PART III

IMMUNOPOTENTIATING ROLE OF IMMUNOMODULATORS
Normally, the immune system of a healthy person confronts well with the invading pathogens leading to their successful elimination from the body. The establishment of the infection could be made possible in situations such as over exposure with the pathogens (in terms of their number as well exposure time), invasion with highly virulent strain or because of the suppressed immune status of the host. Initially, the invasion leads to local or general colonization of the infection, the escaping microbes once find conducive conditions, start multiplying and eventually get disseminated to various parts of the body. Meanwhile immune system also gets activated and applies all possible measures leading to elimination of the pathogens. The war keeps on going unless one overpowers other.

Clinical management should, therefore, involve the restoration of the balanced immune status as the first measure to control of various infectious diseases. Some bioactive substances may offer a strategy to manipulate or alter the state of the immune system in favor of host (Hancock and Chappie, 1999). These substances better referred as immunomodulators are able to influence all vital physiological processes and can play an important role to normalize homeostasis. Restoration of the balanced immune system can be achieved by using the immunomodulators that can support other chemotherapeutic agents for their ability to eliminate infections. Moreover, these immunostimulatory agents or immune adjuvants are not only able to restore the normal response in immunocompromised conditions but can boost the immune status of the subjects susceptible to infective invasions.

Advances, made in recent years regarding the understanding of molecular or cellular basis of immune responses, suggest a much vital role of immunomodulators in fighting various infections. Till date a number of immunomodulators, including small peptides, have been recognized modulate the natural immune response either specifically or non-specifically. These immunomodulators can provide clinical help not only in diseases such as tuberculosis, leishmania and fungal infections, where macrophages, the prime immune cells are impaired by parasites, however also in common viral infections and in immunologically related neural disorders. Tuftsin, an established immunomodulator, for example brings morphological changes in the neurons of visual and sensorimotor cortices and also affects their locomotory activity (Chebotareva, 1990).
The immunoregulatory therapy is a preventive therapy and if used in combination with chemotherapy, can help in treatment of infectious diseases as well. It would be even more valuable in patients who are in immunocompromised state due, either to infection itself or surgical trauma, irradiation, cancer chemotherapy and severe burns etc (Turanek et al., 1997; Talmadge et al., 1984). In combination therapy, it is likely that the antibiotics/antiviral agents would reduce the magnitude of the infection, while the immunomodulatory agents would stimulate the natural immune response of the host for better fight. This eventually would help in complete elimination of the infection and may also reduce the chances of recurrence of infection. Since phagocytes are important in clearing the infectious agents, immunomodulators capable of stimulating their activity would be more helpful in such cases.

Moreover, keeping in view the fact that non-specific defense mechanisms are important in controlling tumor growth and reducing the probability of metastasis, the immunoadjuvants that can enhance the cytotoxic activity of natural killer cells and macrophages could help in cancer therapeutics (Abe et al., 1984). In spite of the fact that they are not so effective as first line of therapy, however, could prove very useful as supplement to cytoreductive therapies like surgical resection, irradiation etc (Turanek et al., 1997). For example, Thymostimulin and thymic extract are found to reduce the chemotherapy induced toxicity and prolongs the survival of the cancer patients (Machhiarini et al., 1989).

Therapeutic use of such immunomodulators would be much precise and advantageous if their mode of action is understood well and is taken into account before using them in combination with particular vaccine/chemotherapeutic agent against a particular disease.

TUFTSIN

In 1970, Najjar and Nishioka demonstrated that Leukokinnin, a leukophilic fraction of immunoglobulin IgG, splits under the action of specific enzyme (Leukokininase) located in the outer membrane of neutrophils. The biological activity of leukokinin rests in a peptide tuftsin- so called because it was discovered at Tufts University (Najjar et al., 1970). Tuftsin is a 289-292 (Thr-Lys-Pro-Arg) sequence in the CH2 domain of the Fc fraction of the IgG molecule.
The tetra-peptide is released physiologically as a free peptide fragment after enzymatic cleavage (Najjar et al, 1987). Two enzymes are responsible for the production of tuftsin from leukokinin, tuftsin endocarboxypeptidase, a specific enzyme that cleaves the heavy chain at the Arg-Glu bond between residues 292-293, and the membrane enzyme leucokininase acts on the bound leucokinin-S to cleave it at the amino end of threonine between residues 288 and 289. Tuftsin is known to bind specifically to macrophages, monocytes and PMN leukocytes and possess a broad spectrum of activities related to the function of immune system primarily (Najjar et al, 1987; Fridkin et al, 1989). These include potentiation of various cell functions, such as phagocytosis, pinocytosis, motility, immunogenic response, fungicidal, bactericidal and tumoricidal activity (Fridkin et al, 1989). The features of tuftsin coupled with its low toxicity make the tetra-peptide a promising candidate for immunotherapy (Nishioka et al, 1986; Khare et al, 1997).

Tuftsin capacity to augment cellular activation is mediated by specific receptors that have been identified, characterized and isolated from rabbit granulocytes (Bump et al, 1986). Tuftsin and many of its analogs have been chemically synthesized and studied extensively for structure-function relationship (Nishioka et al, 1995; Gershonov et al, 1996).

The grafting of tuftsin on the liposomal surface would, therefore, enabled it not only in homing the liposomised-drug to the cells possessing receptors to recognize it, however also stimulate these key cells of the immune system non-specifically against various infections (Singhal et al, 1984). Structure-function studies of tuftsin indicate that its binding and consequent MPS activation is dependent upon rather strict conservation of its molecular structure. Thus the modification of the peptide at its N-terminus or within the chain leads to a significant reduction or even loss of biological activity and also its ability to bind to PMN leukocytes is reduced (Fridkin et al, 1981). As tuftsin is a hydrophilic molecule, it would preferentially reside in the aqueous compartment of liposomes and would not have accessibility to its putative receptors present on the surface of various immune cells. Therefore, the tuftsin was grafted on liposomal surface by attaching a long hydrocarbon fatty acyl residue to the C-terminus through an Ethylene-diamine spacer arm (Thr-Lys-Pro-Arg-NH-(CH\textsubscript{2})\textsubscript{2}-NH-CO-C\textsubscript{15}H\textsubscript{31}) (Singhal et al, 1984).
The incorporation of tuftsin at a percentage of >10 mol% tuftsin in the egg PC/cholesterol (7:3; mol/mol) liposomes was not possible as the resulting mixture could not be dispersed even by the long sonication (Singhal et al., 1984). On the other hand, the liposome containing lower mol% of tuftsin was only poorly bound to PMN leukocytes. Therefore, 7-8 mol% tuftsin in the liposomes is important for the optimal effect. The leakage rate of 6-Carboxy-fluorescin from egg PC/Cholesterol/tuftsin liposomes in which buffer of pH 7.4 at 37 °C was about 2-4%/hour. Incidentally, this leakage rate was dramatically enhanced upon incorporating another tuftsin derivative, Thr-Lys-Pro-Arg-NH-C\textsubscript{18}H\textsubscript{37} in the liposome bilayer (Singhal et al., 1984). This was probably due to binding of the dye with positively charged Arg residue in the analogue. Since this amino acid residue should be aligned just at the bilayer interface, the effect of its binding with the 6-CF on the liposomes permeability must have been mediated through perturbation of the egg PC head group packing in liposomes bilayer (Hauser et al., 1981). The binding/uptake of the egg PC/Chol/tuftsin liposomes to PMN leukocytes was saturable, time dependent and the cell bound liposomes are apparently taken up by the cells by receptor-mediated endocytosis without losing their structural integrity. This was further supported by the fact that endocytosis was inhibited by lowering the incubation temperature to 0° C (Singhal et al., 1984). The specificity of these liposomes was also examined with other blood cells i.e. erythrocytes, lymphocytes and found that no binding with erythrocytes was observed but there appeared some binding with lymphocytes which was presumably due to the presence of PMN leucocytes/monocytes as contamination in the lymphocyte preparation (Singhal et al., 1984).

It has been demonstrated that tuftsin could enhance non-specific defense against infections by activating the macrophages (Singh et al., 1992). The biological activity of the peptide was due to the induction of the macrophage respiratory burst and activated macrophages exhibited enhanced levels of NADPH oxidase, O\textsubscript{2}, H\textsubscript{2}O\textsubscript{2} and myeloperoxidase (MPO). Both super oxide and H\textsubscript{2}O\textsubscript{2} damage proteins, nucleic acids and membranes sufficiently to kill the cell or even the whole organism. Moreover, for macrophages hypohalous acid produced by action of MPO on H\textsubscript{2}O\textsubscript{2}, has been identified as the major killer agent (Klebanoff et al., 1980).
3.1 Anti-tumor activity.

Many workers have reported the anti-tumor activity of tuftsin against experimental tumor models (Nishioka et al, 1981; Nishioka et al, 1983). Florentin et al stated that tuftsin is able to potentiate various types of immune response when injected into mice, and can be used as a potent activator of macrophages in cancer therapy (Florentin et al, 1978). This was also confirmed by the finding that tuftsin treated mouse peritoneal macrophages exert cytostatic activity for tumor cell proliferation (Bruley-Rosset et al, 1981). Tuftsin was also reported to enhance the cytotoxic response of human monocytes against K562 tumor cell line at the doses of $5 \times 10^{-2}$ to $5 \times 10^{-1}$μg/ml. In contrast, the natural killing activity of lymphocytes against that particular cell line was not affected by tuftsin (Caroll et al, 1982). Tuftsin was also used for the treatment of cancer in humans with corresponding experiments in animals (Catane et al, 1983). Tuftsin at the doses ranging between 50 and 500μg/kg of the body weight enhances the cytotoxic activity of mononuclear cells in mice and human, and, in mice, also shows antitumor activity. The effect of tuftsin was accompanied by leucocytosis induction (Catane et al, 1983). It was also stated that tuftsin significantly increases survival rates among Rauscher virus leukemia infected mice and demonstrates antitumor activity against murine melanoma in vivo (Knyszynski et al, 1983; Noyes et al, 1981).

3.2 Immunopotentiating effect

The primary effect of tuftsin, after binding to receptors, consists of stimulation of macrophages and polymorphonuclear (PMN) cells. Specific binding sites of tuftsin are also localized on human monocytes. Tuftsin administered to the cell cultures stimulates the production of some cytokines. Intraperitoneal (i.p.) injection of tuftsin increases the production of TNF-α in serum and supernatants of cultured splenic and peritoneal cells (Wleklik et al, 1987). Robey et al showed that tuftsin as well as its analogs, [Gly$^1$]-tuftsin, [Leu$^4$]-tuftsin, and [Gln$^4$]-tuftsin (all being fragments of human C-reactive protein), induce monocytes to produce IL-1 (Robey et al, 1987). Recently, it was also found that treatment of mouse peritoneal macrophages with tuftsin or tuftsin-THF-γ2 chimeras in the presence of antigen augments the IL-6 production (Granoth et al, 1997). In this way, tuftsin may perform its immunoregulatory functions and may influence
inflammatory processes by enhancing the IL-2 formation induced by IL-1. It was initially found that tuftsin stimulates phagocytosis after binding to PMNs. Subsequently phagocytosis stimulating activity of tuftsin in monocyte-macrophages was also demonstrated by some workers (Coleman, 1986).

Tuftsin also potentiates antibody levels following the simultaneous injection along with a T cell-dependent antigen to mice with genetically controlled defect in affinity maturation, however did not affect antibody affinity in the same mice (Holland et al, 1990). Since peritonitis caused by Candida albicans is a major complication of continuous ambulatory peritoneal dialysis (CAPD), the activation by tuftsin of peritoneal macrophages may be considered a potential therapeutic option in that disease (Kain et al, 1989). Kazanowska et al studied the influence of tuftsin on the functions of granulocytes of children with acute lymphoblastic leukemia (Kazanowska et al, 1987). Tuftsin was found to increase the phagocytic activity of cultured Sertoli cells of rats as well in cultures of murine Kupffer cells (Filippini et al, 1989; Kubo et al, 1994).
3.3 Applications of tuftsin bearing liposomes in various infections

3.3.1 Fungal Infections

Opportunistic fungal infections remained to be a major problem in management of immuno-compromised patients. The presence of any fungal disease implies that host defense system has been compromised. Of late opportunistic fungal infections considered to be major cause of morbidity in immuno-compromised human subjects. Patients with acute leukemia, especially following hospitalization and administration of antibiotics, are prone to various fungal infections and their early diagnosis in cancer patient still remains elusive (Horn et al., 1985). Chemotherapy with anti-fungal agents is a priory for both systemic as well as superficial fungal infections in humans. Various commonly used anti-fungal chemotherapeutic agents include; Polyenes, Azoles, Allylamines, Morpholines, Flucytosin, Griseofulvin, Iodides, Hydroxy-stabamine and imidazole classes of drugs. The elimination of the fungi from the tissues of normal healthy persons is often accompanied with stimulation of cell-mediated immune response, which involves activation of mononuclear phagocytes by sensitising T cells. Treatment with anti fungal drugs in combination with tuftsin provoke macrophages/monocytes must find useful application in fungal chemotherapy (Owais et al., 1993).

Amphotericin B, a polyene antibiotic, has been widely used in clinical practice to treat various fungal infections e.g. candidiasis, cryptococcosis, histoplasmosis and aspergillosis (Gallis et al., 1990; Lopez-Berestein et al., 1989). Besides, mild side effects associated with Amp B such as headache, chills, severe hemolytic anemia, nephrotoxicity is the most serious problem with amphotericin B therapy (Walsh et al., 1992). The toxicity of Amp B to cells originates from its binding to sterol present in the cellular membrane which involves disorganization of membranes by formation of specific pores composed of small aggregates of Amp B and sterol (Vanden et al., 1994). These defects cause depolarization of membrane and consequently an increase in the membrane permeability to protons and monovalent cations, which leads to cell death.

With a view to further increase the efficacy of liposomised-Amp B, the drug was incorporated in tuftsin bearing liposomes and studied for its potential against A.
fumigatus infections in murine model (Owais et al, 1993). The results of these studies revealed that the percent survival of A. fumigatus infected mice increased (70-75%) by treating them with tuftsin bearing liposomal Amp B as compared to the animals that received treatment only with liposomal Amp B. The animals from the Tuft-lip-Amp B treated group were found free of infection whereas animals treated with liposomal Amp B still had some fungal load. These results strongly indicate that efficacy of Tuft-lip-Amp B increased remarkably against A. fumigatus infection due to tuftsin mediated activation of macrophages/monocytes, the key cells of host defense system.

3.3.2 Malaria

Malaria is still considered the most prevalent and devastating disease worldwide affecting about 300-500 million people and claims 1.5-2.7 million human lives. Furthermore, one-third of world human population dwells in the areas infested with the disease (Butler et al, 1997). Numerous efforts have been made for the development of the effective vaccines against malaria as effective vaccine may elicit a protective immune response in individuals of diverse genetic make up and could complement other strategies for prevention and control of this serious and most common public health problem in future. Although these strategies can provide crucial knowledge of nature of the protective host, immunological mechanisms and their respective target antigens but still there is no effective malaria vaccine, only chemotherapy remains the major practical option for managing all forms i.e. exo-erythrocytic and erythrocytic stages, of infection. Moreover, the situation is aggravating as the malarial parasites are rapidly developing resistance to the existing malarial drugs when given in classical pharmaceutical forms (Basco et al, 1998; Peters et al, 1998).

It has been reported that pretreatment of mice with tuftsin, rendered them at least partially resistant to lethal P. berghei infection (Gupta et al, 1986). This effect of tuftsin gets further increased by incorporating tuftsin in the liposomes bilayer where both the mortality and parasitemia in the animals that received pretreatment with liposomal tuftsin were significantly reduced as compared to those pretreated with saline or control liposomes. The mean survival time of mice pretreated with 50 or 100 µg doses of liposomal tuftsin were about 16 or 19 days, respectively, which were greater than that
observed with free tuftsin (Gupta et al, 1986). Since suppression of host immune response seems to be the reason in almost all Parasitic infections (Clayton, 1979), the immunomodulating agent such as tuftsin may help in bringing the immune status to normal, which in turn may further enhance the efficacy of chemotherapy.

3.3.3 Tuberculosis

Tuberculosis is the single most disease that results in the largest number of deaths worldwide; nearly 3 million people are killed every year (Noordeen et al, 1988). The association of tuberculosis with HIV infection has further exacerbated the already complicated situation in developed and developing countries (Weiss, 1992). This infection also increases the risk to new tuberculosis infection that will progress to disease (Tuberculosis control and research strategies for the 1990s; WHO, 1992; Weiss, 1992).

The most important factor in the treatment of tuberculosis is prolonged chemotherapy, for a minimum period of 6-12 months, which is often associated with serious and undesirable side effects e.g. hepatotoxicity (Girling, 1978; Raleigh, 1972). It is therefore desirable to develop an approach, which allows the use of lower drug doses with the use of delivery systems to the infected cells, thereby improving efficacy and potentially reducing toxicity.

Liposomes can solve some of the above problems by serving as carriers of drugs for site specific or sustained delivery. Passive targeting of liposomes to MPS (Alving, 1986; Bakker-Woudenberg et al, 1994) could be utilized as M. tuberculosis resides and proliferates primarily with in the mononuclear phagocytes, which normally serve as the first line of defense against infections. Rifampicin, isoniazid, streptomycin, pyrazinamide, thioacetazone, ethambutol and p-amino salicylic acid are the front line drugs in the treatment of tuberculosis (Mitchison et al, 1988; Raleigh, 1972). A number of studies have demonstrated that the efficacy of these anti tubercular drugs can be increased by encapsulating or incorporating them in the liposome. Liposomes besides delivering the drug to the infected site could also act as drug reservoirs to provide slow and sustained release of the drug. This would not only reduce the cost of the treatment but may also minimize the duration of the treatment, two major drawbacks associated with the therapy of the tuberculosis. The use of liposomes as vehicles of rifampicin for
the treatment of tuberculosis has been demonstrated in mice (Agarwal et al, 1994). The drug acts on DNA dependent RNA polymerase in the bacterial cell to block the protein synthesis and kills the microorganisms. The anti-bacterial activity of rifampicin is high because it also kills the semidormant bacilli. The drug is thus comparatively safe, but its half-life in circulation is relatively short (3 hours) and most of the drug following oral administration is metabolized and excreted, leaving only a limited amount available for activity against mycobacterium. Thus daily dose of 600 mg of RFP is required for effective treatment, which often leads to serious side effects (Raleigh, 1972).

The anti-tubercular activity of liposomised drug was considerably increased, as compared to free drug, because of the ability of liposomes to localize preferentially in macrophages/monocytes, leading a high intracellular drug concentration. This was further supported by the findings that intermittent treatment (twice weekly), with these preparations, was significantly more effective rather than the continuous treatment. The activity of the drug was further increased when the drug was loaded in tuftsin bearing liposomes. Rifampicin delivered in these liposomes was at least 2000 times more effective than the free drug in lowering the load of lung bacilli in infected animals (Agarwal et al, 1994). The dynamics of the distribution of RFP-liposomes in healthy and tuberculous mice showed that a greater liposome concentration in the liver, spleen and lungs of healthy mice was achieved, although the retention in tuberculous mice was longer. Besides tuberculosis RFP is also effective in leprosy and MAIS complex infections, thus widening the scope of RFP-loaded liposomes in treatment of a variety of mycobacterium infections.

3.3.4 Leishmaniasis

Leishmaniasis is caused by the haemoflagellate protozoan and represents four major clinical syndromes; visceral, cutaneous, mucocutaneous and diffuse cutaneous leishmaniasis. It is estimated that worldwide more than 12 million people are infected and approximately 350 million at the risk. The most devastating clinical form, visceral leishmaniasis (kala azar) is caused by L. donovani characterized by fever, hepatosplenomaegly, anemia and leukopenia. The major front line drugs for treatment of leishmaniasis are toxic. Several doses need to be given over a prolonged time period and
development of drug resistance is becoming a major problem. Since leishmaniasis affects the MPS cells, a number of studies exploit the liposomes as drug carriers for the treatment of leishmaniasis (Alving, 1986; Coukell and Brogden, 1998).

The efficacy of several liposomal formulations of stibanate has been shown against *L. donovani*. The encapsulation of sodium stibogluconate, a major front line drug for leishmaniasis, in tuftsin bearing liposomes demonstrated that efficacy of the drug was markedly increased against *L. donovani* infections in hamsters (Guru et al, 1989). The improvement in the therapeutic efficacy of the liposomised drug may result from the respiratory burst-inducing activity of tuftsin, in addition to the effect of the targeted drug delivery of drug to the macrophages (Guru et al, 1989). The pretreatment of animals with free and liposomal tuftsin enhances their resistance to leishmania infection (Guru et al, 1989). The susceptibility of peritoneal macrophages from pre-treated animals to Leishmania infection was examined *in vitro*. The parasite multiplication inside such macrophages was considerably decreased, as compared to the macrophages of untreated animals (Guru et al, 1989). The resistance of the Parasite to antimonials is creating difficulty in management of leishmaniasis (Bryceson, 1987). The second front line drug Amp B is quite effective as an anti-leishmanial agent but it suffers from serious side effects. The toxic effects of Amp B can be minimized, keeping intact the antileishmanial property, by encapsulating this drug in the liposomes. The LD$_{50}$ of Amp B was increased after liposomisation (Berman et al, 1986). The anti-leishmanial activity of Amp B is further enhanced when tuftsin was incorporated on the surface of Lip-Amp B liposomes (Guru et al, 1989). These results clearly indicate that Amp B in liposomes helps not only helps in reducing the drug toxicity but also increases the drug efficacy against *L. donovani* infections. The Tuft-Lip-Amp B was more effective than Lip-Amp B against Leishmaniasis even at a low single dose of 0.5 mg/kg. The tissue distribution study demonstrates the higher and faster uptake of Tuft-Lip-Amp B liposomes from circulation with most of the liposomes being cleared from the circulation within one hour after administration as compared to Lip-Amp B.