Journal of Peptide Science
J. Peptide Sci. **9**: 405–418 (2003)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.467

JOURNAL OF PEPTIDE SCIENCE

Review

Lipid and Carbohydrate Based Adjuvant/Carriers in Immunology

ROSS P. McGEARY,[a,b] COLLEEN OLIVE[c] and ISTVAN TOTH[a,b]*

[a] School of Molecular and Microbial Sciences, The University of Queensland, Brisbane, Queensland, Australia
[b] School of Pharmacy, The University of Queensland, Brisbane, Queensland, Australia
[c] Division of Infectious Diseases and Immunology, Queensland Institute of Medical Research, Brisbane, Queensland, Australia

Received 27 February 2003
Accepted 28 February 2003

Abstract: This review discusses various issues regarding vaccines; what are they and how they work, safety aspects, the role of adjuvants and carriers in vaccination, synthetic peptides as immunogens, and new technologies for vaccine development and delivery including the identification of novel adjuvants for mucosal vaccine delivery. There has been a recent increase of interest in the use of lipids and carbohydrates as adjuvants, and so a particular emphasis is placed on adjuvants derived from lipids or carbohydrates, or from both.

Keywords: lipids; carbohydrates; adjuvants; carriers; immunology

INTRODUCTION

Vaccination is one of the major achievements of modern medicine. Recent approaches to vaccine design include the use of live attenuated microorganisms, killed microorganisms, and recombinant and non-recombinant protein subunit vaccines [1]. These vaccines differ in their mechanisms of action and often have serious delivery problems [2]. The vaccines available today are used successfully for protection against diseases such as smallpox, rabies, measles, yellow fever, tetanus, diphtheria and haemophilus influenza type B [3,4]. However, there remains a lack of safe and efficacious vaccines to protect against other infectious diseases including malaria, AIDS, herpes, dengue fever and some forms of viral hepatitis, which kill or maim millions of people each year.

Immunization can be divided into two types: systemic and mucosal immunization [5]. The systemic immune system functions differently to the immune system of the mucosal surfaces. Systemic immunization aims to induce protective immunity. On the other hand, mucosal immunity has the ability to produce pathogen-specific antibodies at the primary mucosal site of pathogen exposure, and to impede future infection at sites where the pathogen might next present itself [6]. Mucosal immunization is able to induce both mucosal and systemic immunity simultaneously and hence provides the optimal

Definitions: Antigen: a substance that provokes an immune response. Antibody: soluble protein molecule produced and secreted by B cells in response to an antigen and capable of binding to that specific antigen. Epitope: a unique shape carried on the antigen's surface which triggers a corresponding antibody response. Immunoadjuvant: a substance that enhances the immune-stimulating properties of a vaccine. Peyer's patches: a collection of lymphoid tissues in the intestinal tract. Protective immunity: complete resistance to disease, whether long lasting or temporary. Virulent: toxic, causing disease.

*Correspondence to: Istvan Toth, School of Molecular and Microbial Sciences and School of Pharmacy, University of Queensland, Brisbane 4072; e-mail: i.toth@pharmacy.uq.edu.au

BIOGRAPHIES

Dr Ross P. McGeary

Dr McGeary is a lecturer at The University of Queensland, where he holds joint appointments in the Chemistry Department and The School of Pharmacy. His main research interests are in the area of medicinal/biological chemistry — carbohydrate chemistry, peptide chemistry and drug delivery, as well as new synthetic methodologies. Before taking up his lectureship, Dr McGeary held postdoctoral positions in Cambridge, (UK), The IMB (Australia) and in Professor Toth's group at The University of Queensland. Dr McGeary has published over 30 papers, as well as several patents and book chapters. He is regional editor for *Mini-Reviews in Medicinal Chemistry*.

Dr Colleen Olive

Research Fellow Dr Olive has a strong standing in medical research from an immunological perspective. Through research at the John Curtin School of Medical Research, Australian National University, and the Department of Medicine, University of Queensland and Princess Alexandra Hospital, she has published extensively on the characterization of T cell receptors in autoimmune disease, kidney transplantation and cancer. She has been the recipient of research and travel grants including an Australian Academy of Science Travel Fellowship as part of the scientific visits to the United States of America, Canada and Mexico programme. She is a reviewer for several scientific journals and scientific granting bodies, and has been invited to the editorial board of *Gene Vaccines and Therapy Journal*. Over the past three years Dr Olive has focused on group A streptococcal vaccine research at The Queensland Institute of Medical Research and has made significant progress in this area. She has established a number of international and national collaborations, and was recently awarded the prestigious NHMRC R Douglas Wright Career Development Award. She also holds an adjunct appointment with The Australian Centre for International and Tropical Health and Nutrition, University of Queensland/QIMR. Dr Olive's current areas of interest are peptide-based vaccines, mucosal vaccines and adjuvants, and understanding the immunopathogenesis of rheumatic heart disease.

Professor Istvan Toth

Chair in Biological Chemistry and Professor of Pharmacy Professor Toth's major research interests are drug/peptide and vaccine delivery. New developments in drug/vaccine delivery are clearly likely to have enormous economic impacts upon the pharmaceutical and biotechnology industries.

At the School of Pharmacy, University of London and presently at UQ Toth built up a strong, very productive research group where the research orientation well suited the direction of modern multidisciplinary pharmaceutical sciences. He has refereeing duties for many international scientific journals and for scientific granting bodies. He recently obtained a Business/Higher Education Round Table (BHERT) Award: Outstanding Achievement in International Collaborative R&D. The award recognizes Professor Toth's research in the area of liposaccharides in drug/vaccine delivery. Toth has about 150 peer-reviewed publications and 40 patents. His book co-edited with G Keri, *Molecular Pathomechanism and New Trends in Drug Research* has just been published by Taylor & Francis. He is the Editor-in Chief of *Current Drug Delivery* and Board Member of the *Mini-Reviews in Medicinal Chemistry*. He regularly gives invited presentations.

immune protection in both systems. Mucosal immunizations are currently under enormous investigation as an alternative to conventional parenteral vaccination [7]. The gastrointestinal (GI), respiratory and genital tracts which are part of the mucosal surfaces, contain an abundance of immunocompetent cells such as B and T lymphocytes that present to be the main components of the mucosal immune system. Both the oral and intranasal routes of administration have been investigated in several studies with mixed results of success and failure.

The importance of childhood vaccination as one of the most cost-effective public health interventions has undeniably been demonstrated by the global eradication of smallpox, which has saved millions of lives. As part of the childhood vaccination regime, children are now routinely vaccinated against pertussis (whooping cough), tetanus, diphtheria, polio, measles, mumps, rubella (German measles) and haemophilus influenza type B. There is little doubt that disease prevention by vaccination is the key to public health. In addition to infectious diseases, autoimmune disorders and some cancers

may also be amenable to prophylactic and therapeutic treatment by vaccines.

This review will discuss various issues regarding vaccines; what are they and how they work, safety aspects, the role of adjuvants and carriers in vaccination, synthetic peptides as immunogens, and new technologies for vaccine development and delivery including the identification of novel adjuvants for mucosal vaccine delivery. There has been a recent increase of interest in the use of lipids and carbohydrates as adjuvants, and so a particular emphasis will be placed on adjuvants derived from lipids or carbohydrates, or from both. Prior to a discussion of vaccines, however, it is important to review the general principles of how an immune response is generated.

INDUCTION OF AN IMMUNE RESPONSE

The body continually encounters many different antigens; those that are present within the body are classified as 'self' antigens whereas those that are foreign to the body, such as contained within a virus or bacterium, are classified as 'non-self' antigens. During the development of the immune system, the immune system learns to discriminate between these two type of antigens, a process called self–non-self discrimination, such that an immune response is generated when the body is exposed to foreign antigens, whereas self antigens are essentially ignored due to immunological tolerance. The immune response generated involves the induction of protective antibodies by B cells (humoral immunity) and the activation of cytotoxic T cells (CTL) (cell-mediated immunity) [reviewed in [8–10]]. Following exposure to a foreign antigen, the first step in the generation of an immune response is the recognition of antigen by a specific cell surface immunoglobulin (Ig) receptor, followed by internalization by antigen-presenting cells (APC) and B cells. The antigen is subsequently broken down into small peptides containing epitopes, some of which become bound by class II molecules of the major histocompatibility complex (MHC) and are transported to the APC and B cell surface. Helper T cells express T cell receptors (TCR) which interact with the epitope/class II MHC complex on the APC. Additional cell–cell interactions involving co-stimulatory molecules allow the T cell to become fully activated. Once activated, the T cell recognizes B cells with the same specific epitope/class II MHC complex on the cell surface. This interaction between T cells and B cells triggers the B cell to differentiate into a plasma cell which produces antibody of the same specificity as that of the original Ig receptor. The antigen therefore must contain a helper T cell epitope, a short linear sequence recognized by the TCR, and a B cell epitope, usually a three-dimensional conformational structure that is recognized by the Ig receptor, to elicit an antibody response. Immunocompetent cells also produce cytokines which influence the type of immune response elicited.

The activation of CTL and cell-mediated immunity is required for protection against viral infections and in many cases of cancer. Similar to helper T cells, CTLs become activated following interaction with APC that have a specific epitope on the cell surface, but in this case the epitope is presented in association with MHC class I molecules. In a complex of cellular interactions, helper T cells also stimulate the APC to increase the expression of co-stimulatory molecules. The fully activated CTL recognizes cells bearing the specific epitope/class I MHC complex and causes the virus-infected or cancer cell to undergo apoptosis and cell death.

Vaccines: What are they and How do they Work?

Vaccines essentially mimic an attack on the body by an infectious agent which results in immunological memory that provides protection following a natural infection. They do this by inducing the immune system to recognize a non-virulent form of a pathogen (for example a virus or bacterium), that is contained within the vaccine, or a small portion of the pathogen such that when the immune system encounters the natural pathogen, a strong immune response is rapidly stimulated to prevent infection and subsequent illness. This complex immune defence system leads to the production of pathogen-specific antibodies (humoral immunity) and CTL (cell-mediated immunity) specific for epitopes within the native antigen, as described above in the recognition of foreign antigens, which are the body's armament against infections agents. A detailed review of the mechanisms of action, however, leading to protective immunity is beyond the scope of this review. In brief, antibodies bind to complement C3b receptors on the surface of a bacterial infectious agent. This activates the complement cascade which leads to the activation of the terminal complement membrane attack complex and bacterial lysis, prior to clearance by phagocytosis. In the case of viral infections and in many cases of cancer, the immune

system responds by inducing CTL which kill the virus-infected or cancer cell.

The design of vaccines is often complicated by the polymorphism of MHC molecules [11] — which present antigens to the immune system — and the variability of pathogenic antigens [12]. Ideally, a vaccine should provide coverage against all host MHC types as well as multiple pathogenic serotypes. Conventional vaccine preparations consist of live attenuated or killed organisms or components of these organisms. As vaccine candidates, antigens or parts of antigens may be represented in several forms which include recombinant proteins, purified proteins or synthetic peptides (reviewed in [13]).

Vaccines: Are they Safe?

Vaccines are one of the safest disease prevention measures and usually only cause minor side effects such as a local inflammatory reaction at the site of administration. Very serious adverse effects resulting from vaccination are extremely rare and it is clear that the benefits of vaccination outweigh the potential health risks to the individual. Cessation of mass vaccination campaigns due to unsubstantiated health concerns could have disastrous effects on public health and disease eradication efforts. There has been some concern regarding an association with the measles-mumps-rubella (MMR) vaccine and autism and the potential for triggering autoimmunity, although epidemiological studies do not support such associations [14,15]. The use of defined antigenic determinants that play an important role in protective immunity is often preferable to the whole organism or whole antigen for several reasons, one of which is to eliminate the possibility of the induction of autoimmunity due to immunological cross-reactivity between foreign and host self antigens [16].

The Role of Adjuvants and Carriers in Vaccination

The administration of a vaccine formulation requires both an adjuvant and a carrier to induce effective stimulation of the immune system and protective immunity. This is a critical issue particularly with newer generation vaccines such as subunit, recombinant and synthetic peptide vaccines which, despite containing purer antigens, tend to be poorly immunogenic when compared with live attenuated vaccine formulations [17,18]. The actions of an adjuvant are the depot formation of antigen within tissues, the activation of macrophages, and the facilitated targeting of antigen to APC resulting in the induction of an enhanced antigen-specific immune response [17]. Thus, an adjuvant is designed to facilitate effective uptake and presentation of antigens by APC. The most effective experimental adjuvants have an immunostimulatory component such as killed bacteria or a bacterial cell wall component such as bacterial lipopeptide, the lipid A portion of bacterial lipopolysaccharide or muramyl dipeptide from bacterial peptidoglycan. These components are potent activators of macrophages [19,20]. The efficacy of conventional vaccine formulations, administered parenterally and mucosally in experimental animal models has required the use of adjuvants such as FCA [21] and cholera toxin [22], respectively, that are not suitable for use in humans due to their toxicity. Current vaccine formulations licensed for human use mainly contain alum-based adjuvants (as aluminium hydroxide or phosphate) [23]. This limited choice of adjuvants for human vaccination reflects a compromise between a requirement for adjuvanticity and an acceptable low-level of toxicity. In addition, not all adjuvants are effective for all antigens, and there are currently no mucosal adjuvants licensed for human use. Therefore, recent research has focused on the identification of new adjuvants for human vaccination and the improvement of existing ones.

As discussed in the induction of an immune response to a foreign antigen, a helper T cell epitope is required, in addition to a B cell epitope, in order to stimulate T cells which are required to help the antigen-specific B cell mature into a plasma cell and produce antibody of the correct specificity. Essentially, a carrier provides a source of helper T cell epitopes which are necessary for successful vaccine efficacy. The most common carriers are large carrier proteins although recent studies indicate the potential of using synthetic helper T cell epitopes in vaccines.

Carrier Proteins

Unlike whole organisms and antigens, small synthetic peptides usually do not contain an appropriate helper T cell epitope to induce an antibody response and are therefore not effective vaccines by themselves. Such peptides, however, can be rendered immunogenic by conjugation to a carrier molecule which contains helper T cell epitopes. Carrier proteins are traditionally used as a source of helper T cell epitopes, but there are significant

disadvantages to their use including epitope suppression [24] and modification of the antigenic determinant(s) due to the chemical coupling reaction [25].

Synthetic Helper T Cell Epitopes

The assembly of vaccines containing synthetic T cell epitopes may solve some of the problems associated with the use of carrier proteins as a source of helper T cell epitopes. Immune responses to peptide-based immunogens have been achieved in experimental models using a number of different strategies including non-specific polymerization of peptides [26] and assembly of linear tandem (T cell epitope) — (B cell epitope) constructs [27]. There is the potential, however, for antibodies to be generated against epitopes formed at the T cell and B cell epitope junctions [28]. The use of synthetic peptides representing T cell epitopes, however, may alleviate the problem of carrier protein-induced epitope suppression [29] and the use of universal T cell epitopes that bind to different MHC molecules could potentially overcome MHC polymorphism and pathogenic antigen variability [30].

Synthetic Peptides as Immunogens

Synthetic peptides representing individual B cell epitopes are generally short sequences of amino acids that are recognized by an antibody and are poorly immunogenic, either because (1) they may be rapidly degraded before recognition by the immune system; (2) they do not contain an appropriate helper T cell epitope required for antibody production; or (3) conformational integrity, that is the correct three-dimensional structure, is lacking in the B cell epitope. Approaches to mimicking the conformational integrity of peptides have proved successful. An example of a conformational-dependent epitope is found in the M protein of group A streptococci (GAS) [31]. The immunogenicity of peptide vaccine candidates can be enhanced with an adjuvant. Recent advances in peptide vaccine technology, however, indicate the potential of novel delivery systems that are self-adjuvanting and hence do not require the addition of toxic adjuvants. This will be discussed later in this review.

The route of vaccination is an important determinant of success [32]. The parenteral route has several disadvantages, such as acceptance, systemic immunity coverage and most importantly, the route of administration may not follow the route of entry of many pathogens. Parenteral immunization, while effectively inducing systemic responses and clearing systemic infections, fails to provide protection at the mucosal surface where the majority of infections occur. Induction of mucosal responses after local exposure of antigens to the mucosal-associated lymphoid tissues, especially those in the upper respiratory tract and the gastrointestinal tract, leads to the production of secretory IgA (sIgA) antibodies which are not usually produced by systemic immunization.

Oral and nasal delivery are the most desirable means of drug and vaccine administration [32,33] but are not widely used due to the many barriers — mechanical (e.g. epithelial cells) and chemical (e.g. mucins) — posed by the gastrointestinal tract. In addition, preexisting secretory antibodies complex with antigens at mucosal surfaces and this further reduces antigen uptake [34]. Immunogenic sequences on protein molecules are mostly hydrophilic. Hydrophilicity prevents the immunogenic proteins from undergoing passive transcellular absorption across the GIT epithelium and from interacting with the lipid bilayer of the epithelium cell membrane, making transcellular absorption very difficult [35,36]. An additional serious problem of peptide-based vaccines is rapid proteolytic degradation. The GIT has evolved to breakdown dietary peptides and proteins into smaller amino acids. Vaccines must be protected from this proteolytic

Table 1 Different Vaccine Types and Problems[a]

Vaccine type	Problems
Live vaccine	Controlled attenuation normally required Risk of reversion to pathogenicity Certain risk of transmission Poorly defined composition
Inactivated vaccine	Multiple doses normally required Poorly defined composition Antigen must be produced by cultivation of a pathogen Mainly humoral responses Adjuvants normally needed
Subunit vaccine and nucleic acid vaccine	Antigen must be produced and purified by cultivation of a pathogen Multiple doses normally required Adjuvants needed

[a] Adapted from Hansson M, Nygren Pre-Ake, Stahl S. *Biotechnol. Appl. Biochem.* 2000; 96.

degradation in the GIT in order to ensure an adequate dose of antigen for effective immunization (Table 1).

Research is focused on the development of vaccine adjuvants with improved immunogenicity, reduced toxicity, universal efficacy, and the potential for delivery via other routes, particularly mucosal delivery for vaccination against many pathogens that infect mucosal surfaces. Recent advancements in vaccine immunology have seen progress in the development of sophisticated antigen delivery systems and the development of alternative adjuvants for vaccine delivery.

VACCINE-ADJUVANT SYSTEMS

Oil-in-Water Emulsions

Freund's incomplete adjuvant (FIA) is one of the oldest adjuvants used for immunization. It is composed of approximately 85% mineral oil and 15% emulsifier (mannide monooleate). This is then mixed in equal quantities with an aqueous antigen phase to give a water-in-oil emulsion ready for immunization. FIA is to be differentiated from Freund's *complete* adjuvant (FCA), an extremely potent adjuvant that additionally contains heat-killed mycobacteria. FIA produces good stimulation of humoral immunity, but is less reactogenic than FCA. FIA was used as an adjuvant in humans in Britain, up until the early 1960s [37]. The main disadvantages of Freund's incomplete adjuvant is its relatively weak adjuvanticity, and the occurrence of possible side effects including abscesses, muscle indurations and granulomas at the site of injection.

Another oil-in-water emulsion adjuvant is MF59. In this formulation the oil component is squalene, to which polyoxyethylene sorbitan monooleate (Polysorbate 80) and sorbitan trioloeate are added. Administration of MF59 as an adjuvant has been shown to lead to the recruitment of antigen-presenting cells to the site of injection, and to increase the uptake by these cells of soluble antigen. MF59 has recently been approved for use in an influenza vaccine in Italy [38].

Lipopeptides

Lipopeptides derived from bacterial lipoprotein have been shown in several animal species to be potent immunoadjuvants, macrophage activators and polyclonal B-lymphocyte stimulators [39]. For example, the lipopeptide P_3CSK_4 was shown to function as an adjuvant when administered to mice parenterally, nasally or orally, leading to marked increases in serum immunoglobulin responses [40,41].

The immunogenicity of antigenic peptide sequences can often be greatly enhanced by the incorporation of simple lipidic groups into the construct. For example, the incorporation of a palmitic acid onto the ε-amino group of a lysine residue on synthetic lipopeptides derived from *Plasmodium falciparum* has been shown to induce B cell, T-helper cell and CTL responses without additional adjuvant. Mucosal delivery (intranasal and sublingual) of these lipopeptides induces high serum antibody levels, and strong specific T-helper cell responses from the spleen and the inguinal lymph nodes [42]. Lipidated synthetic peptides derived from *Plasmodium falciparum* were shown to induce strong B- and T-helper cell responses in chimpanzees when administered without adjuvant [43], and lipopeptide formulations have been shown to induce antiviral cytotoxic lymphocyte responses in animals and in human clinical trials [44].

The mechanisms of the adjuvant activities of these lipopeptides is unclear, but it is likely that the long lipidic tails of the lipopeptides embed in the cell membranes, allowing access for the antigenic peptides into the cytoplasm [42].

Lipid A

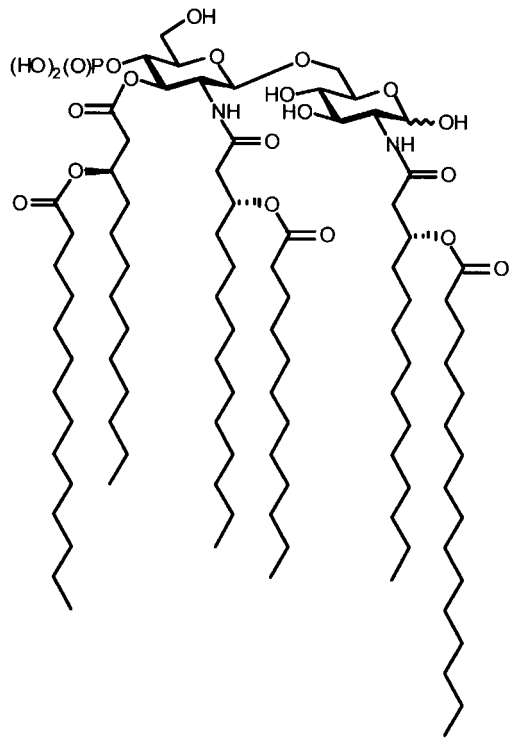
The toxicity and immunomodulating activity of the cell-surface lipopolysaccharide (endotoxin) from gram-negative bacteria have been long recognized [45]. It possesses the unusual property of acting as an adjuvant even when administered at a different site and at a different time than the antigen. The active agent of lipopolysaccharide has been shown to be lipid A. This is a disaccharide composed of two glucosamine units, two phosphate groups and five or six fatty acid chains (generally C_{12} to C_{16} in length). Lipid A is a potent adjuvant for both protein and carbohydrate antigens, and can lead to marked increases in both humoral and cell-mediated immunity. However, lipid A can induce a sepsis-like systemic inflammatory response syndrome, and so the high toxicity and pyrogenicity of lipid A has precluded its use as an adjuvant in human vaccines [46,47].

Monophosphoryl Lipid A (MPL)

Monophosphoryl lipid A is related to lipid A, but lacks the 1′-phosphate group. It is prepared

from the lipopolysaccharide of *Salmonella minnesota* R595 by acid hydrolysis. Subsequent mild alkaline treatment leads to specific removal of the fatty acid at position 3, resulting in the product known as MPL, which has even lower toxicity but strong immunostimulatory activity [48]. MPL is much less toxic than lipopolysaccharide, and yet leads to enhanced antibody and T cell responses. Like lipopolysacharide, MPL possesses the ability to enhance the generation of specific immunity without being directly associated with the antigen [49]. MPL has been used extensively as an adjuvant in human clinical trials for several infectious diseases and cancer. It is well tolerated and produces little, if any, local tissue reaction at the injection site. Its side effect profile is similar to aluminium salt adjuvants [46]. In mice, MPL has been shown to be an effective mucosal adjuvant for influenza vaccine, when administered both intranasally and orally [46]. MPL shows some heterogeneity of the lengths of the fatty acid sidechains. The major component is shown below (**1**).

Lipid A and MPL are thought to act via interactions with a particular member of the Toll-like receptor family, TLR-4, leading to induction of the innate immune response [50].

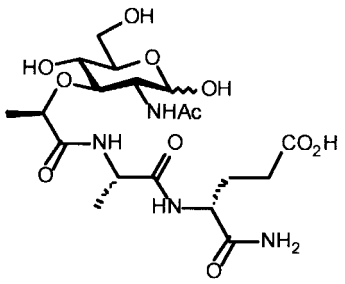


1

Muramyl Dipeptide

N-Acetyl muramyl-L-alanine-D-isoglutamine (**2**, muramyl dipeptide, MDP) is the adjuvant component of a peptidoglycan extracted from mycobacteria. MDP has a variety of physiological effects, including adjuvanticity, pyrogenicity and leucocytopoietic activity [51]. MDP and its derivatives induce the production of interleukin-1 [52].

MDP and its analogues have potent *in vivo* adjuvant activity when administered as water-in-oil emulsions, but MDP itself is a poor adjuvant when administered as an aqueous solution, due to its rapid excretion in the urine [45]. As a result, a number of lipophilic derivatives of MDP have been prepared, and their bioactivities have been reviewed [53]. MDP and its derivatives have been shown to be potent inducers of the cytokines interleukin-1, interleukin-6, interferon-γ and colony-stimulating factors (CSFs) in mice [54].



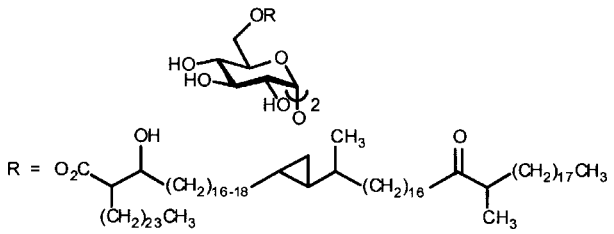
2

Trehalose-6-6′-dimycolate

The structure of the so-called cord factor from *Mycobacterium tuberculosis* has been shown to be the 6,6-dimycolate ester of trehalose (**3**) [55]. This compound has a number of biological activities, including high toxicity, antitumour activity and stimulation of host resistance against infections. A number of analogues of trehalose-6-6′-dimycolate have been synthesized for structure–activity studies using mycolic acid isolated from *Mycobacterium tuberculosis*. Some attenuation of the toxicity was possible, while retaining adjuvant activity [45].

Saponins

Triterpenoid glycosides (saponins) from the bark of *Quillaja saponaria* (the South American soap

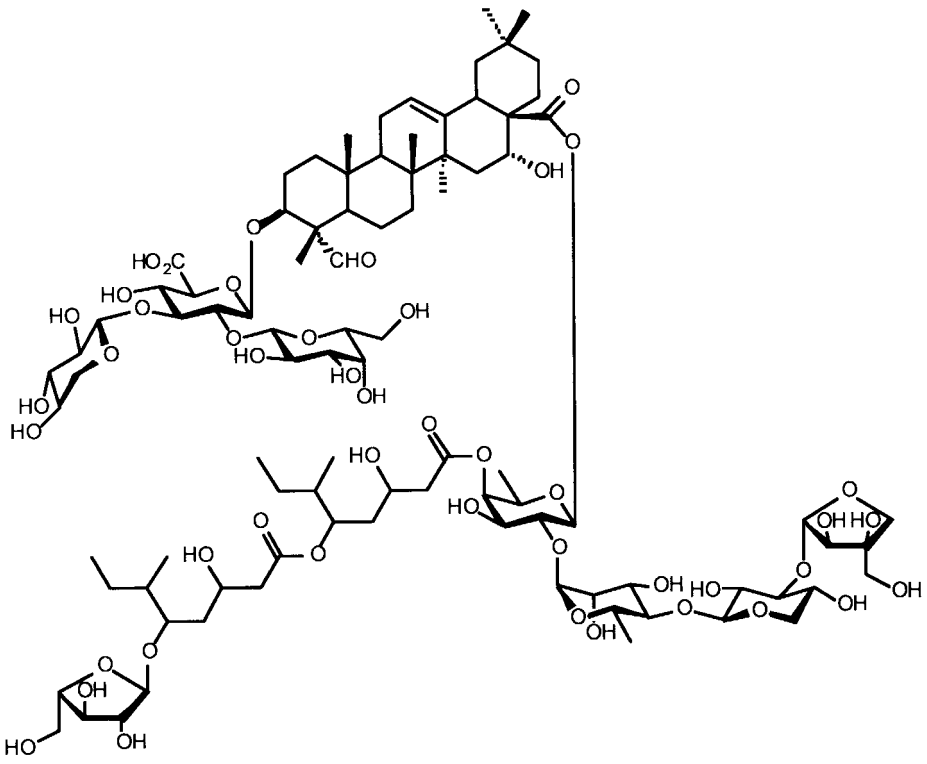


3

tree) have a long history of use as vaccine adjuvants for animals [56]. The crude extracts are complex mixtures of glycosylated triterpenoids, tannins and polyphenols, but the adjuvant activity is associated with the saponin fractions [57]. Partially purified saponins (Quil A and Spikoside) and defined entities or mixtures (QS-21 (Stimulon™) and ISCOPREP™ 703) are available [52]. The water-soluble QS-21 in particular, has been the subject of much study, because of its low toxicity and potent adjuvant activity, in particular its ability to induce the T-helper 1 cytokines (IL-2 and IFN-γ) and IgG2a antibodies [58,59]. It has also been used as an adjuvant for DNA vaccines, via both intramuscular and intranasal administration [60]. Sjölander and Cox have reviewed the use of saponins as adjuvants for orally delivered vaccines [61].

QS-21 (**4**) has been used successfully in a number of animal vaccination experiments, with a range of antigens. It was shown to elicit higher antibody responses than aluminium hydroxide, and similar antibody responses to FCA [62]. More recently it has been the subject of human Phase I and Phase II clinical trials as an adjuvant for vaccines, including cancer immunotherapeutics (breast, prostate and melanoma), HIV recombinant envelope, influenza, herpes, hepatitis B and malarial antigens. It is currently in Phase III trials as an adjuvant for the ganglioside portion of the GM2-KLH melanoma antigen conjugate. Typical doses are 50–100 μg per patient, administered either subcutaneously or intramuscularly. The major problems associated with QS-21 and other saponins appears to be their ability to cause haemolysis of red blood cells, and dose-dependent short-term pain at the injection site experienced by some participants in clinical trials [63].

Saponins are known to intercalate into cell membranes, leading to the formation of pores.



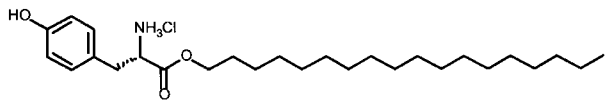
4

Although it is not known whether this phenomenon is related to the ability of saponins to act as adjuvants, O'Hagan *et al.* have suggested that this mechanism may allow antigens to access the cytoplasm, and so promote the endogenous pathway for antigen presentation, for CTL induction [64].

A delivery system for antigens and Quillaja saponin adjuvants, called immune stimulating complexes (ISCOMS) has been described [65–67]. ISCOMS are supramolecular structures, about 30–40 nm in diameter, and are composed of Quillaja saponins, cholesterol and phospholipid in a molar ratio of approximately 1 : 1 : 1. Antigens may be integrated into these structures by mixing antigens and saponins with detergent-solubilized cholesterol and phospholipid, followed by removal of the detergent by dialysis or ultracentrifugation, resulting in the spontaneous formation of ISCOMS. A defined composition called Iscoprep 703™ is in human clinical trials [68]. The advantages of this formulation of the saponin adjuvant are that lower doses of both antigen and adjuvant are required to achieve the same immunological response, and local reactions at the site of injection can be avoided, and the haemolytic activity of the saponins is significantly reduced. Iscoms induce good T-helper 1 and T-helper 2 responses, and strong CTL responses [52].

Stearyl Tyrosine

Stearyl tyrosine (**5**), the octadecyl ester hydrochloride salt of the amino acid tyrosine, is a synthetic, low molecular weight adjuvant. It was designed as a mimic of surface-active adjuvants, with the aim of reducing the toxicity associated with this detergent-like class of molecule, while retaining or improving the adjuvanticity [69]. Stearyl tyrosine retains a cationic charge and a lipophilic tail, and its poor water solubility (<0.01% w/w) allows it to adsorb soluble antigens to form insoluble complexes, thus functioning as a depot, or slow-release system.



5

Stearyl tyrosine is biocompatible and biodegradable. It has very low acute or chronic toxicity, and is composed of two naturally occurring and non-toxic components: stearyl alcohol and tyrosine. It has been studied in a number of animal models, without any observable toxicity [69].

Gupta and Siber compared the adjuvant activities of stearyl tyrosine and aluminium phosphate for tetanus toxoid. They found that while aluminium phosphate elicited higher toxin-neutralizing and IgG antibodies after primary immunization, this difference was no longer present after secondary immunization. They further showed that stearyl tyrosine adsorbed toxoid induced relatively higher IgG2a and IgG2b responses than aluminium phosphate, which induced the highest IgE antibodies [70]. Stearyl tyrosine has also been shown to stimulate T-helper 1 responses [71].

Liposomes

Liposomes are another class of oil-in-water emulsion that can transport antigens to lymphoid tissues following local injection. When given orally, they are also able to be endocytosed by M cells, allowing the antigen to be transported to the lymph cells in the Peyer's patches [72]. Liposomes are single or multilamellar bilayer membrane vesicles that can vary in size from 20 nm to 3 μ m. The lipid components are usually phospholipids or other amphiphiles, often supplemented with cholesterol and other charged lipids [73]. Liposomes can entrap both hydrophobic and water-soluble antigens, either within, or between the lipid bilayers [74,75].

The first description of the use of liposomes as immunological adjuvants was reported by Allison and Gregoriadis, who showed that diphtheria toxoid encapsulated in liposomes elicited a stronger humoral immune response after injection into mice, than the free toxoid [76]. Gregoriadis [77] has since presented a great deal of work that has demonstrated the ability of liposomes (phospholipid vesicles) to produce humoral and cell-mediated immunity to a large collection of antigens. Liposomes are poorly immunogenic themselves, but are useful for presenting antigens to the immune system, either encapsulated within the liposome, or adsorbed on the surface [78]. Zigterman *et al.* have shown for example, that the attachment of simple sugars to liposomes led to an increase in the humoral immune response to these antigens. The liposomes most probably act by supplying macrophages with a steady supply of entrapped antigens, at a rate to favour its efficient processing [79].

Related to the liposomes are the 'immunopotentiating reconstituted influenza virosomes' (IRIVs) which function as both carrier and adjuvant. IRIVs

are similar to liposomes, but contain influenza-derived neuraminidase and haemagglutinin on their outer surface which makes them much more fusogenic than ordinary liposomes, and thus able to deliver antigen to host cells more efficiently. This approach to vaccine development has been very successful, with two virosomal vaccines currently on the market. The first is effective against influenza (Inflexal® BERNA) while the second offers protection against hepatitis A (Epaxal® BERNA) [80]. In addition, veterinary vaccines based on liposomes have been approved for Newcastle disease virus and avian rheovirus [72].

Polysaccharides

The adjuvant activities of the polysaccharides mannan and glucan have been studied, and these have been shown to up-regulate T-helper 1 responses [81,52]. High-molecular weight sulfated- and diethylaminoethyl-dextrans have been used as veterinary adjuvants [82].

Chitosan, a polymer of D-glucosamine and *N*-acetyl-D-glucosamine, obtained by partial deacetylation of chitin, exhibits a range of effects on the immune system. It has been shown to activate macrophages, induce cytokines, and increase antibody production [83]. Nevertheless chitosan has very low toxicity, is non-allergenic, and is biodegradable.

Inulin is the term used to describe a family of low molecular weight unbranched polymers of fructose and glucose. It is found in *Compositae* where it serves as the storage carbohydrate, replacing the normal starch as a reserve food. Gamma inulin, an insoluble inulin polymorph, acts as an immune modulator by initiating the production of the complement protein C3. This protein then acts as an effector molecule, leading to the enzymatic cascade of the alternative complement pathway [84].

Multiple Antigenic Peptides

The multiple antigenic peptide (MAP) system [85] allows the assembly of multiple, but usually identical, peptide sequences attached to a core of branching lysine residues to yield a multivalent construct. Significantly higher antibody titres have been obtained by coupling immunogenic peptides to a polylysine core to form a MAP when compared with carrier protein-conjugated peptides in the presence of adjuvant [85].

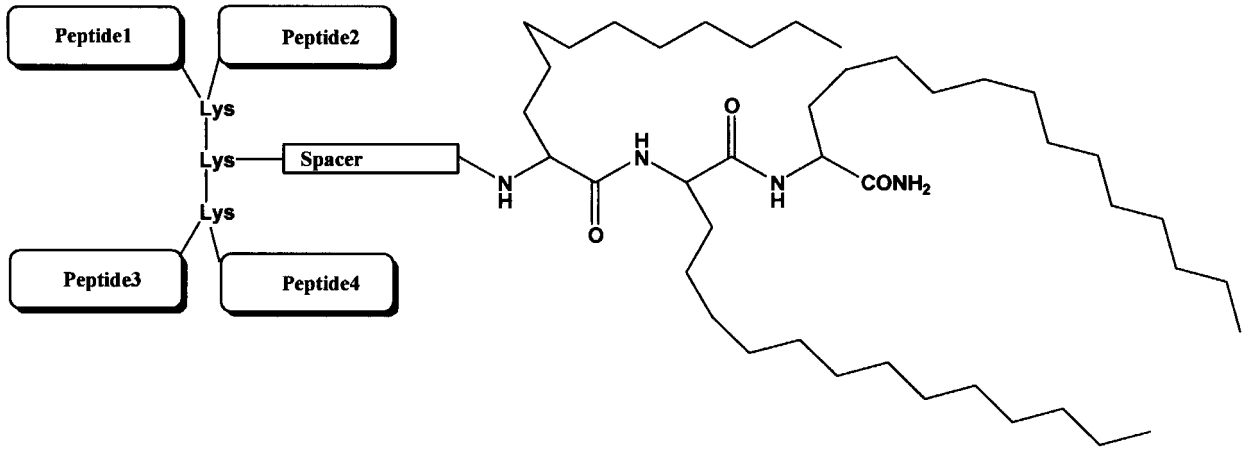
Lipid Polylysine Core Peptides

The lipid polylysine core peptide (**6**, LCP) system essentially combines the MAP and tripalmitoyl-S-glyceryl cysteine (Pam_3Cys) [86] systems. The LCP system incorporates lipoamino acids coupled to a polylysine core containing up to two different antigenic peptides [87], and is uniquely designed to incorporate antigen, carrier and adjuvant in a single molecular entity. Tam has also described a variation of his MAP system ('lipidated MAP') that incorporates lipids in order to boost mucosal immunization [88].

LCP-based vaccine candidates incorporating variable domains of *Chlamydia trachomatis* outer membrane protein have been shown to significantly enhance peptide immunogenicity when compared with peptide monomers given alone in adjuvant [87], and an LCP compound incorporating a foot-and-mouth disease viral peptide was immunogenic, resulting in the induction of anti-peptide antibodies in the absence of additional adjuvant [89]. Recently we investigated [90] the LCP system as a vaccine delivery strategy for group A streptococci (GAS) — the causative agents of rheumatic fever (RF) and subsequent rheumatic heart disease (RHD) [91] — diseases for which currently no available vaccine exists. The bacterial surface anti-phagocytic M protein [12] and major GAS vaccine candidate, was the targeted antigen. Mice immunized parenterally, in the absence of conventional adjuvant, with an LCP formulation containing a protective C-region determinant of the GAS M protein elicited high-titre, heterologous opsonic antibodies that did not cross-react with human heart tissue proteins [90], indicating the potential of such a vaccine in inducing broadly protective immune responses.

Proteosomes

The use of proteosomes is a promising approach for vaccine delivery. Proteosomes are based on the outer membrane proteins of bacteria such as meningococci to which peptides can be incorporated forming vesicular structures which facilitate antigen recognition by the immune system (reviewed in [92]). Proteosomes serve as both a carrier and adjuvant, and have been used to enhance the immunogenicity, particularly at the mucosal level, of a variety of vaccine antigens including proteins, peptides and lipopolysaccharides [93,94]. They are also suitable for human use [95,96].



6

CONCLUSION

The development of synthetic peptide-based immunogens is emerging as a possible approach for human vaccination in the future, as a replacement for conventional vaccines that use killed or attenuated whole microorganisms. The advantages of such synthetic vaccines (high potency, low adverse reactions, low cross-reactivity and high stability) are offset somewhat by the poorer inherent immunogenicity of these constructs. There is a greater need therefore to develop adjuvant/carrier systems to increase the immunogenicity of these newer vaccine candidates. Lipids and carbohydrates, used either separately or in the same construct, have a long history of high adjuvanticity and (often) low toxicity. Recent advances in carrier adjuvant have demonstrated the potential for such systems as effective immunopotentiators, as indicated by several compounds now in clinical trials.

REFERENCES

1. Minichiello V. New vaccine technology, what do you need to know? *J. Am. Acad. Nurse Pract.* 2002; **14**: 73–81.
2. Ellis RW. Technologies for the design, discovery, formulation and administration of vaccines. *Vaccine* 2001; **19**: 2681–2687.
3. Stephenson JR. Genetically modified viruses: vaccines by design. *Curr. Pharm. Biotechnol.* 2001; **2**: 47–76.
4. Hardman JG, Limbird LE, Gilman AG (eds). *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 10th edn. McGraw-Hill: New York, 2001; 1478–1479.
5. Fitzgerald D, Mrsny RJ. New approaches to antigen delivery. *Crit. Rev. Ther. Drug Carrier Syst.* 2000; **17**: 165–248.
6. Chen H. Recent advances in mucosal vaccine development. *J. Con. Rel.* 2000; **67**: 117–128.
7. Medina E, Guzman CA. Use of live bacterial vaccine vectors for antigen delivery: potential and limitations. *Vaccine* 2001; **19**: 1573–1580.
8. Parker D. T cell-dependent B cell activation. *Annu. Rev. Immunol.* 1993; **11**: 331–360.
9. Allison JP. Structure, function, and serology of the T-cell antigen receptor complex. *Ann. Rev. Immunol.* 1987; **5**: 503–540.
10. Rotzschke O, Falk K, Deres K, Schild H, Norda M, Metzger J, Jung G, Rammensee H-G. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* 1990; **348**: 252–254.
11. Marsh SG. Nomenclature for factors of the HLA system, update October 2000. *Hum. Immunol.* 2000; **61**: 1404–1405.
12. Fischetti V. Streptococcal M protein. *Sci. Am.* 1991; **264**: 32–39.
13. Hess J, Schaible U, Raupach B, Kaufmann SH. Exploiting the immune system: toward new vaccines against intracellular bacteria. *Adv. Immunol.* 2000; **75**: 1–88.
14. Peltola H, Patja A, Leinikki P, Valle M, Davidkin I, Paunio M. No evidence for measles, mumps, and rubella vaccine-associated inflammatory bowel disease or autism in a 14-year prospective study. *Lancet* 1998; **351**: 1327–1328.
15. Taylor B, Miller E, Farrington CP, Petropoulos M-C, Favot-Mayaud I, Li J, Waight PA. Autism and measles, mumps, and rubella vaccine: no epidemiological evidence for a causal association. *Lancet* 1999; **353**: 2026–2029.

16. Karlsen AE, Dyrberg T. Molecular mimicry between non-self, modified self and self in autoimmunity. *Semin. Immunol.* 1998; **10**: 25–34.
17. Berzofsky JA, Ahlers JD, Belyakov IM. Strategies for designing and optimizing new generation vaccines. *Nat. Rev. Immunol.* 2002; **3**: 209–219.
18. Singh M, O'Hagan DT. Recent advances in vaccine adjuvants. *Pharm Res.* 2002; **6**: 715–728.
19. Muhlradt PF, Kiess M, Meyer H, Sussmuth R, Jung G. Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*. *Infect. Immun.* 1998; **66**: 4804–4810.
20. Sacht G, Marten A, Deiters U, Sussmuth R, Jung G, Wingender E, Muhlradt PF. Activation of nuclear factor-kappa B in macrophages by mycoplasmal lipopeptides. *Eur. J. Immunol.* 1998; **28**: 4207–4212.
21. Brandt ER, The T, Relf WA, Hobb RI, Good MF. Protective and nonprotective epitopes from amino termini of M proteins from Australian aboriginal isolates and reference strains of group A streptococci. *Infect. Immun.* 2000; **68**: 6587–6594.
22. Holmgren J, Lycke N, Czerkinsky C. Cholera toxin and cholera B subunit as oralmucosal adjuvant and antigen vector systems. *Vaccine* 1993; **11**: 1179–1184.
23. Gupta RK, Siber GR. Adjuvants for human vaccines — current status, problems and future prospects. *Vaccine* 1995; **13**: 1263–1276.
24. Schutze M-P, Leclerc C, Jolivet M, Audibert F, Chedid L. Carrier-induced epitopic suppression, a major issue for future synthetic vaccines. *J. Immunol.* 1985; **135**: 2319–2322.
25. Briand JP, Muller S, Van Regonmortel MHV. Synthetic peptides as antigens: pitfalls of conjugation methods. *J. Immunol. Meth.* 1985; **78**: 59.
26. Jackson DC, O'Brien-Simpson NM, Ede NJ, Brown LE. Free radical induced polymerization of synthetic peptides into polymeric immunogens. *Vaccine* 1997; **15**: 1697–1705.
27. Ghosh S, Jackson DC. Antigenic and immunogenic properties of totally synthetic peptide-based antifertility vaccines. *Int. Immunol.* 1999; **11**: 1103–1110.
28. Sharma P, Kumar A, Batni S, Chauhan VS. Co-dominant and reciprocal T-helper cell activity of epitopic sequences and formation of junctional B-cell determinants in synthetic T : B chimeric immunogens. *Vaccine* 1993; **11**: 1321–1326.
29. Etlinger HM, Knorr R. Model using a peptide with carrier function for vaccination against different pathogens. *Vaccine* 1991; **9**: 512–514.
30. Panina-Bordignon P, Tan A, Termijtelen A, Demotz S, Corradin G, Lanzavecchia A. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur. J. Immunol.* 1989; **19**: 2237–2242.
31. Relf WA, Cooper J, Brandt ER, Hayman WA, Anders RF, Pruksakorn S, Currie B, Saul A, Good MF. Mapping a conserved conformational epitope from the M protein of group A streptococci. *Peptide Res.* 1996; **9**: 12–20.
32. Horie H, Miyazawa M, Ota Y, Wakabayashi K, Yoshida H, Doi Y, Hashizume S. Analysis of the accumulation of mutants in sabin attenuated polio vaccine viruses passaged in vero cells. *Vaccine* 2001; **19**: 1456–1459.
33. Janeway CA, Travers P, Walport M, Capra JD. *Immunobiology: The Immune System in Health and Disease*, 5th edn. Elsevier Science: London, 2001; 579–592.
34. Ciardi JE, McGee JR, Keith JM, (eds). *Genetically Engineered Vaccines*. Plenum Press: New York, 1992; (Advances in experimental medicine and biology; vol. 327).
35. Brown F, Dougan G, Hoey EM, Martin SJ, Rima BK, Trudgett A, (eds). *Vaccine Design*. Wiley: Chichester, 1993.
36. Brayden DJ, O'Mahony DJ. Novel oral drug delivery gateways for biotechnology products: polypeptides and vaccines. *Pharm. Sci. Tech. Today* 1998; **1**: 291–299.
37. Lindblad EB. Freund's adjuvants. In *Vaccine Adjuvants: Preparation Methods and Research Protocols*, O'Hagan DT (ed.). Humana: New Jersey, 2000.
38. Ott G, Radhakrishnan R, Fang J-H, Hora M. The adjuvant MF59: a 10-year perspective. In *Vaccine Adjuvants: Preparation Methods and Research Protocols* O'Hagan DT (ed.) Humana: New Jersey, 2000.
39. Esche Uvd, Ayoub M, Pfannes SDC, Müller MR, Huber M, Wiesmüller KH, Loop T, Humar M, Fischbach KF, Strünkelnberg M, Hoffmann P, Bessler WG, Mittenbühler K. Immunostimulation by bacterial components: I. Activation of macrophages and enhancement of genetic immunization by the lipopeptide P_3CSK_4. *Int. J. Immunopharmacol.* 2000; **22**: 1093–1102.
40. Baier W, Heinevetter L, Huber M, Wiesmüller KH, Jung G, Bessler WG. The lipopeptide P_3CSK_4 constitutes an adjuvant in parenteral and oral immunization. *Vaccine Res.* 1997; **6**: 127–140.
41. Baier W, Masihi N, Huber M, Hoffman P, Bessler WG. Lipopeptides as immunoadjuvants and immunostimulants in mucosal immunization. *Immunobiology* 2000; **201**: 391–405.
42. BenMohamed L, Belkaid Y, Loing E, Brahimi K, Gras-Masse H, Druilhe P. Systemic immune responses induced by mucosal administration of lipopeptides without adjuvant. *Eur. J. Immunol.* 2002; **32**: 2274–2281.
43. BenMohamed L, Thomas A, Bossus M, Brahimi K, Wubben J, Gras-Masse H, Druile P. High immunogenicity in chimpanzees of peptides and lipopeptides derived from four new *Plasmodium falciparum* pre-erythrocytic molecules. *Vaccine* 2000; **18**: 2843–2855.
44. Le Gal FA, Prevost-Blondel A, Lengagne R, Bossus M, Farace F, Cheboissier A, Gras-Masse H, Engelhard VH, Guillet JG, Gahéry-Ségard H. Lipopeptide-based

melanoma cancer vaccine induced a strong MART-27-35-cytotoxic T lymphocyte response in a preclinical study. *Int. J. Cancer* 2002; **98**: 221–227.
45. Azuma I. Synthetic immunoadjuvants: application to non-specific host stimulation and potentiation of vaccine immunogenicity. *Vaccine* 1992; **10**: 1000–1006.
46. Persing DH, Coler RN, Lacy MJ, Johnson DA, Baldridge JR, Hershberg RM, Reed SG. Taking toll: lipid A mimetics as adjuvants and immunomodulators. *Trends Microbiol.* 2002; **10**: S32–S37.
47. Johnson DA, Keegan DS, Sowell CG, Livesay MT, Johnson CL, Taubner LM, Harris A, Myers KR, Thompson JD, Gustafson GL, Rhodes MJ, Ulrich JT, Ward JR, Yorgensen YM, Cantrell JL, Brookshire VG. 3-*O*-Desacyl monophosphoryl lipid A derivatives: synthesis and immunostimulant activities. *J. Med. Chem.* 1999; **42**: 4640–4649.
48. Ulrich JT, Myers KR. Monophosphoryl lipid A as an adjuvant: Past experiences and new directions. In *Vaccine Design: The Subunit and Adjuvant Approach*, Powell MF, Newman MJ. (eds). Plenum: New York, 1995.
49. Ulrich JT. MPL immunostimulant: adjuvant formulations. In *Vaccine Adjuvants: Preparation Methods and Research Protocols*, O'Hagan DT (ed.). Humana: New Jersey, 2000.
50. Kaisho T, Akira S. Toll-like receptors as adjuvant receptors. *Biochim. Biophys. Acta* 2002; **1589**: 1–13.
51. Tamura M, Yoo YC, Yoshimatsu K, Yoshida R, Oka T, Ohkuma K, Arikawa J, Azuma I. Effects of muramyl dipeptide derivatives as adjuvants on the induction of antibody response to recombinant hepatitis B surface antigen. *Vaccine* 1995; **13**: 77–82.
52. Cox JC, Coulter AR. Adjuvants — a classification and review of their modes of action. *Vaccine* 1997; **15**: 248–256.
53. Azuma I, Seya T. Development of immunoadjuvants for immunotherapy of cancer. *Int. Immunopharmacol.* 2001; **1**: 1249–1259.
54. Yoo YC, Yoshimatsu K, Koike Y, Hatsuse R, Yamanishi K, Tanishita O, Arikawa J, Azuma I. Adjuvant activities of muramyl dipeptide derivatives to enhance immunogenicity of hantavirus-inactivated vaccine. *Vaccine* 1998; **16**: 216–224.
55. Noll H, Bloch H, Asselineau J, Lederer E. The chemical structure of the cord factor of *Mycobacterium tuberculosis*. *Biochem. Biophys. Acta* 1956; **20**: 299–309.
56. Bomford R. Will adjuvants be needed for vaccines of the future? In *Modulation of the Immune Response to Vaccine Antigens. Developments in Biological Standardization*, Brown F, Haaheim LR (eds). Karger: Basel, 1998; **92**: 13–17.
57. Kensil CR. Saponins as vaccine adjuvants. *Crit. Rev. Ther. Drug Carrier-Systems* 1996; **13**: 1–55.
58. Singh M, O'Hagan D. Advances in vaccine adjuvants. *Nature Biotechnol.* 1999; **17**: 1075–1081.
59. Kensil CR, Wu J-Y, Anderson CA, Wheeler DA, Amsden J. QS-21 and QS-7: Purified saponin adjuvants. In *Modulation of the Immune Response to Vaccine Antigens. Developments in Biological Standardization*, Brown F, Haaheim LR (eds). Karger: Basel, 1998; **92**: 41–47.
60. Sasaki S, Sumino K, Hamajima K, Fukushima J, Ishii N, Kawamoto S, Mohri H, Kensil CR, Okuda K. Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes. *J. Virol.* 1998; **72**: 4931–4939.
61. Sjölander A, Cox JC. Uptake and adjuvant activity of orally delivered saponin and ISCOM™ vaccines. *Adv. Drug Deliv. Rev.* 1998; **34**: 321–338.
62. Kensil CR. QS-21 Adjuvant. In *Vaccine Adjuvants: Preparation Methods and Research Protocols*, O'Hagan DT (ed.). Humana: New Jersey, 2000.
63. Waite DC, Jacobson EW, Ennis FA, Edelman R, White B, Kammer R, Anderson C, Kensil CR. Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21. *Vaccine* 2001; **19**: 3957–3967.
64. O'Hagan DT, MacKichan ML, Singh M. Recent developments in adjuvants for vaccines against infectious diseases. *Biomol. Engineer.* 2001; **18**: 69–85.
65. Morein B, Villacrés-Eriksson M, Lövgren-Bengtsson K. Iscom, a delivery system for parenteral and mucosal vaccination. In *Modulation of the Immune Response to Vaccine Antigens. Developments in Biological Standardization*, Brown F, Haaheim LR (eds). Karger: Basel, 1998; **92**: 33–39.
66. Osterhaus ADME, Rimmelzwaan GF. Induction of virus-specific immunity by iscoms. In *Modulation of the Immune Response to Vaccine Antigens. Developments in Biological Standardization*. Brown F, Haaheim LR (eds). Karger: Basel, 1998; **92**: 49–58.
67. Lövgren-Bengtsson K, Morein B. The iscom technology. In *Vaccine Adjuvants: Preparation Methods and Research Protocols*, O'Hagan DT (ed.). Humana: New Jersey, 2000.
68. Lacaille-Dubois M-A. Saponins as immunoadjuvants and immunostimulants. In *Immunomodulatory Agents from Plants*, Wagner H (ed.). Birkhauser: Verlag, 1999.
69. Penney C. Stearyl tyrosine: An organic equivalent of aluminum-based immunoadjuvants. In *Vaccine Design: The Subunit and Adjuvant Approach*, Powell MF, Newman MJ (eds). Plenum: New York, 1995.
70. Gupta RK, Siber GR. Comparison of adjuvant activities of aluminium phosphate, calcium phosphate and stearyl tyrosine for tetanus toxoid. *Biologicals* 1994; **22**: 53–63.
71. Penney CL, Donne G, Nixon-George A, Bona CA. Further studies on the adjuvanticity of stearyl tyrosine and amide analogues. *Vaccine* 1994; **12**: 629–632.
72. Sallovitz JM, Menghini MIZ, Lanusse CE. Impact of liposomes as delivery systems in veterinary medicine. *Veter. Res.* 1998; **29**: 409–430.

73. Gregoriadis G, McCormack B, Obrenovic M, Perrie Y, Saffie R. Liposomes as immunological adjuvants and vaccine carriers. In *Vaccine Adjuvants: Preparation Methods and Research Protocols*, O'Hagan DT (ed.). Humana: New Jersey, 2000.
74. Gregoriadis G. Liposomes as immunoadjuvants and vaccine carriers: antigen entrapment. *Immunomethods* 1994; **4**: 210–216.
75. Allison AC. The mode of action of immunological adjuvants. In *Modulation of the Immune Response to Vaccine Antigens. Developments in Biological Standardization*, Brown F, Haaheim LR (eds). Karger: Basel, 1998; **92**: 3–11.
76. Allison AC, Gregoriadis G. Liposomes as immunological adjuvants. *Nature* 1974; **252**: 252.
77. Gregoriadis G. Liposomes as immunological adjuvants for peptide and protein antigens. In *Liposomes in Drug Delivery*, Gregoriadis G, Florence AT, Patel HM (eds). Harwood: Switzerland, CHUR, 1993.
78. Snippe H, Verheul AFM. Liposomes as immunoadjuvants for saccharide antigens. *J. Liposome Res.* 1995; **5**: 453–465.
79. Zigterman GJWJ, Verheul AFM, Snippe H. Liposomes as immunoadjuvants for saccharide antigens. In *Liposomes in Drug Delivery*, Gregoriadis G, Florence AT, Patel HM (eds). Harwood: Switzerland, 1993.
80. Hunziker IP, Zurbriggen R, Glueck R, Engler OB, Reichen J, Dai WJ, Pichler WJ, Cerny A. Perspectives: towards a peptide-based vaccine against hepatitis C virus. *Mol. Immunol.* 2001; **38**: 475–484.
81. Chinnah AD, Baig MA, Tizard IR, Kemp MC. Antigen dependent adjuvant activity of a polydispersed β-(1,4)-linked acetylated mannan (acemannan). *Vaccine* 1992; **10**: 551–557.
82. Bradfield JWB, Souhami RL, Addison IE. The mechanism of the adjuvant effect of dextran sulphate. *Immunology* 1974; **26**: 383–392.
83. Seferian PG, Martinez ML. Immune stimulating activity of two new chitosan containing adjuvant formulations. *Vaccine* 2001; **19**: 661–668.
84. Cooper PD. Vaccine adjuvants based on gamma inulin. In *Vaccine Design: The Subunit and Adjuvant Approach*, Powell MF, Newman MJ (eds). Plenum: New York, 1995.
85. Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Natl Acad. Sci. USA* 1988; **85**: 5409–5413.
86. Metzger JW, Wiesmuller KH, Jung G. Synthesis of N_α-Fmoc protected derivatives of S-(2,3-dihydroxypropyl)-cysteine and their application in peptide synthesis. *Int. J. Peptide Protein Res.* 1991; **38**: 545–554.
87. Zong G, Toth I, Reid R, Brunham RC. Immunogenicity evaluation of a lipidic amino acid based synthetic peptide vaccine for *Chlamydia trachomatis*. *J. Immunol.* 1993; **151**: 3728–3736.
88. Tam JP, Mora AL, Rao C. Lipidation as a novel approach to mucosal immunisation. In *Modulation of the Immune Response to Vaccine Antigens. Developments in Biological Standardization*, Brown F, Haaheim LR (eds). Karger: Basel, 1998; **92**: 109–116.
89. Toth I, Flinn N, Gibbons WA, Good M, Hayman W, Brown F. Immunological evaluation of the Lipid-Core-Peptide (LCP) adjuvant/carrier system. In *Peptides: Chemistry, Structure and Biology*, Pravin T, Kaumaya P, Hodges RS (eds). Mayflower Scientific: Kingswinford, 1996.
90. Olive C, Batzloff MR, Horváth A, Wong A, Clair T, Yarwood P, Toth I, Good MF. Immunisation of mice with a lipid core peptide construct containing a conserved region determinant of group A streptococcal M protein elicits heterologous opsonic antibodies in the absence of adjuvant. *Infect. Immun.* 2002; **70**: 2734–2738.
91. Bisno AL. Group A streptococcal infections and acute rheumatic fever. *N. Engl. J. Med.* 1994; **325**: 783–793.
92. Lowell GH. Proteosomes for improved nasal, oral, or injectable vaccines. *New Generation Vaccines*, 2nd edn, revised and expanded. 1997; 193–206.
93. Levi R, Aboud-Pirak E, Leclerc C, Lowell GH, Arnon R. Intranasal immunization of mice against influenza with synthetic peptides anchored to proteosomes. *Vaccine* 1995; **14**: 1353–1359.
94. Mallett CP, Hale TL, Kaminski RW, Larsen T, Orr N, Cohen D, Lowell GH. Intranasal or intragastric immunization with proteosome-*Shigella* lipopolysaccharide vaccines protects against lethal pneumonia in a murine model of *Shigella* infection. *Infect. Immun.* 1995; **63**: 2382–2386.
95. Haneberg B, Dalseg R, Wedege E, Hoiby EA, Haugen IL, Oftung F, Andersen SR, Naess LM, Aase A, Michaelsen TE, Holst J. Intranasal administration of a meningococcal outer membrane vesicle vaccine induces persistent local mucosal antibodies and serum antibodies with strong bactericidal activity in humans. *Infect. Immun.* 1998; **66**: 1334–1341.
96. Oftung F, Naess LM, Wetzler LM, Korsvold GE, Aase A, Hoiby EA, Dalseg R, Holst J, Michaelsen TE, Haneberg B. Antigen-specific T-cell responses in humans after intranasal immunization with a meningococcal serogroup B outer membrane vesicle vaccine. *Infect. Immun.* 1999; **67**: 921–927.

