Introduction
Vaccines can be defined as the agents that induce protective immunity against the pathogen. They have significant effect on both human and animal health. For more than 200 years, vaccines have traditionally been consisted of live attenuated organism, whole inactivated organism, “killed” pathogenic organism, i.e. viral and bacterial cells or purified components isolated from organisms and inactivated toxins.

The traditional approaches to vaccine development are as follows:

<table>
<thead>
<tr>
<th></th>
<th><strong>Viral</strong></th>
<th><strong>Bacterial</strong></th>
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<tbody>
<tr>
<td>Live attenuated pathogens</td>
<td>Measles</td>
<td><em>Mycobacterium</em> (BCG)</td>
</tr>
<tr>
<td></td>
<td>Vaccinia</td>
<td><em>Salmonella typhi</em></td>
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<tr>
<td>Inactivated pathogens</td>
<td>Rabies</td>
<td><em>Bordetella pertussis</em></td>
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<td></td>
<td>Hepatitis A</td>
<td><em>Vibrio cholerae</em></td>
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<tr>
<td>Toxoids</td>
<td>-</td>
<td><em>Tetanus toxoid</em></td>
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<td></td>
<td></td>
<td><em>Diphtheria toxoid</em></td>
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<tr>
<td>Combination vaccines</td>
<td>MMR</td>
<td>DPT</td>
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MMR - Measles, Mumps and Rubella vaccines
DPT - Diptheria, Tetanus and Pertuis (whole cell) vaccine

Despite considerable success of traditional approaches to vaccine development, alternative approaches for vaccine delivery are required due to number of reasons;

- Some live attenuated vaccines can cause disease in immunosuppressed individuals.
- Whole-inactivated vaccine (e.g. Bordetella virus, Influenza virus) contains reactogenic compounds, which can cause undesirable side effects in some individuals.
- Some pathogens (i.e., Hepatitis B, Hepatitis C) can not grow in the culture.

The challenges for vaccine development are;
(i) Need to improve the existing vaccines by making them more potent and safe
(ii) Need for the development of vaccines against diseases for which no vaccine is available
(iii) Need to improve vaccine to extend their wider coverage in population (O’Hagan et al., 1998)

Vaccination has proven to be the most efficient and cost-effective means of preventing the outbreak and spread of epidemics caused by insidious bacterial, parasitic and viral diseases such as tuberculosis, polio, hepatitis B, etc. This is well proved by the concerted immunization of smallpox throughout the world. Except for few vaccines, most of them are administered as part of routine childhood immunization programs. However, the greatest problem faced in the effective delivery of such vaccines is that multiple dose primary immunization regimens are to be followed essentially. In addition, periodic boosters are required throughout the life to maintain the desired immunity. For better implementation of the immunization program a rational approach would seemingly rely on the development of a delivery system that effectively converts multiple dose vaccines into a single dose vaccine. In our present vaccine systems, except for oral polio vaccines, typhoid and cholera vaccine, all other vaccine systems are administered through parenteral route. This is because the presentation of these vaccines by oral route leads to loss of their antigenic activity.

The major disadvantages with the existing vaccination/immunization programs are;

- At least three doses are needed to require life long immunity
- Periodic boosters are required to maintain immunity
- Adequate cold storage facilities are required
• For the people living in poorly accessible areas the cost of immunizing an individual is very high
• Not all vaccines are capable of stimulating an appreciable amount of mucosal immunity
• Need for potent adjuvants to elicit a strong immune response
• Lack of carriers and adjuvants
• Failure of immunization is still high

The major advantage with a vaccine is prevention of disease. However, unless the vaccine is administered properly, the goal remains unachieved. The application of recombinant DNA technology has allowed for the construction of a wide variety of additional recombinant strains, which may prove useful as either vaccine or sub unit vaccine, or as vectors to deliver vaccine antigens. However, many such highly purified antigens have been found to be non-immunogenic or weakly immunogenic. Sometimes, the use of potent adjuvants is required to elicit a vigorous immune response. To overcome such problems new vaccine delivery technologies need to be developed. The limitations of existing immunization regimens that insist for the development of new or improved vaccines are as follows;

<table>
<thead>
<tr>
<th>Vaccine Type</th>
<th>Description</th>
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<tbody>
<tr>
<td>Hepatitis B vaccine</td>
<td>Recombinant hepatitis B surface antigen expressed in yeast or mammalian cells</td>
</tr>
<tr>
<td>Acellular pertussis vaccines</td>
<td>Including a genetically detoxified pertussis toxin</td>
</tr>
<tr>
<td>Polysaccharide conjugate vaccines</td>
<td>Bacterial polysaccharides from <em>Haemophilus influenza</em> type (normally tetanus or diphtheria toxoids). Similar vaccines for <em>Meningococcus</em> A and C and <em>Pneumococcus</em> are under development stages.</td>
</tr>
</tbody>
</table>
The design of vaccines is governed and directed by numerous considerations that include immunogenicity, safety and stability. There are some major factors affecting immune responses;

- The nature and the dose of the immunogens
- The adjuvant or carriers used in the formulation
- The immunization schedule
- The route of administration
- The immune status of the host being immunized

The immune status of the host/vaccine recipient can be affected by age, genetic constraints, and preexisting deficits in the immune system.

In vaccine therapy need of adjuvants to enhance and modulate the immune response is well appreciated (Gregoriadis et al., 1993; Kay et al., 1997). Many structurally unrelated agents that are capable of improving immune response to vaccine antigens are known as immunological adjuvants. Besides enhancing the immune response, adjuvant in fact can also modulate the response in Th-1 on Th-2 pathway.

In last ten years, a great deal of enthusiasm has emerged in the field of microencapsulation and controlled release of proteins. This is clearly justified by facts that a large number of new drugs and antigens is proteins. A critical issue in this area is that classical oral formulations of spheres are not adequate for administration of therapeutic and antigenic proteins. This is not only due to the limitation of these systems for controlling the release of proteins but also due to the following restrictions for the oral administration of the proteins, which include:

1. Proteins cannot diffuse through the intestinal wall.
2. Proteins are unstable in gastrointestinal compartment.
3. Macromolecules in general have a small half-life.
New formulation strategies are necessary in order to explore great therapeutic potential of proteins. Among various means for improving the administration of proteins, microencapsulation into biodegradable polymers, liposomes and niosomes, etc. represents a practical and promising approach. However, these biodegradable particles/spheres can be injected subcutaneously or intramuscularly and release the entrapped protein for an extended periods of time. It is a well known fact that small particles (microspheres/nanospheres) can transport proteins from gastrointestinal cavity to the immune system.

**IMMUNIZATION**

It is the method by which a host is rendered resistant against infective pathogen for short or long duration of time i.e. provides immunity against a particular disease.

**The Immune System**

The immune system is a remarkably adaptive defense system that has evolved in vertebrates to protect them from invading pathogenic microorganism. The immune system is capable of generating an enormous number and variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders. This is evident from the fact that majority of infections in normal individuals are of limited duration and leave little residual however, active dosage (Kuby 1997; Roitt et al., 1985).

Functionally, an immune response triggers through recognition and response events. Immune recognition is remarkable for its specificity. The immune system can recognize subtle chemical differences that distinguish one foreign pathogen from the other. At the same time it is capable of discriminating between ‘foreign molecules’ and ‘self molecules’. Once these foreign molecules are recognized, the immune
system enlists the participants to mount an appropriate response, known as an effector response, to eliminate or neutralize the organism. Thus, the immune system is able to convert the initial recognition event into different effector responses. These effector responses are uniquely suited to eliminate a particular pathogen. Exposure to same foreign organism at a later stage induces memory response.

**Cells of the Immune System**

Cells involved in the immune response of vertebrates are of different types, which are able to specifically recognize the ‘antigens’ and also help in their elimination.

All the cells of the immune system arise from pluripotent stem cells (Fig. 1.1) and are divided into two differentiation, i.e.,

- The lymphoid lineage – Producing lymphocytes and
- The myeloid lineage – Producing phagocytes & other cells.

The cells of the immune system are as follows:

<table>
<thead>
<tr>
<th><strong>Cell type</strong></th>
<th><strong>Site of origin/maturation</strong></th>
<th><strong>Function</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>Bone Marrow</td>
<td>Humoral immunity</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>Thymus</td>
<td>Cell mediated immunity</td>
</tr>
<tr>
<td>Null cells</td>
<td></td>
<td>Majority of Natural Killer (NK) Cells</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mononuclear</td>
<td>Skin lymph nodes spleen and thymus</td>
<td></td>
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<tr>
<td>Phagocytic cells,</td>
<td>present antigen sensitive lymphoid</td>
<td></td>
</tr>
<tr>
<td>APC (in the blood)</td>
<td>cells</td>
<td></td>
</tr>
<tr>
<td>Macrophages (in the tissues)</td>
<td>Alveolar macrophages (in the lungs)</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td></td>
<td>Histocytes (in the connective tissues)</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td></td>
<td>Kupffer cells (in the liver)</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td></td>
<td>Mesangial Cells (in the kidney)</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td></td>
<td>Microglial cells (in the brain)</td>
<td>Phagocytosis</td>
</tr>
</tbody>
</table>
Granulocytic Cells

Neutrophil  Bone marrow  Active phagocytic cells
Eosinophil  Bone marrow  Active phagocytic cells
Basophil    Bone marrow  Non-phagocytic, Act by releasing contents of their granules.

Mast Cells  Bone Marrow  Important role in development of allergies.

Dendritic Cells Bone Marrow  Process and present antigens to T helper cells.

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Fig. 1.1: Origin of immune cells
Among different cells involved in the immune system, the lymphocytes and antigen presenting cells (APC) are of importance for the present immunological study:

**Lymphocytes**
Lymphocytes are one among the different types of WBC produced in the bone marrow during the process of hematopoiesis. These lymphocytes circulate in blood and lymph system and reside in various lymphoid organs. There are two major populations of lymphocytes – B lymphocytes (B cells) and T lymphocytes (T cells), which are responsible for various functions.

**Antigen Presenting Cells (APC)**
Antigen presenting cells are one among the two types of mononuclear cells. Their role is to present antigen to specific antigen sensitive lymphocytes. They are primarily found in the skin, lymph nodes, spleen and thymus. These APCs are rich in class II MHC antigens and are important for antigen presentation to T cells. The other specialized APCs include the follicular dendritic cells and B Cells found in the secondary follicles of lymph nodes and spleen.

**Organs of the Immune System**
Morphologically and functionally diverse organs and tissues of the immune system have various functions in the development of immune responses. These organs can be divided on the basis of their functions, i.e. primary lymphoid organs and secondary lymphoid organs.

Thymus and bone marrow constitute the primary lymphoid organs, where maturation of lymphocytes occurs. Lymph nodes, spleen and various mucosal associated lymphoid tissues (MALT) constitute the secondary lymphoid organs that
are capable of trapping antigen and provide sites for mature lymphocytes to interact with antigen.

**Immunity**

Immunity involves a specific defensive response whenever a foreign organism or other foreign substances invade a host. The human immune system recognizes foreign substances as not belonging to the body and it develops an immune response against them. Organisms or substances that evoke such a response are called antigens. This immune response involves the production of proteins called antibodies and specialized lymphocytes.

Immunity can be classified into natural and acquired immunity. Acquired immunity is further classified into naturally acquired and artificially acquired immunity, that refers to the protection that a person develops against certain types of microbes or foreign substances. Acquired immunity is developed during an individual's lifetime. Immunity can be acquired either actively or passively. Immunity is said to be acquired actively when a person is exposed to microorganisms or foreign substances and the immune system responds. Immunity is acquired passively when antibodies are transferred from one person to another. Both types of acquired immunity can be obtained by natural or artificial means (Fig. 1.2).

**Humoral and Cell-mediated Immune Response**

**Humoral response**

The name humoral response was derived from the Latin word humor, meaning 'body fluid', thus humoral immunity refers to an immunity that can be conferred on a non-immune individual by administration of serum antibodies from an immune individual.
Humoral immunity or response is carried by B cells (Fig. 1.3). These cells interact with antigen and subsequently proliferate and differentiate into antibody-secreting plasma cells. Antibody functions as the effector unit of the humoral response by binding to antigen and neutralizing it or facilitating its elimination. B cells which are produced in the bone marrow, mature and migrate into the lymphoidal organ, where they encounter antigen, when an appropriate antigen contacts the antigen receptor antibodies on a B cell, the B cell proliferates into a large clone of cells. This phenomenon is also known as "Clonal Selection".
Fig. 1.3: Overview of the humoral and cell-mediated branches of the immune system
Cell-mediated immune response

Cell-mediated immune response/immunity is one in which T cells are involved. Unlike humoral immunity, cell-mediated immunity can be transferred only by administration of T cells from an immune individual. Both activated T helper cells and cytotoxic T lymphocytes serve as effector cells in cell-mediated immune reaction. These effector T cells generated in response to antigen are responsible for cell mediated immunity.

Antigens or Immunogens

Those substances capable of inducing a specific immune response by producing specific antibodies are commonly referred to as antigens, more appropriately immunogen. Most antigens are either proteins or large polysaccharides. Lipids and nucleic acids are also antigenic only when combined with proteins and polysaccharides. Antigenic compounds are components of invading microbes such as the capsules, cell walls, flagella, fimbriae, toxins of bacteria, coats of viruses, or the surfaces of other types of microbes. Most antigens have a molecular weight of 10,000 or higher. A foreign substance that has a low molecular weight is often not antigenic itself unless it is attached to a carrier molecule. These small compounds are called haptens. Haptens are not capable by themselves of inducing a specific immune response, it means they lack immunogenicity. In order to induce an immune response, the hapten requires to be attached to carrier molecule (usually a serum protein i.e., albumin). Generally, antibodies recognize and interact with specific regions in antigens called antigenic determinants or epitopes. Sometimes, there may be confusion between immunogenicity and antigenicity but both are distinct, however
interrelated. Immunogenicity is the ability to induce a humoral and/or cell-mediated immune response.

\[ \text{B cells + antigen} \rightarrow \text{effector B cells + memory B cells (Plasma cells)} \]

\[ \text{T-cells + antigen} \rightarrow \text{effector T cells + Memory T Cells (CTL)} \]

**Antibodies or Immunoglobulins**
Antibodies represent for a group of glycoproteins present in the serum and tissue fluids of all animals. Antibodies are generated in response to an antigen and can recognize and bind to that antigen. Antibodies can therefore help, neutralize or destroy that antigen against which they are produced. Antibodies are highly specific in recognizing the antigen that stimulated their formation. They are the members of a group of soluble proteins collectively known as immunoglobulins (Igs).

**Structure of Immunoglobulin**
A single antibody has two identical heavy chains and two identical light chains (Fig. 1.4). A typical immunoglobulin have similar basic structure and similar chemical properties. An immunoglobulin molecule has four protein chains: two identical light (L) chains and two identical heavy (H) chains. The chains are joined by disulfide links and other bonds to form a “Y” shaped molecule.

The two sections located at the end of “Y” arms are called the Variable "V" regions and Constant "C" regions. Each antibody has at least two identical sites that bind to antigenic determinants. These sites are known as antigen-binding sites. These antigen-binding sites are located at the variable region.
**Classes of Immunoglobulin**

The five distinct immunoglobulins have been recognized in higher mammals namely IgG, IgA, IgM, IgD and IgE. Each class plays a different role in the immune response.

*Immunoglobulin G (IgG)*

IgG is the most abundant class in the normal human serum and constitutes about 75 – 80% of the total serum immunoglobulin. It is a major antibody of secondary immune responses and the exclusive-toxin class. These antibodies are capable of crossing the walls of blood vessels and enter tissue fluids. There are four IgG subclass in humans namely IgG1, IgG2, IgG3 and IgG4. IgG antibodies offer protection against circulating bacteria and viruses, neutralize bacterial toxins, trigger the complement system and when bound to antigen, enhance the effectiveness of phagocytic cells.
**Immunoglobulin M (IgM)**

IgM accounts for about 10% of the total serum immunoglobulin. Only IgM has a pentametric structure, which is produced by plasma cells. Its monomeric form is expressed as membrane bound antibody on B cells. The large size of the molecule prevents IgM from moving about as freely as IgG does. IgM generally remains in blood vessels and does not enter the surrounding tissues. IgM is the predominant type of antibody involved in the response to the ABO blood group antigens on the surface of red blood cells. It is also effective in aggregating antigens. It can also enhance the ingestion of target cells by phagocytic cells as does IgG. IgM antibodies are the first antibodies to appear in response to initial exposure to an antigen and are relatively short-lived.

**Vaccine Adjuvants and Adjuvanticity**

Adjuvants can be defined as the substances, which when mixed with an antigen and injected with it, serve to enhance the immunogenicity of an antigen. Ramon first demonstrated that it is possible artificially to increase levels of diphtheria or tetanus antitoxin by the addition of bread crumbs, agar, starch oil, lecithin or saponin to the vaccines (Ramon, 1925). During the last 70 years, many adjuvant formulations have been developed and a few of these have been evaluated in clinical trials (Edelman, 1990; Chedid, 1985; Warner et al., 1986; Lindblad and Sparck, 1987; Allison and Byars, 1990; Spriggs and Koff, 1991). The term adjuvant was first coined by Ramon, 1926 for substances used in combination with specific antigens that produce more immunity than the antigen alone. The word adjuvant has originated from the latin word “adjuvare”, which means “to help”. Adjuvants are often used to boost the
immune response when antigen has low immunogenicity or when only small amount of an antigen is available limiting the immunization dosage.

These adjuvants are also capable of prolonging immunogenicity. The adjuvanticity of most of the formulations developed so far is associated with diverse side-effects of varying degree, some of the adverse effects are ascribed to the mechanisms involved in the adjuvanticity of these formulations. For example, local reactions may be due to depot formation at the site of injection which is a major mechanism of adjuvanticity of several formulations. Another mechanism of adjuvant action is stimulation of the cells of immune system to secrete various cytokines which may lead to systemic side-effects depending upon the type and amount of cytokines elicited. Other side-effects may be due to general adverse pharmacological reactions of various formulations. Usually, a compromise or balance between toxicity and adjuvanticity is accepted based upon risk-benefit analysis (Gupta et al., 1993). For example, for routine childhood vaccines, safety is biggest concern and adjuvanticity may be restricted to normal healthy babies. An adjuvant for routine immunization of healthy infants children and adults must have a very low rate of adverse side-effects. Severe side effects occuring as rarely as once in several thousand doses would not be acceptable for routine vaccines. On the other hand, for high-risk groups like cancer and AIDS patients or for therapeutic vaccines a certain level of toxicity may be acceptable based upon benefits.

Adjuvants belong to a highly diversified and heterogeneous groups of compounds chemically as well as in regards to their mechanism of adjuvanticity. On the basis of adjuvanticity these can be grouped as follows:
- Causing depot formation at the site of injection (for example, mineral compounds, oil-based adjuvant, biodegradable polymer microsphere > 10 μm)
- Acting as delivery vehicles for the antigens which may help in targeting antigens to immune competent cells (for example, liposomes, oil adjuvant, biodegradable polymer microspheres < 10 μm, non-ionic block polymer surfactants)
- Acting as immunostimulators (for example, Freund's complete adjuvant (FCA). Muramyl dipeptide (MDP), lipopolysaccharide (LPS). Lipid A, monophosphoryl lipid A (MPL), Pertussis toxin (PT), Cytokines
- Adjuvants can be classified according to origin rather than mechanism of action.

Most of the antigens are still not understood completely. Classes of modern vaccine adjuvants, carriers and vehicles can be described as follows:

I. Adjuvants

   A. Aluminum and Calcium salts
      1. Aluminum Phosphate
      2. Aluminum hydrochloride
      3. Calcium phosphate

   B. Bacterial and plant product
      1. Mycobacterial products
         (a) Complete Freund’s adjuvant (CFA, FCA) (Killed M. tuberculosis)
         (b) DETOX (cell wall skeleton on M. Phlei).
         (c) Bacillus Calmette – Guerin (BCG)
         (d) Muramyl dipeptides and tripeptides
      2. Gamma inulin
      3. Beta-glucan
      4. Monophosphoryl lipid A (MPL)
      5. Neuraminidase – galactose oxidase (NAGO)
      6. Klebsiella pneumoniae glycoprotein
      7. Bordetella pertussis
C. Surface – active agents
   1. Saponin
      (a) Quil A
      (b) QS – 21 (stimulon™)
      (c) Immune – stimulating complexes (ISCOMs™)
   2. Deoxycholic acid/alum complex
   3. Dimethyl dioctadecyl ammonium bromide (DDA)

D. Polyanions
   1. Dextran
   2. Double – stranded polynucleotides
   3. Sulfolipopolysaccharide

E. Polycrylics
   1. Polymethyl methacrylate (PMMA)
   2. Acrylic acid – allyl sucrose

F. Vitamins
   1. Vitamin A, D and E

F. Cytokines
   1. Granulocyte – macrophage colony stimulating factor
   2. IFN - α
   3. INF – γ
   4. IL-1,2,7 and 12

G. Hormones
   1. Human growth hormone
   2. Dehydroepiandrosterone (DHEA)

H. Imidazo quinolines
   1. Imiquimod

II. Carriers
   A. Bacterial toxoid (Tetanus, Diphtheria, Pseudomonas, Pertussis)
   B. Meningococcal outer membrane proteins (Proteosomes)
   C. Fatty acids
   D. Cholera toxin, LT toxin, cholera B subunit
E. Nucleic acid vaccines.

F. Living vectors
   1. Vaccinia virus
   2. Adenovirus
   3. Poliovirus
   4. BCG
   5. Attenuated Salmonella
   6. Attenuated *Vibrio cholerae*

III. Vehiciles

A. Mineral Oil emulsions
   1. Incomplete Freund’s adjuvants

B. Vegetable Oil emulsions (Peanut, Olive, Sesame)

C. Squalene and Squalene emulsions
   1. MF – 49.
   2. SAF

D. Lipid – containing vesicles
   1. Liposomes
   2. Sendai Proteoliposomes
   3. Immunopotentiating reconstituted influenza virosomes (IRIV)

E. Bioderadable polymer microspheres
   1. Lactide and glycolide polymers (PLGA, PGA, PLA)
   2. Proteinoid microspheres
   3. Alginates

F. Proteinoid cochleates

G. Edible Plants

A **carrier** is an immunogenic protein bound to a hapten or a weakly immunogenic antigen (Edelman and Tacket, 1990). A carrier may be a living organism bearing gene expression of the foreign hapten or antigen.

A **vehicle** provides the substrate for the adjuvant, the antigen, or the antigen-carrier complex. Unlike carrier, vehicles are not themselves immunogenic. Vehicles,
like carriers can enhance antigen response hence considered as adjuvants belonging to separate class.

Most subunit vaccines are poor antigenic due to variety of reasons:

- Incorrect processing by the immune system.
- Rapid clearance
- Stimulation of an inappropriate immune response
- Lack of critical B or T cell epitopes
- Most of these failures of the subunit vaccines can be alleviated by administering subunit antigens with adjuvants.

**Advantages of Adjuvants**

Some distinctive advantages of adjuvants can be realized and can be summarized as follows:

- Increase the potency of small, antigenically weak synthetic or recombinant peptides.
- Enhance the onset, vigour and persistence of the immune response to stronger antigens.
- Increase the immune response to vaccines in immunologically immature, immunosuppressed or senescent individuals.
- Modulate humoral or cell-mediated immunity.
- Modulate antibody, avidity, specificity, quantity, isotype and subclass against epitopes on complex immunogens.
- Can reduce the cost and increase the availability of vaccines.
- Decrease the amount of antigens in combination vaccines, thus reducing the likelihood of antigen competition and carrier specific epitope suppression.

**Safety**

Over years, many adjuvants have been developed, but they were never accepted for routine vaccination due to their safety concern. Many experimental adjuvants have
high potency but are too toxic for clinical use. The most important attribute of an adjuvant-based vaccine is that it is more efficacious than the aqueous vaccine and that this benefit outweighs its risk. To overcome this safety concern, synthetic vaccines/antigens have been designed, which are not dependent on adjuvants. The potential risks concerned with the administration of vaccine adjuvants are enlisted below (Edelman, 1990; Goldenthal et al., 1993; Bussiere, et al., 1995).

**Safety concerns associated with vaccine adjuvants**

- Local acute or chronic inflammation with formation of painful abscess, persistent nodules, ulcers, or draining lymphadenopathy
- Induction of influenza like illness, with fever malaise, or headache
- Anaphylaxis
- Induction of hypersensitivity to host tissue, producing autoimmune arthritis
- Systemic clinical toxicity to tissue or organs
- Sensitization to tuberculin or to other skin-test antigens
- Immune suppression
- Carcinogenesis
- Teratogenesis
- Abortogenesis

**Characteristics of an Ideal Vaccine Adjuvants**

The characteristics desirable for an ideal vaccine adjuvant include (O’Hagan, 1994):

- The adjuvant must be biodegradable in nature without major toxic manifestations
- They must be defined chemically and biologically so that batch-to-batch vaccinations in the manufactured products can be avoided
- The adjuvant combined with antigens should elicit a more immense protective immune response than do such antigens when used without adjuvant
- Desired immune response should be achieved with the lower and fewer doses of antigen
- The vaccine should be stable in the presence of adjuvants
• The cost of adjuvant should be less
• Adjuvants should have a good shelf-life

Among the variety of adjuvants only Alum (Aluminum salts) has been granted license by FDA (Federal Drug Administration) for use as an adjuvant in human. The major advantage of using aluminum adjuvant is their safety, higher and long lasting antibody after primary immunization as compared to those achieved following administration of soluble vaccines (Edelman, 1990; Gupta and Siber, 1995).

However, aluminum – adsorbed vaccines do not show any advantage over soluble preparations for booster responses. Although it has excellent safety record; yet it poses certain limitations. These include relatively weak adjuvanticity for induction of cell-mediated and humoral immunity, against certain vaccines in human.
• Induction of occasionally painful nodules or swelling and erythema at the inoculation site
• Not readily biodegradable
• Can not be readily lyophilized
• Can not be sterilized by membrane filtration
• Difficult to manufacture in a physiochemically reproducible way

Although newer approaches to vaccine development, particularly the use of recombinant proteins, offer significant advantages over traditional approaches, a general problem is that the newer generation vaccines are often poorly immunogenic. The two adjuvants that have been studied widely are alum and Freud's adjuvants. Freud's adjuvants are water in oil emulsion, which are generally more potent than alum. Freud's complete adjuvant (FCA) contains immunostimulatory compounds from Mycobacterium tuberculosis, while its less toxic Freud's incomplete adjuvant (FICA) contains only mineral oil and the emulsifier Arlacel A.
Due to the toxicity of FCA and FICA, researchers had to look for alternative approaches for the development of adjuvants or delivery systems. This led to recent development in vaccine adjuvant systems which include live bacterial and viral vectors, virosomes, ISCOMs, Cochleates, mucoadhesive polymers, Cholera Toxin B subunit, liposomes, microparticles, niosomes, and DNA.

**Live Bacterial Vectors**
The mucosal surfaces of the intestinal respiratory and urogenital tracts are the site of action or the entrance path to the intestinal tissues for many pathogens. Local immunity induced by the pathogen is the main protector of mucosal surface (Mestecky et al., 1997; McGhee, et al., 1992). To induce an effective protective local and systemic immunity there is an attractive possibility of novel mucosal vaccines for immunization at the mucosal surfaces. Although several bacterial species, including genetically modified strains of Salmonella, *E. coli*, Mycobacterium, etc. (Mestecky and McGhee, 1987) have been studied, majority of the work has been carried out on strains of Salmonella (Michalek et al., 1994; Michalek et al., 1995). These bacteria have been developed as vectors for mucosal immunization. However, the concept of using avirulent salmonella mutants as mucosal vaccine delivery system is now a decade old.

**Live Viral Vectors**
A part of the new vaccine strategies viral vectors has been used to deliver antigens for prophylactic or therapeutic vaccines. Live recombinant vectored vaccines have the advantage that they can stimulate both humoral and cell-mediated immune responses and have great potential for immunization either alone or in combination with subunit vaccine. In recent years, Picornaviruses, the notable one is Poliovirus are utilized for
production of recombinant vaccines (Hogle, et al., 1985). Recombinant adenovirus vectors have been successfully used to induce protective immune response against various viral pathogens like *Herpes simplex*, rotavirus and HIV antigens, when administered via mucosal route (Gallichan et al., 1993; Both, et al., 1993; Berencsi, et al., 1993; Natuk, et al, 1993).

**DNA Vaccine**

Direct DNA inoculation without use of any viral vectors is among the most interesting new additions to subunit vaccine development. This approach is conceptually simple and the most elegant compared to strategy based on sustained protein delivery. Plasmid DNA encoding the antigenic protein is directly introduced into the tissue either by intramuscular or intradermal injection or by bombardment of DNA-coated gold particles through a high voltage gene gun. After plasmid DNA is taken up by nonspecific endocytosis or cell membrane disruption, the host cells produce, process, and present the encoded antigen to the APCs in a manner similar to that happens during the course of natural infection.

This approach has the potential of introducing only the antigenic protein without the unwanted side effects associated with bacterial and viral vaccines. Direct DNA vaccination has generated both humoral (Lai, et al., 1995; Mar et al., 1995; Conry et al., 1995; Conry, et al., 1994; Davis, et al., 1994; Pertmer, et al., 1995; Haynes, et al., 1994; Fuller and Haynes, 1994; Ulmer, et al., 1994; Obterrieder, et al., 1995; Montgomery, et al., 1993) and Cellular (Pertmer, et al., 1995; Fuller, et al., 1994; Ulmer, et al., 1994; Hoffman, et al., 1994; Hau, et al., 1994) protective immunity against a number of pathogens. Antibody responses induced by DNA vaccination were first demonstrated in mice against human growth hormone and
human α-1 antitrypsin after particle bombardment of gold bread coated with DNA-coding for these proteins (Tang et al., 1992).

The effect of DNA mucosal administration has not been extensively investigated and uptake of DNA from epithelial surfaces may not be as effective as in the case of direct injection of DNA into muscle cells. However, it should be possible to enhance transmucosal uptake of DNA using some specific delivery systems such as microspheres, liposomes, virosomes, niosomes, cochleates, or administration of DNA with mucoadhesive polymers.

**Mucoadhesive Polymers**

Polymers with mucoadhesive properties have also been used as film formers and viscosity increasing agents in the pharmaceutical industry for drug delivery. Some mucoadhesives, i.e., sodium alginate and others like sodium carboxymethylcellulose have adjuvant properties when injected, these polymers have been selected for their viscosity to be used as depot forming agents in experimental formulations for parenteral vaccines. The use of alginates as vaccine delivery system has been studied recently (Bowersock, et al., 1996; Cho et al., 1998). The system proves to be a cost effective and safe means of mucosal immunization.

**Cholera Toxin B Subunit Conjugates**

Cholera toxin (CT) is the primary enterotoxin produced by *Vibrio cholerae* bacteria. The CT molecule singularly is well characterized with regard to its structure and function, it consists of five binding B subunits assembled to form a ring. The coupling of antigens to CT or in particular to its nontoxic binding B subunits assemble forming a ring. The coupling of antigens to CT or in particular to its nontoxic binding B subunit (CTB) has proven highly successful in enhancing mucosal (and extramucosal)
immune responses to unrelated antigens. CT alone or in combination might explain their strong adjuvant action in stimulating mucosal IgA and other immune responses. These effects include, depending on animal species and experimental systems, enhanced antigen presentation by a variety of cell types, promotion of isotype differentiation in B-cells leading to increased IgA formation and complex stimulatory as well as inhibitory effects on T-cell proliferation and lymphokine production. This strategy of enhancing mucosal immune response exploits the exceptionally potent mucosal immunogenicity of CT and CTB (Holmgren et al., 1993). Proteins covalently coupled to CTB acquire their mucosal immunogenic properties (Czerkinsky et al., 1989) probably due to the high affinity of CTB for cell-surface GM, ganglioside enhancing uptake by M cells on intestinal Peyer’s patches. In addition, CT and the closely related heat-labile toxin (LT) of *E. coli* and possibly their corresponding B subunits, have mucosal adjuvant effects when mixed with relatively large doses of orally administered antigens (Elson, 1989). However, the intact holotoxins are too toxic to be considered for human application when administered orally in humans.

Better defined and more consistent coupling of CTB to proteins can be accomplished by recombinant DNA technology to yield fusion proteins, which would also facilitate production and reduction in cost.

**Immune-Stimulating Complexes (ISCOMs)**

ISCOMs are cage-like structures into which antigen can be incorporated, resulting in enhanced immune responses after administration (Kersten and Crommelin, 1995; Rimmelzwaan and Osterhaus, 1995; Mestecky, et.al. 1997). ISCOMs are multi-micellar assemblies of lipid saponin micelles that can contain protein antigens. Unlike liposomes, water-soluble antigens are not incorporated in ISCOMs. ISCOMs are
stable particles with the size of a small virus (usually 30-40 nm in diameter) and consists of glycosides of the adjuvant Quil-A, and lipids (cholesterol and phospholipids). The incorporation of viral protein antigens in ISCOMs generally results in enhanced immune response, by stimulating both humoral and cellular immunity. It was shown that ISCOMs, like liposomes and lipopeptides are able to induce class I restricted CTL responses. CD8+ CTLs have been induced in vivo after parenteral immunization of mice with HIV gp 160 and influenza hemagglutinin ISCOMs (Takahashi, 1990), in vitro with human cell lines after activation with ISCOMs containing measles virus F-protein.

The ISCOMs have the advantage over the micelles of an enhanced stability, which increases the probability of reaching the APC in intact form and may result in prolonged presentation of antigen to the APC. High antibody titers have been obtained after immunization with ISCOMs containing hepatitis B virus surface antigen (Howard et al., 1987).

**Liposomes**

Liposomes are phospholipid vesicles that can be used as drug carriers for improving the delivery of “Therapeutic agents”. Structurally liposomes are consisted of one or more concentric spheres of lipid bilayers intercalating aqueous domains.

Liposomes are potential drug carriers for a variety of drugs, therapeutic proteins and diagnostic agents. Liposomes may be defined as spherical, concentric, fluid mosaic made from highly precise self assembly of phospholipids dispersed in aqueous medium (Bangham, 1968). When phospholipids are dispersed in an aqueous medium, the hydrophilic interaction of the lipid head groups with water results in the formation of multilamellar and unilamellar systems (vesicles). These vesicles consist
of simple lipid bilayers. A variety of natural and synthetic phospholipids are available for preparation of liposomes. Because of their entrapping ability, liposomes are being considered as drug delivery structures or vesicles (Gregoriadis, 1976; Juliano and McCollough, 1980).

Liposomes (phospholipid vesicles) were originally introduced in 1965 as model of lipid bilayer membranes (Bangham, 1968). They have been widely applied as models for studying effector phases of immune responses (Alving and Richards, 1983). In 1974, liposomes were proposed as carrier of antigens to augment antibody responses in vivo (Allison and Gregoriadis, 1974; Uemura et al., 1974). In recent years the use of liposomes as potential carriers for vaccines has been extensively explored (Alving, 1987; Van Rooijen and Su, 1989; Gregoriadis, 1990). Applications of liposomes in immunology, and particularly those relating to vaccines, have demonstrated that liposomes may have considerable practical utility as carriers of antigens and adjuvants.

Many studies have demonstrated that liposomes can serve as effective vehicles for inducing humoral immunity to a wide range of antigens (Alving, 1991). There have been many reasons cited for utilizing liposomes in specific situations and are ideally suited for drug delivery by virtue of some of the properties described below:

- Flexibility to entrap both water and oil soluble compounds
- Biocompatible and biodegradable nature of liposomes
- Protection of encapsulated drug from metabolic degradation
- Close resemblance with the natural membrane structure
- Performance as depot releasing their contents slowly and gradually
- Improved drug targeting prospects
- Beneficial modification of pharmacokinetic profile of drugs
- Protective action to plasma unstable drugs
• Facilitation of transport across membranes
• Adjuvanticity of vaccines
• A non-immunogenic substance may be converted to an immunogenic one
• Small amounts of antigen may be suitable as immunogens
• Adjuvants may be incorporated with antigens into the liposomes
• Higher titre of functional antibody activity may be achieved
• Longer duration of functional antibody activity may be induced
• Cell-mediated immunity (inducing cytotoxic lymphocytes) may be induced
• Toxicity of antigens may be reduced or eliminated by inclusion in liposomes
• Soluble synthetic antigens may be represented as membrane associated antigens in an insoluble liposomal matrix

Liposomes can be used to

• Target or transport the drug within the body to the site of action
• Localize the drug when the site of action is close to the site of administration;
• Act as slow release vehicles
• Enhance continuous absorption and decrease drug clearance from the dermis
• Enhance the bioavailability of drug through nasal route using liposomes with bioadhesive property
• Protect labile drugs from gastro-intestinal environment

One of the earliest and well-known observations in the field of liposome research is that parenterally injected liposomes are rapidly ingested by macrophages, particularly in the liver and spleen, where they are gradually degraded in lysosomal vacuoles (Segal et al., 1974). The striking tendency of liposomes to be accumulated by macrophages has served as the major justification and rationale for exploring the feasibility of using liposomes as vehicles for vaccines (Alving, 1987; Van Roaijen and Su, 1989).
Liposomes as Immunological Adjuvants

Twenty years after the discovery of the immunological adjuvant properties of liposome and relation to animal immunization studies, liposomes as adjuvant have come of age with the first liposome based vaccine (against Hepatitis A) being licensed for use in humans. Liposomes are easy to prepare and can have several variations in their compositions as per the requirements of particular protein antigen.

The immunoadjuvanticity of liposomes, first recognized by Allison and Gregoriadis in 1974 has been since repeatedly demonstrated for both, humoral and cell-mediated immunity (Gregoriadis, 1990; Alving, 1991; Van Rooijen, 1990) with unique and obligatory requirement of physical association between antigen and the liposomal vehicle (Shek and Sabiston, 1981; Therien and Shahum., 1989). Using encapsulated and covalently linked antigen, it was demonstrated that not only the association with liposomes but also the mode of antigen association influences the activation of the immune system (Therien and Shahum, 1989; Latif and Bachhawat, 1987; Van Rooijen and Su, 1989). Under the identical conditions of doses and protein : lipid ratios, the encapsulated antigens have shown to stimulate preferentially the production of IgG1. The covalently linked antigen profile favoured a long lasting activation by increased production of IgM, IgG2 and IgG2 along with IgG1.

These overall results suggest that besides their general immunopotentiating effect, liposomal antigens might follow different path in the immune system depending on the mode of antigen-liposome association.

Unlike other potent immunoadjuvants, e.g. complete Freud’s adjuvant, which are unacceptable for human use because of undesirable necrotic reactions at the site of injection, liposome-encapsulated antigens do not induce local granuloma formation (Allison and Byars, 1990). Furthermore, the lipids used in the preparation of the
liposomal vesicles are not foreign to the body and no apparent side effects have been found in injected human subjects (Allison and Gregoriadis, 1974).

Optimal adjuvanticity of liposomes can be achieved by the appropriate choice of vesicle composition (Gregoriadis et al., 1987; Van Rooijen, 1988), surface charge (Latif and Bachhawat, 1987), phospholipid to antigen mass ratio (Davis et al., 1994), ligand-mediated targeting of liposomes to immunocompetent cells and the use of liposome entrapped cytokines such as interleukin 2 (Tan and Gregoriadis, 1989; Mbawuike et al., 1990). Hence the availability of liposomes with variable structural characteristics and mode of antigen enhancement suggest their versatility in immunoadjuvant action and vaccine design.

**Mechanism of Induction of Immune Response by Liposomes**

On the basis of information on liposomal fate in vivo, humoral immunity to liposomal antigens is partly or wholly dependent on the ability of the system to function as an antigen depot, supplying macrophages with free (released) or liposome-entrapped antigen at rates conducive to its efficient presentation to the cells. Involvement of macrophages in liposomal adjuvanticity, a strong possibility in view of T cell participation is further supported by experiments showing its absence in animals depleted of their macrophages. However, macrophage is not an absolute requirement for liposome-induced immunopotentiation. The involvement of macrophagic processing for presentation of liposome-encapsulated antigen is necessary.

An important feature of liposomes as adjuvants is their ability to induce cell-mediated immunity (CMI). This has been shown by positive DTH reactions (Manesis et al., 1979). Liposomes induced CMI can not however, be explained by the antigen-depot mechanism since adjuvants such as alum and oil emulsions, which act in this
way generally induce only a predominantly humoral immunity. Since proteins coupled to lipids are known to induce DTH in proportion to hydrophobicity, it is likely that the increased internalization of hydrophobic antigen lipids by macrophages ultimately improves antigen presentation to T cells. These events may also be favored by the efficient and selective uptake of liposomal antigens into the regional lymph nodes (Tumer et al., 1983).

**Regulation of the Immune System**

There are three significant cellular interactions involved in the regulation of the immune system:

1. Macrophage – T-Cell,
2. T-cell-T-cell and
3. T-Cell-B-cell

**Macrophage – T-cell interactions**

When antigen is first encountered, it is metabolized by macrophages (Skurkovich et al., 1987). The product of this initial step leads to degraded peptide fragments (processed antigen), which is subsequently presented to the helper T-cell population (CD4⁺8⁻) in the framework of the MHC class II molecules. In this process a number of interleukins are generated. The processed antigen is then presented to the helper T-cell subpopulation and is recognized by the CD3/TCR complex.

This clone of T cells is then induced to expand and to produce receptors specific for antigen and for IL-2. T cells are also stimulated to produce IL-2, which can interact and expand as a separate clone of antigen-sensitized T-cells. The latter however, can synthesize other lymphokines such as interferon γ. Interferon γ can stimulate antigens presenting cells (e.g. macrophages) to synthesize class II
molecules. Thus there exists a cascading system of intercellular communication of cellular interaction mediated through signal provided by macrophages, lymphocytes, and interleukins.

**T-cell-T-cell interactions**

This type of regulation involves the interactions of different subsets of T-cells. T-cells can communicate with other subpopulations of T-cells through IL-2 cytokine cascade CD4^8^+. T-cells can also interact with target cells with membrane – associated antigens on target cells in the context of class-I antigens, found on target cells, thus providing antigen presentation to the cytotoxic T-cells.

**T-cell-B-cell interactions**

The third type of regulation occurs between T-lymphocytes and B-lymphocytes. Two major types of communication pathways between T-lymphocytes and B-lymphocytes are involved in regulation: upregulation and downregulation. T-lymphocytes involved in the upregulation of B-cell function are referred to as the helper inducer T-cell population (CD4^8^). Other interactions of interleukins involved in the synthesis of IgE such as IL-4, IL-5, and IL-6 and the inhibitory responses seen with other lymphokines such as interferon-γ.

**Liposomes in Potentiation of Cell-Mediated Immunity**

In addition to their ability to enhance antibody formulation, liposomes are also effective in promoting cell-mediated immunity. Different types of cellular responses induced by liposome associated macromolecules are reported.
Lymphocyte Proliferation Response
It is evident that lymph node cells, primed with liposome-entrapped BSA, elicited a significantly higher proliferative response than cells obtained from the control lymph node primed with free BSA. This selective localization of liposome entrapped antigen may partly explain its apparently better efficacy in providing a higher response. Since the T-cell proliferative response is facilitated by the participation of macrophages (Suzuki and Tomasi, 1980), liposomes encapsulated antigen, being in particulate form, is more likely to enhance the uptake by phagocytic cells, which in turn trigger elevated T-cell response.

Induction of Cytotoxic T-Lymphocytes
Liposomes have been shown to be effective carriers of major histocompatibility complex (MHC) antigens in inducing the formation of MHC-restricted cytotoxic T lymphocytes (CTL) in murine (Finberg et al., 1978) and human beings (Engelhard et al., 1978; Burokoff et al., 1980). The presence of both viral and MHC antigens in liposomes is necessary for the successful stimulation of secondary CTL response against specific viral targets (Finberg et al., 1978). The generation of the CTL response by liposome incorporated MHC antigen requires the active participation of macrophages (Engelhard et al., 1978). Macrophages are involved in processing of the liposome associated antigen and in activating helper T cells, which promote the CTL response (Burokoff et al., 1980).

Macrophage Activation
One of the many lymphokines obtainable from lymphocytes stimulated by antigens or mitogens is the macrophage-activating factor (MAF). Macrophages stimulated by the appropriate lymphokine are more active than resting macrophages in performing
phagocytic functions. The tumoricidal activity of macrophages activated by liposome encapsulated MAF was much enhanced compared to the free factor (Suzuki and Tomasi, 1980). The elucidation of the relationship between the nature of liposome/antigen interaction and the potentiated immune response will facilitate a better design of vaccine carriers for human use.

Non-ionic surfactant vesicles or (niosomes) are now widely studied as an alternative to liposomes. Niosomes consisting of one or more lipid bilayers encapsulating an aqueous core. Niosomes themselves are only weakly immunogenic. The vesicles are able to encapsulate both lipophilic and hydrophilic drugs and to protect them against acidic and enzymatic degradation in the gastrointestinal tract (Yoshida et al., 1992; Finberg et al., 1978; Tomizawa et al., 1993). Engelbhard et al., 1978 have shown that vesicles with a negatively charged surface are preferably taken up via the Peyer's patches alternative to liposomes, especially for controlled drug delivery. They behave similarly to liposomes but have better stability (Rogerson et al., 1987).

They can prolong the circulation of an entrapped drug, resulting in an altered distribution and metabolic stability (Handjani-Vila et al., 1979).

**Microspheres/Microparticles**

O'Hagan (1994, 1998) demonstrated the adjuvant effect achieved through the association of antigens with polymeric microparticles. Encapsulation of antigens into microparticles promotes their entry into lymph nodes and provides a high local concentration of antigen maintained over an extended time-period. Microparticles also promote the interaction of encapsulated antigens with antigen-presenting cells,
e.g. macrophages. The adjuvant effect of microparticles can also be enhanced by co-administration of additional adjuvants (O’Hagan et al., 1998).

Recent studies have shown that micro-particles also exert an adjuvant effect on cell-mediated immunity, including the induction of cytotoxic T-cell response after both systemic and mucosal administration (Maloy et al., 1994; Moore et al., 1995).

Microspheres are solid spherical particles in the size range of few tenths of micrometers to several hundred micrometers. They contain dispersed drug either in homogenous solution or microcrystalline form and can be prepared by various polymerization and encapsulation processes. The size of microspheres ranges from 1-1000 μm and are somewhat bigger in size as compared to the nanoparticles, which range from 1-1000 nm.

Polymers not only influence the biodegradation kinetics but also the mode and rate of antigen presentation, toxicity, tissue compatibility as well as antigen stability under \textit{in vitro} and \textit{in vivo} conditions. Therefore, the selection of polymer needs to be given a fair consideration in formulation of vaccine products. Among a variety of synthetic and naturally occurring polymers, polyesters have been intensively studied e.g., Poly (lactic acid) – PLA, Poly (glycolic acid) – PGA and their copolymer, Poly (lactide-co-glycolide). PLGA are advantageous being biocompatible and less toxic. PLA is more hydrophobic than PGA due to its additional – CH$_3$ group. PLA and PLGA are soluble in organic solvents like chloroform, dichloromethane, acetone, etc.

The polymeric microparticles based controlled release vaccine delivery systems offer many advantages like:

- It reduces the number of vaccine doses needed to achieve effective immunization
- It provides localized or targeted delivery of antigen to the antigen presenting cells or lymphatic systems
• It protects the antigen from the rapid destruction *in vivo*

• It provides a choice over the release of antigen, it can be continuous or pulsatile. Also the release profile can be easily manipulated by controlling the formulation variables

• More than one antigen can be encapsulated, facilitating the design of a formulation that can immunize an individual against more than one infection

• It not only provides controlled release of antigen for long periods of time but also induces long term antibody responses as they also act as adjuvants for entrapped antigen

• It provides a possibility for administering vaccines through non invasive routes (oral and nasal mucosa)

Advantages of Microparticles for Vaccine Development are as follows:

• Biodegradable and biocompatible polymers are safe and acceptable for administration to man

• Controlled – release might enable the development of single dose vaccines

• Adjuvants might be entrapped in the microparticles

• Possibility of being a multiple antigen delivery system

• Administration via various routes, oral administration is also possible

• Antigens are protected from degradation in the intestine

• Antigens are targeted to lymphoid tissues

• Microparticles induce serum and secretory antibodies

• Enhanced stability for entrapped antigens

• Freeze- dried formulations can be obtained

• Being spherical, provide better and enhanced area for antigen-linkage and presentation to the immune system through inducing cell mediated immunity

• Antigen is protected from proteolysis by encapsulating them

The use of microparticles or aggregates in diagnostic circulating studies is based on the assumption that these particles are uniformly mixed with the blood before they reach the organ of interest and that they induce no alteration in blood flow. Hence it is desirable to form uniform sized particles with predictable physiochemical
characteristics. Particle size is one of the main factors affecting immunogenicity. Smaller microparticles (< 10μm) are more immunogenic compared to larger microparticles (> 10μm).

**Microsphere Preparation**
The biodegradable polymeric systems may be further categorized into two systems:

(i) Hydrophilic polymeric systems include gelatin based nano and microparticles and

(ii) Hydrophobic polymeric systems are represented by PLGA-microspheres. Microspheres can be prepared by various techniques including emulsion formation and internal phase spray drying method and solvent evaporation method.

The system required for preparation of microspheres consists of two immiscible phases, i.e., organic phase (methylene chloride, chloroform, hexane etc.) and aqueous phase (double distilled water). Out of these two, the phase which is present in bulk is known as the dispersion phase while the phase which is in smaller amounts is referred as dispersed phase.

Generally, the organic phase is taken in bulk forming the dispersion phase. When the two phases are mixed together an emulsion (w/o) is formed. The primary emulsion formation is the first step in the preparation of microspheres. Various stirring and homogenization methods are used to form the fine dispersion to which the stabilization agents are added for stronger cross-linking and to obtain hardened microparticles.

**Poly (Lactide-co-glycolide) Microparticles/PLG-Microspheres**
The poly (lactide-co-glycolides) are biodegradable and biocompatible polyesters, and are primary candidates for the development of microspheres as vaccines. Due to their
biodegradable nature and safety record, they have been used in man for many years as suture material and as controlled release delivery system for peptide drugs (Wise et al., 1979). However, the adjuvant effect has been achieved by encapsulation of antigen into PLG microparticles (O’Hagan et al., 1991a&b; Eldridge et al., 1991). They have been extensively evaluated for the development of controlled release single dose vaccines.

The limitation with this polymer is the limited range of its solubility in organic solvent. The most commonly used solvent for PLG polymers is dichloromethane. This may lead to the denaturation of the antigen. Nevertheless, a significant number of proteins have been successfully entrapped with full maintenance of structural and immunologic integrity (O’Hagan, 1998).

The polyester resin selected for the present study is poly (D-L-lactide)-glycolide (PLG) copolymer. These copolymers are prepared using typical ion, ring opening, and addition polymerization of cyclic dimers of these materials. The cyclic dimers of glycolic acid (glycolide) and cyclic dimer of lactic acid (lactide) are polymerized by ring opening and addition polymerization to obtain an appropriate molecular weight of polymer and copolymer.

The injectable microspheres can be prepared by several techniques using solvent evaporation and organic phase-separation methods. In the solvent evaporation method the antigen/drug is dissolved, dispersed or emulsified into an external aqueous phase to form the droplets (Bodmeier, et al., 1991). The microspheres are formed, after solvent removal and polymer precipitation around a dispersed drug containing phase induced by addition of a non-solvent or a temperature change, leading to the formation of microcapsules (Ruiz, et al., 1989).
**Gelatin Microspheres**
Gelatin is a naturally occurring polymer, which is obtained from the bones of pig. Gelatin is normally classified as hard or soft depending on the bloom strength of the gelatin solution. It has been well studied for its degradable nature and to serve as the microparticle matrix. It is relatively non-immunogenic and could effectively serve as a matrix for vaccination utilizing the microparticles. Many methods have been documented for the formulation of gelatin microspheres like, coagulation phase separation, salting out, chemical cross-linking, heat cross-linking, etc.

Aqueous solution of gelatin containing antigen is emulsified with corn oil under high stirring at 40 – 45°C. The resultant emulsion is then cooled at 4°C. The gelatin is gelled and hardened to form microspheres. These microspheres are collected by high speed centrifugation, resuspended in water and lyophilized as powder for storage.

**Polymethyl methacrylic acid Microspheres**
In a variation the external phase consisted of polymer organic solution of an appropriate viscosity while internal aqueous phase contains the drug. The aqueous proteinaceous phase is subsequently cross-linked using a cross linking agent in toluene. The cross-linking yields surface reactive hydrophilic protein microspheres.

**HEPATITIS B**
The hepatitis B virus (HBV) is a major cause of infectious liver disease throughout the world. Hepatitis B Virus was first reported in 1885 in Germany. An estimated 3000,000 Americans are infected with HBV each year. At least one of every 1,000 United States citizens are chronically infected with the virus and most are unaware of how or when they caught infection. Five to ten percent of the adult population of the
United States has antibody to hepatitis B virus, indicative of a prior unrecognized infection, and this is probably an unrecognized infection.

The prevalence of antibody to hepatitis B virus infection in the United States increases with increasing age and is inversely related to the socioeconomic status of the population studied: as much as 40% of certain populations of low socioeconomic status has been found to have antibody. Other high-risk populations have also been identified. These include patients and staff of hemodialysis centers, residents and staff of institutions for the mentally retarded, individuals in the health care professionals including physicians (specially surgeons), dentists, nurses, clinical laboratories and pathology laboratory personnel and the sexually active (especially male homosexuals).

In many parts of the world the type B hepatitis problem is of higher orders of magnitude. Prevalence of chronic infection with hepatitis B virus is as high as 2-10% recorded in Asia, Africa, Southern Europe, South America and Oceania. Physicians are now able to accurately diagnose acute and chronic infection, through understanding of viral quantitation and its impact on disease course and treatment response is evolving. Hepatitis B must now be considered as a preventable disease, since acute infection can be effectively prevented by either passive or active immunization. However, when acute infection does occur, it evolves into chronic hepatitis or a chronic infection carrier state in a variable proportion of causes, depending on the age, underlying immune competence of the patient. Chronic infection can also be treated, though the effectiveness of available antiviral and immunomodulatory agents is less than complete.
The Hepatitis B virus was discovered through the identification of its surface protein in the late 1960s. Over the subsequent 30 years, research has led to a clear description of the epidemiology and natural history of infection and a basic understanding of the pathogenesis of liver injury. In addition, physicians can now accurately diagnose acute and chronic infection, prevent infection by passive and active immunization and treat active infection with antiviral and immunomodulatory agents.

Hepatitis B virus is a double stranded DNA virus of the family Hepadnaviridae, which refers to hepatitis DNA viruses and includes a number of similar animal viruses. The viral particle is about 42nm in size and is comprised of an inner viral core containing the HBV nucleic acid (HBV-DNA) and an outer lipoprotein coat referred to as Hepatitis B surface antigen (HBsAg). An outer coat approximately 7nm thick is composed of protein, lipid and carbohydrate. Approximately seven polypeptides have been isolated from the HBV coat: at least two of them are glycoproteins. A number of antigenic specificities have been identified in association with the HBV coat and its peptides, these are collectively known as hepatitis B surface antigen (HBsAg), and consist of a group-reactive antigen within the HBV coat which is a 28nm nucleocapsid that contains a DNA-dependent DNA polymerase and a double stranded circular DNA molecule. The circular DNA molecules contain single stranded regions of various lengths. Using the open strand as a primer, the DNA polymerase appears to fill the single-stranded gaps, resulting in a double-stranded molecule with a molecular weight of about $2.1 \times 10^6$ daltons. On the surface of the nucleocapsid that has distinct antigenic specificity (HBcAg) polymerase probably is associated with an additional antigenic specificity, it
has not been possible to date to extract it from the nucleocapsid without destroying its activity.

Hepatitis Be antigen (HBeAg) also identified in Hepatitis virus, which is associated with soluble protein and does not appear to be physically associated with HB virions. HBeAg itself is a group of at least two distinct antigenic entities that parallel each other in appearance, disappearance and stimulation of an antibody response.

### Nomenclature and source of Hepatitis B virus antigens

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB surface antigens (HBsAg) also called</td>
<td>Outer shell of Dane particle : 22 nm spherical forms and filamentous forms up to 200 nm in length.</td>
</tr>
<tr>
<td>Australian antigen (AuAg) and hepatitis associated antigen (HAA)</td>
<td>HBsAg particle group specific antigen.</td>
</tr>
<tr>
<td>a d/y : w/r</td>
<td>Subtype determinants</td>
</tr>
<tr>
<td>HB core antigen (HBCAg)</td>
<td>Dane particle core with or without DNA polymerase activity.</td>
</tr>
<tr>
<td>HBe antigen (HBeAg)</td>
<td>Possibly part of Dane particle core.</td>
</tr>
<tr>
<td>Delta (δ) antigen</td>
<td>Hepatocytic nuclei of HB patients. It is not found in serum and possesses helper activity for HBV.</td>
</tr>
</tbody>
</table>

The largest morphologic form, the Dane particle, is most likely candidate for the complete infectious HBV. Dane particle cores or hepatitis B core antigen (HBcAg) has been found in the nuclei of infected hepatocytes. These cores contain circular DNA and DNA polymerase activity. HBcAg is only found as an integral part of the Dane particle. It has not been found in the free form in serum. Two populations
of Dane particles are detected after cesium chloride gradient centrifugation, the highly buoyant dense defective particles with cores apparently lacking DNA polymerase.

**Immune Response to Hepatitis B Infection**

Evidence has shown that both humoral and cell-mediated immune responses are induced after hepatitis B infection. The antibody response to the various components of HBV antigens varies a great deal, depending on the course and outcome of the infection. The HBsAg is acquired during the incubation period and two to eight weeks before the onset of clinical symptoms. Greater than 90% of patients develop serum antibody. The first antibody detectable in the serum is directed against HBeAg (anti-HBc), which persists in small amounts for a few years. Antibody to HBsAg (anti-HBs), which is next in order of appearance, persists in the serum presumably for life. Antibody to HBeAg (anti-HBc) appears somewhat later and may persist for years. Under an observation of the sequence and nature of antibody response in patients who are HBsAg carriers with or without chronic disease, there is a striking lack of anti-HBs and often of anti-HBc antibodies and the characteristically high and sustained presence of anti-HBc activity. Some evidence suggest that in the HBsAg carrier state, susceptibility to acquire Chronic Active Hepatitis (CAH) is related to the presence of HBeAg and lack of anti-HBc antibodies in the serum. There are also some suggestions that HBeAg, which may be intimately related to take antigen as a marker of infectivity, as evidenced by the fact that neonatal transmission of HBV from HBsAg carrier mothers correlates well with the presence of HBeAg in the maternal sera.

Cell-mediated immune response to HBV has been documented a number of times. Three *in vitro* correlates of HBV specific lymphocytic sensitization have been
employed to detect cellular response to HBV. They include the transformation of lymphocytes by HBsAg, inhibition of leukocyte migration and production of lymphocytes inhibition factor by lymphocytes exposed to HBsAg. A distinct response is seen in lymphocytes from patients with acute and recovered hepatitis B and in patients with chronic active hepatitis. It has been suggested that the continued presence of HBsAg may be partly related to viral specific T cell hyporesponsiveness, and persistent T cell reactivity to HBV in patients with chronic active hepatitis may represent an ineffective attempt by the host to eliminate the virus.

**Epidemiology of HBV Infection**

Hepatitis B has been reported throughout the world and in virtually every geographical, cultural, and socioeconomic settings. The incidence of HBV infection does not appear to be related to race nor is there a seasonal distribution. In general, the incidence of HBV and the HBsAg carrier state is reportedly higher in males and in the lower socioeconomic groups. A true age distribution for HBV infection has not yet been determined despite the fact that it is usually considered to be an infection of adult. However the incidence of HBsAg carrier states appears to be higher in infections acquired in the neonatal period and early infancy.

People involved in health care occupations, particularly those with direct patient care (hemodialysis technicians, dentists, etc.) appear to have an increased incidence among intrafamilial contacts, drug users, and in institutions for physically or mentally handicapped people.

The parenteral route is the primary mode of transmission of HBV, with contaminated blood and blood products being the most prominent source. In addition to medicinal procedure, parenteral transmission has been associated with tattoo
needles and injection equipment shared among illicit drug users. It is now generally accepted that HBV infection may be spread by other routes after prolonged and close contact as well as familiar parenteral route. There is a significantly increased occurrence among male homosexual compared to the general population. HBsAg has been detected in saliva, menstrual fluid, semen, vaginal fluid, and feces, which support the possibility of other modes of transmission. Vertical transmission of type B hepatitis can occur from mother to offspring either during pregnancy or at the time of birth. The importance of genetic factors in the development of the carrier state and evolution of the clinical response are subjects for continued study.

Although HBV infection has been transmitted to primates in the laboratory, it has not been clearly shown to occur in natural settings. Humans still remain the major reservoir of HBV infection in nature.

RESEARCH ENVISAGED AND PLAN OF WORK

The turn of century is witnessing an explosion in the field of biotechnology with emergence of new bio-products like, therapeutic enzymes, proteins and vaccines. Increased understanding of the molecular nature of immune responses and advances in the technologies of gene sequencing and molecular biology have resulted in new approaches to vaccine development. And yet, in many ways vaccine development against many diseases is in its juvenile stage. As most of the vaccines currently in use are of proteinaceous origin, their oral administration is ineffective due to degradation by GIT pH/enzymes, therefore parenteral route is preferred. However, present vaccines are inefficient in providing sufficient immunological response from single administration and also they require relatively higher doses of antigen for developing sufficient immunological response. Novel antigen delivery systems provide means of
controlled delivery of antigen with less amount of antigen giving a cheaper and more efficient mode of immunization. In addition, controlled delivery novel carrier systems also act as adjuvants that augment the immune response to antigens.

Hepatitis B virus (HBV) causes significant health problems worldwide and chronic carriers of this virus often die from active hepatitis and liver cirrhosis. HBV is involved in chronic hepatocellular carcinoma and is one of the few cancers known to be caused by a virus. Any attempts to reduce the incidence of Hepatitis B infection on a global scale require the availability of large quantities of a suitable and affordable vaccine. Two approaches to produce hepatitis B vaccine (HBV) on a commercial scale have proven successful, the purification of hepatitis B surface antigen (HBsAg) and the production of HBsAg using recombinant technology. The application of recombinant DNA technique to produce a HBV with expression of HBsAg in yeast offers a promising alternative to the previously used plasma-derived vaccines in terms of cost, supply and safety. Despite the availability of an effective vaccine, the high cost of the vaccine, the geographical location of the population at risk and the multiple injection schedule of the currently available vaccines have resulted in under utilization of the recombinant vaccines. Therefore, it has been envisaged to develop an efficient means of presenting the recombinant technology derived hepatitis surface antigen (HBsAg), which enhances the effectiveness, reduced the amount of antigen and frequency of application vis-à-vis increases the stability of vaccine.

Vesicular systems (liposomes/niosomes) in general are capable of incorporating both water as well as lipid soluble compounds. In the present study phospholipid vesicles (liposomes) and non-ionic surfactant vesicles (niosomes) were prepared. Particulate systems especially PLG microspheres and gelatin microspheres
were selected in order to develop systems with reduced frequency of administration for achieving a particular immune response. These systems (vesicular/particulate) effectively stimulate immune response at cellular and humoral level. The effect of entrapped antigen in delivery system was compared with respect to plain counterpart.

The proposed work was carried out on the following lines

1. Selection of antigen and its estimation
2. Preparation of vesicular systems (liposomes and niosomes) loaded with HBsAg
3. Optimization of process parameters
   (1) Various phospholipids and non-ionic surfactants used
   (2) Lipid/non-ionic surfactant:cholesterol ratio
   (3) Time and temperature of hydration
4. \textit{In vitro} characterization of vesicular systems (liposomes/niosomes)
   (1) Vesicles shape and size
   (2) Entrapment efficiency
   (3) \textit{In vitro} release profile
5. Preparation of particulate systems (PLG and gelatin microspheres)
6. Characterization of particulate systems
   (1) Size and shape
   (2) Encapsulation efficiency
   (3) Surface associated protein
   (4) \textit{In vitro} release profile
7. Storage stability studies at 4°C and 37±1°C
8. \textit{In vivo} immunological response
   (1) Measurement of serum IgG levels
   (2) Measurement of IL-2, IL-4, IL-5 & IL-6 levels
   (3) Determination of antibody secreting cells
9. Compilation and presentation of data