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 $\gamma$-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 elevation 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 $\gamma$-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 therefore, 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 itself 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.