Carlo, E., Ferrini, S., Musiani. P. (2000). Gene transfer of a secretable form of IL-15 in murine adenocarcinoma cells: effects on tumorigenicity, metastatic potential and immune response. *Int. J. Cancer,* **87**: 574-81.
83. Miller, E.C.; Miller, J.A. (1981). Mechanism of chemical carcinogenesis.*Cancer*, **47**: 2327-45.
84. Miller, M. R., *et al* (1985). Interaction of monoclonal directed against bromodeoxyuridine with pyrimidine bases, nucleosides, and DNA. *J. Immunol.,* **136**: 1791-1796.
85. Miller, R. A., Maloney, G. D. Warnke, R. and Levy, R. (1992). Treatment of B cell lymphoma with monoclonal anti-idiotype antibody. *N. Eng. J. Med.,* **306**: 517.
86. Miltenburger, H. G., Sachse, G., and Schleimann, M. (1987). S-phase cell detection with a monoclonal antibody. *Develop. Biol. Standard* **66**: 91-99.
87. Mitchell, M. S., Kan-Mitchell, J., Kempf, R. A., Harel, W., Shau, H., and Lind, S. (1988). Active specifi immunotherapy for melanoma: Phase I trial of allogenic lysates and a novel adjuvant. *Cancer Res.* **48**: 5883.
88. Mizrahi, I. J., and Emmelot, P. (1963).*Biochem. Pharmacol.,* **12**, 55.
89. Mizrahi, I.J. and Emmelot, P.(1962). *Cancer Res.,* **22**,339.

90. Moore, M. A., Mayer, D., Bannasch, P. (1982). The dose dependant and sequential appearance of putative preneoplastic populations induced in the liver by stop experiments with N-nitrosomorpholine. *Carcinogenesis* **3**(12): 1429-36.
91. Morton, R. K. (1950). Seperation and purification of enzymes associated with insoluble particles. *Nature,* **266**: 1092.
92. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assays. *J. Immunol. Meth.,* **65**: 55-63.
93. Naoyuki Taniguchi (1974). Purification and some properties of γ-glutamyl transpeptidase from azo dye-induced hepatoma, J.Biochem, 75: 472-480.
94. Nepom, G. T., Hellstrom, H., and Hellstrom, K. E. (1983). Suppression mechanism in tumor immunity. *Experentia,* **39**: 235.
95. North, R. J., DiGiacomo, A., and Dye, E. S. (1987). Suppression of antitumor immunity.In: Research Monographs in Immunology, Vol. **11**, Den Otter, W., and Ruitenberg, E.J., Eds., Elsever, Amsterdam, 125.
96. Papahadjopoulos, D., and Klimberg, H. K. (1973). Phospholipid vesicles (liposomes) as model for biological membrane: A recent review on reconstitution of membrane proteins etc. on liposome protein. In: Davidson, S.G. (ed). *Progress in surface science*, Vol. **4** (part 2), pp 141, Pergamon press, Oxford.
97. Peraino, C., Staffeldt, E.F., Haugen, D. E., Lombard, L. S., Stevens, F. J., and Fry, R. J. M. (1980). Effects of varying the dietary concentration of phenobarbital on its enhancement of 2-acetylaminofluorene-induced hepatic tumourigenesis. Cancer Res., **40**: 3268-3273
98. Pieters, R, Huismans D. R., Leyva, A., and Veerman, A. J. P. (1988). Adaptation of the rapid automated tetrazolium dye based (MTT) assay for chemosensitivity testing in childhood leukemia. *Cancer Letters,* 41: 323.
99. Presant, C. A., Scolaro, M., Kennedy, P., Blayney, D.W., Flanagan, B., Lisak, J., and Presant, J. (1978). Liposome daunorubicin treatment of HIV-associated Kaposa's sarcoma. *Lancet,* **341**: 1242.
100. Preussmann, R., Stewart, B. W. (1984). In: Chemical Carcinogens, Searle Charles, E., Ed.; Vol. **2**, ACS MONOGRAPH SERIES NO. **182:** Washington, D. C., pp.643-828.
101. Ragupathi, G. (1996). Carbohydrate antigens as targets for active specific immunotherapy. *Cancer immunol. Immunother.,* **43**: 152.
102. Ratcliffe, N. A. (1983). *Practical Illustrated Histology.*
103. Remandet, B., Gouy, D., Berthe, J., Mazue, G., Williams, G. M. (1984). Lack of initiating or promoting activity of six benzodiazepine tranquilizers in rat liver limited bioassays monitered by histopathology and assay of liver and plasma enzymes. *Fund. Appl.Toxicol.* (in press).
104. Riddell, S. R., Greenberg, P. D. (1995): Principles for adoptive T cell therapy of human viral diseases. *Annu Rev Immunol.,* **13**: 545-586.
105. Rizvi, S. Y., Kundu, B., Mathur, K. B., Alam, A. and Talwar, G. P. (1998). A process for the synthesis of N-acetyl nor muramyl- L-N-methyl alanyl-D-isoglutamine-N-substituted amines possessing high immuno-adjuvant activity. Patent (No. 11F/ 106/ 88) *Council of scientific and Intrustrial research*, New Delhi.
106. Rodolfo, M., Zilocchi, C., Melani, C., Cappetti, B., Arioli, I., Parmiani, G., and Colombo, M. P.(1996). Immunotherapy of experimental metastasis by vaccination with interleukin gene transduced adenocarcinoma cells sharing tumor-associated antigens. *J. Immunol.,* **157**: 5536.
107. Scherer, E., Hoffman, M., Emmelot, P., Friedrich-Freksa, M. (1972). Quantitative study on foci of altered liver cells induced in the rat by a single dose of diethylnitrosamine and partial hepatectomy. JNCL *J.Natl.Cancer Inst.* 1972, **49**, 93-106
108. Sarker, D. P., and Das, M. K. (1984). Immunogenicity of galactosylated liposomes. *Immunol. Commun,* **13**: 5.
109. Schmahl, D., Preussmann, R., Hamperl, H. (1960). *Naturwissenschaften* **47**: 89.
110. Sharan, R. N., Alam, A. and Sanghamitra, C. Saikia, J. R. and Srivastava, P. N. (1995). 2-mercaptopropeonyl glycine affords enhanced radio protection after a liposome encapsulation. *J. Rad. Res.,* **36**: 31.
111. Simmons, R. L., Rious, A., Lundgren, G., Ray, P., Mc Khann, C. and Haywood, G. (1971). Immunospecific regression of methlycholanthrene fibrosarcoma with the use of neuraminadase. *Surgery.* **70**: 38.
112. Singh, O., Sharma, N.C., Rao, L. V., Alam, A., Talwar, G. P. (1989). Antibody response and characteristics of antibody in women immunized with three formulations of contraceptive vaccine inducing antibodies against human chorionic gonadoropin. *Fert. Sterl.* **52**: 739.
113. Skornick, Y., Kurman, C.C. and Sindelar, W.F. (1984). Active immunization of hamsters against pancreatic carcinoma with lipid-treated cells or their shed antigens.*Cancer Res.* **44**: 946.
114. Solt, D. B., Menline, A., and Farber, E. (1977). Rapid emergence of carcinigen-induced hyperplastic lesions in a new model for the sequential analysis of liver carcinogenesis. Am. J. Pathol., **88**: 595-618.
115. Srivastava, P. K., Old, L. (1988). Individually distinct transplantation antigens of chemically induced mouse tumors. *Immunology Tod.,* **9**: 78-83.

116. Stewart, H. L., Williams, G.M., Keysser, C. H., Lombard, L.S., Montali, R. J. (1980). *JNCI, J. Natl. Cancer Inst.* **64**: 177-207.
117. Tada, H., *et al* (1986). An improved colorimetric assay for interleukin-2. *J. Immunol. Methods,* **93**: 157-165.
118. Tatematsu, M., Shirai, T., Tsuda, H., Miyata, Y., Hirose, M., Ito, N., (1979). *JNCI, J. Natl. Cancer Inst.* , **63**, 1411-16.
119. Topalian, S. L., Gonzales, M. I., Parkhurst, M., Li, Y. F., Southwood, S., Sette, A., Rosenberg, S. A., Robbins, P. F. (1996). Melanoma-specific CD4+ T cells recognize nonmutated HLA-DR-restricted tyrosinase epitopes. *J. Exp. Med.,* **183**: 1965-1971.
120. Topalian, S. L., Rivoltini, L., Mancini, M., Markus, N. R., Robbins, P. F., Kawakami, Y., Rosenberg, S. A. (1994). Human CD4+ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. *Proc. Natl. Acad. Sci .*, U.S.A., **91**: 9461-9465.
121. Upadhyay, S. N., Alam, A. and Talwar, G. P. (1989). Functional morphology of testis and its excurrent ducts in rats immunized with synthetic luteinizing hormone releasing hormone conjugated to tetanus toxoid. *J. Reprod. Immunol.*, **16**: 151.
122. Van Rooijen, N. (1990). Liposomes as carrier and immunoadjuvant of vaccine antigens. In: Bacterial vaccines. *Advances in Biotecnological processes,* Vol. **13** (ed. Mizrahi, A), pp. 255-279. New York: Alan, R. Liss.
123. van Rooijen, N., and vanNieuwmegan, R. (1980). Endotoxin enhanced adjuvant effect of liposomes, particularly when antigen and endotoxin are incorporated within the same liposome. *Immunol. Commun.,* **9**: 243.
124. Vanderlaan, M., and Thomas, C. B. (1985). Characteristerics of monoclonal antibodies to Bromodeoxuridine.*Cytometry* **6**: 501-505.
125. Vistica,D.T. et al. (1991). Tetrazolium-based assays for cellular viability: A critical examination of selected parameters affecting formazan production. *Cancer Res.,* **51**: 2515-2520.
126. Walden, P., Nagy, Z. A. and Klein, J. (1986). Major Histocompatibility Complex-restricted activation of helper T cell lines by liposome-bound antigens. *J. Mol. Cell. Immunol.* **2**: 191.
127. Weinberg, R. A. (1982). Oncogenes of spontaneous and chemically induced tumors. *Adv. Cancer Res.,* **36**: 149-63.
128. Weisburger, J. H and Williams, G. M., (1981). Carcinogen testing: Current problems and new approaches. *Science* , **214**: 401-7.
129. Weisburger, J. H. and Williams, G. M. Bioassay of carcinogens: *In Vitro* and *in Vivo* Tests. In *Chemical Carcinogen,* (1984) Vol. 2; 2nd ed., ACS Monograph 182: p. 1329-1331.
130. Weisburger, J. H., Williams, G. M., (1980). In *Cassarett and Doull's Toxicology: The Basic Science of Poisons,* Doull, J.; Klaassen, C.; Amdur, M., Eds.; Macmillan: New York; 2nd ed., p. 84.
131. Williams, G. M.(1980). The pathogenesis of rat liver cancer caused by chemical carcinogens. *Biochim.Biophys. Acta.,* **605**: 167-89.
132. Wright, D. L., and Plummer, D. T. (1972). *Biochim. Biophys. Acta,*. **261**, 398-401.
133. Zakour,R. A., Kunkel, T. A., Loeb, L. A., (1981).Metal induced infidelity of DNA synthesis. *Environ Health Prespect* **40**: 197-206.
134. Zhang, S., Graeber, L. A., Helling, F., Raghupati, G., Adlure, S., Lloyd, K. O., and Livingston, P. O. (1995). Augmenting the immunogenicity of synthetic MUCI peptide vaccines in mice. *Cancer Res.,* **56**: 3315.

Curriculum Vitae

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EDUCATION

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Research Experience

Isolation and Purification of proteins and glycoproteins, Entrapment of biomolecules into Liposomes, Tumor Antigens delivery using liposome as carrier.

Techniques Used

Electrophoresis, Spectrophotometry, Microtomy, ELISA, Western Immunoblotting, *in vitro* bioassays.

Research Publications

1. Anis Alam, Longkumer Imliwati, Chowphi Rapthap and Vinod Singh. Liposome Encapsulated Tumor-Associated Antigens Elicited Humoral And Cellular immune Responses In Mice Bearing Tumor. *Ind. J. Expt. Biol.*,Vol. 39, March 2001, pp. 201-208.

2. C. Rapthap, L.K. Imliwati, R.N. Sharan and Anis Alam. Tumor-associated antigen expression in mice exposed to diethylnitrosamine, In, *Trends in Radiation and Cancer Biology*, Ed. R.N. Sharan, Vol. 29, pp 177-180, Forschungszentrum Julich GMBH, 1998.

3. L.K. Imliwati, C. Rapthap, R.N. Sharan and Anis Alam. Expression of a high molecular weight glycoprotein in mice exposed to diethylnitrosamine (abstract) In, 17th Annual Convention of Indian Association for cancer Research, Chittaranjan National Cancer Institute, Calcutta, January 21-24, 1998.

Symposia Participated

1 17th Annual Convention of Indian Association for cancer Research, Chittaranjan National Cancer Institute, Calcutta, January 21-24, 1998.

2. International conference on Radiation Biology: DNA Damage, Repair and Carcinogenesis, North-Eastern Hill University, Shillong, April 1998

3. 19th Annual Convention of Indian Association for cancer Research, Amala Cancer Research Institute, Thrissur, January 21-23, 2000.