CHAPTER I

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1. Vaccines and Vaccination:

1.1. Vaccination: a prophylactic measure to control infectious diseases.

Development of vaccine constitutes the greatest achievement in the field of modern medical sciences. It is perhaps one of the most cost effective and economic therapeutic interventions available today. Conventional vaccines consisted of live attenuated pathogens, whole inactivated organisms, or inactivated toxins. Although such forms of vaccine have proved successful in past, several drawbacks limited their use against more challenging diseases such as hepatitis C and AIDS etc. First, certain live-attenuated vaccines can cause disease in immunosuppressed individuals by reverting to a more virulent phenotype. Second, whole inactivated vaccines (e.g. *Bordetella pertussis*) contain reactogenic components that can cause undesirable side effects. Third, some pathogens are difficult or even impossible to grow in culture (e.g. hepatitis B, hepatitis C, and human papilloma virus etc.) making preparation of a vaccine problematic. During recent past, several approaches in vaccine development have emerged that may have significant advantages over traditional approaches. These new strategies include recombinant protein subunits, synthetic peptides and plasmid DNA. Although they offer advantages such as reduced toxicity, they are poor immunogenic when administered alone. This is particularly true for the vaccines based on recombinant proteins and peptides. Further, traditional vaccines are heterogeneous and contain many epitopes, some of which can provide additional T-cell help or function as adjuvants (e.g. bacterial DNA in whole cell vaccines). Therefore, a great need exists for immunological adjuvants that are potent, safe, and compatible with new generation vaccine, including DNA vaccine.

Vaccines can be broadly classified into four major groups: live, killed/inactivated, subunit and DNA based vaccine. Each of these groups can be further divided into various subclasses, which include recombinant derived antigens as well as native microorganisms and their components. In addition, there are new enabling technologies such as delivery systems and vectors, which can be exploited as potential adjuvant to achieve coveted goals. Most pathogens targets, whether infectious or noninfectious in origin, can be approached by the application of several different vaccine technologies and can be tested during the discovery phase of vaccine development.
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Table: 1

Comparative properties of active vaccines

<table>
<thead>
<tr>
<th>Type of vaccine</th>
<th>Salient features</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live vaccine</td>
<td>Able to replicate in the host</td>
<td>May elicit broader immune responses</td>
</tr>
<tr>
<td></td>
<td>Attenuated devoid of pathogenicity</td>
<td>May require fewer does</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Generally longer and lasting protection</td>
</tr>
<tr>
<td>Subunit vaccines</td>
<td>Unable to replicate in the host</td>
<td>Cannot multiply or revert to pathogenicity</td>
</tr>
<tr>
<td>(killed inactivated)</td>
<td></td>
<td>Generally less reactogenic</td>
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<tr>
<td></td>
<td></td>
<td>Non transmissible to another person</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Usually more feasible technically</td>
</tr>
<tr>
<td>Genetic vaccines</td>
<td>Stimulate synthesis of antigens only upon</td>
<td>Elicit cellular immune responses</td>
</tr>
<tr>
<td>(DNA- based)</td>
<td>administration in the cells</td>
<td>Standardized method of production</td>
</tr>
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1.2. Live vaccines:

Conventional live viral and bacterial vaccines are produced by selecting avirulent mutants, which can establish themselves in host, however fail to invoke full blown disease. The mutations are generally induced by treatment of organisms with mutagens, chemicals, heat or passage in vitro. Following induction of mutational events, a virulent mutant is selected and tested in vivo. In most cases, multiple mutations led to the development of attenuated form of pathogen although it is often
unknown what constellation of genes are altered to achieve attenuation. Using this approach, it is difficult to modulate the degree of attenuation and if the attenuation happens to be due to single-point mutation, there is high probability of reversion to the virulence upon administration to the host (Minor et al 1986). This reversion can result into full blown disease, especially to the underlying immunosuppressive conditions. Secondly, some of the viral vaccines shed virulent virus into the environment, which can infect non-vaccinated contacts.

Nevertheless, some live vaccine come very close to meeting the criteria for an ideal vaccine and elicits life long protection with minimal reactogenicity. Such vaccines consist of microorganisms (usually viruses) that replicate similarly to the natural microorganism in vivo, thereby eliciting an immune response similar to that of natural infection. The live vaccine is attenuated by technical or biological manipulations for eliminating its disease causing capacity. The live vaccine should be neither over attenuated, such that it no longer replicates sufficiently to function as a vaccine, nor under attenuated, whereby it retains even limited pathogenicity or ability to revert to pathogenicity.

1.3. Subunit vaccine:

Subunit vaccines are defined as those containing one or more pure or semi-pure antigens. In order to develop subunit vaccines, it is critical to identify the individual components out of myriad of proteins and glycol-proteins of the pathogen, that are involved in inducing protection. Indeed, some of the proteins present in pathogens are immunosuppressive, whereas immune responses to certain other proteins may actually enhance disease. Thus, it is crucial to identify antigenic proteins that are important for imparting protection and help in elimination of pathogens. The potential advantages associated with the subunit vaccines are increased safety, less antigenic competition. Although, subunit vaccines can be produced by conventional technologies, the purification is generally not economic due to low quantities of protective antigens produced by most of the infectious agent. For example, Pasteurella haemolytica produces low level of extra cellular toxin (leukotoxin), which is involved in pathogenesis and tissue damage in the lungs of cattle (Lo et al 1987, Shewen and Wilkins, 1982). This makes the development of neutralizing antibodies a very costly affair. In addition, some bacterial proteins required for scavenging nutrients such as
iron are not expressed at sufficient level to allow purification of protective component. By identifying the proteins involved in inducing protective immunity, or the genes coding for these proteins, it is impossible to use recombinant DNA technology or synthetic peptide technology to produce sufficient quantities of these protective epitopes for incorporation into vaccines.

In addition to identifying potential targets of pathogens, it is also possible to develop subunit vaccines, which reduce vector transmission of some pathogens by immunization against the vector. For example, many infectious agents are transmitted by blood feeding vectors. Studies have shown that immunization of animals against antigens present in the gut of the vector results in the disruption of their intestinal cell function when exposed to antibodies rich blood meal directed against these intestinal antigens. Relying on the recombinant DNA technology to express protective antigen and its use in development of pathogen specific vaccine is demand of the ordeal. The identification and subsequent expression of such antigen is likely to shorten the time required to develop subunit vaccines for a large number of pathogens.

1.4. Genetic Vaccines:

A recent novel approach for successful immunization exploits usage of DNA that encodes specific protective antigen. The in vitro paradigm for this approach lay in the transformation of mammalian cells in the culture with a plasmid that directs the expression of a vaccine antigen from the cells that take up the plasmid DNA. Subsequent to its uptake the administered DNA encodes antigen (s) that can be secreted or be associated with the cell surface in a way that would trigger a humoral or cellular immune component of the host. The uptake of DNA can be facilitated by chemical formulation or delivery by a virus or bacteria. Several strategies have been exploited for immunization with genetic vaccine.

1.4.1. Purified DNA:

In the early nineties, intramuscular administration of solution of uncoated or naked DNA encoding a vaccine was found to induce desirable immune response (Wolf et al 1990). Cells take up the DNA, transcribe it to induce desirable antigen, which may be processed in a similar way to that of administration of DNA subsequent to viral
attack. Both humoral and cellular immune responses to the encoded antigen were elicited. The advantages of using DNA based vaccine involve relatively technically easy way of preparation and ability to direct the synthesis of multiple copies of mRNA that eventually results in amplification of both antigen synthesis and immune responses. Such vaccine has been shown to be effective in many animal models. DNA vaccines are particularly proficient at eliciting cellular immune responses. One strategy has been the presentation of DNA on gold micro projectiles that are shot directly into cells that eventually results in expression of antigen to stimulate an immune response (Williams et al 1991). DNA also has been coated with cationic lipids, lipospermines or other molecules, which neutralize its charge, while surplus cationic lipid moieties facilitate cellular uptake (Remy et al 1994).

1.4.2. Virus mediated DNA delivery:

For delivery of DNA by fowl pox or canary pox virus, the expression cassette for the recombinant protein is integrated into the viral genome. Although able to infect avian species to produce disease, these poxviruses can infect mammalian (human) cells without inflicting any infection; hence can be considered as a safe genetic approach for immunization (Kent et al 1994). This single round of self – limiting infection may be sufficient to elicit broad immunity against pathogen whose recombinant polypeptide is expressed by these avian poxviruses in infected cells. The approach has some basic advantages over Vaccinia virus as these viruses have considerably less reactogenicity mostly because of their inability to spread with in host.

1.4.3. Bacterial delivery:

The intracellular bacteria can be engineered to deliver plasmid DNA into host cells for the expression of recombinant proteins. *S. flexneri* has been attenuated by making a deletion mutation in the essential asd gene (Sizemore et al 1996). The attenuated strain can be prolonged *in vitro* in the presence of diaminopimelic acid (DAP) and retains its potential to invade cells (as long as it maintains a plasmid encoding invasion – associated polypeptides). However, it cannot replicate *in- vivo* because of the absence of DAP. A plasmid harboring a prokaryotic promoter and recombinant gene can be transformed into this strain. The resultant recombinant *S. flexneri* strain was shown to be able to invade mammalian cells *in-vitro* and to express the plasmid-
encoded protein as a potential vaccine antigen. Since, *S. flexneri* replicates in the intestine and stimulate mucosal immunity; its oral administration can effectively deliver DNA to the cells to evoke mucosal immunity.

1.5. Combination vaccines:

In this strategy, combination of more than one vaccine can be administered to the hosts. There are two general categories of combination vaccines.

**Multi Disease combination vaccines** include individual vaccines for several diseases. A multi disease combination is usually developed after each individual vaccine in the combination has been developed and licensed separately, so that this becomes a combination of licensed components.

**Multivalent combination vaccines** are directed against more than one type (serotypes or serogroups) of viral or bacterial pathogen. A multivalent combination is developed as such from the outset rather than developing and licensing each individual serotype vaccine separately. While multivalent combinations invariably are mixed during the manufacturing process. Multi disease combinations may be mixed at one of three stages: (i) during the manufacturing process such that the combination is filled into vials or syringes. (ii) At the time of administration by prior mixing of different vaccines in a vial then using the mixture for injection or (iii) mixing of individual vaccine preparation at the time of administration using dual chambered syringe. While the first alternative is clearly preferred for ease of use, it is not always possible in cases where the combination is not compatible giving rise to fully unstable preparation.

Till date only three different types of combination vaccine are available. Live attenuated vaccines consisting of viruses or bacteria, which have been cultivated *in vitro* in a way which attenuates their infectivity and pathogenicity. Such strains of viruses or bacteria are able to infect and replicate *in vivo* to some limited extent but do not cause disease. The second type of combination vaccine is referred to as non live (inactivated, killed or subunit vaccine). Such vaccines may be chemically inactivated viruses or bacteria (inactivated or killed vaccine), individual antigens purified from viruses or bacteria or expressed by recombinant host cells (subunit), or synthetic components such as peptides (subunit). Vaccines may be combinations of live or non-live components, but there are not yet any examples of successful combination of live
and non live components together. The third type of vaccine has been categorized as genetic or DNA based, however as this type of vaccines have entered clinical trials in recent past only, there are not yet examples of combinations of such vaccines. There are six combination vaccines, both multi disease and multivalent, which have been available for periods of 2 to 6 decades for routine immunization as discussed below. Some of these have been mixed with other vaccines and further developed into combination vaccine.

1.5.1. DTP (Diptheria- Tetanus- Pertussis) vaccine:

This multi - disease non- live combination vaccine is available for over last fifty years and required as pediatric vaccine in most major countries worldwide. It is typically being given three to four times in first two years of life with subsequent boosters. (Ramon 1923, Plotkin and Cadoz 1997).

1.5.2. Influenza vaccine:

The trivalent inactivated combination vaccine has been available for over last forty years (Kilbourne 1994). It is widely used, especially in older adults, for the prevention of acute disease through annual immunization. The vaccine consists of two HA antigen serotypes of influenza virus; one of serogroup A and serogroup B.

1.5.3. Polio vaccine:

This multivalent combination vaccine has been available in two different forms. The inactivated vaccine (IPV; Salk vaccine) is a combination of three serotypes of poliovirus, each is purified and inactivated prior to mixing. The vaccine is indicated for immunization up to four times in first two years of life (Salk and Gori 1960). The live attenuated oral polio vaccine (OPV; Sabin vaccine) is trivalent, consisting of a combination of three serotype viruses, each of which has been attenuated through in vitro passage (Sabin 1957). The vaccine is indicated for immunization three to four times in first two years of life, or is given in a mixed regimen with the first two doses as IPV and next two doses as OPV (Plotkin et al 1968).
1.5.4. MMR (measles, mumps, rubella) vaccine:

This multi disease live combination has been available for more than 30 years (Weibel et al 1980). Each of the three components of the vaccines was attenuated through in vitro passage and developed as an individual vaccine prior to the combination being made and developed. In areas where trivalent vaccine has been widely utilized, there has been a >99% reduction in the incidence of each of three diseases (Paunio et al 1991).

1.5.5. Pneumococcal vaccines:

This multivalent combination has been available for over last two decades (Austrian et al 1989). Even though there are > 80 known serotypes of Streptococcus pneumoniae (Pn), it was recognized that only limited population is responsible for most cases of disease in susceptible adults as well as children.

1.5.6. Meningococcal vaccine:

This multivalent vaccine has been available for more than two decades. Among the multiple serotypes of Neisseria meninitidis, five serotypes were recognized as responsible for infliction of disease in children and adults. The serotype specificity is carried on polysaccharide (Ps). Among them type B, is non immunogenic. Therefore, the vaccine was developed as a mixture of remains four capsular Ps (types A, C W-135, Y) for the immunization of children and adults (Armand et al 1982).

1.6. Marker vaccine:

A marker vaccine can be either a subunit or a gene- deleted vaccine and is defined as one which can be used in conjunction with a diagnostic test to differentiate a vaccinated animal versus a carrier animal. These vaccines are gaining popularity in veterinary medicine where eradication of specific diseases is of national interest. The technology for developing marker vaccines has been used most extensively for eradication of Herpes- virus in pigs and cattle. The basis of marker vaccine is that animals develop immune responses to the antigens present in the vaccine but not to antigens of the pathogen origin that have been excluded from the vaccine, since these vaccines reduce shedding from latently infected animals, it is possible to immunize a herd for a number of years and eventually reduce the shedding to such a level that only the older animals are latently infected (Bosch et al 1998). One important
criterion for choosing a marker protein is that it must be one that induces a rapid and long lived antibody response in infected animals but not in vaccinated animals even after repeated immunization.

2. Attributes of the successful vaccination:

The requirement of eliciting a protective immune response against pathogens requires involvement of specific components of immune system for their successful clearance from systemic circulation. In general, pathogens may simply be eliminated by specific antibodies, however, certain classes of pathogens adapt intracellular shelter to avoid antibody on slaught. The elimination of intracellular pathogens e.g. *Mycobacterium, Leishmania, Candida sps* is executed with the help of CD8^+ T-cells of the host immune system (Flynn *et al* 1993, Muller *et al* 1991). Interestingly, requirement of vaccination to evoke characteristic immune responses against some pathogens may even vary against different stages of their life cycle. For example, taken example of malaria parasite *Plasmodium sps*, it has been seen that the protective immunity required against sporozoites and liver stages of parasite may be generated by specific CD8^+ CTL responses (Suss and Pink 1992), while the require CD4^+ Th1 type immune response successfully suppress blood stages of the parasite without involving CD8^+ T-cells (Vander-Heyde *et al* 1993). Thus before designing a vaccine, one should keep into consideration not only the type of pathogen but the specific immuno-protective mechanism also that is involved in eliminating the pathogen. As suggested by Ada and Ramsay (1997) the general attributes of successful vaccination include:

I. The vaccine must be able to activate antigen presenting cells (APCs) for the processing and presentation of antigen by the lysosomal/cytoplasmic pathways, the expression of co-stimulatory factors and chemokine receptors at the cell surface, and the secretion of certain other important cytokines.

II. The vaccine formulation should enhance the replication and differentiation of T and B lymphocytes that leads to the generation of large pools of memory cells of desirable phenotypes.

III. Vaccine preparation should include both sufficient B-cell epitopes to generate strong neutralizing antibody responses as well as T-cell determinants that bind with high affinity to at least the major regional HLA haplotypes to be recognized by the T-cell receptor.
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IV. The long term persistence of conformationally intact antigen is desirable, preferably as aggregates complexed with antibodies and held at the surface of FDCs (Follicular Dendritic Cells) in lymphoid tissues. This allows the continuing production of cells those secret antibody with increasing higher affinity, and memory B-cells responses.

3. Immunological adjuvant:

Immunological adjuvants were originally described by Ramon as substance used in combination with a specific antigen that produce more immunity than the antigen alone (Ramon 1924). This broad definition encompasses a very wide range of materials that can adjuvenate immune system of the host upon their administration (Vogel and Powell 1995). However, despite extensive evaluation over many years, the only adjuvants currently approved by US Food & Drug Administration (Rockville, MD) are aluminium- based mineral salts [generically called alum (Gupta 1998). The most important issue in adjuvant development is its safety, which has restricted the development of newer adjuvant (Eldman 1997). Many experimental adjuvant have advanced to clinical trials and some have demonstrated high potency, but most have proved too toxic for routine clinical use. For standard prophylactic immunization in healthy individuals, the adjuvant that induce minimal side effect is likely to be acceptable. In contrast, for adjuvant that are designed to be used in life threatening situations (e.g. cancer), the acceptable level of adverse events is likely to be higher. Additional issues that are important for adjuvant development include biodegradability, stability, ease of manufacture, cost and applicability to a wide range of vaccines. Ideally, for ease of administration and patient compliance, an adjuvant should allow a vaccine to be given by a mucosal route. Some of the adjuvant that has been evaluated in clinical trials are enlisted in (Table 2), Although the mechanisms of action of most of the adjuvant are often poorly understood, they can be broadly classified on the basis of their basic principal of action. Hence, the most convenient way to classify the wide range of adjuvant includes their mode of action as mentioned below (Ada and Ramsay 1997)

I. Some adjuvant (e.g. Freund’s adjuvant) forms a depot of antigen for its long persistence at the site of the application primarily and from where the antigen is released over a period of time.
II. The activation and maturation of APCs, particularly DCs and macrophages, may be influenced by some adjuvant (e.g. liposomes) resulting in expression of different co-stimulatory molecules chemokines receptors.

III. Many adjuvant are shown to increase the synthesis and secretion of enhancing factors, such as cytokines and chemokines that act principally for evoking immune response, but not only on cells of the immune system, especially T and B lymphocytes.

3.1. Mineral salts:

The mineral salts, which have been used as adjuvant, include aluminum hydroxide/phosphate (alum adjuvant) and calcium phosphate. Alum has historically served as immuno-potentiater in vaccine development and continued to be the most widely used adjuvant (Lindblad 1995). Alum has a good safety record, but comparative studies show that it’s a weak adjuvant for antibody induction against protein subunits and a poor adjuvant for cell mediated immunity. Not to be on very positive side, alum adjuvants can induce immunoglobulin E (IgE) antibody responses and have been associated with some allergic reactions in human subjects (Gupta 1998, Relyveld et al 1998).

3.2. Microbial components:

3.2.1. Muramyl dipeptide:

N-acetyl muramyl-1 alanyl-D isoglutamine is smallest subunit of the mycobacterial cell wall that retains immuno-adjuvant activity and referred as Muramyl dipeptide (MDP). MDP solution administered in saline induces humoral immune responses mainly, while water in oil emulsion (e.g. Syntax, emulsion of threonyl MDP and squaline) increases antibody and cell mediated immunity (Ellouz et al 1974, Warren et al 1986).

3.2.2. Monophosphoryl Lipid A:

Monophosphoryl lipid A (MLA) is a less toxic but active derivative of lipopolysaccharide (Ribi et al 1984). The adjuvant promotes IFN-gamma production
by T-cells and thus indirectly helps in the production of antibodies (Tomai and Jhonson 1989). MLA preferentially triggers Th-1 cells and direct secretion of IgG2a isotype (Takayama et al. 1991). Because of its safety and

Table 2

Selected examples of vaccine adjuvants

<table>
<thead>
<tr>
<th>Adjuvant type</th>
<th>General examples</th>
</tr>
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<tbody>
<tr>
<td>Mineral salts</td>
<td>Aluminium hydroxide/phosphate, Calcium phosphate</td>
</tr>
<tr>
<td>Microbial</td>
<td>Muramyl dipeptide, bacterial exotoxins (e.g. cholera enterotoxin)/ endotoxins (e.g. monophosphoryl lipid A, bacterial DNA)</td>
</tr>
<tr>
<td>Particulate</td>
<td>Microspheres, ISCOMS, Liposomes, niosome, virosomes.</td>
</tr>
<tr>
<td>Oil emulsion and surfactant-based</td>
<td>Freunds adjuvant, MF 59, SAF</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Saponins (e.g. QS 21), Muramyl peptide derivatives, non-ionic block copolymers, synthetic polynucleotides</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IL-2, IL-12, GM-CSF, IFN-γ</td>
</tr>
<tr>
<td>Genetic</td>
<td>Cytokine genes or genes encoding costimulatory molecules delivered as plasmid DNA</td>
</tr>
</tbody>
</table>

extensive clinical evaluation, MPL is strong candidate for human preventive and therapeutic vaccines against infectious and neoplastic diseases.

3.2.3. Bacterial DNA:

One of the most promising classes of new adjuvant is the immunostimulatory sequence in bacterial DNA. These are short nucleotides of DNA containing un methylated
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guanine and cytosine dinucleotides, so called CpG motifs. They trigger B cell activation and induce cytokine response leading to Th-1 response. CpG oligodeoxynucleotide work for both systemic and mucosally administered antigens (McCluskie and Davis 2000, Weeratna et al 2000).

3.3. Cytokines:

Several cytokines have been used as experimental vaccine adjuvants, including IL-2 and IFN-γ (Nohira and Rubin 1994). Recently IL-12 and IL-18 have also been incorporated in various newly developed vaccines (Schott and Trinchieri 1997). Cytokine mixtures, including GM-CSF, TNF-alpha and IL-12 emulsified in IFA have also been employed to steer the immune response in desired direction (Ahlers et al 1997).

3.4. Oil emulsion and surfactant based vaccine:

3.4.1. Freund's Adjuvant:

The powerful immunologic adjuvant is composed of water- in- mineral oil emulsion and containing killed mycobacterium as an additional immunomodualtor (Freund et al 1937). This adjuvant is known as Freund' s complete adjuvant (FCA). Although FCA is one of the most effective adjuvants known, is highly reactogenic and cannot be used in human vaccines. However, Freund, s incomplete adjuvant (FIA), which does not contain mycobacteria was employed in an influenza vaccine licensed in the United Kingdom and is used in several HIV vaccines that are under clinical evaluation (Jancen et al 1998).

3.4.2. MF59 Adjuvant:

MF59 is oil in water microfluidised emulsion composed of stable droplets (250nm) of the metabolizable oil squaline (4.3 %w/v) and two surfactant polyoxyethylene sorbitan monooleate (Tween 80; 0.5 % w/v) and sorbitan trioleate (Span 85; 0.5 % w/v). MF59 is safe, practical and potent adjuvant for use with human vaccines, with more than five million doses distributed. The adjuvant has been shown both in animal models and in humans to be a potent stimulator of cellular responses to subunit antigens. Toxicology studies in animal models and phase I through IV studies in
humans have demonstrated the safety of MF 59 with HSV, HIV, and influenza vaccines (Podda 2001, Ott et al 1995).

4. Immunological perspective of controlled antigen delivery:

The response to various vaccine antigens is routinely optimized by assessing a variety of delivery methods that include variation of the adjuvant and delivery systems used or manipulation in the dose and number of injections as well as the route of vaccine delivery etc. The recent advances in the antigen delivery have resulted in the development of controlled delivery systems (such as liposomes) that can offer a further parameter for vaccine assessment and may provide means of enhancing and manipulating the host immune response to a significant extent. Nevertheless, application of such delivery systems for vaccination would allow effective utilization of vaccine antigens that have previously not been able to induce adequate or appropriate immune responses thus, improving the responses to existing vaccines.

4.1. Induction of affinity maturation and isotype switching

The maturational changes in the immune response include antibody shift from IgM to a predominant IgG isotypes, development of a high affinity antibody response as well as presence of memory lymphocytes that will mount a rapid response following further antigenic challenge. This process is generally described as occurring after at least two doses of antigen administration. Furthermore, prolonged antigen exposure assisted by controlled delivery system can also allow immune maturation to occur and may even enhance this process many folds. This property of the delivery systems is desirable as most of the controlled release mechanisms result in delivery of low levels of antigen over time, which will first stimulate high affinity B-cell clones to proliferate. For example, induction of higher affinity antibody responses by low doses of antigen has been demonstrated (Gonzalez-Fernandez and Milstein 1998).

4.2. Induction of immune memory

Research on controlled antigen delivery has facilitated the memory immune responses, which is the aim of most of the vaccine formulations. For example, delivery of *Yersinia pestis* V antigen through PLA microspheres as well as BSA by archaeosomes has resulted in generating significant memory immune responses in
mice (Eyles et al 2001). Apart from such type of antigen delivery systems, injectable silicone implants that delivered avidin and IL-1β as adjuvant were useful in generating a strong memory antibody responses as well (Kemp et al 2002). Interestingly, in these studies, the effector response induced a mixture of IgG1 and IgG2 isotypes of antibodies, in contrast to the memory response generated by soluble antigen alone that showed predominance of IgG1 isotype.

Besides activating effectors arms of immunity, the persistence of an antigen for longer duration may contribute in maintaining immune memory as well, although its exact role is yet to be resolved. This condition may arise due to differential antigen requirements of different cell types for the formation of memory cell repertoires (Eyles et al 2001, Marumaya et al 2000). Thus, controlled antigen delivery facilitates the low level of antigen and continuous drainage of the antigenic materials to the FDCs present in the lymph nodes that eventually helps in maintaining immune memory.

4.3. Manipulation of Th1/Th2 responses:

Recently, it has been demonstrated that a difference in antigen exposure in vivo may direct different precursors to produce IL-4 secreting CD4+ T-cells (Foucras et al 2000 and 2002). In contrast, some other studies performed using a range of different antigen delivery mechanisms have shown preponderance of both Th1 and Th2 responses depending on the type of antigen and more importantly upon the type of delivery system used. For example, Moore et al (1995) induced strong CTL responses following vacccination of mice with a soluble recombinant HIV protein by encapsulating the antigen in polymer microspheres. Similar responses were induced by delivery of ovalbumin releasing microspheres (Newman et al 1998). More importantly, delivery of various antigens encapsulated in different types of liposomes has resulted in the induction of diversity in immune responses. These include a Th1 dominant response following liposome encapsulation of various allergens (Sehra et al 1998), and a Th2 dominant response using Leishmania antigens, characterized by poor DTH responses and predominant IgG1 antibody responses that resulted in limited protection (Afrin et al 2000). In absence of polarization the simultaneous induction of both Th1 and Th2 responses has also been reported from different kinds of liposomes (Krishnan et al 2000b; Babai et al 1999). Hence, the rational
manipulation of delivery systems, in combination with variation of other parameters such as dose, route of administration as well as use of adjuvant or delivery systems is likely to result in significant improvement in the effectiveness of many existing vaccines.

Liposomes, also known for their potential and actual applications in targeted drug and gene delivery, appear to satisfy many of these criteria and therefore have potential for their future use in vaccine delivery (Gregoriadis 1990, Allison and Gregoriadis 1974). Delivery of antigens using liposome may therefore be used to rationally manipulate the type of immune response where liposome may be tailored to obtain a desired immune response (Owais and Gupta, 2000). Nevertheless, various other factors including intrinsic properties of an antigen, booster doses etc. may play important role during elicitation of immune responses. Therefore, a case-by-case basis in combination with variations in dose, routes of administration and schedule of delivery may be desired to develop effective vaccines. The structural versatility of liposomes, their ability to incorporate a wide variety of antigens regardless of size and solubility and a favorable bio-distribution profile have rendered the system an effective mean for the immunopotentiation and delivery of peptides and microbial vaccines alone or in conjunction with other co-adjuvants such as cytokines. The promise of the system as a vaccine carrier has recently been substantiated with the first liposome based vaccine (against hepatitis A) (Epaxal-Bema) licensed for use in humans (Glicht 1994) and by the encouraging results of phase I and phase II clinical trials with a variety of other liposomal vaccines (e.g. trivalent influenza, hepatitis B, diphtheria, and tetanus toxoid vaccines) (Gregoriadis 1995).

5. Particulate antigen delivery system:
5.1. Liposomes:

Liposomes were originally introduced in 1965 as models of lipid bilayer membrane (Bangham 1968). Since then, they have been widely applied as models for studying effectors phases of immune responses (Alving and Richards 1983). In 1974, liposomes were proposed as carriers of antigens to augment antibody responses in vivo (Allison and Gregoriadis 1974). In recent years, use of liposomes as potential carriers for vaccines has been extensively explored (Alving 1987, Gregoriadis 1990, Alving 1991, Alving et al, 1995) and applications of liposomes in immunology, and
particularly those relating to vaccines, have demonstrated that liposomes may have considerable practical utility as carriers of antigens. Besides antigen delivery, liposomes have also been shown to serve as carriers of a variety of adjuvants and mediators, including lipid A, muramyl dipeptide and its derivatives (Alving 1991).

Among the various factors involved e.g. location of an antigen either on the surface or encapsulation within liposomes, fluidity of the lipid bilayers, number of lamellae (uni-vs. multilamellar), size and surface charge of liposomes have proven most imperative in imparting variety in immune responses \textit{in vivo} (Latif and Bachhawat 1984, Alving 1987, Gregoriadis \textit{et al}, 1989).

5.2. Suitability of liposomes as vaccine carriers:

Many studies have demonstrated that liposomes can serve as effective vehicles for inducing humoral immunity to a wide range of liposomal antigens (Alving 1987, Gregoriadis 1990). The most common reason for using liposomes has been to convert a poorly immunogenic or even non-immunogenic protein into a highly immunogenic one. Some investigations have suggested that for certain antigens or under certain circumstances liposomes can be stronger adjuvants than CFA (Gregoriadis and Manesis 1980, Gregoriadis and Panagiotidi 1989, Brynestad \textit{et al} 1990). Liposomes have also been shown to induce antibody having conformational specificities (Orgert \textit{et al} 1990). \textit{e.g.} an unconjugated synthetic peptide containing 25 amino acid residues was encapsulated in liposomes containing lipid A, and the liposomes were successfully used to produce MAbs that recognized widely separated sequences of amino acid epitopes (Geysen \textit{et al} 1984). It may be presumed from the study that this type of specificity was caused by the conformation of the peptide as it interacted with the liposome bilayer.

As mentioned earlier, a considerable amount of \textit{in vitro} research has demonstrated that interaction of certain types of liposomes containing antigens with APCs can result in the generation of antigen specific MHC-restricted CTLs, and the similar results have also been obtained \textit{in vivo}. Furthermore following immunization of mice with the liposomised-antigen, CTL response was induced that blocked the formation of tumor mass from Hantaan NP transfected B16 melanoma cells in C57BL/6 mice and delayed the growth of pre-inoculated melanoma cells (Chang \textit{et al} 2001).
5.3. Strategies for optimization of liposomal adjuvanticity

Approaches to further improve the immuno-adjuvant action of liposomes include receptor-mediated targeting to macrophages (Garqon et al 1988), use of a variety of co-adjuvants (Alving 1991), modification of the structural characteristics of vesicles (Davis and Gregoriadis 1987) and the use of cytokines (Mbawiuke et al 1990, Abraham and Shah 1991). Importantly, liposomes have also been modified to target antigens to the desired APCs such as macrophages and DCs. For instance, liposomes coated with a mannose-terminating ligand promoted greater IgG responses in mice against entrapped tetanus toxoid than ligand-free liposomes (Nair et al., 1992), presumably because of improved targeting of vesicles to the dendritic cells which are known to express mannose receptors on their surface. Recently, the effect of targeting strategies for improving the interaction of liposomal PorA (a major antigen of *Neisseria meningitidis*) with DCs was investigated (Arigita et al 2003). In this study, purified PorA was reconstituted in different types of targeted liposomes *i.e.* by using mannose or phosphatidylserine as targeting moieties and such targeted liposomes were found to enhance maturation of murine DCs. Moreover, presentation of encapsulated proteins in sterically stabilized liposomes to the human DCs has been demonstrated to initiate CD8+ T-cell responses (Ignatius et al 2000). Administration of antigens into the liposomes together with interleukin-2 (IL-2) has been proved to be an effective way to augment immune responses against a variety of antigens. These include tetanus toxoid, bacterial polysaccharide (Abraham and Shah 1991), influenza A virus (Mbawiuke et al., 1990), inactivated influenza virus and HSV-recombinant glycoprotein D vaccines.

Structural characteristics of liposomes play a decisive role (both qualitatively and quantitatively) in the expression of immuno-adjuvant activity (Gregoriadis, 1988). There are for instance, numerous studies on the extent to which membrane fluidity (Kinsky 1978, Gamier et al 1991, Davis et al 1987), number of bilayers (Shek 1982), vesicle size (Francis 1985), surface charge (Kraaijeveld 1984), and location of antigen (Shek 1982) can affect adjuvanticity of liposome based delivery systems.
6. Fusogenic liposomes derived from microbial lipids:

Since the inception of the concept of using liposomes as antigen carriers, numerous attempts have been made to develop liposome based particulate delivery systems. Among various strategies employed to improve liposome mediated antigen delivery, the fusogenic-liposome based vaccines remained more convincing approach to deliver antigens to the proteasome machinery through fusion of liposome with membrane of the target cells (Owais and Gupta 2000, Reddy et al 1991 Straubinger et al 1985), that eventually leads to MHC class I mediated presentation of the antigen ensuing specific CTLs generation. One method for imparting fusogenicity to liposomes is to incorporate charged phospholipids, for example in their preparation; thereby positively charged liposomes are capable of delivering encapsulated soluble antigens to the cytosol for class I MHC presentation unlike neutral lipid liposomes (Nakanishi et al 1997). Moreover, apart from intrinsic fusogenic property of the lipids, various glycoproteins such as hemagglutinin (HA) of influenza virus and fusion protein (F-protein) from Sendai virus, which are responsible for the entry of virus into the host cells, have also been used for imparting fusogenicity to the conventional liposomes (Kunisawa et al 2001).

Recently, it has been demonstrated that liposomes made up of polar lipids extracted from *Saccharomyces cerevisiae* undergo membrane-membrane fusion with the APCs to elicit antigen specific CTL responses (Owais and Gupta 2000). Some other groups have also reported the fusogenicity of total polar lipids unique to *Archaea* to prepare archaeosomes that can deliver entrapped solutes to the cytosol of the target cells. This eventually led to the elicitation of antigen specific Th1/Th2 cytokine as well as CD8\(^+\) CTL response against entrapped soluble antigens (Krishnan et al., 2000a & 2000b; Sprott et al 1997 & 2003).

Various strategies that have exploited microorganisms to impart fusogenicity to the antigen delivery vehicles are as follows.

6.1. Virosomes:

The interest to analyze the adjuvant effect of liposomes on one hand, and curiosity to know morphological and immunological aspects of influenza virus, on the other, led to the creation of the first so-called virosomes (Almeida et al 1975). The name itself
reflects the structural similarities between the viral liposomes and the actual influenza virus particles. The fusion potential of influenza virosmome is based on the major viral envelop glycoprotein hemagglutinin (HA) and neuraminidase (NA) in which HA acts as a targeting device as it binds to sialic acid residues present on APCs and directs a passage for viral entry into the host cells (Skehel and Wiley 2000, Matlin et al 1981). After endocytic uptake, the acidic environment in the endosome induces a conformational change in the HA component of the virosomes that leads to the fusion of the virosomes with the endocytic membrane. Keeping in view the significance of HA conformation in the fusion mechanism, the protocols for influenza virosmome preparation have been modified to conserve the properties of HA (Stegmann et al 1987). The virosomes thus produced retain the receptor binding and membrane fusion activity of the native virus, by preserving the conformational integrity of the viral HA. These functionally reconstituted influenza virosomes have the capacity to deliver encapsulated macromolecules to the cytosol of the target cells (Bron et al 1994, Schoen et al 1993). An additional advantage of influenza derived virosomes is that HA may also activate immune system of the host thereby acting as a potential adjuvant (Watts 1997).

The detailed in vitro studies suggests that virosomes efficiently deliver their entrapped material to DCs for MHC class I and class II presentation (Bungener et al 2002). In these studies, influenza virosomes have been shown to induce DC maturation as measured by up-regulation of CD40, ICAM-1, B7.1 and B7.2. Moreover, insertion of HA as well as NA from influenza virus into immunostimulating complexes (ISCOMS) (Morein et al 1979, Coulter et al 2003), conventional liposomes (Almeida et al 1975), liposomes containing immunomodulators (Nerome et al 1990, Linuma et al 1995) or liposomes having cationic lipids (Gloqck and Zurbriggen, 2003), supports the notion that both HA and NA contribute significantly to the fusogenic properties of virosomes rather than envelop lipids.

The earlier studies with influenza virosomes focused on enhancing its immunogenicity in vivo by using antigens from various sources. For instance, immunization of mice with virosomes containing a peptide derived from the influenza nucleoprotein (NP) resulted in successful priming of NP-specific CTL activity (Arkema et al 2000). Recently, immunopotentiating reconstituted influenza virosomes

~ 20 ~
(IRIV) as vehicles have been evaluated to deliver HLA-A*0201-restricted hepatitis C virus (HCV) peptides (core 35–44 and 131–140) into the cytoplasm of at least three different target cell types [including T2, a transporter associated with antigen processing (TAP)-deficient cell line] resulting in class I MHC presentation of the antigen and lysis by peptide-specific CTL lines (Hunziker et al 2002). Virosomes containing influenza matrix peptide 58–66 can efficiently restimulate in vivo primed CTL and importantly, IRIV containing HCV core peptides can even prime CTL from peripheral blood mononuclear cells of HCV-healthy blood donors in vitro. In another study in vivo induction of cellular immune responses was demonstrated against influenza virosome-encapsulated ovalbumin (OVA) in mice (Bungener et al 2005). It was demonstrated that a small amount of OVA (0.75 µg) delivered by fusion-active virosomes was sufficient to induce strong class I MHC restricted CTL response. Because of their success rates, influenza virosome based vaccines have also been evaluated in clinical studies (Gluck 1999). In addition to the observed CTL responses elicited by influenza virosomes, incorporation of hepatitis A virons (HAV) in influenza-derived virosomes strongly stimulated HAV-specific antibody responses (Nerome et al 1990), in fact a virosomal HAV vaccine (Epaxal) is currently in the market as well.

Apart from influenza, virosomes from Sendai virus have been generated by reconstitution of the Sendai fusion protein (F-protein), with or without the hemagglutinin–neuraminidase protein (HN-protein) in viral lipids (Bagai and Sarkar, 1993 & 1994). Unlike HA & NA proteins of influenza virus, the F proteins of sendai virus are responsible for direct fusion with lipid layer of the cell membrane to induce cell fusion (Mizuguchi et al 1999). It was found in earlier studies that such virosomes can efficiently fuse with conventional liposomes as well as cell membranes at 37 °C (Nakanishi et al 1985). Furthermore, the fact that the Sendai virus receptors (sialic acid residues) do not exist on the conventional liposome surface suggests that in the fusion process of Sendai virosomes this receptor is not mandatory. In addition to this unique fusion mechanism between the Sendai virus and liposomes, the Sendai virus-liposome fusion vehicle has been shown to fuse with mammalian cells (Nakanishi et al 1985). The cytoplasmic delivery of macromolecules by these vehicles was established in vitro using ‘diphtheria toxin A’ subunit (DTA) as a probe. Sendai virus virosomes carrying DTA suppressed protein synthesis in cultured cells indicating its...
cytoplasmic delivery by these virosomes (Nakanishi et al 2000, Mizuguchi et al 1996a). Beside their use as antigen delivery systems, Sendai viroosome mediated delivery of anti-tumor drugs facilitated total disappearance of S-180 tumors from the abdominal cavity without any side effects (Mizuguchi et al 1996a & 1996b).

Further studies demonstrated that Sendai virosomes may potentially induce CTL response even better than complete Freund’s adjuvant (CFA) (Nakanishi et al 2000) in conjunction to MHC class II response for enhanced antibody production (Hayashi et al 1999). Moreover, in a recent study nasal administration of model antigen (OVA) using fusion glycoproteins of Sendai virus on the surface of liposome membranes was found to efficiently deliver antigen to antigen-sampling macrophages in nasopharyngeal-associated lymphoreticular tissue and elicited strong immunological responses as well (Kunisawa et al 2001). Additionally, these vesicles also facilitated an adjuvant activity against mucosal epithelial cells to enhance MHC class II expression as demonstrated by induction of OVA-specific CD4+ Th1 and Th2 cell responses. Furthermore, antigen-specific CTL and humoral response were also elicited at both mucosal and systemic sites by nasal immunization with these OVA-encapsulated fusogenic liposomes (Kunisawa et al 2001). However in another study, primary human B and T lymphocytes (CD4+ or CD8+) and the human B cell line were not permissive to this kind of fusogenic liposome-mediated delivery (Watabe et al 1999).

Apart from these two extensively explored virosomes, reports are available where other viruses have been exploited to prepare fusogenic liposomes e.g. Rubella virus based virosomes were prepared by incorporating E1 and E2 envelope glycoproteins into liposomes (Orellana et al 1999) and vesicular stomatitis virus (VSV) virosomes were generated by adding the G-protein of VSV to preformed liposomes (Loh et al 1979 Shoji et al 2004). In addition, virosomes have also been generated using Epstein–Barr virus (Grimaldi et al., 1995), human immunodeficiency virus (Corret et al 1990), Semliki Forest virus (Helenius et al 1977), Friend murine leukemia virus (Schneider et al 1983), herpes simplex virus (Johnson et al 1984) and Newcastle disease virus (Kapczynski and Tumpey 2003) etc. However, fusion activity of these virosomes was not always determined and the main emphasis of using such vehicles...
remained restricted to study induction of humoral responses against the specific viruses from which they have been derived.

6.2. Archaeosomes:

Liposomes prepared from polar archaebacterial glycerolipids (archaeosomes) have been shown to induce strong adjuvant action in mammals. Membranes of archaebacteria are reported to contain lipids that are chemically distinct from that of eukaryotic or other prokaryotic organisms. The saturated, branched C-20, C-25 and C-40 phytanyl chains form liposomes with unique properties in context to physical and chemical stability and uptake by APCs (Patel and Sprott, 1999) and it was speculated that such properties of lipids are the possible factor for observed adjuvanticity to occur (Sprott et al 2004). Immune responses comparable to immunization with CFA and superior to conventional liposomes have been reported after immunization with archaeosomes (Krishnan et al 2000b, Conlan et al 2001). Authors have shown the potential of liposomes composed of archaebacterial lipids of various archaebacteria in evoking CTL as well as antibody responses to their entrapped antigens (Krishnan et al 2000a, 2001 & 2003). In these studies, ether glycerolipids extracted from various archaebacteria were formulated into liposomes and mice of varying genetic backgrounds, immunized via various parenteral routes with archaeosomes containing BSA demonstrated markedly enhanced serum anti-BSA antibody titers. These titers were often comparable to those achieved with CFA and considerably more than those with alum or conventional liposomes (PC/PG/chol, 1.8:0.2:1.5 molar ratio). Furthermore, antigen-specific IgG1, IgG2a, and IgG2b isotype antibodies were all induced. Apart from BSA, encapsulation of OVA or hen egg lysozyme within archaeosomes showed similar immune responses (Krishnan et al, 2000b). Moreover, antigen-archaeosome immunizations induced strong cell-mediated immune response as evident from antigen-dependent proliferation and substantial production of both Th1 (IFN-γ) as well as Th2 (IL-4) cytokine responses. In contrast, conventional liposomes induced little cell-mediated immunity, whereas alum stimulated IL-4 response only. Further, in contrast to alum and CFA, archaeosomes composed of Thermoplasma acidophilum lipids evoked memory antibody responses to the encapsulated antigen as observed at ≈300 days after two initial immunizations (days 0 and 14). The observed response was shown to be correlated with increased antigen-
specific cell cycling of CD4$^+$ T cells as revealed by flow cytometry. In further studies, a comparison between liposomes prepared from the lipids extracted from archaebacteria *Haloferax volcanii*, *Planococcus sp.* and a eubacterium *Bacillus firmus* was made on their ability to influence immune system to evoke effective immune responses (Sprott et al. 2004). It was hypothesized that occurrence of isoprenoid neutral lipids was the possible factor for the adjuvanticity of *B. firmus* crude lipid-SDS micelles. On the other hand, due to similarities of lipid head groups (predominantly PG lipids, sulfoglycolipids and cardiolipin) between *H. volcanii* and *Planococcus spp.*, either kind of vesicles delivered their encapsulated antigens in vivo to both MHC class I and class II compartments of APCs. Interestingly, out of these three liposome formulations, archaeosomes promoted a greater memory recall antibody responses in immunized mice that were found to be highly significant as compared to *Planococcus* liposomes. Surprisingly, CTL response generated by *Planococcus* liposomes were of short term, while liposomes made up of lipids from *H. volcanii* consisting of almost same lipid composition, elicited stronger CTL response. Moreover, *Planococcus* liposomes led to the activation of DCs to secrete inflammatory cytokines (IL-12 and IL-6) that may be the possible reason for observed short-term CTL responses (Dudani et al. 2002). In other set of experiments, liposomes made up of archaeal lipids from *M. smithi* enhanced costimulation on DCs to facilitate T-cell activation (Krishnan et al. 2001). However, the response was independent of IL-12 production (Krishnan et al. 2003), which supports the adjuvant activity of archaenal lipid liposomes by providing both signals required to activate T-cells: efficient antigen delivery as well as co-stimulation (Zinkernagel 2002). Furthermore, archaeosome-entrapped listeria antigen elicited rapid and prolonged specific immunity against *L. monocytogenes* in the mice model (Conlan et al. 2001). In this regard, superiority of tested archaeosomes to conventional liposomes (made up of PC/PG/chol) further emphasizes significant contribution of unique features of archaeosomes in antigen delivery.

In the context of the lipid vesicles, possible routes of their entry into the cell include either direct fusion with the plasma membrane or phagocytosis by APCs (Owais and Gupta 2000). Alternatively, some other routes like endocytosis mediated by mannose receptor, FcRs, and those recognizing apoptotic cells, like PS-specific receptors, may be responsible for their uptake (Somersan and Bhardwaj 2001, Li et al. 2003).
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of several reports available on archaeosome mediated antigen delivery, no intercession has been developed towards the fusogenicity of these liposomes. Information deciphering the exact mechanism of archaeosome uptake by APCs was behind the screen until recently; an article demonstrating role of PS in uptake of such liposomes by APCs suggests that the whole process is dependent on phosphatidylserine receptor present on macrophages and DCs (Gurnani et al 2004). Archaeosomes from \textit{M. smithii} have been fully characterized and found to comprise of 30 mol \% of the archaetidyl, which is equivalent of PS. These types of liposomes are striking in forming archaeosomes with surface-exposed PS head groups (Sprott et al 2003). Hence, antigen presentation results corroborate PS-specific recognition as triggering archaeosome entry into the phagosomal compartment, leading to antigen delivery for MHC class I (Gurnani et al 2004).

6.3. Yeast lipid liposomes:

An interesting correlation between the plasma membrane lipid composition of the living organisms and their generation time can be made. For example, bacteria such as \textit{Escherichia coli}, \textit{Bacillus megaterium} and \textit{Bacillus subtilis} have preponderance of anionic lipids \textit{viz.} PG and DPG (in combination of PE) in their plasma membranes and have very short generation time of the order of 20-25 minutes (Rattray 1988; Jain 1988). On the other hand, membranes of relatively more evolved \textit{Saccharomyces cerevisiae} or \textit{Candida albicans} have greater variety of phospholipids with lower percentage of anionic lipids (e.g. PG, PI, PS, DPG) and the organisms have a generation time of approximately two hours. Since both the classes of organisms multiply by binary fission, it can be presumed that the presence of anionic lipids facilitates the fusion of the membranes essential for high duplication rates (Owais and Gupta, 2000). Unlike the lower organisms, the more evolved eukaryotes have neutral phospholipids as major membrane components and have generation times of the order of days (cf. \textasciitilde 22 hrs). The plasma membrane of lower organisms is mainly composed of amino phospholipids along with cardiolipin and PG. The eukaryotic plasma membrane lipid composition is different from that of lower organisms and earlier work revealed the composition and distribution of phospholipids in eukaryotic cells (Kumar and Gupta, 1983). They contain all classes of phospholipids, distributed in a set fashion in the two leaflets of the bilayer. The amino-phospholipids are mainly
confined to the inner leaflet and play a major role in exocytosis, which involves membrane-membrane fusion.

In the earlier studies, it was demonstrated that asymmetric distribution of phospholipids is also maintained in yeast cells (Balasubramanian and Gupta, 1996; Dixit and Gupta, 1998). However, similar to other lower organisms the baker's yeast (Saccharomyces cerevisiae) undergoes rapid cell duplication, in which the two opposite sites of the plasma membrane inner leaflet come close to each other and fuse under physiological conditions. Therefore, the fusogenic properties of the vesicles formed from yeast lipids were evaluated to deliver antigen to APCs (Owais and Gupta 2000). In this study, the fusion efficiencies of various types of membrane vesicles were compared using epifluorescence as well as other conventional membrane-membrane fusion assay methods. In the content mixing assay, marked decrease in fluorescence results when ANTS containing fusogenic vesicles (yeast lipid liposomes) fuse with the excess of DPX containing vesicles of same lipid composition. Among various types of vesicles used in this study, quenching of fluorescence was highest in yeast lipid liposomes indicating their strong fusogenic potential.

The membrane lipid composition of yeast exhibits a great majority of anionic phospholipids (PC 48.1%; PE 23.1%; PI 16.1%; PS 6.0%; PG 3.9%; and cardiolipin 1.6%) that play pivotal role in membrane-membrane fusion. Like yeast, Bacillus subtilis contains majority of anionic lipids in their plasma membrane (mainly PG & PE) and have a short generation period (Rattray 1988). Recently, membrane lipids from B. subtilis were extracted to demonstrate their fusogenic potential (Deba et al 2005) that were found to posses greater fusogenicity than erythrocyte vesicles (RSO and ISO) or conventional egg PC/chol vesicles as revealed by conventional fusion assays. Moreover, to reconfirm that such vesicles interact via membrane-membrane fusion as a major mode of interaction with the target cells (J774 A.1 cells), the toxic effect of 'ricin A' was observed by incubating J774 A1 cells with 'ricin A' loaded vesicles. It was shown that effect of 'ricin A' is more remarkable in case of subtilosomes followed by ISO and RSO erythrocyte vesicles (Deba et al 2005). Therefore, both yeast lipid liposomes as well as subtilosomes (liposomes made up of Bacillus subtilis lipids) offer a novel approach to deliver the entrapped antigen into the cytosol of the APCs.
6.4. Niosomes:

Apart from these fusogenic liposomes used as antigen delivery vehicles, few reports are available on fusogenic vesicles that belong to other classes of vesicle family (e.g. niosomes, transfersomes, proteosomes etc.). Niosomes are small unilamellar vesicles made from non-ionic surfactants. Hence, they are also called non-ionic surfactant vesicles (NISV) (Brewer and Alexander 1994) or Novasomes (Gupta et al 1996). BSA entrapped within niosomes was reported to be as immunogenic as BSA with CFA (Brewer and Alexander 1992) and the chemical stability of niosomes is higher than the stability of conventional phospholipid liposomes. Transfersomes are ‘ultra deformable’ liposomes with enhanced skin penetrating properties. These vesicles consist of PC/cholate (9:2 molar ratios). Studies with model antigen have shown that transfersomes have an upper hand than conventional liposomes when administered epicutaneously (Paul and Cevc 1995). Cochleates are non-vesicular bilayer sheets consisting of PE/PS/chol. Calcium ions are added which intercalate with the bilayers. This results in a rolled up bilayer sheet without internal volume. Results obtained with protein- and DNA-cochleates have been reviewed elsewhere (Gould-Fogerite et al 1998). Proteosomes are also considered as outsiders in the liposome group as they are comprised of protein mainly. These vesicles are of bacterial origin (outer membrane) and are prepared by solubilization of bacterial membranes, followed by ammonium sulphate precipitation and dialysis against detergent containing buffer (Lowell et al 1988). Electron micrographs revealed that these vesicles have a size of about 100 nm, but the protein: lipid ratio is higher than that can be achieved with purified protein incorporated in liposomes. Proteins and peptides are non-covalently complexed to the proteosomes, making them highly immunogenic (Lowell et al 1997). Moreover, fusogenic liposomes have been prepared from artificial lipid membranes consisting of synthetic arenavirus ‘fusion peptide’ (Glushakova et al 1992). In addition, it has been demonstrated that non-phospholipid liposomes composed primarily of dioxyethylene acyl ether and cholesterol have capacity to fuse with membranes composed primarily of phospholipids (Baraka et al 1996).
7. Plasmodium infection:

7.1. Life cycle of *Plasmodium* parasite:

All strains of the malaria parasite have a complex life cycle that begins in the mammalian hosts when the female mosquito deposits 5 to 20 sporozoites subcutaneously during a blood meal (Rosenberg *et al* 1990, Ponnudurai *et al* 1991) (figure 1). The sporozoites enter the blood stream and within few minutes migrate to the liver and invade hepatocytes (Mota *et al* 2001 & 2002). Within this relatively immunoprotected intracellular environment, each sporozoite develops over a period of 6 to 16 days into tens of thousands of schizonts. The schizonts rupture, releasing 20,000 to 30,000 merozoites per original sporozoite into the hepatic venous circulation and from there they disseminate systemically. *Plasmodium vivax* and *P. ovale* can develop into hypnozoites, which might remain quiescent in the liver from months to years. It is only after the parasites erupt from the hepatocytes that symptoms of clinical disease are apparent. In the blood stream, each merozoite that has escaped from phagocytic cells then invades, multiply, mature over 24 to 72 hours in the erythrocytes, and finally lyse them to invade further new erythrocytes (the blood stage of malaria). The blood stage culminates in death of the host. Finally, some of the merozoites develop into sexual stage gametocytes, typically about 10 days after *P. falciparum* enters the blood stage (3 days for *P. vivax*). When these are taken up during blood meal by mosquitoes, they can sexually combine and develop into sporozoites (10 to 22 days) and this leads to the restarting of the cycle.
Figure 1: Malaria vaccines by life-cycle target

**Target 1** (Sporozoites) = RTS, S, ICC-1132, NYU CS, Lausanne CS;

**Target 2** (Liver Stage) = DNA and viral vectors (NMRC, Vical, and Oxford), *RTS,S, * Lausanne CS

**Target 3** (blood stages) = MSP1, MSP2, RESA, MSP3, GLURP, FMP1, AMA-1;

**Target 4** (sexual stages) = Pvs 25

The * in front of RTS, S and Lausanne CS indicates that these vaccines might also partly act against the liver stage as well as against sporozoites. (Moorthy *et al* 2004)
7.2. Pathogenesis and Epidemiology:

The classical signs and symptoms of malaria in human beings include acute febrile episodes and rigors that occur every 48 to 72 hours, which in fact coincide with the synchronized lysis of erythrocytes by newly matured merozoites. Systemic signs and symptoms associated with *P. vivax* include fever, malaise, headache, photophobia, muscle aches, anorexia, nausea, and vomiting (Markell *et al* 1992). Severe disease occurs most frequently with *P. falciparum* because of its ability to infect a greater percentage of erythrocytes and to cytoadhere to capillary walls (Markell *et al* 1992, Miller *et al* 2002). Clinical sequelae (such as acute renal failure, anemia, hypoglycemia, cerebral malaria, and pulmonary edema) occur most commonly in populations that are relatively naive to the parasite, such as children and travelers from non-endemic area (Chen *et al* 2000). Pregnant women are at special risk for severe disease and sequelae, having a unique mechanism of pathogenesis (Reeder 1999).

Quantification of the malaria parasite burden in affected populations is imprecise, impacted mainly by their access to diagnostic and therapeutic health centers, species prevalence, case definition, and low specificity of diagnosis (Breman 2001, Murphy and Breman 2001). It is thought that there are 300 to 500 million clinical cases per year globally, about 25% of which are caused by *P. vivax*. Estimated annual malaria attributed deaths vary from 1.4 to 2.7 million (Breman 2001, Snow *et al* 1999) with the majority of deaths occurring in children in the African continent (Snow *et al* 1999). This translates into 39 million disability-adjusted life years lost annually (WHO 1999), which in turn tells about the impact of the disease on economic and social disruption in developing countries (Gallup and Sachs 2001).

The number of malaria cases diagnosed within the United States usually stable and ranges at approximately 1000 to 1500 per year over the past decade, with the vast majority of cases are reported among travelers and military personnel overseas. However, the United States has very small number of congenital, blood transfusion, and locally acquired cases (Holtz *et al* 2001, Sunstrum *et al* 2001). On the other hand, European countries has a much higher rate of malaria, with 37,000 autochthonous and 13,000 imported cases as per survey made in 1999 (Sabatinelli *et al* 2001).
7.3. Vaccinology:

Multiple lines of evidence suggest that a malaria vaccine for humans is feasible. Immunization of humans with irradiated (and thus attenuated) sporozoites confers sterile protection in roughly 90% of naïve volunteers when they are subsequently exposed to malaria (Clyde 1990, Egan 1993, Rieckmann et al 1979). Because this model requires each volunteer to be bitten by hundreds of mosquitoes over a period of several months, it is not a practical vaccination strategy but is useful in probing immune responses (Hoffman et al 2002). Naturally acquired clinical immunity occurs during the first two decades of life in people living in malaria-endemic areas (Trape et al 1994, Baird 1991). In general, this immunity is imperfect, can wane rapidly, appears to be linked to intensity of exposure, and primarily impacts the severity of the clinical disease (Day and Marsh 1991, Baird 1998, Hogh 1996, Smith et al 2001). Additionally protection can be mimicked by the passive transfer of hyperimmune immunoglobulin into human volunteers (Sabchareon et al 1991).

The immunologic correlate for clinical protection has yet to be identified. Through its complex life cycle, the malaria parasite exists in various immunologic “compartments.” It is advisable to explore nature of immune responses that might impact various stage life cycle of parasite in specific manner. Furthermore, it is possible to extrapolate from other malaria intervention studies how exactly the various stage specific vaccines might work. Ultimately, it might be necessary to combine antigen from various stages of the Plasmodial life cycle into a single vaccine. Presently, there are three intermediate goals of vaccine research (Beverley 2002): identification of protective antigens for stage specific immunity; successful combination of candidate immunogens; and finally, the induction of strong, strain-transcending, durable immune responses. Whereas, the main strategy for development of a malaria vaccine falls under following categories:

I. Blocking the sporozoite from invading or developing within hepatocytes;
II. Blocking merozoite invasion of red cells and inhibiting development of schizonts;
III. Blocking the adverse pathology-inducing effects of cytokines and parasite sequestration; and
IV. Developing an ‘altruistic’ vaccine that would block host-mosquito transmission by immunizing against the sexual stages or gametes.

7.3.1. Pre-Erythrocytic vaccines:

Vaccine strategies targeting the sporozoites aim to generate a humoral immune response to neutralize the sporozoites and prevent it from invading the hepatocytes. Once the sporozoite has entered the relative immunoprotected intracellular environment of a hepatocyte, a successful vaccine strategy would rely upon elimination of infected hepatocytes with the help of CTL (Doolan and Hoffman, 2000). Experiments in ortholog model systems and irradiated sporozoite challenge studies have implicated CD4⁺, CD8⁺, natural killer T-cell, and γδ T-cells in the inhibition of intrahepatic parasites (Gonzalez-Aseguinolaza et al 2000, Nardin and Nussenzweig 1993, Tsuji et al 1994, Good and Doolan 1999, Doolan and Hoffman 1997, Nardin et al 1999). The leading candidate vaccine of this category is RTS,S – a recombinant protein vaccine (Stoute et al 1997).

The passive transfer of antibodies specific to the sporozoite was found to be protective in the murine ortholog model systems (Marussig et al 1997). Moreover, a dominant feature of the immune response induced by infected humans with irradiated sporozoites is an antibody response to the circumsporozoite protein (CSP), the major component of the sporozoite surface coat of Plasmodium sp (Herrington et al 1991, Egan et al 1993). The role of antibodies in mediating protection was elegantly demonstrated with the use of MAbs that neutralized the infectivity of sporozoites in a rodent model (Potocnjak et al 1980). Initial clinical studies using either a synthetic peptide or recombinant protein containing multiple copies of the immunodominant repeat sequences found in the CSP (Herrington et al 1987, Ballou et al 1987). For example, the peptide (NANP)³ was conjugated to tetanus toxoid, whereas the recombinant protein containing 32 CSP repeats fused to an out-of-frame 32 amino acid sequence from the gene encoding bacterial tetracycline resistance. A small number of subjects immunized with each of these CSP vaccines, formulated with alum, were challenged with infected mosquitoes. One of the three high titer responders to the peptide vaccine was protected (Herrington et al 1987), whereas only one of six individuals challenged after receiving the recombinant CSP vaccine was protected (Ballou et al 1987). Ironically, the following studies including numerous
additional trials were carried out over a period of ten years with disappointing results (Rogers and Hoffman 1999).

More recently, results with a recombinant hepatitis B surface antigen vaccine incorporating repeat and non-repeat CSP sequences have been much more encouraging. When the hybrid hepatitis B particles were formulated in an oil-in-water emulsion containing monophosphoryl lipid A and QS21, six of the seven immunized volunteers were protected against subsequent malaria challenge (Stoute et al 1997). This vaccine was designated as RTS, S and assessed with thrombospondin-related anonymous protein (TRAP) which failed to impart protective immunity against malaria (Stoute et al 1997). In a randomized controlled field trial of three-dose RTS, S in Gambian adults, vaccine efficacy was 34% (p=0.014) during the 15-week surveillance period, but with 71% efficacy in the first 9-weeks and 0% in the next 6 weeks (Bojang et al 2001). Moreover, the protection was not strain specific (Alloueche et al 2003), although the duration of the efficacy was short, RTS, S is the first pre-erythrocytic vaccine to show clear protection against natural *P. falciparum* infection. Another vaccine candidate ICC-1132 is a hepatitis B core particle, includes a region of CS for high antibody production. High titers of biologically active CS antibodies have been noted in preclinical studies (Birkett et al., 2002). Although, latest trials with DNA vaccination proved to be efficient priming vaccines but failed to boost efficiently (Schneider et al 1999), still intense efforts are being made to develop effective DNA based vaccines, each encoding a pre-erythrocytic antigen, have undergone phase I studies (McConkey et al 2003).

Other CS-based candidate vaccines that have reached phase-I studies include a multiple antigen peptide, a type of synthetic delivery system, which induced strong antibody responses; a polyoxime construct, containing a universal T-cell epitope; and a long synthetic peptide in an oil-based adjuvant, which induced detectable antibodies and CD4^+^ and CD8^+^ T-cell responses with a good safety profile (Nardin et al, 2000 & 2001, Lopez et al 2001).

### 7.3.2. Asexual Blood-Stage Vaccines:

There are two possible classes of blood-stage vaccine: anti-invasion and anti-complication. Merozoites enter the blood stream after erupting from the hepatocyte.
Within seconds they invade red blood cells that do not express MHC molecules. Hence, a vaccine that could prevent invasion would prevent malaria disease. For this reason, the vaccine strategy has classically been considered antibody dependent, either by inactivating the merozoites or by targeting malarial antigens expressed on the red blood cell surface to antibody dependent cellular inhibition (or possibly complement lysis) (Bouharoun-Tayoun 1995, Long 1993, Holder et al 1999). Recently, ortholog malaria models have implicated CD4^ T-cells, nitric oxide, and γδ T-cells in helping control blood stage parasites (Good 2001, Bouharoun-Tayoun 1995, Long 1993, Holder et al 1999), and there is increasing evidence that CMI can play a role in human protection (Plombo et al 2002). Vaccines developed against this stage would serve as disease-reduction vaccines, mimicking naturally acquired immunity by suppressing the exponential multiplication of merozoites (Tsuji et al 2001, Richie and Saul 2002).

The two leading asexual blood stage vaccine candidates against Plasmodium are merozoite surface protein 1 (MSP-1) and apical membrane antigen-1 (AMA-1). Both of these antigens have been identified in all Plasmodium spp and examined for of their homologues in rodent and simian parasites that eventually has facilitated the assessment of MSP-1 and AMA-1 vaccines in several animal models. MSP-1 is the most well characterized antigen involved in invasion, and is the basis of several candidate vaccines. Most interest in MSP-1 is currently focused on fragments that include the two C-terminal epidermal growth factor (EGF)-like domains. Inhibitory MAbs react with a 19 kDa fragment (MSP-119) but this fragment is poorly immunogenic (Chappel and Holder 1993). Immunity induced by immunization with C-terminal fragments of MSP-1 appears to be mediated by antibodies reacting with conformational epitopes in the EGF-like domains. The effective antibodies to MSP-19 block invasion of merozoites, while the other antibodies reacting with the EGF-like domains are either neutral or block the action of the inhibitory antibodies (Blackman et al 1994). In a small efficacy study in Papua New Guinea, a blood stage vaccine incorporating the antigen MSP-2 and two other blood stage antigens reduced parasite density in vaccine recipients (Genton et al 2002). Participants were protected from infection with the vaccine strain of malaria, suggesting that polymorphic antigen such as MSP-2, a vaccine including just one allelic form of the antigen is not likely to give sufficient protection. Immunization with recombinant forms of AMA-1 has protected
monkeys and mice against challenge with *P. fragile* and *P. chabaudi*, respectively (Collins *et al* 1994, Crewther *et al* 1996).

A recombinant viral vaccine, NYVAC Pf-7 encoding seven antigens from different stages of plasmodium life cycle (Ockenhouse *et al* 1998) has been shown to suppress parasitemia upon challenge with sporozoites. The immune response generated by this vaccine included some humoral as well as CTL immune response. Two more blood-stage candidates, glutamate rich protein (GLURP) and MSP-3, have been clinically assessed (Oeufray *et al*. 2000 & 1994). Main emphasis of all such protein candidates is the identification of a safe, immunogenic adjuvant, since the traditional adjuvant alum seems to be insufficiently immunogenic for many malaria proteins. In addition, this adjuvant induces a Th-2 response rather than a desirable Th-1 response.

The erythrocyte membrane protein-1 antigen (PfEMP-1), the ligand responsible for adherence of malaria parasite to vascular endothelial cells in the brain, kidneys and placenta, is being used in experimental laboratory vaccine trials. However, due to high degree of variability, rapid antigen variation and high copy number within each parasite, use of this antigen as a vaccine becomes complicated as researchers believe in using a conserved part of the antigen to be more promising. The *P. falciparum* glycosyl phosphatidylinositol (GPI) molecule that is an inflammatory mediator released during schizont rupture has shown its efficacy as vaccine candidate in experimental animals (Schofield *et al* 2002, Molano *et al* 2000). Two more GPI-anchored membrane proteins of trophozoite and merozoites stages of *P. yoelii* offered protection against lethal malaria infection in BALB/c mice (Burns *et al* 2000). On sequencing these antigens were found to contain two epidermal growth factor (EGF)-like domains.

7.3.3. Transmission-Blocking vaccines:

Transmission-blocking vaccine relies on the mosquito imbibing both antibody and complement along with the parasite during a blood meal. Within the mosquito, the antigens become exposed to antibodies during the parasite’s maturation, thus neutralizing the sexual stages (Kaslow 1993, Lobo *et al* 1999, Hisaede *et al* 2000). This type of vaccine would not prevent illness or infection in the vaccinated individual, but would prevent the further spread of malaria by mosquitoes feeding.
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from the vaccinated host (Kaslow 1997, Stowers and Carter 2001). The vaccine could be useful in areas of low endemicity to halt transmission or as an important component of a multi-antigen vaccine.

Only few reports are available addressing the exciting developments with DNA vaccines (Hedstrom et al 1998), multiple antigenic peptides (Moreno et al 1999), or artificial proteins containing multiple epitopes and or domains (Shi et al 1999). There has been a remarkable increase over a past decade in our understanding and knowledge of the malaria parasite and the disease that it causes. Never before has one been in the position to appreciate what a sophisticated and complex organism we are attempting to defeat through vaccine development. Nevertheless, we believe that pre-erythrocyte and asexual blood stage vaccines are now a reality and priority of malaria vaccine development is to field-test the large number of vaccine candidates that have been identified with various adjuvant combinations.

7.3.4. Subunit vaccination:

The production of live attenuated or killed inactivated vaccines is not practical for many diseases. In subunit vaccination, part or complete antigen are identified from a pathogen's proteomic complement, which can induce protective immunity to the whole pathogen on vaccination. However, for successful elimination of the parasite, induced antibodies must have the correct avidity, specificity, biological activity, and be produced at a high enough titer to block the infection. To this end, varying immunogenicity of the proteins may be the key factor for successful vaccination. Increased understanding of antigen processing, adjuvant and their effects on innate immunity, genetic engineering techniques, and novel antigen delivery systems have facilitated the increased immunogenicity of proteins. Unfortunately, recombinant protein subunit vaccines are generally poor at induction of effectors T-cell responses, such as CD8$^+$ cytotoxic T lymphocytes, that are necessary for elimination of intracellular pathogens such as liver-stage malaria parasite.

Presently, several laboratories are emphasizing on DNA based subunit vaccines (Ulmer et al 1993, Li et al 1993). The strategies in such kind of vaccination may be either to use DNA sequences from malaria parasites that have been inserted into plasmid DNA molecules (DNA vaccines) or using various recombinant attenuated
DNA viruses (recombinant viral vaccines) to generate candidate vaccines (Schneider et al 1998). One of the benefits associated with DNA vaccines may be in the induction of memory T-cell population (Gurunathan et al 2000). Recombinant viral vaccines actively infect cells and express the recombinant malaria proteins before aborting infection (Miyahira et al 1998). DNA or recombinant viral subunit vaccines can induce high levels of effectors T-cell immune responses, but poor antibody responses limits use of such vaccines (Gurunathan et al 2000, Paoletti 1996).

7.3.5. Liposomal malaria vaccines:

The identification of protective antigens and adjuvant is a challenge for vaccine development in the fight against the world’s most common infectious killers, including malaria. In this regard, merely identification of antigen does not help in solving some key problems in malaria vaccine development while induction of a strong and durable immune response is prerequisite for successful vaccination against this deadly parasite. Adjuvant imparts variety in humoral as well as cell mediate immune responses or may even stimulate a differential IgG isotype expression (Richards 1998, Kenney et al 1989). Hence, the selection of adjuvant or antigen delivery systems is crucial for achieving high efficiency of malaria subunit vaccines. In this regard, new vaccine delivery methods and adjuvants can be brought into practice that may eventually lead to the induction of desirable humoral as well as cellular immunogenicity.

By using experimental animal models, number of workers have tried to achieve various levels of protection with crude preparations of blood-stage antigens with adjuvants against Plasmodium sp. (Burns et al 1997, McCollm et al 1982, Playfair and De Souza 1987, Playfair et al 1977, ten Hagen et al 1993, Patterson et al 1999, Hunter et al 1995, Desowitz 1975, Murphy and Lefford 1978, Kumar et al 1990, Reese et al 1978, Siddiqui 1977 & 1978, Targett and Fulton 1965, Mitchell et al 1979, Brown et al 1970). Adjuvants used in these studies included Freund’s adjuvant, saponin, alhydrogel, IFN-γ, nonionic block polymers, detoxified LPS, Quil A as well as bacteria, including Bordetella pertusis, Corynbacterium parvum and Salmonella typhimurium all of which produced mixed results. Apart from these adjuvants, protective immunity against experimental P. falciparum challenge was achieved after immunization of humans with Al(OH)₃-adsorbed recombinant proteins, or a peptide-
Hence, the selection of adjuvants or antigen delivery systems without any side effect is crucial for achieving high efficiency of subunit vaccines. In an effort to improve immunogenicity of repeat based malaria antigen, variety of Al(OH)$_3$-adsorbed candidate liposomal vaccines containing lipid A or monophosphoryl lipid A (MPLA) were evaluated in animal models (Alving et al 1986, Richards 1988 & 1989, Alving and Richards 1990). Moreover, recombinant antigen (R32NS$_{181}$) was found to be more effective in humans in its liposomal form rather than with Al(OH)$_3$ or other adjuvants (Fries et al 1992). Purified parasite gp 195 entrapped in liposome B30-MDP with LA-15-PH (synthetic equivalent of E. coli derived MPLA), evoked higher humoral response than FCA in mice (Hui et al 1991). In a study on MSP-1 derived 83.1 portion spf66 on its encapsulation into influenza virosomes elicited higher immune response than commercial whole virus influenza vaccine (Poltl-Frank et al, 1999). Interestingly, efficacy of liposome as antigen delivery vehicle against malaria was also found effective against other stages of P. falciparum as CSP antigen entrapped in MPLA/liposomes revealed the higher efficacy of liposomal vaccine as compared to previously used CSP/Al(OH)$_3$ formulation (Alving et al 1986 Richards et al 1988).

The use of adjuvant can critically influence the quality of immune responses. For example, it is well known that the Ig subclasses can be influenced by the use of adjuvants. Studies on rodent and humans malarias have suggested that immunity may be associated with differential expression of malaria specific Ig classes (White et al 1991, Bouharoun-Tayoun and Druilhe 1992). Enhancement of certain Ig isotypes (e.g. IgG2a/IgG2b) can be achieved by adjuvant formulations such as Thr-MDP and or nonionic, pluronic polymer vesicles, liposomes, lipid A derivatives, and their use may favor induction of a protective antibody population. Nevertheless, CTL response has been demonstrated to play a key role in immunity to the liver stages of malaria.
Taking into consideration the need of desired immune response against malaria, choice of an adjuvant formulation is likely to have a major impact on immunologic recognition of parasite and help in designing of subunit vaccines. Since most basic studies on immune specificity and mechanisms of immunologic recognition require in vivo priming of animal hosts by delivery of the antigen together with an adjuvant, the nature and characteristics of immune responses observed and the interpretation of the results may largely be restricted within the context of the adjuvant used. Along the same line, in the design of subunit vaccines, it may not be useful to exhaustively define immune responses to vaccine antigen using FCA adjuvant because it is too toxic to be used clinically. Therefore it will be necessary to characterize the immunogenicity of antigens using clinically acceptable adjuvant such as liposomes.

8. Immunopotentiating Role of Immunomodulator

8.1. Immunomodulators:

Immunomodulators may be defined as substance biological or synthetic which can stimulate, suppress or modulate any of the components of immune system. The basic function of immune system is to protect individual against infectious agents and potential pathogens which puts the immune system in a vital position between a healthy and diseased state of the host. Networking and interaction with in the immune system are so complex that modulation of immune system to achieve designed therapeutic success is still in the realm of philosophical editorials rather than definite efficacy studies (Benson 1993). The use of immunostimulants, particularly as an adjunct to chemotherapy for control and prevention (Chatterjee et al. 1988) holds great promise but has not yet received the attention that it deserves.

Immunomodulators can be classified immuno-adjuvants, immunostimulants and immuno-supresants. Immuno-adjuvants are used to increase the efficacy of vaccines and since specific immunoadjuvants are used with specific vaccines, therefore could be considered as specific immunostimulants. Lack of availability of suitable adjuvant for human use has been one of the important stumbling blocks in our ability to develop various prophylactic means to control the infection (Allison 1997). Immunostimulants by definition are inherently non-specific in nature as they are...
envisaged to enhance body's resistance against infection. They can act through innate as well as adaptive immune response. In healthy individuals the immunostimulants are expected to serve as prophylactic or promoter agents i.e. immunopotentiater by enhancing the basic level of immune response. Immunosuppressants could be used for control pathological immune response and are active in autoimmune diseases, immediate and delayed type of hypersensitivity immune reactions and graft rejections.

Objective of immunomodulation as observed by modern researchers are many. In clinical medicine, both aspects of immunomodulation viz. immunostimulation and immunosuppression are equally important. In conventional chemotherapy immunopotentiation is an ideal choice when the host defense mechanisms are to be activated under conditions of impaired immune response.

8.2. Plant derived products as immunomodulator:

Natural products offer resource for the maintenance of life for ages. Already in the earliest written traditions, e.g. the Rigveda of South Asia (ca. 1500-900 BC), plants had been considered as best of source of medicine. One of the best-known examples is Soma, the juice extracted from the plant, which was used as a medicine (Mukhopadhyaya, 1922-1929, Mahdihassan and Mehdi 1989). The interest in medicinal plants has never ceased since. Even today, natural products become increasingly important as a source of pharmacotherapeutics, either directly, for example in the application of herbal drugs for the treatment of chronic diseases, or as raw material from which more or less complex chemical structures with particular biological activity are isolated. Cragg reviewed the role of natural products in drug discovery, and concluded that for the disease indications anticancer and anti-infection, more then 60% of new approved drugs are derived from natural sources. Furthermore, there is a global concern about emerging infectious diseases, and an urgent need to identify novel means for effective treatment thereof. The National Cancer Institute (USA) has screened randomly a hundred thousand plant extracts for antineoplastic, anticarcinogenic, and antitumor activity in several model systems. This strategy did not prove very effective. Traditional systems of medicine, however, provide an extremely vast body of source material for the development of new drugs. Evaluation of traditionally used medicines, keeping into account the traditional principles that are applied in drug therapy, may offer a lead towards effective drug discovery.
Several medicinal plants used in Indian traditional medicines called Rasaynas (devoted to enhancement of body, resistance) have attracted the attention of many scientists. Ironically, most of the investigations were carried independently without any significant interdisciplinary approach. In literature many plants have been listed having immunomodulatory effect and some of them have been proved by using modern scientific methodologies. These plants include *Allium sativum* (Lahsun), *Aloe Vera* (Gharita Kumari), *Andrographis paniculata* (kirayat), Asparagus racemose (satawar), *Azadirachta indica* (neem), *Curcuma longa* (haldi), *Ocimum sanctum* (Tulsi), *Panax ginseng* (ginseng), *Phyllanthus emblica* (amla), *Picrorhiza kurroa* (kutali), *Tinospora cordifolia* (giloe) and *Withania somnifera* (ashwagandha) etc.

9. Picrorhiza kurroa

*Picrorhiza kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhea, and scorpion sting. It is a small perennial herb from the Scrophulariaceae family, found in the Himalayan region growing at elevations of 3,000-5,000 meters. *Picrorhiza kurroa* has a long, creeping rootstock that is bitter in taste, and grows in rock crevices and moist, sandy soil. The leaves of the plant are flat, oval, and sharply serrated. The flowers, which appear in June through August, are white or pale purple and borne on a tall spike, manual harvesting of the plant takes place from October to December. The active constituents are obtained from the root and rhizomes. The plant is self-regenerating but unregulated over-harvesting has caused it to be threatened to near extinction. Current research on *Picrorhiza kurroa* has focused on its hepatoprotective, anticholestatic, antioxidant, and immune-modulating activity (Atal et al 1986, Subedi 2000).

9.1. Active Constituents

Kutkin is the active principal of Picrorhiza kurroa and is comprised of kutkoside and the iridoid glycoside picrosides I, II, and III. Other identified active constituents are apocynin, drosin, and nine cucurbitacin glycosides (Weinges et al, Stupner H and Wagner H 1989). Apocynin is a catechol that has been shown to inhibit neutrophil oxidative burst in addition to being a powerful anti-inflammatory agent. (Simons et al
1990) while the curcubitacin has been shown to be highly cytotoxic and possess antitumor effects (Stuppner H and Wagner H 1989).

9.2. Mechanisms of Action

The hepatoprotective action of Picrorhiza kurroa is not fully understood but may be attributed to Picrorhiza's ability to inhibit the generation of oxygen anions and to scavenge free radicals (Russo et al 2001). Picrorhiza's antioxidant effect has been shown to be similar to that of superoxide dismutase, metal-ion chelators, and xanthine oxidase inhibitors (Chander et al 1992). In malaria infected rats, Picrorhiza restored depleted glutathione levels, thereby enhancing detoxification and antioxidation, and helping maintain a normal oxidation-reduction balance (Chander et al 1992). In this same animal model, Picrorhiza also demonstrated an anti-lipid peroxidative effect (Chander et al 1998). Like silymarin, Picrorhiza has been shown to stimulate liver regeneration in rats, possibly via stimulation of nucleic acid and protein synthesis (Singh et al 1992). Picrorhiza's anti-inflammatory action is attributed to the apocynin constituent, which has been shown to have potent anti-inflammatory properties in addition to inhibiting oxidative burst in neutrophils (Simons et al 1990). Although the mechanism is unclear, animal studies indicate Picrorhiza's constituents exhibit a strong anticholestatic activity against a variety of liver-toxic substances, appearing to be even more potent than silymarin. Picrorhiza also exhibits a dose-dependent choleretic activity, evidenced by an increase in bile salts and acids, and bile flow (Shukla et al 1991).