NEW AGE ADJUVANTS AND DELIVERY SYSTEMS FOR SUBUNIT VACCINES

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ABSTRACT

The dramatic advancements in the field of vaccinology has led to the formulation of chemically well defined vaccines composed of synthetic peptides and recombinant proteins derived from the immunologically dominant regions of the pathogens. Though these subunit vaccines are safer compared to the traditional vaccines they are known to be poorly immunogenic. This necessitates the use of adjuvants to enhance the immunogenicity of these vaccine formulations. The most common adjuvant for human use is alum. Research in the past has focused on the development of systemic immunity using conventional immunization protocols. In the present era, the emphasis is on the development and formulation of alternative adjuvants and delivery systems in generating systemic as well as mucosal immunity. This review mainly focuses on a variety of adjuvants (particulate as well as non-particulate) used with protective antigens of HIV, malaria, plague, leprosy using modified delivery vehicles. The experience of our laboratory and other researchers in this field clearly proves that these new age adjuvants and delivery systems undoubtedly generate enhanced immune response - both humoral and cell mediated. The choice of antigens, the nature of adjuvant used and the mode of delivery employed have a profound effect on the type of immune response generated. Besides the quantity, the quality of the antibodies generated also play a vital role in protection against these diseases. Some of the adjuvants and delivery systems used promoted high titre and affinity antibodies, which were shown to be cytophilic in nature, an important criteria in providing protection to the host. Thus the studies on these adjuvants/ delivery systems with respect to various infectious diseases indicate their active role in efficient modulation of immune response along with safety and permissiblity.

KEYWORDS: vaccine, subunit, adjuvants, delivery systems

INTRODUCTION

Traditional vaccines were based on whole killed or attenuated microorganisms. There are a number of disadvantages associated with these whole cell vaccines such as reversion to pathogenesis, risk of contamination from the infectious material, storage problem, batch to batch variation etc. This led researchers to use defined antigens or tailor-made sequences using peptide chemistry or recombinant technology. Though,

subunit vaccines have shown number of advantages over conventional vaccines they suffer from poor immunogenicity, genetic variability of the immune response in heterogeneous population (MHC restriction) and induction of only partial protective immunity. These drawbacks necessitate the use of adjuvants or delivery vehicles that would target the antigen to the proper immune effector cells thereby activating the appropriate limb of the immune response. Therefore, for the formulation of a highly effective subunit vaccine, the inclusion of strong immunoadjuvants and/or a proper delivery vehicle has become essential to elicit optimal immune response in the host.

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The term adjuvant, first used by Ramon *et al.*, (1) is derived from the Latin word *adjuvare*, which means to *help* or *to aid*. An immunological adjuvant can be described as any substance which when incorporated into a vaccine formulation acts to prolong or enhance the quality of immune response to the vaccine antigen. Despite considerable research and progress made over the years, the only adjuvant approved for human use is aluminium compounds. Alum although being safe, is a relatively weak adjuvant for antibody production and a poor inducer of cell mediated immune responses (CMI), also sometimes it elicits undesirable side-effects (2). Therefore there is an urgent need for the development of potent and safe adjuvant(s) and delivery systems which can be used with new generation vaccines. The characteristics of an ideal vaccine adjuvant are listed in Table 1.

Table 1. The characteristics of an ideal vaccine adjuvant

Simple well defined chemical structure.

Non-antigenic and not immunologically cross-reactive with tissue antigens.

Induces a minimum of nonspecific effects on the immune system

Should not be toxic, carcinogenic, teratogenic or abortogenic.

Effective after a single dose, biodegradable and biocompatible. Induces both humoral and cell-mediated immunity

Easy to mix with antigen or combination of antigens. Acceptable for administration to humans

Safe to administer to young and immuno-compromised individuals.

Capable of being administered orally.

Effective for peptide, protein, polysaccharides and DNA vaccines.

Can be manufactured reproducibly on a large scale.

Long shelf-life, preferably without refrigeration.

Induces systemic and mucosal immunity.

MECHANISMS OF ADJUVANT ACTION

Adjuvants/delivery systems modulates the immunogenicity of antigen(s) either by simply prolonging the half-life in the recipient or through activation of combination of effector mechanisms as listed below:

Depot effect: The delivery systems act as depots by slowly releasing the antigen and so prolonging the time of exposure to the immune system. Gel-like adjuvants such as alum and CFA associate with the antigen and effectively increase the immunological half-lives of the formulations.

Enhanced antigen presentation: Adjuvants have the capability to preserve the conformational integrity of antigen and to present it to the appropriate immune effector cells. The former interacts directly or indirectly with antigen presenting cells such as macrophages and dendritic cells and enhances the immune recognition of the antigens (eg. particulate liposomes).

Immunomodulation: Macrophages can be stimulated by adjuvants to secrete immunomodulatory cytokines, which act on lymphocytes to promote either Th1 or Th2 like responses. These cytokines have been demonstrated to have adjuvant activity, for e.g. IL-2, IFN-γ and IL-12.

Induction of CD8+ cytotoxic T-lymphocytes (CTL): Induction of CTL's plays a vital role in the control of intracellular pathogens. Adjuvants such as saponin, ISCOMs and liposomes have shown to induce MHC class I restricted CD8+ CTL responses. This action is through the transfer of antigen into the cytosol where they can associate with the MHC class I molecules.

Targeting: Adjuvants enhance the production of opsonins which promote the efficient uptake by APC's. Sometimes antigens can be linked to small molecules that recognize cell surface receptors so as to achieve efficient targeting.

The currently available adjuvants and delivery vehicles can be divided into three major classes, namely particulate, non-particulate and others.

Particulate: These include aluminum salts, surface active agents, slow releasing vehicles like FCA, liposomes and microspheres.

Non-particulate: These include non-ionic block copolymers, glycopeptides and lipopeptides and peptides of microbial origin.

Other adjuvants: These include proteosomes, cytokines, Trat, polytuftsin, mucosal adjuvants and many more. As the topic is vast and beyond the scope of this review, the authors have restricted to few adjuvants which are of clinical relevance. This paper deals with adjuvants/ new delivery vehicles being considered and tested for systemic as well as mucosal vaccines. The first part focuses on immunostimulation of systemic immune response while the second part emphasizes on mucosal immunity.

PARTICULATE ADJUVANTS

Freund's Complete Adjuvant (FCA)

The most well documented adjuvants are Freund's complete (FCA) and incomplete adjuvants (IFA). The original FCA was formulated with heat killed *Mycobacterium tuberculosis* incorporated in a mineral oil phase and the antigen in an aqueous phase to form an emulsion.

When surface proteins from different stages of the life cycle of malarial parasites were used individually or in conjugation with either FCA or muramyl tripeptide as adjuvant, it was observed that the formulations were successful in inhibiting the parasite growth at different stages of the life cycle (3). In one of our studies mice were immunized with RESA peptide alone or coupled with promiscuous T cell sequence derived from CS protein of *P. falciparum* emulsified in 1:1 FCA/IFA. Mice with different genetic background immunized with the second formulation showed high antibody levels and secondary immune response as compared to RESA peptides alone (4). In another study, recombinant V antigen of *Y. pestis* in IFA when inoculated intraperitoneally into mice was shown to induce protective immune response

against a subcutaneous challenge with virulent *Y. pestis*. Protection correlated well with the induction of high titer serum antibodies (5).

However, there are several side effects associated with FCA, which include abscess formation, severe pain, fever, possibility of organ damage or induction of autoimmune disease. Though FCA adjuvanted vaccines were found to be highly effective in enhancing immunity, their use has been more or less discontinued in humans in view of the associated deleterious effects. Now, FCA is mainly used as a reference standard in laboratory animals for testing new adjuvants.

Aluminium salts

Aluminum compounds are widely used as they are the only adjuvants licensed for human use. They can be used in the hydroxide or phosphate form and can be added to the antigen as such or *in situ* precipitated in the presence of antigen. They act through slow release of antigen adsorbed at their surface due to depot formation at the injection site. Aluminium compounds activate complements that may lead to local inflammatory responses, thus facilitating role in development of B cell memory (6).

It is interesting to note that alum could be used effectively with peptide vaccines. An alum adjuvanted synthetic HIV-1 V3 branched peptide was found to be not only safe and well tolerated by healthy adult volunteers but also induced high levels of neutralizing antibodies against HIV-1 MN strain in 90% of immunized individuals (7).

The safety and immunogenicity of yeast-derived, blood-stage malaria vaccines was evaluated in phase I trial. Healthy adults were given two or three doses of alum-adsorbed vaccine containing the 19 Kda carboxy-terminal fragment of the merozoite surface protein-1 (MSP-1(19)) of *Plasmodium falciparum* fused to tetanus toxoid T-helper sequences. The first two doses of MSP-1(19) were well tolerated with minor hypersensitive reactions. Both MSP-1(19) vaccines were immunogenic in humans, but changes in formulation

will be necessary to improve further safety and immunogenicity properties (8). In another study when the purified recombinant V antigen from *Y. pestis*, expressed in *E. coli* was adsorbed onto aluminium hydroxide, the anti-V antibodies protected the mice from lethal bubonic and pneumonic plague caused by CO92 strain (9). Recently, co-administration of F1 and V antigens adsorbed to alhydrogel given intramuscularly in mice caused an increase in specific IgG1 titre which was maintained till 8 months post-boost and they were also fully protected against a challenge with virulent *Y. pestis* (10).

Though alum formulations showed immunopotentiation with a number of antigens of different pathogens, they still suffer few deficiencies a) they favor immune response towards Th2 type (11), b) inability to induce cell mediated immune response is a major limitation, c) does not show immunostimulation with some of the antigens, d) granuloma formation at the site of injection. These inherent deficiencies of alum have led to the search for better and more effective adjuvants for use in human vaccine formulations.

Liposomes

Liposomes are artificially prepared lipid spheres composed of phospholipids and other lipids in a bilayer configuration. Allison and Gregoriadis first described the use of liposomes as immunoadjuvants in 1974 (12). Since then many studies have confirmed their capacity to enhance the immune response to various antigens (6,13). Parenterally injected liposomes are rapidly ingested by macrophages particularly in the liver and spleen, where they are gradually degraded in lysosomal vacuoles (14). The uptake of the liposomes by the cells is partly by receptor or non-receptor mediated endocytosis, Fc-mediated endocytosis or phagocytosis, or complement dependent phagocytosis (15). Liposomes have a number of advantages a) they are biodegradable, non-toxic and immunologically inert, b) serve as carriers and depots of antigens enhancing the uptake and presentation of antigens by APC's, leading to efficient stimulation of the T cells (16), c) long

lasting, high titre as well as high affinity functional antibodies can be achieved for antigens administered through various routes (17,18), d) cell mediated immunity can be induced (19), e) other adjuvants such as MPL, LPS and MDP analogs can be co-entrapped along with the antigen to further enhance the quality of the immune response (20), f) toxicity of antigens and adjuvants can be reduced or eliminated by entrapment in liposomes (21), g) liposomes can also be used to enhance the production of cytophilic antibodies viz. IgG2a/IgG2b type (22), h) there are convincing reports to indicate that encapsulation inside the liposome in no way disturbs the native conformation (23).

In another important study, a vaccine containing synthetic repeat epitope of *P. falciparum* CS protein and MPLA (monophosphoryl lipid A) in liposomes and in alum caused a strong specific humoral immunity. This formulation overcame the immunosuppression observed with the antigen alone. Thus much higher and specific antibody response CS Protein was observed using inbuilt adjuvant containing the liposome and this approach proved to be a successful adjuvant strategy in humans (18). To expose the hidden sequences, a recombinant CS protein containing the regions corresponding to the non-repeat sequences of *P. falciparum* (RLF) was tested as a vaccine candidate. The antigen (RLF) alone did not induce any CTL response, but when encapsulated into liposomes produced a good cytolytic response *in vivo*. So the liposomes like the lipid component of lipoprotein facilitate the channeling of antigen to associate with MHC-I (24).

The F1 antigen, one of the protective anigens of *Y. pestis*, when entrapped in liposomes and immunized in mice of different haplotypes induced very high antibody titres as compared to F1 antigen adsorbed onto alum (29). This approach provided an opportunity to delineate the major and minor antigenic determinants on F1 antigen (25).

Asiaticoside, a plant glycoside having microbicidal properties was tested against *M. leprae* and *Mycobacterium tubercuolosis* both *in vivo* and *in vitro*. The results showed that liposomal

asiaticoside had better microbicidal property against *M. leprae* and *M. tuberculosis* when compared to free asiaticoside. Thus it is inferred that appropriate glycosides, if used in liposomal formulation can enhance drug efficacy, reduce toxicity and such glycoside bearing liposomes could be used for chemo-therapeutics for control of several other diseases (26). The soluble antigen(s) of *M. leprae* was coupled to liposomes and was used for skin testing of leprosy patients, to mimic that this mode of antigen presentation would be identical to that of integral lepromin. The liposomized antigen(s) elicited both early (24-48 hr) and late (3-4 weeks) delayed-type hypersensitivity reactions, true to the nature of lepromin which was unlike the soluble antigen(s) alone which elicit(s) only the early reaction (27).

Thus it can be inferred from the above studies that liposome formulated vaccines have the potential to stimulate both antibody as well as T cell responses simultaneously by gaining entry to both the conventional MHC class I and Class II pathways and could prove to be extremely useful in formulation of an effective sub-unit vaccine.

Novasomes

Phospholipid liposomes have certain limitations like they are readily broken down *in vivo* by host phospholipases and are unstable on storage, so limiting their use in slow delivery vaccines. For these reasons there has been an increasing interest in development of non-phospholipid liposomes composed of "membrane mimetic" amphiphiles which are molecules with a hydrophilic head group attached to a hydrophobic tail. One such non-phospholipid liposome (Novasome® vesicles) has been used commercially as a safe and potent immunological adjuvant for two approved poultry vaccines. When DT and TT were encapsulated in these non-phospholipid liposomes, an immune response similar to Freund's and aluminium phosphate adjuvanted formulations was observed. Also addition of squalene considerably enhanced the immune response. Another important observation was the enhanced levels of IgG2a/ 2b antibodies

suggestive of Th1 type cell stimulation (28) which induce CMI in addition to antibody production and so are important for controlling viral and intracellular infections.

So, non-phospholipid liposomes that can be easily scaled up may be a permissive alternative to aluminum adjuvants.

IRIV (Immunopotentiating reconstituted influenza virosomes)

These are fusogenic liposomes created by inserting virus fusion proteins (with the antigen of interest) into the liposomes. Better results are obtained by first priming the recipient with influenza virus vaccine alone.

In a recent study, SPf66 (synthetic malaria vaccine)-phosphatidylethanolamine was incorporated into IRIV and Balb/c mice were immunized twice by intramuscular injection with peptide-loaded virosomes. The reported antibody titres were significantly higher and the required dose of antigen was lower, when mice had been pre-immunized with a commercial whole virus influenza vaccine. These results indicate that IRIV is a suitable delivery system for synthetic peptide vaccines and thus have great potential for the design of molecularly defined combined vaccines targeted against multiple antigens and developmental stages of the same parasite, as well as against multiple pathogens (29).

Microencapsulated liposomes (MELS)

Though the conventional liposomes suffer the drawback of storage as well as instability, this has been overcome by the use of microencapsulated liposomes which was designed to bypass rapid destruction *in vivo* (30). Numerous animal studies have been carried out using a large variety of antigens such as TT (tetanus toxoid) (31), SEB (staphylococcal enterotoxin B) (32) and gp120 native antigen (33). Inspite of the advantages over conventional liposomes, it still suffers from the acidic environment that is created locally within the spheres upon storage and may be deleterious to

the antigen and thereby making it a poor immunogen. Their ability to be lyophilized is a major advantage as well as challenge.

ISCOMs

ISCOMs are non-covalently bound complexes of Quill-A (saponin extracted from *Quillaja saponaria Molina*), cholesterol and amphipathic antigen (34). Immune stimulating complexes (ISCOMs) constitute a more recent and highly interesting approach in stimulating humoral and cell mediated immune response particularly CD8+CTL response (MHC I) to amphipathic antigens (35). ISCOMs increases the efficiency of uptake and internalization of antigen by professional antigen presenting cells (mainly constituted by macrophages and monocytes) as compared to non-phagocytic cells such as splenic dendritic cells and naïve B cells. The high affinity antibody generation can be explained by the selective expansion of B-cell clones which bear high affinity receptors for the antigen.

Immune response to HIV-gp 120 when delivered in ISCOMs showed the induction of ten folds higher antibody titers than gp 120 emulsified in depot adjuvant in rhesus monkeys with generation of potent virus precipitating and neutralizing response to RF and MN isolates (36). Moreover the antisera from ISCOM immunized rhesus monkeys recognized gp 120 in the membranes of HIV-1 infected H9 cells, indicating the preservation of epitope structure in the ISCOM matrix. To understand the best delivery mode, when a model peptide from crown sequence of V3 loop of HIV-gp 120 was delivered in ISCOMs or liposomes and its immunogenicity studied in mice of different haplotypes keeping alum as gold standard, non-MHC restricted immune response was observed. Major IgG subtype elicited were IgG2a/2b suggestive of Th1 response (37).

The adjuvant properties of ISCOMs has also been studied with a variety of viral capsid proteins (38) and repeat sequence of *P. falciparum* Pf155/RESA antigen (39). They modulate the expression of MHC class II molecules by upregulating their

expression and also are able to stimulate CD8+MHC class I restricted cytotoxic T cells against non-infectious antigens. It was also reported that entrapment of otherwise poorly immunogenic synthetic peptides of RESA in ISCOMs resulted in increased immunogenicity as well as generation of strong anamnestic response without significant MHC restriction in different inbred strains of mice. Also interestingly there was a shift from noncytophilic antibodies to cytophilic antibodies (40).

Polymer Microspheres

Controlled release antigen delivery systems offer a promising addition to existing delivery systems and is a priority with WHO because of their potential in reducing number of injections as well as avoiding the cold chain. These delivery systems are designated to release the antigen within their matrix at regular intervals into the tissue. Various polymers have been used for the preparation of such microspheres viz ethylene vinyl acetate (41), Lactic-co -glycolic (42), poly amino acid or proteinoid (43) both for mucosal and parenteral use.

NON-PARTICULATE ADJUVANTS

Glycoproteins and peptides of microbial origin

Attempts at characterizing the molecular entities responsible for the immunopotentiating activities of the myocobacterial cells in Freunds adjuvant reached a turning point when water soluble adjuvant active fraction could be isolated from the *mycobacterium*. Ellouz *et al.*, (44) showed that the smallest sub-unit of the mycobacterial cell wall that retains immunoadjuvant activity is N-acetyl muramyl-L-alanyl-isoglutamine, called muramyl dipeptide (MDP).

MDP possess a broad spectrum of pharmacological and immunological activities including the abilities to stimulate macrophages, polymorphonuclear leukocytes, mast cells, endothelial cells and fibroblasts (45) while increasing phagocytosis (46) and chemotaxis (47). They help in the secretion of variety of lymphokines

including IL-1, B-cell growth factor, CSF, fibroblast activation factor and also increase the production of superoxides, prostaglandin and collagenase (48) and increases mitogen induced T cell proliferation (49). Finally MDPs enhance carrier specific helper T cell function (50).

But many of the immunological activities result in unacceptable side effects. MDP is considered toxic, especially pyrogenic for human use (6). A number of derivatives of MDP are being developed that retain its adjuvanticity but are devoid of its toxicity.

The most promising derivatives include murametide, threonyl-MDP and murabutide (51,52). Murabutide (MDP-n-butyl ester) was found to be as active as the parent compound as an adjuvant for inducing antibody production and enhancement of non specific resistance to infections (53). It also did not induce any toxic effects of MDP such as fever, leukocytosis, adjuvant arthritis and acute phase reactions (45). Some of the new synthetic MDP derivatives (β-butyl-MDP, MTPO-26β-cholesterol-MD) and one saponin (Taurosid 1) have been shown to induce strong humoral immune response to HIV-1 envelope glycoproteins rgp 160 and rgp 120. (54). In one of our studies on the env and gag peptides of HIV, high antibody titres were obtained with peptides entrapped in liposomes alongwith MDP analog (MA729). It was also observed that these antisera had greater than 90% inhibition of syncytia formation indicating the protective and neutralizing nature of these antibodies (Lokesh *et al.*, manuscript communicated). The same peptide antigens with identical delivery systems and with the same adjuvant induced high CMI response and the major cytokines generated were IFN-γ and IL-2 suggestive of being beneficial immunity to the host (Lokesh *et al.*, manuscript communicated).

In continuation of our effort to develop a peptide based immunogen, RESA peptides conjugated to CS.T3 were administered in mice of different haplotypes alongwith non-toxic adjuvants like norMDP/ LTP. It was observed that the adjuvanted formulations generated high titre and affinity antibodies and also high proportion of cytophilic IgG 2b isotype. Also, the peptide anti-sera showed 76-96% protection *in vitro* (55).

Lipopeptides

Lipopeptides are non-toxic, non-immunogenic analogs of bacterial cell walls and do not induce tissue damage when injected. Lipopeptides are able to act as potent *in vitro* immunoadjuvants in mice, rabbits and other species when administered in combination with antigens (56). Synthetic lipopeptide vaccines have been shown to induce both humoral and cell mediated immune response (57).

Lauroyl tetrapeptide

Chemical coupling between lauric acid and a biologically inactive tetrapeptide, L-Ala-D-glu [LL,A2pm(gly)]NH2 (which has been isolated from immunostimulating crude extracts of a *streptomyces* strain) yielded a compound (Lauroyl tetrapeptide, LTP) with marked *in vivo* and *in vitro* immunopotentiating activities. LTP stimulates *in vitro* the phagocytic and bactericidal activities of mouse macrophages and of human blood monocytes and in mouse enhance the clearing activity of reticuloendothelial system (58).

Non-ionic block polymer surfactants

The NBP copolymer adjuvants are simple linear chains or polymers of polyoxypropylene, which is hydrophobic, flanked by two chains of polyoxyethylene, which is hydrophilic. Larger hydrophobic copolymers are most effective adjuvants in inducing a higher proportion of IgG2a/2b isotypes in mice. Mechanism of action of copolymers adjuvants: They function as adhesion molecules that binds antigens and host components to hydrophobic surfaces by combination of hydrogen bonding and hydrophobic interaction. The copolymers are resistant to degradation and are excreted from the body largely, intact without causing systemic organ toxicity (59). All the copolymers with adjuvant activity augment the expression of class II MHC by macrophages *in vivo*.

When administered in oil-water emulsions, they elicit humoral responses against viral, parasite or bacterial peptides, proteins and polysaccharides (59). The repeat region, (NAGG) of *P. cynamolgi* was used as a vaccine candidate in monkey model

The different copolymers tested were L121, L141 and L180.5. Of these formulations, only LPS along with L121 showed good antibody response. L121/L141 induced IgG2a whereas presence of LPS stimulated increased IgG2b subclass levels. Also changing just the adjuvant alters the specificity of antibody to antigen. This may be due to acquisition of a specific configuration by the vaccine molecule depending on size of copolymer used resulting in specific B cell epitope recognition (60). Though such polymers are being focused upon as new age vaccine adjuvants, their acceptability for human use needs further study.

OTHER ADJUVANTS

Cytokines

Cytokines are glycoproteins generated by activated T-cells, macrophages which are proposed as human and veterinary vaccine additives. They have various actions, e.g. IL-1 (T and B cell maturation), IFN-γ (Th1 upregulation, enhanced MHC expression), IL-2 (Th1 upregulation), IL-4 (Th2 upregulation) and GM-CSF (co-migratory signal for dendritic cells). IL-12 has been shown to induce strong Th1 shifts and may have potential as an adjuvant in human vaccines (61). IL-1 is known to have diverse immunological functions besides acting as a first costimulatory molecule during T cell activation. IL-1 possess inflammatory and adjuvant property, so we used a bioactive segment of IL-1 (IL-1β) to modulate the immunogenic function of an asexual blood stage antigen of *P. falciparum* (62) as well as modulating the immunogenicity of HIV peptide antigens (63). It was seen that a nine amino acid fragment of IL-1 could substitute the entire IL-1 molecule in mounting an effective immune response against these candidate antigens.

In case of intracellular pathogens, a strong CMI is needed to eliminate them. To augment such an effect, Th1 type of response, IL-2 and IFN-γ have been advocated to activate the cells of immune system to reduce the parasitemia. IFN-γ differentially regulates IL-12 and IL-10 production (64). IFN-γ is being clinically used to boost the host antimicrobial defense. The beneficial effects are being extrapolated to diseases like cutaneous leishmaniasis, HIV and non HIV related disseminated atypical mycobacterial infections (65).

Polytuftsin

Polytuftsin (TKPR) is a polymer of tuftsin tetrapeptide (thr-lys-pro-arg)[40] derived from the immunoglobulin heavy chain. It is known to have diverse biological and pharmacological functions. In our lab repeat sequences of RESA of *P. falciparum* have been coupled to polytuftsin and administered in alum and FCA in mice of varying haplotypes. The results of the study indicated that such formulations have increased immunogenicity and antigen induced T cell proliferation and the results were comparable to native antigen. Furthermore, these antibodies have shown 80-90% protection *in vitro* (66). The work had been extended subsequently by incorporating the above formulation in liposome containing a promiscuous T cell sequence (CS.T3). After analyzing the previous data with the present work, it clearly showed enhancement of both humoral and CMI response. This approach generated high affinity, high titre and cytophilic antibodies (67). There was enhanced T cell response and the cytokine profile was of CD4+TH1 type (i.e. IL-2 and IFN-γ). The enhanced immune response seen with such weak antigens containing polytuftsin has shown to be due to increased Ia antigen expression or class II molecule on APC thereby increasing processing and presentation to the T cells and secondly, releasing a costimulatory molecule IL-1 that helps in T-cell proliferation (68).

Mice immunized with liposome containing RESA peptide(s)-CS.T3 conjugate along with polytuftsin showed the highest antibody levels in all the strains, whereas the RESA peptide(s) alone, adsorbed on alum or entrapped in liposomes, showed either poor or moderate antibody levels (69).

Chimeric immunogens were constructed by chemical linkage between synthetic peptides of HIV and polytuftsin. These were employed for immunization of mice of different MHC haplotypes. A significantly stronger immune response was observed in mice immunized with peptide polytuftsin conjugates compared to mice receiving peptide dimers. Peptide polytuftsin conjugates induced IgG2a 2b isotype switching in secondary response. In addition there was a positive correlation between amounts of cytokines and shift in the IgG isotypes. Interestingly, polytuftsin proved to be non-immunogenic during these studies. These results suggests that the use of polytuftsin as a carrier may increase the immunogenicity of otherwise poorly immunogenic synthetic peptides and thereby will prove helpful in developing a peptide based vaccines (70).

QS-21

QS-21 is a purified derivative of triterpene glycoside saponin derived from the bark of *Quillaja saponaria* tree (71). QS-21 is a known enhancer of cellular and humoral responses in human's (72) and increases passive protective immunity when included in veterinary vaccines (73). QS-21 has been shown to enhance response to alum adjuvanted vaccine formulations (74). QS-21 has been included as a component of vaccine that induced protective immunity in 6 of 7 volunteers immunized with CS recombinant vaccine (75). It has been reported that immunization with MAP (multiple antigen peptides)/ alum/ QS-21 elicit antibody titres 30-45 folds higher in mice and 10 folds higher in *Aoutus* monkeys as compared to MAP-alum alone. Recently, shifting of immune response from Th2 to Th1 using an adjuvant combination of Monophosphoryl lipid A and QS21 with a recombinant HIV protein has been reported (76).

Montanide ISA720

This adjuvant has been used mostly in emulsion with a number of antigens derived from *P. falciparum* and from HIV. It has shown to enhance both humoral and cellular immunity and its effect has proved to be better than alum. Montanide ISA

720 is (SEPPIC, Paris, France) composed of natural metabolizable oil and highly refined emulsifier from mono-oleate family which forms a stable w/o emulsion. Its use with several recombinant malaria proteins has resulted in high antibody levels in mice, rabbits and sheep (77). Also it was used in a challenge trial with *Saimiri* monkeys which were vaccinated with *P. fragile* malarial antigen AMA-1. Three of the four monkeys generated high titres antibodies and were protected (78). This oil adjuvant was also shown to provide a priming effect as compared to Alhydrogel in a study using the HIV-1 derived multi-epitope polypeptide TAB9, and thus eliminating undesirable reactions characteristic of these compounds (observed only during boosters) while achieving equivalent levels of specific antibodies.

MF59

This adjuvant is an oil/water emulsion which is licensed for human use. The chemical composition of MF 59 is oil squalene (a terpenoid cholesterol precursor) and the surfactant sorbitan trioleate (Span85) and polyoxy ethylene sorbiton mono-oleate (Tween 80). Two principal viral targets, HIV and HSV, were selected. The protection strategy was based on generation of neutralizing antibody against viral coat proteins for both the vaccines. A decade after initiation of the studies which led to development of the MF59 adjuvant, the clear conclusion is that this adjuvant is very safe for intramuscular use in humans and that it is an effective way to generate significant antibody titre against subunit antigens. When this adjuvant was tried in humans using HIV recombinant antigens (env gp120 and p24 gag) it generated high antibody titers with virus neutralizing properties. Its superiority was further enhanced when delivered with microspheres leading to both CTL activity as well as humoral response across the species. Similar results were observed in baboons with PLG/ p24 in MF59 as compared with MF59 alone plus p24(79).

Trat

Trat protein, known popularly as "ISCAR" (Immunostimulatory carrier) is one of a family of

integral memabrane proteins (Imps) of E. coli (Serum resistant) representing powerful carrier molecules which when injected into experimental animals generates substantial antibody and T proliferative responses to molecules conjugated to it (80). It was observed that T cell epitope peptides (T2, T4 and T6 from Trat protein) can be employed as carriers in subunit vaccines to overcome the unresponsiveness observed in animals and humans as a result of MHC restriction. So, they are attractive candidates for the production of subunit vaccines because of the induction of immune response in majority of outbred population (81).

Though the mechanism of action is still unknown we have extrapolated the above findings to our study in leprosy. PBMC's of BT/TT and BL/LL patients have been stimulated in vitro with various mycobacterial antigens physically mixed with Trat derived peptides and entrapped in different delivery vehicles. This approach has generated high proliferation of lymphocytes secreting mostly the cytokines of DTH type. This is an example where poorly immunogenic antigen is made into immunogenic form using such a modulator (Sridevi Kurella et al., manuscript under communication).

MUCOSAL IMMUNE SYSTEM AS A POTENTIAL TARGET FOR VACCINE DEVELOPMENT

Numerous lines of evidence emerging over the last two decades have clearly divided immune system into two functionally independent compartments (82). Systemic immune response is represented by bone marrow, spleen and lymph nodes while mucosal is represented by lymphoid tissues in mucosa and external secretory glands. For vaccine development understanding this compartmentalization is essential (83). While parenteral immunization induces poor mucosal immunity, mucosal immunization offers the advantage that same delivery mode induces both systemic and mucosal immunity. Furthermore, systemic and mucosal immune system do not show a parallel maturation pattern and the products of immunocytes (i.e. antibodies and cytokines) differ remarkably in their quality and quantity.

Mucosal immunization offers several advantages

1) Safety and minimization of adverse effects can be increased.
2) Reduces the need for personnel and equipment required for injections.
3) The migration of antigen specific lymphoblasts from the organized immune inductive tissues of the GI tract (Peyer's patches) provides a disseminated IgA response at diverse mucosal membranes.
4) May increase vaccine effectiveness in the elderly age group.
5) Generates both humoral and cell-mediated immunity (84).

The normal route for inducing mucosal response is rectal, oral, nasal or vaginal. The antibodies present in the secretions that bathe the mucosal membrane are predominantly secretory form of IgA (s-IgA) and in virtually all cases their induction requires direct mucosal immunization. Systemic and vaginal antibody response is also generated after intranasal immunization with HIV-1, C4/V3 peptide TISIPIOMN(A) (85). In addition it was observed that TISIPIOMN(A) with CT were able to induce anti-HIV cell mediated immunity in vivo. Also, the induction of HIV-1 specific CTL and DTH by a p24 region peptide has provided the pathway for study of synthetic peptides using mucosal immunizations (84).

Effective oral immunization with purified vaccine antigen has been hampered because of antigen degradation in the gut and the lack of an appropriate adjuvant and delivery system with immunopotentiating activity. Approaches to overcoming these limitations that are currently being investigated include genetically engineered microbial vectors, biodegradable microspheres vaccine delivery system and mucosal adjuvants. Antigens inside microspheres are efficiently targeted to M cells of the Peyer's patches without inducing tolerance (86). The ideal size of microspheres which can induce mucosal response is 5-10μm as they are taken up by the M cells of the Peyer's patches and are transported to the T-

B- cell zones. Microspheres < 5μm are ingested by the cells and are taken to the systemic lymphoid tissue such as spleen where the released antigen elicits a serum antibody response, whereas microspheres >5 μ m remain in Peyer's patches and provides a sustained release of antigen, eliciting sIgA response (87).

Particulate Vaccine Delivery System

The use of a particle as a mucosal vaccine delivery system is intended to restrict antigen uptake to the M cell route. An appropriate particle should deliver the antigen in its native form, protect it from premature degradation, exhibit minimum antigen leakage and display an adjuvant like activity. A large array of synthetic particulates such as liposomes (88). ISCOMs (89) or biodegradable polymers microparticles (90) are being developed to ferry selectively to O-MALT.

Mucosal adjuvants

Cholera toxin-B

Cholera toxin is a enterotoxin produced by Vibrio Cholera which is a heterodimeric protein molecule where A-subunit carries the toxic effect and the pentameric B-subunit provides the receptor binding site. The receptor is the ganglioside GM1 an integral membrane component of all nucleated mammalian cells (91). It was observed that on immunization of female rhesus macaques nasally with p55 core antigen of SIV using CTB as mucosal adjuvant, antigen-specific IgA and IgG antibodies were induced in the mucosal secretions (eg. cervico-vaginal secretions, rectal washes and saliva) and serum. These studies have provided future direction in developing protective mucosal immunity to SIV.

CpG DNA

CpG ODN (synthetic oligodeoxy nucleotide containing immunostimulatory CpG motifs) has been found to be a potent adjuvant with DNA

vaccines and protein antigen. The success with this particular antigen has been linked with the production of appropriate cytokines as well as the expression of some of the co-stimulatory molecules on the immune cells (92). This adjuvant has been contemplated to be an adjuvant of choice in future vaccine strategies (93).

LT(R192G)

The heat labile enterotoxin (LT) is produced by E. coli and has a potential to function as mucosal adjuvant. LT(R192G), a mutant form when co-administered with certain inactivated bacteria or viruses or with subunits of relevant virulant determinants from these pathogens or conjugated against peptide antigens of oligomeric HIV-gp160 and delivered intranasally, stimulated antigen specific humoral and cellular immune responses, in both systemic and in mucosal compartments (94). It is safe and nontoxic at adjuvant effective doses. Interestingly the antibody in the secretion as well as sera showed protection with laboratory adapted virus. This approach also provides shifting of non-protective isotype antibodies to protective antibody isotypes.

Lectins

The ability of several proteins to induce immune responses following oral administration was done with protein with lectin like activity, acting as adjuvant because of their binding capacity to glycoproteins and glycolipids at the intestinal mucosa. The glycocalyx of M cells and enterocytes is rich in complex carbohydrates, which could serve as receptors for lectin like molecules. The general capability of lectins to act as M cell targeting molecules was demonstrated in transport studies using rabbit Peyers patches where lectin-conjugates of macromolecules that adhere to M cell apical membranes were transcytosed much more efficiently (95). These molecules interact directly with the cell surface rather than with the mucous layer, and are subsequently internalized. These molecules are good immunological adjuvants and include bacterial adhesins, live bacterial vectors (Salmonella typhi and S.

typhimurium) toxin binding subunits bacterial pilli, lipopolysaccharide and viral neamagglutinins.

IgA

Various types of immunoglobulins are able to bind to the M cell apical surface but the nature of the binding site involved is unknown. Monoclonal IgA, polyclonal secretory IgA and IgA antigen complexes were shown to adhere selectively to M cell surfaces and transported into the intraepithelial pocket (96). In this way sIgA would serve as a vaccine-targeting molecule.

Cochleates

These fusogenic proteoliposomes prepared by protein cochleate method have been recently developed and tested for their immunogenicity after oral and parenteral administration (97). They consist of stable phospholipid-calcium precipitates. Synthetic peptide (12aa) from SIV gag protein that contains an epitope for CTL has been incorporated in such structures. This apparently highly effective vaccine delivery system has a potential to include not only antigenic proteins but also DNA for mucosal administration.

Conclusion

Acquired immunity to most of the infectious diseases involves both antibodies and antibody independent cell mediated mechanisms. T cells play a major role in this immunity both by regulating the immune response and by acting as effector cells. In recent years, the focus has been on subunit vaccines in which the immunogens are various antigens or fragments assumed to be important for protection or disease prevention. The principle of combining several antigens from different stages of the life cycle of pathogen is also attracting major interest. Though antigenic diversity among the parasite/ virus/ bacterial strains is a major problem, vaccine candidates containing conserved or semi-conserved epitopes are gaining importance to combat these diseases. Secondly, in order to avoid the problem of MHC restriction, promiscuous T cell epitopes reacting with several MHCII haplotypes appear to be particularly useful.

The interplay of mucosal and systemic immunization has also come into major consideration in vaccine designing. Generation of both mucosal and systemic immunity using novel delivery system and mucosal adjuvants has promised a direction for preparation of vaccine formulation acting at the level of mucosal surface where the antigens are first encountered.

Optimizing of subunit vaccines requires efficient adjuvant(s) and/or controlled delivery systems. Controlled released vaccine mimics the effect of booster doses, following a single immunization. So, the main role associated with adjuvants is to prolong the life span of the antigen and target to the appropriate immune effector cells. Both these actions result in generation of enhanced specific immune response for a prolonged period of time. Thus in conclusion, the judicious use of adjuvants and delivery system for subunit vaccines will be the backbone for the formulation of effective subunit vaccines for major diseases.

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