SUMMARY

Out of several drug delivery systems presently in use liposomes have been tried to reduce the toxicity of drugs. Liposomes being nontoxic and biodegradable appears to be the most promising delivery system. Although, they possess inherent tendency to localize mainly towards RES, but they can be targeted to the other cells also by grafting on their surface, the cell specific ligands such as antibodies, peptides, carbohydrate etc. However, in recent years, there has also been a growing interest in using liposomes for immunization. Liposomised antigen can elicit both humoral as well as cell mediated immunity.

In the present study we have exploited liposomal delivery system for targeting of drugs and other macromolecules not only to the desired population of cells but also at the subcellular level within the cell itself. The work presented in this thesis is divided into three parts. The first two parts deals with liposome mediated drug delivery to erythrocytes and macrophages, infected with *P.berghei* and *A. fumigatus* respectively. The third part deals with the cytosolic delivery of model antigens to APCs, for elicitation of cell mediated immunity.

Malaria, once seemingly under control with the advent of drugs like CHQ, is again on the resurgence with the development of resistance against CHQ. The probable cause for the resistance of CHQ seems to be attributed to the nonattainment of the required drug concentration in the erythrocyte, infected with malaria parasite.

Antibodies specific against neoantigens on the surface of malaria infected erythrocytes, can be utilized for specific delivery of drugs to the infected cells. This will not only minimize the drug toxicity but would also help in attainment of optimum drug concentration in the infected cells.

Monoclonal antibodies recognizing neoantigens present on the infected cells were raised with the aim of targeting. Two sets of monoclonal antibodies MoAb F_{10} and MoAb D_2 were used. MoAb F_{10} recognizes surface antigen of the infected erythrocytes derived from parasite itself while MoAb D_2 recognizes altered antigen formed due to structural modification. The specific recognition properties of these antibodies were determined by *in vitro* and *in vivo* binding studies. The antibodies were covalently attached to the surface of [^{14}C] inulin containing liposomes after removing their Fc portion by pepsin treatment and labeling the resulting $F(ab')_2$ fragments with ^{125}I. The antibody bearing liposomes MoAb F_{10}-Lip and MoAb D_2-Lip thus obtained were interacted with normal and *P. berghei* infected mouse erythrocytes *in vitro* in order to assess their binding specificities. The binding was determined by measuring the cell associated ^{125}I and ^{14}C markers. Liposomes bearing $F(ab')_2$ of normal mouse IgG on their surface were used as control.

To further confirm the specificity of MoAb F_{10}-Lip binding with infected erythrocytes, the liposome preparations described above were administered intravenously to healthy and *P. berghei* infected (50-60% parasitaemia) Balb/c mice, and their distribution pattern in various tissues was determined. Tissue distribution pattern of MoAb D_2-Lip and MoAb F_{10}-Lip demonstrated that MoAb F_{10} recognized malaria infected erythrocytes while MoAb D_2 antibody recognized both normal as well as infected erythrocytes.

The efficacy of liposomized preparation of CHQ was assessed on the basis of survival of animals and percent parasitaemia after treatment of malaria infected animals with various liposomal formulations of CHQ. Initial experiments were performed in the animals infected with CHQ sensitive strains of *P. berghei.* Swiss male mice were used as model animals. It was found that the dose of 5mg/kg was most effective in the treatment of CHQ sensitive experimental murine malaria. As an extension of this work it was thought appropriate to examine effectivity of liposomized CHQ formulation in controlling CHQ-resistant *P. berghei* infection in mice. It was found that treatment of CHQ resistant malaria infected animals with two doses of MoAb F_{10}-Lip-CHQ on day 4 and 6 post infection can cure 75-90% animals. No parasites were detected in their blood even 30 days posttreatment. Unlike this observation, only 40-50% of the animals survived in the group, treated with MoAb D_2-Lip-CHQ under identical conditions.

From these results, we conclude that the therapeutic efficacy of CHQ can be markedly increased by delivering this drug through target specific liposomes. The application of target-specific liposomes as CHQ carriers in selective homing of CHQ to the drug resistant *P. berghei* infected mouse erythrocyte can be further extended to treat *P. falciparum* infections as well.

Aspergillosis, an opportunistic systemic fungal infection is of major concern in clinical medicine specially for immuno-compromised persons. Amp-B, although used widely as antifungal drug, does not seem to be an ideal candidate for the treatment because of its severe toxicity.

The present investigation describes the use of tuftsin bearing liposome as drug carrier of Amp-B, to circumvent the toxicity, and also to increase the therapeutic efficacy in treatment of experimental aspergillosis. Tuftsin, an immunomodulator, is recognized by macrophages and monocytes. It plays a major role in elimination of

fungal diseases such as aspergillosis by activating macrophages and monocytes.

The first part of this study deals with the toxicological experiments. Various liposomal preparation of Amp-B were tested for their toxicity on the basis of LD_{50} determination. The LD_{50} values increased markedly both in Lip-Amp-B and Tuft-Lip-Amp-B formulations.

The survival of mice undergoing therapy and the fungal load in the infected tissue such as lung, liver, kidney and spleen were checked to study the comparative therapeutic efficacy of free and liposome intercalated Amp-B. The animals were first challenged with 1.8×10^7 spores of *A.fumigatus* and then treated with various doses of Amp-B. The survival rate was about 35% in the group which was treated with Tuft-Lip-Amp-B while none of the animals survived in group which was treated with Lip-Amp-B. On increasing the dose upto 0.5 mg/kg body weight, the survival of the animals was about 70-75% in the case of Tuft-Lip-Amp-B treated animals after seven days of therapy while survival rate in the animals which were treated with Lip-Amp-B was about 35%. The animals which were left untreated or treated either with drug free liposomes or free drug did not survive after three days of the infection. The extent of eradication of fungal spores in various organs viz. liver, lung, spleen and kidney was determined by counting colony forming units (colonies represent fungal load in particular organ). It was found that the animals which survived infection after treatment with Tuft-Lip-Amp-B were free of any fungal load.

To ascertain the immune status of Tuft-Lip-Amp-B treated animals, these were rechallenged with 1.8×10^7 fungal spores of *A. fumigatus*. The fungal load in different organs demonstrated that the previous exposure with *A. fumigatus* infection might have imparted some degree of protection to the surviving animals.

Immunomodulatory role of tuftsin was determined by pretreating animals with Tuft-Lip for three consecutive days before challenging the animals with infection. Subsequently these animals were treated with various liposomal formulations of Amp-B. The survival data demonstrated that pretreatment with Tuft-Lip can increase the survival rate upto 85% in both Lip-Amp-B and Tuft-Lip-Amp-B treated animals. Colony forming units in different organs, determined after 24h demonstrated that the pretreatment alone can suppress the fungal load to almost 2.5 fold.

The present study further extended the scope of liposomized Amp-B formulation in the treatment of fungal infection by demonstrating a significant improvement in the lip-Amp-B efficacy against experimental aspergillosis after grafting tuftsin onto the surface of liposomes. In conclusion, this study shows that the Tuft-Lip-Amp-B formulation is considerably better than the Lip-Amp-B preparation in the treatment of systemic fungal infection, due to its higher efficacy and comparable toxicity.

The final chapter of thesis deals with the use of liposome for immunization. Liposome can induce cell mediated immunity, which is very much desirable in controlling the diseases like tuberculosis, leprosy, leishmaniasis, toxoplasmosis and various viral diseases. Cytotoxic T lymphocytes, which play role in cell mediated immunity, can only be provoked when they recognize processed antigen alongwith MHC-I molecules. The presentation of the processed antigen mainly depends on the site of processing. Thus antigens which undergo endolysosomal processing are presented along with MHC-II molecules while endogenous antigens or antigens present in the cytosol of antigen presenting cell are presented along with MHC-I molecules.

Liposomes composed of yeast lipids have a strong tendency to fuse with the target membrane and consequently deliver their contents into the cytosol of targeted cells.

Yeast lipids were extracted and purified using Folch process. These lipids were then analysed for their components using two dimensional thin layer chromatography and various chemical analysis methods.

Water soluble fluorescent markers viz 6-CF, FITC-Dextran and FITC-lectin were encapsulated in liposomes and then incubated with J774 A.1 cells/peritoneal macrophages. The cytosolic delivery was demonstrated by epifluorescence microscopy. The diffused fluorescence pattern was observed in case of yeast lipid liposomes in contrast to punctate fluorescence in case of PC/Chol liposomes.

The fusion mode of delivery was further confirmed by using fluorescent membrane probe like NBD-PE incorporated in yeast lipid liposomes and interacting them with erythrocyte ghosts and J774 A.1 cells/peritoneal macrophages. The transfer of membrane probe from liposomes to the erythrocyte ghosts depended on yeast lipid liposome concentration and time of incubation. It increased with an increase in incubation time or the liposomes concentration. The significant transfer of 6-CF or NBD-PE from yeast lipid liposomes to J774 A.1 cells was demonstrated even in the presence of CHQ.

The interactions between yeast lipid liposomes and the adherent cells were analyzed by time and concentration dependent uptake of the yeast lipid DRVs containing ^{125}I lysozyme, by the cultured J774 A.1 cells. For this purpose fixed number of cells (about 10^5) were incubated with varying concentration of yeast lipid DRVs for 1h or with a fixed concentration of yeast lipid DRVs (4.8 μmole lipid P) for varying periods of time. The vesicles uptake by the cell increased upto 60 min but saturated thereafter. No such saturation of the uptake was observed when the cells were incubated for 1h with varying concentrations of the yeast lipid DRVs, the uptake increased linearly with the vesicle concentration at least upto 5 μmol lipid P/ml vesicle

concentration.

The cytosolic delivery of water soluble ^{125}I-lysozyme was demonstrated by incubation of J774 A.1 cell/peritoneal macrophages with yeast lipid DRVs as well as SUVs. About 15% of the total cell associated ^{125}I-lysozyme was localized in the cytosol of the macrophages that were incubated with the yeast lipid DRVs loaded with ^{125}I-lysozyme. This cytosolic fraction increased upto 30% when the same cells were incubated with the ^{125}I-lysozyme containing yeast lipid SUVs. Unlike this finding, only < 2% of the cell-associated radioactivity could be detected in the cytoplasmic compartment of the cells which were incubated with ^{125}I-lysozyme loaded egg PC/Chol vesicles. The intactness of cytosol delivered lysozyme was demonstrated by immunostaining (western blotting) of cytosolic fraction of electrophoretogram with anti-lysozyme antibody.

Finally cytotoxic response was demonstrated by immunizing Balb/c mice with yeast lipid DRVs encapsulating ovalbumin. Ovalbumin specific $CD8^+$ cells thus generated were interacted with ^{51}Cr-labeled mouse peritoneal macrophages. These macrophages were pretreated either with saline or free ovalbumin or protein free yeast lipid DRVs or ovalbumin encapsulated in egg PC/Chol DRVs or ovalbumin encapsulated in yeast lipid DRVs. Cytotoxic T cell mediated macrophage lysis was ascertained by measuring the ^{51}Cr release in the medium. It was found that considerably high degree (about 40%) of macrophage lysis occurred when these cells received pretreatment with yeast lipid DRVs loaded with ovalbumin.

These results indicate that the yeast lipid vesicles could serve as carriers for delivering proteins and other macromolecules to the cytoplasmic compartment of the interacting cells. The amount of proteins thus delivered to the cytosol was sufficient to elicit the desired biological response as judged from the present observation that the

$CD8^+$ T lymphocytes derived from the ovalbumin sensitized mouse could exhibit strong ovalbumin specific cytotoxic activity.