Review Article

Indian J Med Res 138, November 2013, pp 779-795

Novel adjuvants & delivery vehicles for vaccines development: A road ahead

Teena Mohan, Priyanka Verma & D. Nageswara Rao

Department of Biochemistry, All India Institute of Medical Sciences, New Delhi, India

Received July 1, 2013

The pure recombinant and synthetic antigens used in modern day vaccines are generally less immunogenic than older style live/attenuated and killed whole organism vaccines. One can improve the quality of vaccine production by incorporating immunomodulators or adjuvants with modified delivery vehicles *viz.* liposomes, immune stimulating complexes (ISCOMs), micro/nanospheres apart from alum, being used as gold standard. Adjuvants are used to augment the effect of a vaccine by stimulating the immune system to respond to the vaccine, more vigorously, and thus providing increased immunity to a particular disease. Adjuvants accomplish this task by mimicking specific sets of evolutionary conserved molecules which include lipopolysaccharides (LPS), components of bacterial cell wall, endocytosed nucleic acids such as dsRNA, ssDNA and unmethylated CpG dinucleotide containing DNA. This review provides information on various vaccine adjuvants and delivery vehicles being developed to date. From literature, it seems that the humoral immune responses have been observed for most adjuvants and delivery platforms while viral-vector, ISCOMs and Montanides have shown cytotoxic T-cell response in the clinical trials. MF59 and MPL® have elicited Th1 responses, and virus-like particles (VLPs), non-degradable nanoparticle and liposomes have also generated cellular immunity. Such vaccine components have also been evaluated for alternative routes of administration with clinical success reported for intranasal delivery of viral-vectors and proteosomes and oral delivery of VLP vaccines.

Key words Adjuvant - antibody - antigen - epitope - ISCOMs - liposomes - MHC - microsphere - peptide - vaccine

Introduction

The role for adjuvants in human vaccines has been a matter of vigorous scientific debate because for over 80 years, aluminium salts were the only adjuvants approved for research purpose by the Food and drug administration (FDA). Even today, alum-based adjuvants, alone or combined with additional immune activators, remain the only adjuvants approved for human use. To seriously challenge alum's supremacy, a new adjuvant has many major hurdles to overcome, not least being alum's simplicity, tolerability, safety record and minimal cost[1]. Adjuvants can be used for multiple purposes: to enhance immunogenicity, provide antigen-dose sparing, to accelerate the immune response, reduce the need for booster immunizations, increase the duration of protection, or improve efficacy in poor responder populations including neonates, immunocompromised individuals and the elderly[2].

The other studied adjuvants play major signaling roles within the immune system and have the advantage with exception of high biocompatibility and low toxicity. For example, carbohydrates adjuvants are easily metabolized or excreted, with little risk of generating toxic metabolites or long-term tissue deposits. This means that adjuvant accumulation and excessive/chronic immune activation are less likely when using other adjuvants *e.g.* carbohydrate adjuvant, virus-like particles (VLPs), emulsions *etc*. By contrast, aluminium salts have been shown to form long-term deposits at the injection sites, potentially lasting for decades after vaccine administration, condition known as macrophagic myofasciitis[3,4]. A depot effect was initially thought important to alum's adjuvant activity but was long ago disproved by showing adjuvant action even after excision of the alum depot, findings since largely forgotten. Any detrimental pathophysiological effect of long-term aluminium tissue deposits and associated macrophagic myofasciitis remains a matter of debate[5,6]. In this review, we discuss different types of adjuvants/delivery vehicles, with each their own unique physio-chemical and immunological attributes and behaviours, providing a wide range of options in vaccine design.

Adjuvant and delivery system

An adjuvant is defined as any compound that enhances the immune response against a vaccine antigen. The word 'adjuvant' comes from the Latin word 'adjuvare', means 'help' or 'to enhance', can be defined as any product or association of components that increases or modulates the humoral or cellular immune response against an antigen. In many cases, the antigen itself is very weakly immunogenic; therefore an adjuvant is needed to intensify the immune response. Adjuvant can also be included in vaccine to guide the type of immune response generated. This may be especially important when developing vaccine for cancer, human immunodeficiency virus (HIV) or mucosal immune system. In contrast, a more immunogenic antigen may benefit from a specific delivery vehicle. This component may facilitate targeting and/or controlled release of the antigen to dendritic cells (DCs)[7]. Recent studies, utilizing Toll-like receptor (TLR) ligands, have shown that antigens associated with their ligands can produce exceptionally high antibody and rapid immune responses[8,9]. Adjuvants have also been shown to protect antigens from degradation, although this generally depends on the nature of adjuvant. For example, chitosan-adjuvate nanoparticles were found to stabilize ovalbumin while on the other side, the model protein antigens are actually destabilized by the traditional aluminium salt adjuvants. The adjuvants can be classified based on their five potential modes of action: *(i)* immunomodulation (modification of cytokine networks), *(ii)* presentation (maintenance of antigen confirmation), *(iii)* cytotoxic T-lymphocytes (CTL) induction, *(iv)* targeting specific cells, and *(v)* depot generation[10].

An adjuvant can act in more than one way, contributing to elicit a productive immune response against an antigen. The combination of one or more adjuvants plus the antigen has been studied in detail[11]. In the last few years, the adjuvant properties of immunomodulation have been attributed to several macromolecular components of microorganisms which are recognized by pathogen-associated molecular patterns (PAMPs), present on cells of innate immune system. These components are called molecular patterns because these are structures frequently encountered in microorganisms that facilitate the innate immune response against them. Examples of immune modulation by these components include binding of compounds like lipopolysaccharides (LPS), lipopeptides and CpG motifs to distinct members of TLR family, leading to macrophages and DCs activation and the binding of glycoproteins or glycolipids to mannose receptor on phagocytes[12-14]. Although many components of this class have been purified and tested with different vaccine formulations targeting to elicit a suitable immune response against a specific antigen, yet to perform the adjuvant effect, the antigen and the adjuvant should be together at the same site since the antigen-presenting cells (APCs) which process the antigen should also be activated for a posterior activation of a naïve T-cell. To solve these problems, several formulations and carrier systems have been developed such as emulsion, liposome, microspheres, immune stimulating complexes (ISCOMs) and nanospheres. These carriers share some of the following properties: protection of antigen from degradation following its administration by different routes including mucosal, ability to sustain the antigen release over an extended period of time, intracellular delivery of antigen contributing to cytotoxic T-cell stimulation and targeting at APCs. Hence, with the aim of eliciting broad immune response especially with strong cellular compounds, the trend has been to combine adjuvant or to formulate these to achieve depot formation, recruitment and activation of APCs in the presence of the desired antigen[15].

Why use an adjuvant?

As discussed earlier, adjuvants have been traditionally used to increase the magnitude of an immune response to a vaccine, based on antibody titre or ability to prevent infection, but a second role for adjuvants has become increasingly important *i.e.* guiding the type of adaptive response to produce the most effective forms of immunity for each specific pathogen. Thus, there are two distinct reasons to incorporate an adjuvant into a vaccine. First as adjuvants are currently used clinically to *(i)* increase the response to a vaccine in the general population, increasing mean antibody titres and/or the fraction of subjects that become protectively immunized, *(ii)* increase seroconversion rates in populations with reduced responsiveness because of age (both infants and the elderly), disease, or therapeutic interventions, as in the case of MF59 adjuvant to enhance the response of older subjects to influenza vaccine[16,17], *(iii)* facilitate the use of smaller doses of antigen[18-20], because the ability of an adjuvant to permit comparable responses with substantially lower amounts of antigen could be important in circumstances in which large-scale vaccination is urgent and production facilities limiting, as in the emergence of a pandemic influenza strain, and *(iv)* permit immunization with fewer doses of vaccine. The requirement of many vaccines for multiple injections presents compliance issues and, in much of the world, significant logistic challenges[18,20,21].

The second reason for incorporating an adjuvant into a vaccine is to achieve qualitative alteration of the immune response. For vaccines currently under development, adjuvants are increasingly used to promote types of immunity not effectively generated by the non-adjuvanted antigens. For example, adjuvants have been used in pre-clinical and clinical studies to *(i)* provide functionally appropriate types of immune response (*e.g.* Th1 versus Th2 cell, $CD8^+$ versus $CD4^+$ T-cells, specific antibody isotypes), *(ii)* increase the generation of memory; especially T-cell memory[22-24], *(iii)* increase the speed of initial response, which may be critical in a pandemic outbreak of infection[25-27], and *(iv)* alter the breadth, specificity, or affinity of the response[26,28].

Adjuvant selection

Some of the features involved in adjuvant selection are the antigen, the species to be vaccinated, the route of administration and the likelihood of side effects[29,30]. Ideally, adjuvants should be stable with long shelf-life, bio-degradable, cheap to produce, not induce immune responses against themselves and promote an appropriate immune response (*i.e.* cellular or humoral immunity depending on requirements for protection)[31]. There are marked differences on the efficacy of adjuvants depending on the administration route (*e.g.* between mucosal and parenteral routes). Hence, new vectors, antigen delivery systems and adjuvants compounds need to take into account the characteristics of the proposed administration routes[32].

Adjuvants/delivery systems in vaccine research

Potent adjuvants can improve the effectiveness of vaccines by accelerating the generation of robust immune responses, sustaining responses for a longer duration, inducing local mucosal immune responses, generating antibodies with increased avidity/affinity and neutralization capacity, eliciting CTLs, enhancing immune responses in individuals with weakened immune systems (*e.g.* children, elderly or immunocompromised adults), increasing the response rate in low-responder individuals and reducing the amount of antigen needed, thus reducing the cost of vaccination programmes. Adjuvants are functionally defined as components added to vaccine formulations that enhance the immunogenicity of antigens *in vivo*. Adjuvants can be divided into two classes (delivery systems and immunopotentiators) based on their dominant mechanisms of action. Immunopotentiators activate innate immunity directly (*e.g.* cytokines) or through pattern recognition receptors (PRRs) (such as bacterial components), whereas delivery systems (*e.g.* microparticles and nanoparticles) concentrate the antigen and display antigens in repetitive patterns, target vaccine antigens to APCs and help co-localize antigens and immunopotentiators. Thus, both immunopotentiators and delivery systems can serve to augment antigen-specific immune response *in vivo*. For subunit vaccines, it is highly desirable that the combination of delivery systems, immunopotentiators and isolated antigens will be required to elicit optimal immune responses.

Currently licensed adjuvants were developed using empirical methods, thus these are not optimal for many of the challenges in vaccination today. In particular, the historical emphasis on humoral immune responses has led to the development of adjuvants with the ability to enhance antibody responses. As a consequence, most commonly used adjuvants are effective at elevating serum antibody titres, but do not elicit significant Th1 responses or CTLs. The ability

of an adjuvant to qualitatively affect the outcome of the immune response is an important consideration, because the need for vaccines against chronic infections [*e.g.*, HIV, hepatitis C virus (HCV), tuberculosis and herpes simplex virus (HSV)] and cancer has shifted the focus to generation of cellular immune responses and adjuvants specifically geared towards eliciting this effect[17,18,21]. To this end, many new and existing adjuvant formulations are being tested in various pre-clinical and clinical trials. An expanded understanding of the immunobiology of TLRs and other PRRs, immunoregulatory cells, DCs and the importance of specific T helper cell responses (Th1 versus Th2) in resolving particular diseases provides a framework for their continued optimization[9,22].

Major adjuvant groups

Alum based adjuvants: Alum salts principally aluminium phosphate and hydroxides have been the most widely used human adjuvants. Alum salts, *per se*, are relatively week adjuvants and rarely induce cellular immune responses but, these slow down the rate of release of the antigen and also increase the duration of antigen interaction with the immune system, thus enhancing the immune response against the antigen. Although, the last two decades of systematic research into the nature of these adjuvants has contributed significantly to understand their nature and limitations as the stimulators, the more detailed mode of action of these adjuvants is still not completely understood[29].

Other mineral salt adjuvants: The salt of calcium, iron and zirconium has also been used to absorb antigens, although not to the extent of alum salts. In particular, calcium phosphate has been used for diphtheria tetanus pertussis (DTP) vaccines. While, having similar properties to alum salts, calcium phosphate has the advantage that it is a natural compound to the human body and is, therefore, exceptionally well-tolerated[33]. It has a reasonable capacity to absorb antigens, induces high levels of IgG antibodies and does not increase IgE production[34]. Neurological reactions to pertussis vaccines absorbed to calcium phosphate are rare[35].

Complete Freund's adjuvant (CFA): For several decades, Freund's adjuvants have been considered the most effective adjuvants available for raising antibodies in test animals. Complete Freund's adjuvant contains heat-killed mycobacteria, which is a primary agent responsible for stimulating antibody production, but has also been attributed to a number of undesirable side effects[36]. The undesirable side effects attributed to CFA use include increased pain and suffering and morbidity in inoculated test animals and potentially serious health and safety threats. Even for animal research there are currently guidelines associated with its use, due to its painful reaction and potential for tissue damage. Complete Freund's adjuvant is effective in stimulating cellular immune response and may lead to the potentiation of the production of IgG and IgA[37].

Adjuvants emulsions: This class includes *oil-in-water* (o/w) or *water-in-oil* (w/o) emulsions such as IFA (incomplete Freund's adjuvant), montanide, MF 59 and Adjuvant 65. In general, these adjuvants are considered toxic for routine human prophylactic vaccines. Frequent side effects of emulsion include inflammatory reactions, granulomas and ulcers at the injection site. Various types of emulsions have been used with different natural oils to find more stable, potent and less toxic formulations.

Incomplete Freund's adjuvant (IFA): Incomplete Freund's adjuvant, *water-in-oil* (w/o) emulsions is prepared from non-metabolizable oils. The IFA induces predominantly a Th2-biased response through the formation of a depot at the injection site and the stimulation of antibody producing plasma cells. The antibody response towards many antigens is greatly enhanced when these are administered with IFA[38]. With regard to cellular hypersensitivity, immunization with a variety of antigens in IFA primes animals for a transient form of delayed-onset, lymphocyte-mediated cutaneous reactivity characterized by extensive infiltrates of basophilic leucocytes[39].

Adjuvant 65: Adjuvant 65 offers the advantage over the mineral oil used in IFA that it can be metabolized. Different emulsions like *oil-in-water* and *water-in-oil* have been developed with the w/o being as potent as IFA, but more stable, less viscous and easier to administer, with less resulting granulomas.

Montanide: Montanide is a family of oil based adjuvants that have been used in experimental vaccines in mice, rats, dogs and cats using natural, recombinant and synthetic antigens. In humans, montanide has been used in trial vaccines against HIV, malaria and breast cancer. There are several different types of montanides including ISA, 50V 20G and 720. Emulsions of montanide ISA, 50V and 720 are composed of metabolizable sequence based oil with a mannide mono-oliate emulsifier and shown to induce high antibody titre and CTL responses in a variety of animal species. In one recent study, it was concluded that the Montanide ISA-201 adjuvanted

foot-and-mouth disease (FMD) vaccine induces enhanced immune responses and protective efficacy in cattle[40].

MF59: MF59 is a submicron *oil-in-water* emulsion which contains squalene and varying amounts of muramyl tripeptide phosphatidyl-ethanolamine (MTP-PE), and activates non-TLR sensing receptors. Published data suggest that the addition of MF59 induces a modest increase in antibody levels in the elderly and no difference in younger individuals when compared to unadjuvanted vaccine[41]. MF59 has been shown to be superior to alum in inducing antibody responses to hepatitis B vaccine in baboons and humans[42]. The molecular effects of MF59 have been described in the mouse model, following injection into the muscle, demonstrating recruitment of APCs and upregulation of multiple inflammatory cytokines, chemokines, and receptors. In addition, APCs were recruited in response to MF59 and genes responsible for antigen processing and presentation were upregulated. Effort has been done to develope TLR agonists that are more compatible with emulsions, including lipid-associated imidazoquinoline, leading to local adjuvant effects with decreased systemic immune activation[43].

Oil-in-water emulsions have also been used successfully with influenza vaccines, primarily those produced in eggs. Pandemic influenza vaccines with *oil-in-water* emulsion adjuvants have been prioritized because of antigen dose-sparing and enhancing cross-reactive antibody titres which could be critical in the event of a pandemic[44].

Bacterially derived adjuvants

(i) Toxins

(a) Cholera toxin: Cholera toxin (CT) is a protein complex secreted by the bacterium *Vibrio cholerae*. Cholera toxin is responsible for the massive, watery diarrhoea characteristic of cholera infection. Cholera toxin has been shown to enhance the immunogenecity of relatively poor mucosal immuogens when mixed or conjugated together with them and given intranasally; thus, CT and its β-subunit have generated a great deal of interest as potential adjuvants for oral vaccines[45].

(b) Pertussigen: The killed *Bordetella pertussis* has been used experimentally as a parenteral adjuvant. Obviously, this material is a complex mixture including LPS as well as variable amount of pertussis toxin (PT). In particular, it enhances the cellular immune response as measured by delayed skin test responses to soluble antigens and increases inflammatory responses such as foot pad swelling after injection. Like LPS, pertussigen can be given by a different route at a different time than the antigen and still exerts its adjuvant effects[46].

(c) Clostridium difficile toxin: Toxin A and Toxin B of *C. difficile* have been evaluated for their ability to act as mucosal adjuvants. Mice were immunized intranasally with antigen containing toxin A/B, elevating the levels of salivary and serum IgA. Formalin inactivation of *C. difficile* toxins completely eliminated their ability to act as adjuvants, suggesting that biological activity was important for this function[47].

(d) Shiga toxin: Shiga toxin (STx) is a protein toxin of *Shigella dysenteriae*, Type-I, a causative agent of severe diarrhoea. Mice vaccinated oro-gastrically with various doses of STx developed serum and gastro-intestinal antibodies to STx in a dose dependant manner. In a recent study, the immunomodulatory potential of recombinant *Shiga* toxin B subunit (rStxB) protein in BALB/c mice was evaluated. Animal protection with recombinant StxB was conferred through both humoral and cellular immune responses[48].

(e) Staphylococcal enterotoxins: Staphylococcal enterotoxins are basic proteins produced by certain *Staphylococcus* strains in a variety of environments, including food substrates. These structurally related, toxicologically similar proteins are produced primarily by *Staphylococcus aureus*. The ability of staphylococcal enterotoxin to act as mucosal adjuvants has not been specifically explored but has been examined for its immunogenicity by an intranasal route of administration[49].

(ii) Non-toxin proteins

(a) Muramyl dipeptide (MDP): N-aceylyl muramyl-L-alanyl-D-isoglutamine is derived from the cell wall of mycobacteria. It is the smallest structural component of the cell wall that still retains adjuvant activity and is one of the active components in CFA. In several instances, oral administration of MDP has been used to stimulate non-specific immunity to bacteria and to tumour cells. The increase in non-specific immunity was probably due to induction of cytokine. MDP is known to be a potent inducer of interleukin-1 (IL-1), which can activate macrophages and T-cells. Although, not directly relevant to mucosal immunity these results show that MDP is absorbed by the gut and, therefore, may affect the immunoregulatory environment of mucosa-associated lymphoid tissue (MALT). The mechanism of action is unknown, but is probably due at

least in part, to the ability of MDP to induce basophils production and increase processing and presentation of antigens by macrophages[50].

(b) Lipopeptides: Lipopeptides derived from bacterial lipoproteins have been shown to be potent adjuvants for parenteral immunization. One synthetically produced lipopeptide N-palmitoyl - S - [2,3 - bis(palmitoyloxy) - (2R,S) - propyl] - (R) - cysteinyl - seryl - (lysyl) 3-lysine (P3CSK4), has been shown to be an effective adjuvant for oral immunization[51]. This compound has been observed to stimulate murine lymphocytes from peyer's patches in a dose dependent manner without any apparent toxicity.

(c) Proteosomes: Proteosomes are multi-molecular preparations of meningococcal outer membrane protein. Intranasal immunization of mice with proteosome toxoid vaccine combinations elicited high level of anti-toxin IgA in lung and intestinal secretions, whereas toxoid without proteosome did not[52]. Furthermore, proteosome toxoid delivered intranasally afforded significant protection against challenge by a lethal aerosol exposure to toxin[52].

Liposome adjuvant: Liposomes are synthetic spheres consisting of lipid layers that can encapsulate antigens and act as both vaccine delivery vehicle and adjuvants. Liposomes have been used widely in experimental vaccine. The potency of liposome depends on the number of lipid layers, electric charge, composition and method of preparation. These enhance both humoral and cellular immunity to proteins and polysaccharide antigens[53]. Liposomes help to extend the half-life of antigens in blood ensuring a higher antigen exposure to APCs after vaccination. Stability, manufacturing and quality assurance problems seem to have been major factors behind the fact that as yet no adjuvant based on liposome has been registered for human use[54].

Tenso-active adjuvants: Quil-A is a component of saponin, a detergent derived from the plant *Quillaja saponaria* molina, which has been shown to have adjuvant activity. Quil-A is one of the biologically active components of ISCOMs, but it has also been employed alone as an adjuvant. Mainly QS21 has been studied as an alternative to alum when strong cellular responses are required for a particular vaccine. Saponins are tenso-active glycosides containing a hydrophobic nucleus of tri-terpenoid structure with carbohydrate chains linked to the nucleus[55]. Saponins induce a strong adjuvant effect to T-dependent as well as T-independent antigens. The usefulness of Quil-A as an adjuvant has been hampered by its apparent toxicity. However, non-toxic immunostimulatory fractions of Quil-A have been identified. While, Quil-A by itself does not appear to be highly effective as a mucosal adjuvant, its use as one of the components of ISCOMs appear to be critical for the effectiveness of this system[56].

Immunostimulating complexes (ISCOMs): The term ISCOM was coined to describe 40nm cage-like particles that form spontaneously when cholesterol is mixed with Quil-A. Protein antigens can be incorporated into such particles, with Quil-A serving as a built in adjuvant. The incorporation of antigens into ISCOMs occurs via hydrophobic interactions, which potentially limits the utility of this adjuvant for protein antigens[57]. ISCOMs stimulate a strong response for all immunoglobulin classes. These also stimulate cellular immune response as measured by T-cell responses and delayed-type hypersensitivity. Perhaps a unique feature of ISCOMs is their ability to induce $CD8^+$ specific cytotoxic responses. A single subcutaneous immunization of mice with ISCOMs containing either purified HIV gp160 or influenza haemagglutinin resulted in priming of antigen specific $CD8^+$ MHC class-I restricted CTLs. On the other hand, when administered intranasally, influenza ISCOMs vaccines were found to elicit strong mucosal (IgG and IgA) responses[58].

Carbohydrate adjuvants: Several complex carbohydrates of natural origin stimulate cells from the immune and reticulo-endothelial system. γ-inulin, a carbohydrate derived from the plant roots of the Compositae family. It is a potent adjuvant inducing humoral and cellular immunity without the toxicity. γ-inulin can be combined with a variety of other adjuvant components, *e.g.* aluminium hydroxide, to produce a range of adjuvants with varying degree of Th1 and Th2 activity. In addition, the other glucose and mannose based polysaccharides having adjuvant action include glucans, dextrans, lentanans, glucomannans and galactomannans. Another example of carbohydrate adjuvants is acemannan, a natural polysaccharide extracted as a mucilaginous gel of *Aloe barbadensis*, and stimulates generation of CTLs and the cytotoxic activity of natural killer (NK) cells[59].

CpG oligodeoxynucleotide (ODN): Immunostimulatory DNA sequences containing unmethylated CpG dinucleotide in the context of particular base sequence (CpG motifs) exert a strong stimulatory influence on the immune system. Such sequences which are either found naturally in bacterial DNA or produced as synthetic oligonucleotides directly activate human

B-cells and plasmacytoid DCs via TLR9. CpG oligos act as polyclonal activator, directly activate B-cells to proliferate and differentiate into IgG producing cells[60]. CpG oligos also indirectly activate other cells such as monocytes and macrophages to produce a variety of proinflammatory cytokines and in particular those associated with these stimulatory influences. CpG ODNs were capable of enhancing $CD4^+$ T-cells, $CD8^+$ T-cytotoxic cells and antibody response to a wide variety of antigens. As a result of their strong adjuvanticity and low reactogenicity, CpG ODNs are currently considered as one of the most promising adjuvants for the development of future vaccines against diverse conditions including infectious diseases, allergies or cancer[61].

Innate molecules as adjuvant: Different antimicrobial peptides including defensins are providing the first line of defense by rapidly clearing a wide variety of microbes, prior to the development of an adaptive immune defense system. Defensins enhance phagocytosis, stimulate prostaglandins release, neutralize the septic effect of LPS, promote recruitment and accumulation of various immune cells at the inflammatory sites, and induce wound repair. These peptides also display immunostimulatory activities including a chemotactic effect for T-lymphocytes, monocytes, immature DCs and induction of cytokine production[62,63]. Recently, we have also examined the potential role of human defensin as mucosal adjuvant for eliciting strong and long lasting humoral and cellular immunity against HIV-1 antigen. The results demonstrated the effectiveness of synthetic defensin peptides to induce significant increase in T-lymphocyte proliferation response at lamina propia (LP), spleen (SP) and Peyer's patches (PP) with increase in IgG/IgA antibody at systemic and mucosal secretions[64,65].

Cell-based adjuvants / delivery systems: Dendritic cells are able to prime potent lymphocytes responses and are increasingly being tested for their ability to act as adjuvant in therapeutic vaccines. To effectively use DCs as an adjuvant, these must be of the appropriate phenotype to optimally present antigenic peptides and express co-stimulatory molecules. Mouse DCs have been shown to aid migration and recruitment of NK cells to the lymph nodes to provide an early source of interferon-γ (IFN-γ)[66].

Cytokines as adjuvants: A large number of cytokines have been evaluated alone or in combination for their effects on immunity. Different cytokines were studied as adjuvants to induce antigen specific serum/ mucosal antibody and cell-mediated immunity. The most notable cytokine adjuvants studied to date include granulocyte/macrophage colony stimulating factor (GM-CSF), IFN, IL-1, IL-2, IL-6, IL-12, IL-15, IL-18 and chemokines[67].

(i) GM-CSF: The mechanism of the adjuvant effect of GM-CSF is that it mobilizes DCs into the tissues after injection, thus enhancing the ability of the co-injected vaccine antigen to be presented to the cells of the immune system. The generated DCs expressed higher levels of MHC class-I molecules and produced equally high levels of IL-12. For example, systemic co-administration of a DNA vaccine encoding the *env* gene of HIV with GM-CSF expressing plasmids into mice induced both vaginal and faecal IgG and IgA, with levels of IgA exceeding those of IgG in both the vaginal wash fluids and faeces[68].

(ii) Type-I IFN: Though type-I IFNs are frequently thought of as primarily involved in antiviral immune responses, these have several other roles as well, including T-cell proliferation, NK cell activation, and cytokine induction. Previous studies have linked the adjuvant activities of a TLR9 agonist and the presence of interferon alpha receptor -1 (IFNAR-1)[69].

(iii) IL-1: IL-1 is a potent proinflammatory cytokine with a wide range of effects on the host immune system. These effects include the up- and downregulation of many genes, cytokine and chemokine molecules and their receptors, and adhesion molecules, resulting in the trafficking of cell populations (*e.g.* neutrophils) into areas of inflammation[70].

(iv) IL-2: IL-2 is involved in T-cell proliferation and the induction of T-cell regulatory responses. As such, it has been investigated for its ability to induce cellular response, but its ability to induce serum antibody production has also been examined. IL-2 is a lymphoproliferative cytokine mainly produced by $CD4^+$ T-cells. The mechanism of action appears to be through upregulation in the expression of CD48 and CD80 on DCs as well as upregulation of their respective ligands, CD2 and CD28, on CD^+ T-cells[71].

(v) IL-6: IL-6 is a potent inflammatory cytokine, and has also been shown to play a role in shaping adaptive immune responses as both B-cell stimulating factor and Th17-inducing cytokine[72].

(vi) IL-12: IL-12, a Th1 cytokine, induces NK cells, T- and B-cells, and is also involved in Th1 differentiation. It is a proinflammatory cytokine that is heterodimeric

in structure and is produced by phagocytes and DCs in response to infection by pathogens. Initially, IL-12 plays a significant role in the modulation of the CTL response and is central to immunity against pathogens that are controlled by cell mediated mechanisms driven by Th1 cells. IL-12 biases the naïve T-cells to the Th1 pheonotype both alone and through directed IFN-γ production by NK cells[73].

(vii) IL-15: It is known to share several overlapping activities with IL-2, which is likely due to their extremely high homology and structural similarities. In addition to its ability to promote both NK and T-cell development and proliferation, IL-15 is also known to augment B-cell antibody production[74].

(viii) IL-18: IL-18 is produced by macrophages and kupffer cells and is a potent pleiotropic cytokine. It induces the production of IL-2 or IL-12 and enhances proliferation and activity of NK and $CD8^+$ T-cells. Overall, this cytokine is promoter of a Th1 immune response. Although, it does not itself induce differentiation of Th1 cells, it influences Th1 cells to produce IFN-γ[75].

(ix) Chemokines: Chemokines are small molecules secreted by different cells, have ability to induce directed chemotaxis in nearby responsive cells, and the also called as chemotactic cytokines. Like many other cytokines, the macrophage inflammatory protein-1α (MIP-1α) family of chemotactic cytokines, is known best for its roles in innate immune responses, including the recruitment of pro-inflammatory cells. These also modulate Th cell differentiation, as the MIP-1α has been shown to promote Th1 responses. On the other side, the blocking of MIP-1α has been shown to reduce Th1 responses to *Cryptococcus neoformans* infection[76].

Polymeric particles

(i) Biodegradable: A variety of polymers exists from which nanoparticles for drug delivery can be synthesized, however, the most commonly studied polymers are poly (D,L-Lactide-co-glycolide) (PLG) and poly lactide (PLA). These biodegradable, biocompatible polymers have been approved for use in humans (*e.g.* as sutures, bone implants and screcus as well as implants for sustained drug delivery) and have been extensively studied for the use in the formulation of vaccine antigens (*i.e.* proteins, peptides, DNA, *etc.*)[64,65]. In these formulations, antigen can either be entrapped or adsorbed to the surface of the particles. These can act as depot from which the encapsulated antigen is gradually released. Additionally, the polymeric particles may offer protection to encapsulate the antigens delivered and facilitate uptake by M-cells in the MALT, thus serving as a vehicle for mucosal immunization[77]. The adsorbed antigen may offer improved stability and activity over encapsulated antigen by avoiding formulation and acidic pH conditions caused by the degradation of the polymer[78,79].

The pre-clinical studies have shown that PLG micro/nanoparticles can induce systemic antibody titres comparable to those of aluminium salts. Additionally, a study using tetanus toxoid (TT) found that a synergistic immune response (*i.e.* four fold higher mean serum anti-TT IgG response) could be achieved by injecting TT bound to an aluminium salt along with TT loaded nanoparticles[80].

Non-degradable: Among the various non-degradable nanoparticles that are being evaluated for their use as vaccine adjuvants and delivery system are gold, latex, silica and polystyrene. Since, these materials may remain in the tissues for extended period of time; it is thought the antigen may be presented to the immune system over similar time periods thereby enhancing immunogenicity. Gold particles have frequently been described for vaccine delivery both with and without the aid of electroporation which has shown to often dramatically enhance the potency of DNA vaccine, by improving delivery into cellular interiors. Combining electroporation with intradermal delivery of DNA and gold particles, an enhanced and accelerated immune response has been observed in mice; however, electroporation may not be applicable in humans due to the cell mortality resulting from the high voltage electrical pulses. A study in humans using these particles without electroporation produce a relatively low immune response after vaccination with DNA-gold particles-GM-CSF transfected analogues tumour cells[81]. Another approach for DNA delivery is through particle bombardment or Particle Mediated Epidermal delivery (PMED) or the "Gene Gun" approach. While the delivery efficiency of this technique is quite low, only small amounts of DNA are required to achieve a significant immune response. Clinical trials have shown that this approach can elicit both humoral and cellular immune responses, making it one of the only consistently successful DNA vaccine delivery approaches[82].

Cholesterol bearing hydrophobized pullulan nanoparticles: Cholesterol can be conjugated to a variety of carbohydrate including pullulan, dextran and mannose, rendering the molecules amphiphilic. Such

molecules have been shown to self-assemble with and without protein into 30-40 nm colloidally stable nanoparticle whose size and density can be modified by altering the degree of substitution of cholesterol groups on the polysaccharides. Pullulan is the most popular polysaccharide, to which cholesterol has been conjugated. There are numerous *in vitro* reports[83] while only one in humans is published to prove these pullulan nanoparticle as delivery vehicle and adjuvant.

Currently, there is only one report of cholesterol hydrophobized pullulan (CHP) nanoparticle being evaluated in clinic. A complex of CHP and a cancer testis antigen *i.e.* NY-ESO-1, was shown to enhance the humoral immune response. In this study, the cellular immune response was not evaluated due to seropositive patients possessing activated $CD8^+$ T-cells[84]. Previously, an *in vitro* study showed that DCs loaded CHP/NY-ESO-1 complexes induced both $CD8^+$ and $CD4^+$ T-cells. A pre-clinical study in mice showed that CHP induced both humoral and $CD8^+$ T-cell responses. In all these studies, vaccination with CHP seems to be both safe and well tolerated[84].

Saponins: Saponin, a natural product derived from the tree bark, was used to make ISCOMs, which are immunostimulatory complexes incorporating protein antigen into saponin. This technology has led to the development of ISCOMATRIX, which can be combined with a variety of antigens, and has been reported to induce $CD8^+$ T-cell responses via the MyD88 pathway. Association of saponin with cholesterol to form ISCOMATRIX reduces reactogenicity[85], and enhances its adjuvant effects possibly by improving bioavailability.

Virosomes: Virosomes are unilamellar structures composed of membrane lipids and viral membrane proteins. These empty enveloped particles are physically associated with vaccine antigen which results in enhanced immunogenicity. Virosome technology has been most advanced in influenza, in association with protein or peptides, but it is rapidly being used for other antigens as well. A potential advantage or application of this technology is to take advantage of the physical properties of virosomes in terms of uptake by APCs, as well as the chemical composition, and compatibility with adjuvant molecules derived from lipid-A[86]. A number of virosome based vaccines have already reached the market. The first of these was Epaxal™, a hepatitis A vaccine registered in 1994 in several European, Asian and South American countries. Another influenza vaccine utilizing virosome is Invivac™, one of the successful virosomal influenza vaccines for elderly subjects and registered in the Netherlands and Switzerland, is also available in the market[87].

Virus-like particles: Virus like particles (VLPs) use the nature's own mechanisms and structural principles to trigger the immune system for protective effects. These macromolecular complexes stimulate an immune response by delivering a material that mimics certain viral properties. VLPs are essentially non-infective virus consisting of self-assembled vial envelope proteins without accompanying the genetic material. Virus like particles maintain a morphology and cell-penetrating ability similar to infective viral particles. The VLPs have also been shown to stimulate both cellular and humoral immunity. Several recombinant HBV-VLP vaccines have been licensed. The first licensed recombinant HBV vaccines; Recombivan™ and energin-B™, were composed of the viral small envelope protein which upon expression in yeast formed 22 nm VLPs. While effective, these suffered from a lack of immunogenicity (5-10% non-responders), which was determined to be due to an absence of pre-S epitopes on the surface of VLPs. A more immunogenic VLP vaccine was subsequently described that contained Pre-S1, Pre-S2 and HBV surface antigen. This potential third generation HBV vaccine, Bio-HepB™ was found to elicit a strong antibody response and 100 per cent sero-conservation and seroprotection rates[88].

One recently approved VLP vaccine is Gardozil™ for immunization against human papilloma virus (HPV) and subsequent prevention of cervical cancer. This vaccine is composed primarily of self-assembled particles of L1 (the major capsid protein) from HPV types 6, 11 and 18 and also contains an aluminium salt adjuvant. It has been shown to reduce infection of HPV by 90 per cent and is apparently almost 100 per cent effective against these types since two of the four antigens in the HPV vaccine (HPV types 16 and 18) are implicated in 70 per cent of the cervical cancers. This vaccine is expected to drastically reduce the occurrence of this life threatening disease in women[89].

Viral-vectored vaccines: Viral-vectored vaccines consist of a non-replicating virus that contains some defined genetic material from the pathogen to which immunity is desired. Such vaccines are also commonly referred to as live recombinant vaccines since the immune system has evaluated to respond to viruses, this would seem to be an ideal way to deliver an antigen. Advantages of viral-vectored vaccines include

their ease of production, a good safety profile (at least in some cases), ability to potentiate strong immune responses, potential for nasal or epicutaneous delivery and mucosal immunization[90].

Adenovirus which has been administered orally as its own vaccine for decades also provides a frequent viral-vector platform for many of these types of vaccines including delivery systems for Alzheimer's disease, influenza, tetanus and HIV based vaccines. Such systems are also being used for alternative routes of administration (*i.e.* not the parental route, which is typically used for immunization). A recent phase-I clinical trial of an adjuvant-vectored influenza vaccine administered intranasally and epicutaneously was found to elicit high serum antibody titres with a good safety profile. This study was the first of its kind to show that adenovirus-vectored vaccines are safe for intranasal and epicutaneous administration in humans[91]. Pre-clinical studies of an adenovirus-vectored tetanus vaccine reported similar results[92]. In addition to adenovirus, a variety of other vectors have shown success in both pre-clinical and clinical studies. A modified vaccinia virus Ankara (MVA) was well tolerated and produced a good safety profile in humans infected with HIV-1 undergoing highly active anti-retroviral therapy (HAART). Additional viral-vector technologies that are currently being pursued for vaccine delivery include proxy-viruses, measles virus, vesicular stomatitis virus, HSV and alpha virus among others[93].

Mechanism of action

Depot and slow release: Adjuvants like aluminium hydroxide gel and oil emulsions were considered to exert their effects by a protracted release from the site of injection. Aluminium hydroxide has proved beneficial for priming immune responses to soluble antigens *e.g.* toxins and gp120 of HIV-1, but less effective for boosting. This effect might be due to the adsorption of antigen to the gel phase, a substitute for the particulate form. It is likely that the local reactions particularly caused by oil emulsions induced inflammatory response, which attract mainly antigen presenting macrophages. Further, granulocyes and neutrophils contribute to the adjuvant activity by producing cytokines. Negative side effects on the other hand are well documented in the form of granuloma and abscesses. IFA also excite the draining lymph nodes which become enlarge[94].

Modern versions of depot adjuvant are micro capsules and biodegradable nanosphere. These nanosperes are composed of biodegradable, biocompatible synthetic polymer in which the antigen is dispersed. Examples of biodegradable substances used are polyesters, polyorthoesters, polyanhydride and various natural polymers including proteins and polysaccharides. Most attention has been paid to co-polymers of PLG or their homopolymers. The proportion of co-polymer in the PLG affects the degradation of the micro/ nanospheres and thus the rate of antigen release. A combination of quickly and slowly degrading micro/ nanosphere can provide primary and booster doses with a single administration of vaccine.

Micro/nanspheres, the delivery vehicles, in general do not have immunomodulatory effects, if an immunomodulator is not built into the particle. One exception is the stimulation of IL-1 production of polyacryl starch microparticles. The primary mode of action of microparticles seems to be targeted macrophages mediated by their hydrophobic surfaces, which particularly applies to PLG nanospheres. The nature of PLG coat protects its content from proteolytic degradation during this time[95].

Uptake and intracellular distribution of antigen in APCs: The first stage by which the adjuvant can influence the immune processing of the antigen is its attachment to APCs and its internalization. Amine containing compounds like dimethyl dioctadecyl ammonium (DDA) bromide and Avridine are reported to act by positive charge electrostatic attachment of antigen or by hydrophobic interaction. Similarly, the serum amyloid A-activating factor-1 (SAF-1) formulation would attack antigen by blocking polymer component[96]. These compounds are poorly soluble in water, but are well suited for incorporation into liposomes. Limitations of its proteolytic activity on the antigen may enhance its capacity to present antigen thereby giving more room for DCs to handle antigen. The DCs are professional APCs and are more effective in antigen presentation to lymphocytes than macrophages. While there is a vast literature on antigen processing and presentation by APCs to T-cells, there are limited numbers of reports about the influence of adjuvants. *In vitro* studies are difficult to perform as many adjuvants are not suitable for cell culture work. A unique property is the strong binding between Quillaja triterpenoids and cholesterol which may explain the stability of the complex[97].

Influence of adjuvant on the distribution of antigen following parenteral immunization: From the site of injection, antigens are transported to the draining lymph nodes and subsequently to various lymphatic

tissues, *e.g.* spleen and bone marrow. This process can be influenced by the adjuvants. Complete Freund's adjuvant causes a failure or delay in the transfer of antibody producing cells from draining lymph nodes to bone marrow due to granulopoiesis induced by CFA in the bone marrow. The antibody formation was concluded to be dependent on the migration of antigen activated lineages cells from elsewhere. In contrast, aluminium hydroxide not causing granulopoiesis, did not influence the antibody formation in the bone marrow. It is not clear whether aluminium hydroxide enhanced the bone marrow memory response either[98].

Adjuvant as antigen presenting systems and their influence on T- and B-cells response: Many substances have been shown to have adjuvant activity, but the adjuvant activity is poorly characterized. Besides the depot effect, an adjuvant should be evaluated by its capacity to influence the B-cell response by prompting induction of antibody of desired isotypes and subclasses. The modulation of the T-cell response is evaluated by the profile of cytokine evoked and the capacity to induce immune response under MHC class-I and class-II restriction. Most adjuvant studies are done in mice where the classification of immune response is easier to perform than in various other species.

In our recent studies[64,65], we have also shown defensins as mucosal adjuvants, which work under the above category. Defensins display immunostimulatory activities including a chemotactic effect for T-lymphocytes/immature DCs and secretion of proinflammatory cytokines. The reported results demonstrate the effectiveness of synthetic defensins to induce strong and long lasting B- and T- immune response through intranasal route using PLG-microsphere as a delivery vehicle. The studies have highlighted that defensin peptides have a potential role as mucosal adjuvant, might be responsible for the induction of cellular and humoral immunity when administered in mice through intranasal route with HIV-1 peptide antigens[64,65].

Some adjuvant formulations have a clear delivery function, as the ISCOMs, liposomes and nanoparticles. These formulations present soluble antigens in a particulate form and thereby exert a delivery function. A particulate form emphasizes the recognition of the antigen by APCs, particularly macrophages focusing on to the lymphatic system. Several strategies to form particulate antigen have been used to improve the B- and T-cell immune response to proteins or peptide epitopes by genetically engineering these peptides into self assembling particles.

Adjuvant and delivery systems for induction of mucosal immunity: In recent years, there has been a remarkable attention in adjuvant and vaccine delivery system for the induction of mucosal immune response, mainly by the oral and respiratory tract routes. The oral route is desired for convenience. However, there are three problems to overcome for oral vaccines; the acid pH in the stomach, the mucosal barrier, and the induction of tolerance which is clearly observed with subsequent parenteral immunization. CT produced by *Vibrio cholerae* induces strong secretary antibody response and a long-term immunological memory in mice to added unrelated antigen. The B-subunit of CT is good for targeting, but it has a weak adjuvant activity in contrast to the whole toxin[99]. Therefore, considerable efforts were laid down to modulate the A-subunit to abolish the toxicity, but to keep adjuvant activity. The respiratory tract is the second desired target for a mucosal vaccine. Furthermore, in this tract a locally applied antigen may induce tolerance possibly by γ/δ T-cells; this should be taken into consideration for prospective vaccines[100].

How do adjuvants engage the immune system?

Adjuvants in widespread clinical or experimental use have long been regarded as either immunostimulatory agents or as passive depots or vehicles. Most immunostimulatory adjuvants are ligands for PRR, although some act by providing a key component of the innate response (cytokines) or by stimulating an activation pathway directly, by passing the innate receptor (toxins). It is now becoming clear that adjuvants once thought to act primarily as depots or formulations, such as alum and emulsions, trigger innate responses and these responses are central to their adjuvant activity[101]. For these widely used adjuvants extensive efficacy data, and substantial human safety databases for vaccines with alum, MF59, AS03, and AS04 are available. For this reason, it is important to define the innate receptors and pathways utilized by these existing, empirically derived adjuvants and to try to establish correlations between observed safety and efficacy and mechanisms of action.

Adjuvant safety issues

The benefits from adjuvant incorporation into any vaccine formulation have to be balanced with the risk of adverse reactions[102]. Adverse reactions to adjuvants can be classified as local or systemic. Important local

reaction include pain, local inflammation, swelling, injection site necrosis, lympho-adenopathy, granuloma formation, ulcers and the generation of sterile abscesses. Systemic reactions include nausea, fever, adjuvant arthritis, uveitis, eosinophilia, allergy, anaphylaxis, organ specific toxicity, immunosuppression or autoimmune diseases and liberation of different cytokines[103]. Unfortunately potent adjuvant action is often correlated with increased toxicity as exemplified by the case of CFA which although potent is toxic for human use. Thus, one of the major challenges in adjuvant research is to gain potency while minimizing toxicity. The difficulty of achieving this objective is reflected in the alum despite being initially discovered over 80 years ago, remains the dominant human adjuvant in use today.

Innate immunity and adjuvant safety

The adoption of new adjuvants into licensed vaccines has been slowed by a variety of hypothetical safety concerns, especially the possibility of an increased risk of autoimmune disease. These concerns are based in part on two sets of observations. First, the infections can trigger or exacerbate some autoimmune diseases, and this can often be tied to elements of the innate response. For example, IFN are important in the pathogenesis of lupus, and disease flares are often triggered by viral infections[104]. Second, PRR ligands are capable of breaking tolerance in animal models, *e.g.* by overcoming inhibition by regulatory T-cells. Repeated injection with IFN-inducing PRR ligands can also enhance the growth and pathogenicity of *M. tuberculosis* in mouse models[105]. Consideration of several important differences between live infections and adjuvanted subunit vaccines can put these theoretical concerns in perspective. Innate immune stimulation with non-living vaccines is short-lived and focused on a local injection site and its draining lymphatic. Also, adjuvants are engineered to enhance the response to immunogenic nonself-antigens and only a few, if any, provide all of the activities needed to render a self-antigen sufficiently immunogenic to trigger autoimmunity, even if autoreactive T-cells are present. Perhaps the most compelling argument is the fact that many of the widely used and safest vaccines- the live, attenuated viral and bacterial vaccines- rely on activation through multiple PRR, yet have not been linked to an increased risk of any autoimmune disease. Similarly, the large human safety databases obtained with vaccines using either MF59 or AS04[106], both approved for human use in multiple countries, as well as more limited experience with several advanced experimental vaccines, have failed to support an increase in autoimmune or infectious diseases with these newer adjuvants.

Adjuvant regulatory requirement

Regulations of the human use of adjuvant are far more rigorous than those applied to veterinary vaccines. In addition to pre-clinical studies on the adjuvant itself, the combined antigen-adjuvant formulation also need to be subjected to toxicology prior to commencement of phase-I clinical trials[107]. The toxicological evolution is normally conducted in small animal species such as mice, rats or rabbits and should use the same administration route proposed for human use. The dose and frequency of vaccination for pre-clinical toxicology should be similar to or higher than the proposed human dose to minimize the ability to identify potential safety problems. Pre-clinical studies may also help in selecting the optimal vaccine dose[108].

Adjuvant limitations

In spite of progress made in the identification of mechanisms of adjuvant action, alum remains the dominant adjuvant for the human vaccines. Although many other adjuvants have been proposed over the years, these have failed to be successful in human largely because of toxicity, and problems related to stability, bioavailability and cost. Because of effects of size, electric charge and hydrophobicity which regulate the incorporation of proteins into the adjuvant formulation, it is difficult to predict on an empirical basis which adjuvant will work most effectively with a particular protein or peptide. Moreover, epitope modification may occur during formulation or conjugation. In the case of carrier proteins, a pre-existing immunity to the carrier protein is the major limitation[109]. Furthermore, each adjuvant generates a characteristics immune response profile. For example, the inability of alum based adjuvant to induce Th1 antibody isotype or cellular immune responses and their poor adjuvant effect on polysaccharide antigens limits their applicability to many vaccines.

Future perspectives

Several forces are converging to drive increased research and development efforts in adjuvant design and discovery. First and foremost are the recent and dramatic breakthroughs in theoretical and mechanistic understanding of innate immunity and how it drives antigen-specific responses and the generation of

immunological memory. This new appreciation of innate defense mechanisms provides a foundation for rational approaches to immunopotentiator discovery and optimization. Several first-generation candidates (*e.g.*, CpG, monophosphoryl lipid-A and imidazoquinolines) have shown some efficacy in experimental animals and in phase-1 studies in humans. Second, the trend in vaccine development away from traditional whole-cell or virus vaccines to subunit vaccines has shown that isolated antigens often lack sufficient immunogenicity, thus requiring the addition of potent adjuvants. Finally, the lack of vaccines for important disease targets such as HIV, hepatitis C virus (HCV), herpes simplex virus (HSV), *Neisseria meningitides* and others increases the need for improved vaccine adjuvants capable of boosting the antigen-specific immune response to protective levels against these insidious pathogens. Although there is a growing acceptance by regulatory agencies and commercial vaccine producers that improved vaccine adjuvants are needed to meet the infectious diseases, at present, the safety and regulatory hurdles that will be encountered with the addition of novel immunopotentiators and delivery systems to final vaccine formulations may be significant and are still largely ill defined. The key focus should be on separating the potential increases in immune toxicity from improved immunogenicity provided by vaccine adjuvants. It is likely that improved formulation and controlled release of potent immunopotentiators will limit toxicities while increasing efficacy. In addition, the growing numbers of immunopotentiators, targeting diverse innate immune mechanisms, should allow for the identification of candidates with improved therapeutic indices. Thus, the long-term goal should focus on selection of the optimal platforms and identification of the key innate immune targets for induction of potent, but safe, immune responses. The mechanistic understanding of the innate immune system and the tools to manipulate it are growing, and together these will make a significant impact on vaccine development in the near future.

What have we learned from studies of vaccines and adjuvants?

The immune system is optimized to generate adaptive responses to microbial antigens delivered to APCs in intimate association with PRR ligands, as would be the case for microbial infections and live attenuated vaccines. For subunit vaccine candidates, co-delivery has been accomplished by covalent coupling of TLRs to purified proteins or by constructing recombinant fusion proteins consisting of antigen and the TLR ligand[110]. In these examples, the potency of the linked vaccine is 10-100 times that of a comparable mix of separate components. In the case of CpG-ODN conjugates, coupling of an ODN enhances antigen uptake and cross-presentation in DCs, although the enhanced uptake is not TLR dependent[111]. Co-delivery of antigens and PRR ligands can also be accomplished by association (covalent or noncovalent) of both within a large particulate structure, *e.g.* VLPs and synthetic nano- and microparticles[112].

The enhanced efficiency of the co-delivery may be simply quantitative; uptake of enough linked antigen for effective presentation will inherently provide a stimulatory amount of the linked PRR ligand, and enhanced uptake would lead to preferential presentation of the linked antigen. However, co-delivery may also lead to preferential handling of antigens associated with PRR ligands, by facilitating antigen presentation at the level of individual lysosomes. Many vaccine candidates with this strategy have reached early stage clinical studies, and this represents one of the most promising new directions in vaccine development.

Conclusion

Adjuvants have long been of great interest for vaccine development in the clinical and basic immunology. Advances of the past decade in understanding innate immunity have brought a wider interest in understanding how existing adjuvants work and how these may be improved. All adjuvants appear to stimulate components of the innate immune system, but the diversity of mechanisms used by even a short list of well-studied adjuvants is impressive. Adjuvants currently used in humans enhance humoral immunity, but many new adjuvants in clinical or pre-clinical development are focused on enhancing specific types of T-cell responses and generating the multi-faceted immune responses that may be needed for challenging diseases such as malaria and HIV. Although well-defined ligands for PRR have attracted most of the attention, it is clear that strategies for formulation and delivery of subunit vaccines can profoundly influence T-cell immunity, most notably by facilitating cross-presentation of antigen by DCs. Along the path of development of new vaccines and adjuvants lies an unparalleled opportunity to study the immune responses of large populations of healthy humans. No other form of defined "experimental" challenge can be as easily and ethically given to humans, and mechanistic studies incorporated as part of the clinical development of new adjuvants can teach us a great deal about the human immune system.

References

1. Petrovsky N, Cooper PD. Carbohydrate-based immune adjuvants. *Expert Rev Vaccines* 2011; *10* : 523-37.
2. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. *Immunol Cell Biol* 2004; *82* : 488-96.
3. Rivas E, Gomez-Arnaiz M, Ricoy JR, Mateos F, Simón R, García-Peñas JJ, *et al.* Macrophagic myofasciitis in childhood: a controversial entity. *Pediatr Neurol* 2005; *33* : 350-6.
4. Gherardi RK, Coquet M, Cherin P, Belec L, Moretto P, Dreyfus PA, *et al.* Macrophagic myofasciitis lesions assess long-term persistence of vaccine-derived aluminium hydroxide in muscle. *Brain* 2001; *124* : 1821-31.
5. Holt LB. Quantitative studies in diphtheria prophylaxis; the primary response. *Br J Exp Pathol* 1949; *30* : 289-97.
6. Freund J. The effect of paraffin oil and mycobacteria on antibody formation and sensitization; a review. *Am J Clin Pathol* 1951; *21* : 645-56.
7. Kataoka K, Fujihashi K. Dendritic cell-targeting DNA-based mucosal adjuvants for the development of mucosal vaccines. *Expert Rev Vaccines* 2009; *8* : 1183-93.
8. McCartney S, Vermi W, Gilfillan S, Cella M, Murphy TL, Schreiber RD, *et al.* Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. *J Exp Med* 2009; *206*: 2967-76.
9. Steinhagen F, Kinjo T, Bode C, Klinman DM. TLR-based immune adjuvants. *Vaccine* 2011; *29* : 3341-55.
10. Cox JC, Coulter AR. Adjuvants - a classification and review of their modes of action. *Vaccine* 1997; *15* : 248-56.
11. Degten WG, Jansen T, Schijns VE. Vaccine adjuvant technology: from mechanistic concepts to practical applications. *Expert Rev Vaccines* 2003; *2* : 327-35.
12. Verthelji D, Klinman DM. Immunoregulatory activity of CpG oligonucleotides in human and non-human primates. *Int Immunol* 2003; *109* : 64-71.
13. Seya T, Akazawa T, Vehori J, Matsumoto M, Azuma I, Toyoshima ZK. Role of toll-like receptors and their adapters in adjuvant immunotherapy for cancer. *Anti Cancer Res* 2003; *23* : 4369-76.
14. Agarwal S, Agarwal A, Doughty B, Gertuitz A, Blenis J, Van Dyke T, *et al.* Different toll-like receptor agonists instruct dendritic cells to induce distinct Th responses *via* differential modulation of extracellular signal-regulated kinase- mitogen-activated protein kinase and C-Fos. *Immunology* 2003; *171* : 4984-9.
15. Sasaki S, Takeshita F, Xin KQ, Ishiii N Okuda K. Adjuvant formulations and delivery systems for DNA vaccines. *Methods* 2003; *31* : 243-54.
16. Beran J. Safety and immunogenicity of a new hepatitis B vaccine for the protection of patients with renal insufficiency including pre-haemodialysis and haemodialysis patients. *Expert Opin Biol Ther* 2008; *8* : 235-47.
17. Podda A. The adjuvanted influenza vaccines with novel adjuvants: Experience with the MF59- adjuvanted vaccine. *Vaccine* 2001; *19* : 2673-80.
18. Banzhoff A, Gasparini R, Laghi-Pasini F, Staniscia T, Durando P, Montomoli E, *et al.* MF59-adjuvanted H5N1 vaccine induces immunologic memory and heterotypic antibody responses in non-elderly and elderly adults. *Plos One* 2009; *4* : e4384.
19. Boyle J, Eastman D, Millar C, Camuglia S, Cox J, Pearse M, *et al.* The utility of ISCOMATRIX adjuvant for dose reduction of antigen for vaccines requiring antibody responses. *Vaccine* 2007; *25* : 2541-4.
20. Schwarz TF, Horacek T, Knuf M, Damman HG, Roman F, Drame M, *et al.* Single dose vaccination with AS03-adjuvanted H5N1 vaccines in a randomized trial induces strong and broad immune responsiveness to booster vaccination in adults. *Vaccine* 2009; *27* : 6284-90.
21. Halperin SA, Dobson S, McNeil S, Langley JM, Smith B, McCall-Sani R, *et al.* Comparison of the safety and immunogenicity of hepatitis B virus surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide and a licensed hepatitis B vaccine in healthy young adults. *Vaccine* 2006; *24* : 20-6.
22. Galli G, Medini D, Borgogni E, Zedda L, Bardelli M, Malzone C, *et al.* Adjuvanted H5N1 vaccine induces early CD4+ T-cell response that predicts long-term persistence of protective antibody levels. *Proc Natl Acad Sci USA* 2009b; *106* : 3877-82.
23. Leroux-Roels I, Roman F, Forgus S, Maes C, De Boever F, Drame M, *et al.* Priming with AS03 A-adjuvanted H5N1 influenza vaccine improves the kinetics, magnitude and durability of the immune response after a heterologous booster vaccination: An open non-randomised extension of a double-blind randomised primary study. *Vaccine* 2010; *28* : 849-57.
24. Vandepapeliere P, Horsmans Y, Moris P, Van Mechelen M, Janssens M, Koutsoukos M, *et al.* Vaccine adjuvant systems containing monophosphoryl lipid A and QS21 induce strong and persistent humoral and T-cell responses against hepatitis B surface antigen in healthy adult volunteers. *Vaccine* 2008; *26* : 1375-86.
25. Galli G, Hancock K, Hoschler K, DeVos J, Praus M, Bardelli M, *et al.* Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine. *Proc Natl Acad Sci USA* 2009a; *106* : 7962-7.
26. Khurana S, Chearwae W, Castellino F, Manischewitz J, King LR, Honorkiewicz A, *et al.* Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. *Sci Transl Med* 2010; *2* : 15ra5.
27. Huleatt JW, Jacobs AR, Tang J, Desai P, Kopp EB, Huang Y, *et al.* Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. *Vaccine* 2007; *25* : 763-75.
28. Malherbe L, Mark L, Fazilleau N, McHeyzer-Williams LJ, McHeyzer-Williams MG. Vaccine adjuvants alters TCR-based selection thresholds. *Immunity* 2008; *28* : 698-709.
29. Lindblad EB. Aluminium compounds for use in vaccines. *Immunol Cell Biol* 2004; *82* : 497-505.
30. Byars NE, Allison AC. Immunologic adjuvants: general properties, advantages and limitations. Laboratory methods in Immunology, *Boca Raton, CRC Press* 1990; 39-51.
31. Edelman R. Vaccine Adjuvants. *Res Infect Dis* 1980; *2* : 370-83.

32. Nentra MR, Pringault E Krachenbuhl JP. Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Ann Rev Immunol* 1996; *14* : 275-300.
33. Goto N, Kato H, Mayeama JI, Eto K Yoshihara S. Studies on the toxicities of aluminium hydroxide and calcium phosphate as immunological adjuvants for vaccines. *Vaccine* 1993; *11* : 914-8.
34. He Q, Mitchell A, Morcol T, Bell SJD. Calcium phosphate nanoparticles induce mucosal immunity and protection against herpes simplex virus type 2. *Clin Diagn Lab Immunol* 2000; *9* : 899-903.
35. Jiang D, Premchandra GS, Johnson C, Hem SL. Structure and adsorption properties of commercial calcium phosphate adjuvant. *Vaccine* 2004; *23* : 693-8.
36. Jackson LR, Fox JG. Institutional policies and guidelines on adjuvants and antibody production. *ILAR J* 1995; *37* : 141-50.
37. Bomford R. The comparative selectivity of adjuvants for humoral and cell-mediated immunity. II. Effect on delayed-type hypersensitivity in the mouse and guinea pig, and cell-mediated immunity to tumour antigens in the mouse of Freund's incomplete and complete adjuvants, alhydrogel, Corynebacterium parvum, Bordetella pertussis, muramyl dipeptide and saponin. *Clin Exp Immunol* 1980; *39* : 435-41.
38. Freund J. The mode of action of immunologic adjuvants. *Adv Tuberc Res 1956; 7* : 130-48.
39. Dvorak HF, Dvorak AM, Simpson BA, Richerson HB, Leskowitz S, Karnovsky MJ. Cutaneous basophil hypersensitivity II: A light and electron microscopic description. *J Exp Med* 1970; *132* : 558-82.
40. Dar P, Kalaivanan R, Sied N, Mamo B, Kishore S, Suryanarayana VV, *et al.* Montanide ISA™ 201 adjuvanted FMD vaccine induces improved immune responses and protection in cattle. *Vaccine*. 2013; *31* : 3327-32.
41. Frey S, Poland G, Percell S, Podda A. Comparison of the safety, tolerability, and immunogenicity of a MF59-adjuvanted influenza vaccine and a non-adjuvanted influenza vaccine in non-elderly adults. *Vaccine* 2003; *21* : 4234-7.
42. Traquina P, Morandi M, Contorni M, Van Nest G. MF59 adjuvant enhances the antibody response to recombinant hepatitis B surface antigen vaccine in primates. *J Infect Dis* 1996; *174* : 1168-75.
43. Seubert A, Monaci E, Pizza M, O'Hagan DT, Wack A. The adjuvants aluminium hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. *J Immunol* 2008; *180* : 5402-12.
44. Mbow ML, De Gregorio E, Valiante NM, Rappuoli R. New adjuvants for human vaccines. *Curr Opin Immunol* 2010; *22* : 411-6.
45. Lee JB, Jang JE, Song MK, Chang J. Intranasal delivery of cholera toxin induces Th17- dominated T-cell response to bystander antigens. *PLoS One* 2009; *4* : e5190.
46. Orozco-Morales M, Sánchez-García FJ, Guevara-Salazar P, Arrieta O, Hernández-Pedro NY, Sánchez-García A, *et al.* Adjuvant immunotherapy of C6 glioma in rats with pertussis toxin. *J Cancer Res Clin Oncol* 2012; *138* : 23-33.
47. Ghose C, Kalsy A, Sheikh A, Rollenhagen J, John M, Young J, *et al.* Transcutaneous immunization with Clostridium difficile toxoid A induces systemic and mucosal immune responses and toxin A-neutralizing antibodies in mice. *Infect Immun* 2007; *75* : 2826-32.
48. Gupta P, Singh MK, Singh Y, Gautam V, Kumar S, Kumar O, *et al.* Recombinant Shiga toxin B subunit elicits protection against Shiga toxin via mixed Th type immune response in mice. *Vaccine* 2011; *29* : 8094-100.
49. Lowell GH, Kaminski RW, Grate S, Hunt RE, Charney C, Zimmer S, *et al.* Intranasal and intramuscular proteosome-staphylococcal enterotoxin B (SEB) toxoid vaccines: immunogenicity and efficacy against lethal SEB intoxication in mice. *Infect Immun* 1996; *64* : 1706-13.
50. Qiu HN, Wong CK, Chu IM, Hu S, Lam CW. Muramyl dipeptide mediated activation of human bronchial epithelial cells interacting with basophils: a novel mechanism of airway inflammation. *Clin Exp Immunol* 2013; *172* : 81-94.
51. Bessler WG, Baier W, Esche U, Hoffmann P, Heinevetter L, Wiesmüller KH, *et al.* Bacterial lipopeptides constitute efficient novel immunogens and adjuvants in parenteral and oral immunization. *Behring Inst Mitt* 1997; *98* : 390-9.
52. Burt D, Mallett C, Plante M, Zimmermann J, Torossian K, Fries L. Proteosome-adjuvanted intranasal influenza vaccines: advantages, progress and future considerations. *Expert Rev Vaccines* 2011; *10* : 365-75.
53. Nakanishi T, Kunisawa J, Hayashi A, Tsutsumi Y, Kubo K, Nakagawa S, *et al.* Positively charged liposomes function as an efficient immunoadjuvant in inducing cell-mediated immune response to soluble proteins. *J Control Release* 1999; *61* : 233-40.
54. Katre NV. Liposome-based depot injection technologies; how versatile are they? *Am J Drug Deliv* 2004; *2* : 213-27.
55. Ronnberg B, Fekadu M, Morien B. Adjuvant activity of non-toxic *Quillaja saponaria* Molina components for use in ISCOM matrix. *Vaccine* 1995; *13* : 1375-82.
56. Fossum C, Bergström M, Lövgren K, Watson DL, Morein B. Effect of ISCOMs and their adjuvant moiety (matrix) on the initial proliferation and IL-2 responses: comparison of spleen cells from mice inoculated with iscoms and/or matrix. *Cell Immunol* 1990; *129* : 414-25.
57. Sjolander S, Drave D, Davis R, Beezum L, Pearse M, Cox JC. Intranasal immunisation with influenza- ISCOMATRIX adjuvanted influenza vaccine. *Vaccine* 2003; *21* : 946-9.
58. Rimmelzwann GF, Baars M, VanAmerongen G, VanBeek R, Oestrhaus AD. A single dose of an ISCOM influenza Vaccine induces long- lasting protective immunity against homologous challenge infection but fails to protect cynomologus macaques against distant drift variants of influenza A (H3N2) viruses. *Vaccine* 2001; *20* : 158-63.
59. Mukherjee C, Mäkinen K, Savolainen J, Leino R. Chemistry and biology of oligovalent β-(1→2)-linked oligomannosides: New insights into carbohydrate-based adjuvants in immunotherapy. *Chemistry* 2013; *19* : 7961-74.
60. Pun PB, Bhat AA, Mohan T, Kulkarni S, Paranjape R, Rao DN. Intranasal administration of peptide antigens of HIV with mucosal adjuvant CpG ODN co-entrapped in microparticles enhances the mucosal and systemic immune responses. *Int Immunopharmacol* 2009; *9* : 468-77.

61. Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM. CpG DNA as a vaccine adjuvant. *Expert Rev Vaccines* 2011; *10* : 499-511.

62. Yang D, Biragyn A, Hoover DM, Lubkowski J, Oppenheim JJ. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu Rev Immunol* 2004; *22* : 181-215.

63. Yang D, Biragyn A, Kwak LW, Oppenheim JJ. Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol* 2002; *23* : 291-6.

64. Mohan T, Mitra D, Rao DN. Nasal delivery of PLG microparticle encapsulated defensin peptides adjuvanted gp41 antigen confers strong and long-lasting immunoprotective response against HIV-1. *Immunol Res.* In press 2013.

65. Mohan T, Sharma C, Bhat AA, Rao DN. Modulation of HIV peptide antigen specific cellular immune response by synthetic α- and β-defensin peptides. *Vaccine* 2013; *31* : 1707-16.

66. Young JW, Inaba K. Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. *J Exp Med* 1996; *183 :* 7-11.

67. Taylor CE. Cytokines as adjuvants for vaccines: antigen-specific responses differ from polyclonal responses. *Infect Immun* 1995; *63* : 3241-4.

68. Nambiar JK, Ryan AA, Kong CU, Britton WJ, Triccas JA. Modulation of pulmonary DC function by vaccine encoded GM-CSF enhances protective immunity against *Mycobacterium tuberculosis* infection. *Eur J Immunol* 2010; *40* : 153-61.

69. LeBon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type-I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells *in-vivo*. *Immunity* 2001; *14* : 461-70.

70. Staats HF, Ennis Jr FA. IL-1 is an effective adjuvant for mucosal and systemic immune responses when co-administered with protein immunogens. *J Immunol* 1999; *162* : 6141-7.

71. Abraham E, Shah S. Intranasal immunization with liposomes containing IL-2 enhances bacterial polysaccharide antigen-specific pulmonary secretory antibody response. *J Immunol* 1992; *49* : 3719-26.

72. Kishimoto T. Interleukin-6: discovery of a pleiotropic cytokine. *Arth Res Ther* 2006; *8* (Suppl 2): S2.

73. Berm´udez-Humar´an LG, Cortes-Perez NG, Lef`evre F, Guimarães V, Rabot S, Alcocer-Gonzalez JM, *et al.* A novel mucosal vaccine based on live lactococci expressing E7 antigen and IL-12 induces systemic and mucosal immune responses and protects mice against human papillomavirus type 16-induced tumors. *J Immunol* 2005; *175* : 7297-302.

74. Shanmugham LN, Petrarca C, Frydas S, Donelan J, Castellani ML, Boucher W, *et al.* IL-15 an immunoregulatory and anti-cancer cytokine: Recent advances. *J Exp Clin Can Res* 2006; *25* : 529-36.

75. Eberl M, Beck E, Coulson PS, Okamura H, Wilson RA, Mountford AP. IL-18 potentiates the adjuvant properties of IL-12 in the induction of a strong Th1 type immune response against a recombinant antigen. *Vaccine* 2000; *18* : 2002-8.

76. Huffnagle GB, Strieter RM, McNeil LK, McDonald RA, Burdick MD, Kunkel SL, *et al.* Macrophage inflammatory protein-1α (MIP-1α) is required for the efferent phase of pulmonary cell-mediated immunity to a *Cryptococcus neoformans* infection. *J Immunol* 1997; *159* : 318-27.

77. Manocha M, Pal PC, Chitralekha KT, Thomas BE, Tripathi V, Gupta SD, *et al.* Enhanced mucosal and systemic immune response with intranasal immunization of mice with HIV peptides entrapped in PLG microparticles in combination with Ulex Europaeus-I lectin as M cell target. *Vaccine* 2005; *23* : 5599-617.

78. Malyala P, Singh M. Microparticle adjuvants: preparation and formulation with antigens. *Meth Mol Biol* 2010; *626* : 91-101.

79. Oyewumi MO, Kumar A, Cui Z. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. *Expert Rev Vaccines* 2010; *9* : 1095-107.

80. Esparza I, Kissel T. Parameters affecting the immunogenicity of microencapsulated tetanus toxoid. *Vaccine* 1992; *10* : 714-20.

81. Rakhmilevich AL, Imboden, Hao Z, Macklin MD, Robert T, Wright KM, *et al.* Effective particle-mediated vaccination against mouse melanoma by co-administration of plasmid DNA Encoding Gp100 and granulocyte-macrophage colony-stimulating factor. *Clin Cancer Res* 2001; *7 :* 952-61.

82. Kemp MM, Kumar A, Mousa S, Park TJ, Ajayan P, Kubotera N, *et al.* Synthesis of gold and silver nanoparticles stabilized with glycosaminoglycans having distinctive biological activities. *Biomacromolecules* 2009; *10* : 589-95.

83. Jiang L, Li X, Liu L, Zhang Q. Cellular uptake mechanism and intracellular fate of hydrophobically modified pullulan nanoparticles. *Int J Nanomed* 2013; *8* : 1825-34.

84. Kawada J, Wada H, Isobe M, Gnjatic S, Nishikawa H, Jungbluth AA, *et al.* Heteroclitic serological response in esophageal and prostate cancer patients after NY-ESO-1 protein vaccination. *Int J Cancer* 2012; *130* : 584-92.

85. Lövgren Bengtsson K, Morein B, Osterhaus AD. ISCOM technology-based Matrix M adjuvant: success in future vaccines relies on formulation. *Expert Rev Vaccines* 2011; *10* : 401-3.

86. Moser C, Amacker M, Zurbriggen R. Influenza virosomes as a vaccine adjuvant and carrier system. *Expert Rev Vaccines* 2011; *10* : 437-46.

87. De Bruijn IA, Nauta J, Gerez L, Palache AM. The virosomal influenza vaccine Invivac: immunogenicity and tolerability compared to an adjuvanted influenza vaccine (Fluad in elderly subjects. *Vaccine* 2006; *24* : 6629-31.

88. Sominskaya I, Skrastina D, Dislers A, Vasiljev D, Mihailova M, Ose V, *et al.* Construction and immunological evaluation of multivalent hepatitis B virus (HBV) core virus-like particles carrying HBV and HCV epitopes. *Clin Vaccine Immunol* 2010; *17* : 1027-33.

89. Hernandez BY, Ton T, Shvetsov YB, Goodman MT, Zhu X. Human papillomavirus (HPV) L1 and L1-L2 virus-like particle-based multiplex assays for measurement of HPV virion antibodies. *Clin Vaccine Immunol* 2012; *19* : 1348-52.

90. Van Kampen KR, Zhang J, Foster JW, Chen DT, Marks D, Elmets CA, *et al.* Safety and immunogenicity of adenovirus vectored nasl and epicutaneous influenza vaccines in humans. *Vaccine* 2005; *23* : 1029-36.

91. Cantazaro AT, Koup RA, Rodeerer M, Bailer RT, Enama MF, Moodie Z, *et al.* Phase I safety and immunogenicity evaluation of a multiclade HIV-I candidate vaccine delivered by a replicon-defective recombinant adenovirus vector. *J Infect Dis* 2006; *194* : 1638-49.
92. Berenesi K, Guyali Z, Gonezol E, Pincus S, Cox WI, Michelson S, *et al.* A Canarypox vectorexpressing cytomegalous (CMV) phosphoprotien 65 induces long-lasting cytotoxic T-cell responses in human CMV-seronegative subjects. *Infect Dis* 2001; *183* : 1171-9.
93. Zak DE, Andersen-Nissen E, Peterson ER, Sato A, Hamilton MK, Borgerding J, *et al.* Ad5/HIV induces broad innate immune activation that predicts $CD8^+$ T-cell responses but is attenuated by preexisting Ad5 immunity. *Proc Natl Acad Sci USA* 2012; *109* : E3503-12.
94. HogenEsch H. Mechanisms of stimulation of immune response by aluminium adjuvants. *Vaccine* 2002; *20* : 534-9.
95. Yoo JY, Kim JM, Seo KS, Jeong YK, Lee HB, Khang G. Characterization of degradation behavior for PLGA in various pH condition by simple liquid chromatography method. *Biomed Mater Eng* 2005; *15* : 279-88.
96. Ray A, Shakya A, Kumar D, Ray BK. Overexpression of serum amyloid A-activating factor 1 Inhibits cell proliferation by the induction of cyclin-dependent protein kinase Inhibitor p21WAF-1/Cip-1/Sdi-1 Expression. *J Immunol* 2004; *172* : 5006-15.
97. Korsholm KS, Agger EM, Foged C, Christensen D, Dietrich J, Andersen CS, *et al.* The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes. *Immunology* 2007; *121* : 216-26.
98. Hamilton JA, Byrne R, Whitty GJ. Particulate adjuvants can induce macrophage survival, DNA synthesis, and a synergistic proliferative response to GM-CSF and CSF-1. *Leukoc Biol* 2000; *67* : 226-32.
99. Odumosu O, Nicholas D, Payne K, Langridge W. Cholera toxin B subunit linked to glutamic acid decarboxylase suppresses dendritic cell maturation and function. *Vaccine* 2011; *29* : 8451-8.
100. Yamamoto M, Kiyono H, Kweon MN, Yamamoto S, Fujihashi K, Kurazono H, *et al.* Enterotoxin adjuvants have direct effects on t cells and antigen-presenting cells that result in either interleukin-4-dependent or -independent immune responses. *J Infect Dis* 2000; *182* : 180-90.
101. Maraskovsky E, Schnurr M, Wilson NS, Robson NC, Boyle J, Drane D. Development of prophylactic and therapeutic vaccines using the ISCOMATRIX adjuvant. *Immunol Cell Biol* 2009; *87* : 371-6.
102. Warren HS, Chedid LA. Future prospects for vaccine adjuvants. *Crit Rev Immunol* 1986; *4* : 369-85.
103. Allison AC, Byans NE. Immunological adjuvants. Desirable properties and side effects. *Med Immunol* 1991; *28* : 279-84.
104. Zandman-Goddard G, Shoenfeld Y. Infections and SLE. *Autoimmunity* 2005; *38* : 473-85.
105. Antonelli LR, Gigliotti Rothfuchs A, Goncalves R, Roffe E, Cheever AW, Bafica A, *et al.* Intranasal Poly-IC treatment exacerbates tuberculosis in mice through the pulmonary recruitment of a pathogen-permissive monocyte/macrophage population. *J Clin Invest* 2010; *120* : 1674-82.
106. Verstraeten T, Descamps D, David MP, Zahaf T, Hardt K, Izurieta P, *et al.* Analysis of adverse events of potential autoimmune aetiology in a large integrated safety database of AS04 adjuvanted vaccines. *Vaccine* 2008; *26* : 6630-8.
107. Goldenthal KL, Covagnaro JA, Alving CR, Vogel FR. Safety evaluation of vaccine. Development Working Group. *AIDS Res Hum Retrovirus* 1993; *9* : 545-9.
108. Morein B, Lövgren K, Höglund S. Immunostimulating complex (ISCOM). In: Gregoriadis G, Allison AC, Poste G, editors. *Immunological adyuvants and vaccines*. New York: Plenum Publishing Corporation; 1989. p. 153-61.
109. Jennings R, Simms JR, Health AW. Adjuvants and delivery systems for viral vaccines. Mechanisms and potentials in modulations of the immune response to vaccine antigens. *Dev Biol* 1998; *92* : 19-28.
110. Tighe H, Takabayashi K, Schwartz D, Van Nest G, Tuck S, Eiden JJ, *et al.* Conjugation of immunostimulatory DNA to the short ragweed allergen *Amb a* 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* 2000; *106* : 124-34.
111. Heit A, Maurer T, Hochrein H, Bauer S, Huster KM, Busch DH, *et al.* Cutting edge: Toll-like receptor 9 expression is not required for CpG DNA-aided cross-presentation of DNA-conjugated antigens but essential for cross-priming of CD8 T cells. *J Immunol* 2003; *170* : 2802-5.
112. O'Hagan DT, De Gregorio E. The path to a successful vaccine adjuvant- The long and winding road. *Drug Discov Today* 2009; *14* : 541-51.

Reprint requests: Dr D.N. Rao, Professor, Department of Biochemistry, All India Institute of Medical Sciences
Ansari Nagar, New Delhi 110 029, India
e-mail: dnrao311@rediffmail.com, mohan.teena@gmail.com