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are alone sufficient to prevent HBV infection, and that antibody levels of 10 or more mIU/ml in humans will normally result in protection. Mice can be conveniently used to test the immunogenicity of recombinant HBsAg preparations. Although extrapolation to humans is not strictly justified, it is nonetheless possible to put the responses to DNA-mediated immunization for this antigen in mice into perspective. We have found that a single injection of DNA can induce antibody levels which routinely achieve mean values of several hundreds to thousands of mIU/ml (Davis et al., 1993a, 1994) depending on the mouse strain used. On occasion, however, levels will rise to over 10 000 mIU/ml after a few months. This suggests that the very small amounts of protein produced subsequent to DNA transfer (on the order of nanograms) must be very efficiently presented to the immune system to achieve such immune responses.

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Introduction

The term ‘adjuvant’ (Latin: adjuvare, to help) has been used in many ways in biology and
medicine. Most commonly, adjuvants are immunostimulatory compounds or associations of compounds that potentiate or modulate the immune system against an antigen or vaccine beyond the level achieved by administration of the immunogen alone. These compounds have immunostimulatory properties and generally act on one or several of the steps in the immune system in a nonspecific manner, which results in an improvement of the specific immunity to an antigen.

Adjuvants have been extensively used for more than 6 decades in order to increase the immune response in experimental immunology as well as in practical vaccination. A series of simple but elegant experiments performed by Ramon in 1925, showed that the antitoxin response to tetanus and diphtheria was increased by injection of these vaccines together with other compounds, such as metal salts, oil, tapioca, or pyogenic bacteria. These experiments indicated for the first time that components of a vaccine formulation other than the antigen itself are important for a successful biological response and started a search for vaccine adjuvants that continues to this day. The traditional vaccines currently available contain antigen preparations which are frequently impure and poorly characterized, however, these preparations are capable of eliciting protective immunity and have been proven to be safe. In some cases, such as with killed bacterial vaccines, the contaminants themselves may act as immuno-potentiating agents, thus boosting the immune response against the vaccine (as adjuvants do). However, these same contaminants may be responsible for most of the side-effects induced by the vaccine (Munoz, 1964; Stewart, 1985; Griffith, 1989). Advances in DNA technology and biochemistry have led to the production of increasingly pure antigens allowing the induction of more specific immune responses. The culmination of progress in this area was the introduction in the 1980s of the first recombinant subunit vaccine consisting of the hepatitis B surface antigen. This vaccine is the only example of an existing licensed vaccine based on recombinant technology. Unfortunately, many of these highly purified protein preparations have also been shown to be relatively weak immunogens and this major difficulty encountered during development of modern vaccines can be theoretically overcome by the use of adjuvants. Aluminium salts as hydroxides and phosphates are the only adjuvants widely approved for use in humans. However, these compounds are not active with all immunogens and only stimulate humoral responses. Consequently the availability of improved/new adjuvants is crucial for the development of new vaccines.

**What are adjuvants?**

Adjuvants are a group of structurally heterogeneous compounds with the property of being able to increase immune responses to antigens. They affect the immune system in different ways and this most probably depends on their individual chemical and biological properties. Indeed, the hyper-activation of the immune response by adjuvants may be accompanied by adverse reactions that implicate distinct mechanisms involving the immune system, and/or physiological parameters outside the immune system, thus giving variable toxicity profiles depending on the adjuvant used.

Apart from the intended stimulation of the immune response, adjuvants also produce some side effects. For many years research has been focused on finding adjuvants with the ability to potentiate the immune response with minimal or no toxicity.

Adjuvants commonly used or considered for human or veterinary vaccines include
mineral salts, oil emulsions, compounds from bacterial cell walls, saponins, liposomes and ISCOMs. The use of cytokines, which act as modulators through the normal pathway of the immune response, may be also included as promising compounds, especially for human vaccines.

**What should adjuvants achieve?**

There are several properties which an adjuvant should ideally possess in order to be effective, safe and practical for use in vaccines. The requirements for developing new adjuvants for clinical use, apart from the factors involved in vaccine efficacy, are dictated primarily by concerns for safety.

Basically, adjuvants must be capable of stimulating an early and strong immune response (higher antibody titers), and a more prolonged response to an antigen (long-lasting immunity). Adjuvants can also be used to modulate the immune response to an antigen with respect to humoral or cellular immunity, or relative to the antibody class or subclass, in order to improve adequate protection.

**Development of new adjuvants**

To date, our knowledge about the events involved in the generation of protective immunity and the ways in which adjuvants can modulate these events is not sufficient to allow us to tailor these molecules to the requirements of a particular vaccine. Indeed, most of the experimentation of vaccine adjuvants has been empirical, in that an antigen preparation was given with and without an adjuvant and the specific immune responses directed towards the antigen were compared by assessing humoral- and/or cellular-mediated immunity. The advent of improved chemical techniques has allowed the construction of new and well-characterized adjuvants but these are generally based upon the older empirical experiments. As cellular immunology technology and our knowledge of the cell types and cytokines interacting in immune responses increases, so does our understanding of the mode of action of adjuvants, as well as the way in which they produce side-effects. Consequently, it has become possible to study many of the factors that must be considered in the rational design of new adjuvants for use in vaccines, and the side-effects to be avoided. These are crucial points which may determine regulatory approval.

**Mode of action of adjuvants**

The distinct mechanisms by which adjuvants may act can be summarized as: a) depot effect for slow release of antigen; b) effects on antigen-presenting cells (APCs) by targeting the antigen to APCs, activation and/or recruitment of APCs; c) improving antigen presentation, for example, by allowing the exposure of epitopes implicated in pathogen neutralization; and d) induction of immunoregulatory substances, such as cytokines.

For soluble antigens at least 2 additional mechanisms can explain the immunopotentiation activity when aluminium salts and many other adjuvants (emulsions in general, liposomes, ISCOMs, stearyl tyrosine, etc) are used. These adjuvants can transform a soluble antigen partially or completely into a particulate one (ie antigen adsorbed in the aluminium precipitate, or included in the oil microdroplets of emulsions) by physical association. As a result there is a shift from soluble antigen uptake by B cells to particulate uptake by macrophages or dendritic cells. We know that B cells internalize antigens via specific binding to their cell surface immunoglobulins (Abbas et al, 1985) and can present soluble
antigens in concentrations as low as 1 ng/ml (Malynn et al, 1985). On the other hand, APCs such as macrophages and dendritic cells internalize antigens by non-specific phagocytosis and pinocytosis (Unanue, 1985), and present these to T cells as peptides in association with major histocompatibility complex (MHC) class II molecules. Consequently, we can conclude that particulate forms of antigens effectively mediate the complete accessory cell activation of naive T cells and a soluble form of antigen best mediates B cells interacting with helper T cells.

### Compounds with adjuvant activity

#### Mineral compounds

Aluminium salts have been used as an adjuvant worldwide in animal and human vaccines for many decades. Aluminium-based adjuvants continue to be utilized today primarily because they are the most widely used for human vaccines and because they are generally regarded as safe. However, aluminium salt adjuvants are limited in that they preclude lyophilization or freezing of the vaccine, they are not effective with all antigens, and they do not stimulate cell-mediated immunity. There is also a batch-to-batch variation in vaccine preparation, making the assessment of efficacy and quality control difficult. In summary, aluminium compounds are adequate adjuvants if an antibody response is sufficient for protection and the antigen itself is a strong immunogen.

Several mechanisms have been proposed for its action. Basically, aluminium salts act as the other depot-type adjuvants do, by allowing slow release of the antigen, and probably also by increasing antigen stability. Furthermore, recent work suggests that they may also mediate cytokine release (Allison and Byars, 1992).

Calcium phosphate has been used as a vaccine adjuvant in France and has been shown to be safe and efficient in various field trials. However, opinions concerning the use of calcium as an effective substitute of aluminium are controversial.

### Hydrophobic substances and surfactants

Experiments performed using hydrophobic substances and surfactants have been shown to increase cellular and/or humoral immune responses, and have served as the basis for the development of several classes of adjuvants. Examples include emulsions containing water and oil, saponins, synthetic polymers, liposomes, and quaternary amines.

#### Emulsions

The use of emulsions (W/O, water in oil emulsions) started with experiments by Freund in the 1930s who mixed mineral oil and mannitol monooleate (emulsifying agent) with solutions of killed mycobacteria. This compound, Freund's complete adjuvant (FCA), is too toxic to be used either in humans or animals. An oil emulsion without the mycobacteria, Freund's incomplete adjuvant (FIA), is less toxic and has been used in humans in the past for influenza and poliomyelitis vaccines (Salk and Salk, 1977). Hundreds of thousands of doses of FIA-adjuvanted vaccines had already been inoculated into humans before the question of possible carcinogenicity was raised. Long-term follow-up of subjects included in these trials failed to show an increase in mortality, tumours or autoimmune diseases due to oil adjuvants (Beebe et al, 1972). However, from the studies in the United States on the use of oil adjuvants, it was concluded that the use of mineral oil adjuvants in the human population may be hazardous and should not be recommended for general use (Pittman, 1990).
The introduction of novel purified mineral oils and new injectable emulsifying agents over the last decade, has permitted the production of many oil-based veterinary vaccines (foot-and-mouth disease (FMD) and Aujeszky's disease widely benefited from this technological advance) and promising human formulations. Indeed, new FIA-based formulations have been recently used for the HIV-based antigen in human trials.

**Saponins and ISCOMs**

Saponins, which are glycosides from plants, are included in many FMD vaccines. Currently saponins are used as a crude extract (Quil A) or as a highly purified compound (QS21-Stimulon, which is HPLC purified from Quil A). QS21 formulations have been shown to be useful in adjuvanting a subunit vaccine for feline leukemia virus (Kensil et al, 1991) and a GM2 ganglioside-KLH conjugate vaccine in humans (Livingston et al, 1994). This adjuvant strongly stimulates the production of the IgG2a subclass in mice, possibly as a result of TH1 cell type lymphokine induction, and develops CTL responses when it is used in conjunction with soluble protein antigens.

Another approach taken in order to diminish quantities of Quil A used (and thus minimizing toxicity) has been to incorporate antigens into cage-like particles called ISCOMs (immunostimulatory complexes). These structures (35 nm) are generated by mixing the antigen with phospholipids, cholesterol, a biocompatible detergent and Quil A. ISCOMs induce high antibody titers and when complexed with glycoproteins they may also induce CTL responses, perhaps through the delivery of antigen directly into the cytosol thus allowing association with MHC class I molecules. Safety problems concerning Quil A and scale-up production must be resolved before ISCOMs can be administered to humans.

**Non-ionic block copolymers and polymers**

Recent studies utilizing chemically defined non-ionic polymer surfactants (consisting of varying proportions of hydrophobic polyoxypropylene (POP) and hydrophilic polyoxyethylene (POE) administered with antigen in oil-in-water emulsions) suggest that the type of immune response elicited depends upon the size and arrangement of the vaccine components (Hunter et al, 1991). Such studies provide hope that such synthetic surfactants could be specifically designed for different uses if problems of metabolism, toxicity, and stability can be overcome. Other polymers which have also been used to entrap antigens and have demonstrated adjuvant effects are: a) polymethacrylate-based nanoparticles (Kreuter et al, 1992); b) PLGA {poly (d, l-lactide-co-glycolide)}; and c) microspheres of a new class of ion cross-linkable water soluble polyphosphazene (Cohen et al, 1990).

**DDAB**

Dimethyldioctadecylammonium bromide (DDAB) is a lipophilic quaternary amine that stimulates humoral response; it is also a strong adjuvant for cellular immunity, especially DTH responses. The toxicity of DDAB is not well known although severe detrimental side-effects have not been seen. This adjuvant has been used in conjunction with experimental vaccines for veterinary purposes, and may be particularly advantageous if cell-mediated immunity is considered to be important.

**Liposomes**

The use of liposomes as adjuvants has been studied experimentally for many years. The adjuvant activity of liposomes depends upon the charge, the number of phospholipid bilayers, the composition, and the method of
preparation. Depending on these parameters, humoral or cellular immunity, or both, can be increased. Potential problems for liposome-based vaccines are stability and batch-to-batch variation.

Compounds derived from bacteria

Adjuvants from bacteria, such as lipopolysaccharides (LPS), and other components of the cell wall, are too toxic in their natural form for use in humans and so much effort has been made to develop analogs which might be appropriate for use in vaccines. Experiments such as phthalylization or succinylation of the LPS or removal of a phosphate group from lipid A to create monophosphoryl lipid A results in diminished toxicity of the compound while retaining adjuvanticity. The chemical synthesis of an entire family of lipid A analogs should allow analysis of the structure–function relationships of LPS with respect to adjuvanticity. Biochemical studies on the cell-wall compounds of mycobacteria revealed that the smallest subunit that retains immunoadjuvant activity is the N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP). Over the last decade, numerous analogs of MDP have been made and studied (Audibert et al, 1985). Hydrophilic MDP analogs administered in saline induce mainly antibodies, whereas cellular response is induced if MDP is given together with antigen in oil emulsions or liposomes.

Adjuvant combinations

Another approach to further augment the immune response, especially cellular immunity, is to utilize combinations of adjuvants, such as MDP, or LPS derivatives incorporated into liposomes or oil emulsions together with the antigen. Some examples of promising formulations are the following.

i) The Syntex formulation (SAF) contains squalene oil (from sharks), an amino acid derivative of muramyl dipeptide (threonyl-MDP), and non-ionic block polymers, but the side-effects related to the induction of uveitis remain unsolved.

ii) The Ciba-Geigy/Chiron formulations contain squalene and surfactants (MF-59), with or without a fatty-acid derivative of muramyl tripeptide covalently linked to dipalmitoyl phosphatidylethanolamine (MTP-PE) and have been used in HIV clinical trials inducing virus neutralizing antibodies. However, enhanced reactogenicity due to the formulation with MTP-PE (MF59-100) was evidenced in a pilot study in humans, when it was associated with an influenza virus vaccine (Keitel et al, 1993).

iii) The Ribi formulation (containing monophosphoryl lipid A and trehalose dimycolate) has been clinically tested with a candidate malaria vaccine (Rickman et al, 1991). Ribi's MPL is being successfully tested in a 2-dose regimen with a hepatitis B vaccine in humans.

Future prospects

As the availability of highly purified immunogens becomes routine and the discovery of specific epitopes emerges, the obtention of more efficient adjuvants will become increasingly necessary.

Despite the large number of substances known to be capable of increasing specific immunity, only aluminium hydroxide is currently utilized as an adjuvant in humans (emulsions and saponins may be added to the list for veterinary vaccines). The major obstacle to the use of the remaining immunostimulating compounds is that the toxic/therapeutic ratio is too narrow. The documented dissociation of efficacy from severe toxicity with several adjuvants, such as emulsions, monophosphoryl lipid A, non-
ionic polymer surfactants, some MDP analogs and saponins, suggests that acceptable compounds may be developed in the future. Emulsions, principally water in oil emulsions represent an optimal balance of safety, efficacy and cost, especially if more metabolizable oils are employed and less viscous and stable emulsions obtained.

As research progresses providing answers to the many questions that remain concerning the mechanistic aspects of immunostimulation, new and novel approaches to the development of new generations of adjuvants will be within our reach.

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Vaccine delivery systems. J Haensler (Pasteur Méribes Sérums et Vaccins, 1541, av Marcel-Méribes, 69280 Marcy-l’Étoile, France)

Introduction

Important advances in the development of carrier systems for the delivery of macromolecules have made it possible to apply these systems to vaccine delivery. Many strategies for the adjuvantation of poorly immunogenic purified antigens and for the development of mucosal (especially oral) and single shot vaccines rely on micro-