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Review

Vaccine adjuvant systems: Enhancing the efficacy of sub-unit protein antigens

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ABSTRACT

Vaccination remains a key tool in the protection and eradication of diseases. However, the development of new safe and effective vaccines is not easy. Various live organism based vaccines currently licensed, exhibit high efficacy; however, this benefit is associated with risk, due to the adverse reactions found with these vaccines. Therefore, in the development of vaccines, the associated risk–benefit issues need to be addressed. Sub-unit proteins offer a much safer alternative; however, their efficacy is low. The use of adjuvanted systems have proven to enhance the immunogenicity of these sub-unit vaccines through protection (i.e. preventing degradation of the antigen in vivo) and enhanced targeting of these antigens to professional antigen-presenting cells. Understanding of the immunological implications of the related disease will enable validation for the design and development of potential adjuvant systems. Novel adjuvant research involves the combination of both pharmaceutical analysis accompanied by detailed immunological investigations, whereby, pharmaceutically designed adjuvants are driven by an increased understanding of mechanisms of adjuvant activity, largely facilitated by description of highly specific innate immune recognition of components usually associated with the presence of invading bacteria or virus. The majority of pharmaceutical based adjuvants currently being investigated are particulate based delivery systems, such as liposome formulations. As an adjuvant, liposomes have been shown to enhance immunity against the associated disease particularly when a cationic lipid is used within the formulation. In addition, the inclusion of components such as immunomodulators, further enhance immunity. Within this review, the use and application of effective adjuvants is investigated, with particular emphasis on liposomal-based systems. The mechanisms of adjuvant activity, analysis of complex immunological characteristics and formulation and delivery of these vaccines are considered.

Contents

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1. Introduction

In recent years there has been a massive increase in the perceived potential for application of vaccination technology for a diverse range of uses in disease prevention, treatment and therapies. This is in addition to the traditional role of vaccination in prophylactic prevention against infectious agents, as well as potential applications for protection against cancer, allergy and other areas (Alpar and Bramwell, 2002; Mesa and Fernandez, 2004) in therapeutic as well as prophylactic mode. However, like all medicines, vaccines have an associated risk. Due to their strong potency, a large proportion of currently licensed vaccines are based on live organisms (Perrie et al., 2007), although these live systems have associated adverse reactions which can range from simple headache to encephalitis (MMR), intussception (rotavirus), vaccine associated disease (polio) and even death (smallpox) (Huang et al., 2004). Indeed, due to this it is unlikely that vaccines in this format would now be approved by regulatory bodies. Whilst rare, inactivated vaccines can also cause serious adverse effects varying from nausea to anaphylactic reactions and neurological complications (Huang et al., 2004). Alternatively, highly purified sub-unit proteins or synthetic peptides are recognised as offering the best safety profile, unfortunately their effective implementation is limited by their poor immunogenicity when administered without adjuvants (Demana et al., 2005; Vangala et al., 2006).

2. What makes a good vaccine?

It is not surprising that there is no clear recipe for a good vaccine. Rather, this is very much dependent upon the individual application. However, we can subdivide different criteria that apply to vaccines in a general sense. There are some general criteria that either a vaccine must satisfy, or a vaccine would benefit greatly from. In general terms, firstly a vaccine must be capable of eliciting the appropriate immune response; as mentioned, it should be safe to administer, at least in terms of satisfying risk–benefit criteria; a stable and reproducible formulation can certainly be beneficial, which may also relate to another important criteria; that of cost. Lastly, in terms of vaccine coverage and patient compliance, single dose oral administration has long been championed as the holy grail of vaccine researchers. In real terms, anything to reduce administrations or increase patient compliance must be good news. To expand on this, we can examine some examples based on these criteria.

2.1. Eliciting the appropriate immune response

This is particularly complicated for more tricky foes, such as HIV and TB (in terms of vaccines against disease causing agents). Often, correlates of protection are poorly defined (Agger and Andersen, 2001; Aebischer et al., 2008) and, due to host specificity, there is a distinct lack of suitable animal models that makes vaccine development difficult, expensive or even speculative (Di Pietrantonio and Schurr, 2005; Griffin, 2002; Scollard et al., 2006; Guinovart and Alonso, 2007). Even where encouraging results can be achieved in animal models this is not always translated into effective results in humans. For example, the tumour associated antigen mucin 1 (MUC 1) was shown to induce very high cytotoxic T-lymphocyte (CTL) responses and poor antibody responses in mice immunised with mannose receptor targeted MUC 1 peptides (Apostolopoulos et al., 1996). In humans the result was somewhat different: a clinical trial of the targeted antigen involving 25 patients with advanced metastatic carcinoma of breast, colon, stomach, or rectum resulted in high antibody responses in more than half of the patients and CTL responses in 2 from 10 patients tested (Karanikas et al., 1997). It turns out that the high antibody response observed to MUC 1 in humans may have its basis in the differential natural spectrum of antibodies present in mouse and man. Humans have antibody directed towards the terminal disaccharide epitope formed by α(1,3)galactosyl transferase whereas mice, which express this enzyme, do not have antibodies against this epitope. Such antibodies are known to be cross reactive with MUC 1 peptides and it is thought that this immunological difference provides the predisposition towards the largely antibody mediated response seen on transposition of this promising immunotherapeutic technique from mice to humans (Apostolopoulos et al., 1998).

In terms of vaccines against other diseases and for other applications, some are well defined regarding the immune response required for vaccine efficacy. Certain levels of antibody are considered protective in vaccine efficacy for vaccines against hepatitis, diphtheria and tetanus for example—anti-HBsAg antibody levels of >10 mIU/ml, anti-diphtheria antibody levels of >100 mIU/ml and anti-tetanus antibody levels of >100 mIU/ml are quoted as protective levels of antibody in humans (Dentico et al., 2002; Schmitt et al., 2003; Bramwell and Perrie, 2005a,b).

It is clear that eliciting an appropriate immune response is a fundamental vaccine objective or could be defined as a non-negotiable goal directed criterion, with the level of difficulty dictated by the proposed application and its particular complications.

2.2. Safety of administration

This consideration, and the associated risk–benefit issues, are exemplified in the attempted implementation of a rotavirus vaccination program in the U.S. With approximately 20 deaths annually attributable to rotavirus (Tucker et al., 1998), the use of a rotavirus vaccine had limited benefit in the U.S. and with the possibility of a 1 in 10,000 risk of intussusception its use became unacceptable. Introduction of the vaccine was well intentioned and it may well have reduced disease incidence, but the problems incurred in the U.S. from rotavirus are mitigated by a good healthcare system that enables good recovery from rotavirus infections. In contrast, in the third world, where about 20% of the 3 million child deaths caused by diarrhoea are attributable to rotavirus (Parashar et al., 2003, 2006), some element of risk may be tolerable for an effective vaccine. However, any risk associated with vaccination will undoubtedly add controversy to its use and initiate as well as intensify ethical issues. A vaccination program against rotavirus would need to take into account serotypical variation and vaccine trials in developing countries is a sensitive matter, not helped by controversial HIV vaccination trials in the past (Angell, 1997; Lurie and Wolfe, 1997).

The potential eradication of poliomyelitis is solely attributable to effective worldwide vaccination programmes (Plotkin, 2005). In the case of polio vaccination, the shift towards the inactivated vaccine (Salk) in the US and UK, in preference to the live attenuated (Sabin) orally administered vaccine represents, in part, a transformation in risk–benefit analysis in relation to immunisation against polio. The Sabin vaccine is believed to be more effective and may be given orally whilst the Salk vaccine, although recently improved, has to be administered parenterally. The live vaccine, however, has been associated with a small but definite risk of paralytic polio (potentially associated with 57 cases in the United States between 1961 and 1964). The knowledge of this was not seen as sufficient to change the vaccination policy until the risk of vaccine induced poliomyelitis attributable to the live vaccine became an issue of increasing concern in a developed environment of low disease incidence (Blume and Geesink, 2000; Alpar and Bramwell, 2002).

It needs to be said that safety poses a major concern for pharmaceutical companies. It is them who may be liable for any

vaccine related adverse events when a vaccine comes to market, but how do you balance this against the observed benefit? Unfortunately, this is not easy when the benefit has no direct observable impact to a particular individual, but any adverse events certainly do.

These are the overriding considerations, but other criteria may certainly be desirable. Nevertheless, developing a stable, reproducible and inexpensive formulation – or even a single dose oral administration – can be subordinated to eliciting an appropriate immune response in many cases. For example, an effective HIV vaccine that requires multiple parenteral administrations, and is expensive and difficult to make would still be a massive breakthrough in vaccine research. However, it is certain that reducing cost, increasing stability and easy (e.g. oral) administration could have dramatic implications for vaccines worldwide, and especially in the developing world in terms of vaccines against infectious agents. Indeed, they would be desirable facets of a vaccine developed for any purpose.

3. Enhancing the potency of a vaccine through formulation

Taking into consideration the above discussion, it is clear that we require a safe and effective vaccine. Of our options available, sub-unit proteins offer the safest potential but require enhanced efficacy. These vaccines need to be better designed to stimulate the three key interacting elements of our natural defence system: antigen-presenting cells (APCs), thymus-derived lymphocytes (T cells), and bone-marrow derived lymphocytes (B cells). Of the APCs, dendritic cells are the most important as they are designed for the capture and processing of antigens into small fragments with subsequent presentation at the cell surface in association with MHC molecules (Sprent and Webb, 1987; Nossal, 1997). This allows T-cell recognition and activation with subsequent B cell stimulation. If this is the first time the body encounters an antigen the stimulated response can be slow and limited, leaving the body vulnerable to the infectious disease state. However, after this first infection the body develops an acquired immunity against the invading pathogen via cells that survive as highly reactive plasma cells (B cells) or memory cells (B and T cells). Therefore, upon re-infection, the immune system will react quickly to provide a faster and stronger immune response (Perrie et al., 2007; Storni et al., 2005).

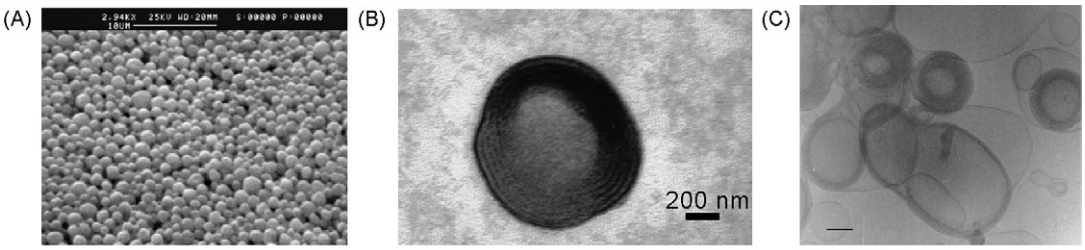
Therefore, a key aim is to formulate an adjuvanted sub-unit vaccine that is able to enhance the delivery of sub-unit antigens by protecting them from degradation in vivo and to enhance their targeting to dendritic cells, thus allowing the above immune cascade to occur. Currently licensed adjuvants include aluminum hydroxide or phosphate (used in, e.g. Diphtheria, Tetanus and Hepatitis B vaccines), MF59 (which consist of squalene droplets (<250 nm) combined with two surfactants, polyoxyethylene sorbitan monooleate and sorbitan trioleate), and AS04 (containing aluminum and the bacterial lipid, monophosporyl lipid A; already licensed in Europe). Toxoids, in addition to being successful vaccines in their own right, are also used to increase the immunogenicity of vaccines including Haemophilus influenza type b (Hib), which contains a polysaccharide unit from the virus conjugated to diphtheria or tetanus toxins.

Aluminum based adjuvants have a demonstrated safety profile of many decades and their role in the implementation of successful vaccine programmes is impressive (Clements and Griffiths, 2002); however, these adjuvants have been associated with severe local reactions such as erythema, subcutaneous nodules and contact hypersensitivity (Baylor et al., 2002). Even publications that point out the positive benefits of aluminium adjuvants concede that the next generation of vaccines will probably require new adjuvants (Clements and Griffiths, 2002). Therefore, there remains a clear need for the further development and application of adjuvants.

However, there is also a role for better exploitation of adjuvants in existing vaccines. At a recent WHO conference the deficiencies of current inactivated influenza vaccines were reviewed (Cassetti et al., 2005). In addition to the genetic drift in the virus which necessitates new vaccines to be prepared annually, the dependence on eggs for their production and other process and development considerations, these vaccines have a relatively low immunogenicity in young children and the elderly, meaning protection is often less than desired. Yet, despite this recognised problem, out of the Influenza vaccines currently available, most are inactivated virus-based vaccines or sub-unit vaccines, with only very few systems using adjuvant systems, e.g. Inflexal V® – which uses a liposome-based system (known as Virosomes) built from viral constituents (Mischler and Metcalfe, 2002) – and Fluad® which uses the oil-in-water emulsion MF59. The advantages of these adjuvanted systems are clearly proven. For example, in a comparison of more than 450 children, the virosome adjuvanted Inflexal V® vaccine showed generally better immunogenicity than Fluvarix® (the preservative-free inactivated split-virion trivalent vaccine) that has been used extensively in the clinic worldwide (Kanra et al., 2004). In a separate study, comparison of a sub-unit virus vaccine adjuvanted with MF59 and a split virus vaccine has shown that the adjuvanted vaccine reveals a better immunogenicity, including the induction of satisfactory antibody levels in the elderly (Baldo et al., 2006). Indeed, the combination of MF59 with influenza vaccine has shown increased immunogenicity following parenteral administration in several clinical trials and analysis of the literature supports increased efficacy for influenza vaccines containing MF59 in the elderly (Atmar et al., 2006; Banzhoff et al., 2003; Giudice et al., 2006).

In terms of mucosal administration, heat-labile *Escherichia coli* enterotoxin (LT) adjuvant was previously incorporated into a nasally administered non-living influenza vaccine used in Europe but was associated with a possible increase in cases of Bell's palsy, leading to withdrawal of the vaccine from the market (Mutsch et al., 2004). However, non-toxic mutants of this powerful mucosal adjuvant could provide a safer and effective replacement. In an interesting phase I evaluation, a supramolecular, nanoparticulate drug delivery system with a positively charged polysaccharide core enclosed by a phospholipid-cholesterol double layer co-adjuvanted with the LT mutant LTK63 demonstrated potential for the elicitation of mucosal (IgA) immune responses following two intranasal administrations in humans (Stephenson et al., 2006). In addition to these adjuvant considerations, the search for effective influenza vaccines also includes the use of conserved internal epitopes that potentially offer heterotypic protection (Saha et al., 2006).

Of the most recently developed vaccines, the HPV vaccines are formulated as virus-like particles. Virus-like particles (VLPs) are formed by the self-assembly of envelope or capsid proteins from viruses, and retain many of the structural characteristics of authentic viruses, whilst being non-infectious and non-replicating due to the absence of the genetic material. These "pseudovirions" can be produced by transfection of DNA plasmids encoding for the necessary proteins into mammalian cells, yeast cells or recombinant baculoviruses in insect cells (Noad and Roy, 2003; Bramwell and Perrie, 2005a,b; Grgacic and Anderson, 2006; Young et al., 2006). However, both of the HPV vaccines are formulated with additional adjuvants; the Merck vaccine is administered with an aluminium adjuvant and the GSK vaccine with AS04. In addition, GSK have an adjuvanted product commercially available in Europe, the hepatitis B vaccine *Fendrix*, and Novartis have a range of MF-59 adjuvanted vaccines in development.



Mechanism of action	Delivery system
Up regulation ofantigen presentation(signal 1)	Niosomes, PLGA (Sun et al., 2003). Lipid vesicles (Breweret al., 2004).
Increased antigen uptake and localisation to lymph nodes	DRV liposomal DNA (Perrie et al., 2001). Liposome-encapsulated peptides (Ludewig et al., 2000). Polymer micro- and nano-particulatedelivery systems.
Cellular distress (signal 0)	Oil emulsions, surfactants,aluminium salts.
Depot effect	Oil emulsions, alum, gels, polymer based particulates, liposomes (Ludewiget al., 2000).

Fig. 1. Examples of particulate delivery systems in vaccine design and particulate delivery systems: Mechanisms of adjuvant activity. (A) Scanning electron micrograph (SEM) of microspheres formulated using water-in-oil-in-water (W/O/W) emulsion techniques, approximate size 1–2 μm; (B) cationic niosomes prepared by the DRV method and imaged by TEM; (C) cationic DRV liposomes entrapping DNA imaged using cryo-EM (image courtesy of Peter Frederik; bar = 200 nm).

4. Choosing your adjuvant

There has been many adjuvant systems investigated and reported. However, categorising these is not actually as clear as would first be perceived, since understanding of their underlying mechanisms of action remains vague and the structural requirements of an effective adjuvant is lacking. However, adjuvants have also been classified in various ways, with probably the most commonly applied grouping being outlined by Schijns (2000) (Fig. 1). This is based on five concepts of immunogenicity neatly summarised by Storni et al. (2005) as;

1. the geographical concept of immune reactivity;
2. the theory of depot effect (emphasising the importance of antigen localisation);
3. the paradigm that adjuvants act as Signal 0, which precedes the induction of the epitope Signal 1 and co-stimulatory Signal 2 (Fig. 2);
4. the role of Signal 2 molecules as natural adjuvants in the activation of naïve T-helper cells which co-subsequently co-ordinate T-cell dependent immune responses;
5. the hypothesis that immunity is activated by exogenous and eventually endogenous danger signals.

The classification of events that lead to immune responses in terms of signals 0, 1 and 2 can be a useful perspective for interpretation of the mechanisms of action of vaccines and their components. Signal 0 refers to the primary activation of the immune system through the transmission of a 'danger-signal', which is brought about as a consequence of intruding micro-organisms displaying certain pathogen associated molecular patterns (PAMPs) on their surface (Hashimoto et al., 1988), which assist the immune system in distinguishing between self and non-self through interaction with pattern recognition receptors (PRR). Molecules capable of pattern recognition can be either soluble (lysozyme, complement) or cell-associated, particularly on the surface of antigen-presenting cells (e.g. macrophages, dendritic cells) (Medzhitov and Janeway, 1998; Goldsby et al., 2003; Playfair and Bancroft, 2004; Storni et al., 2005). These cell-associated receptors have been termed Toll-like receptors (TLRs), and their stimulation activates important mediators of innate and adaptive immunity. Signal 1 refers to antigen related parameters – including the processing and subsequent delivery of antigen to secondary lymphoid organs – and can be seen as encompassing geographical concepts outlined by Zinkernagel et al. (1997). Signal 2, facilitated through the perception of possible microbial danger by signal 0, relates to co-stimulatory signals as molecules on the membrane of antigen-presenting cells or as host-secreted soluble immunologically active molecules (Schijns, 2003). It is easy to see that individual adjuvants may be seen as falling into any of these categories. Liposomes or microspheres and other vaccine carriers may enhance signal 1 by providing effective delivery of antigen to secondary lymphoid organs. Signal 0 is enhanced by toll-like receptor agonists and powerful adjuvants such as *E. coli* heat-labile toxin and its safer mutant derivatives, whilst signal 2 is effectively realised by the use of immunologically active host molecules such as IL-2 (O'Hagan et al., 2001).

4.1. Toll-like receptors and their adjuvant ligands

The increasing understanding of Toll-like receptors and their role in immunological signalling will also allow for better adjuvants. A list of TLR implicated in the function of specific adjuvants

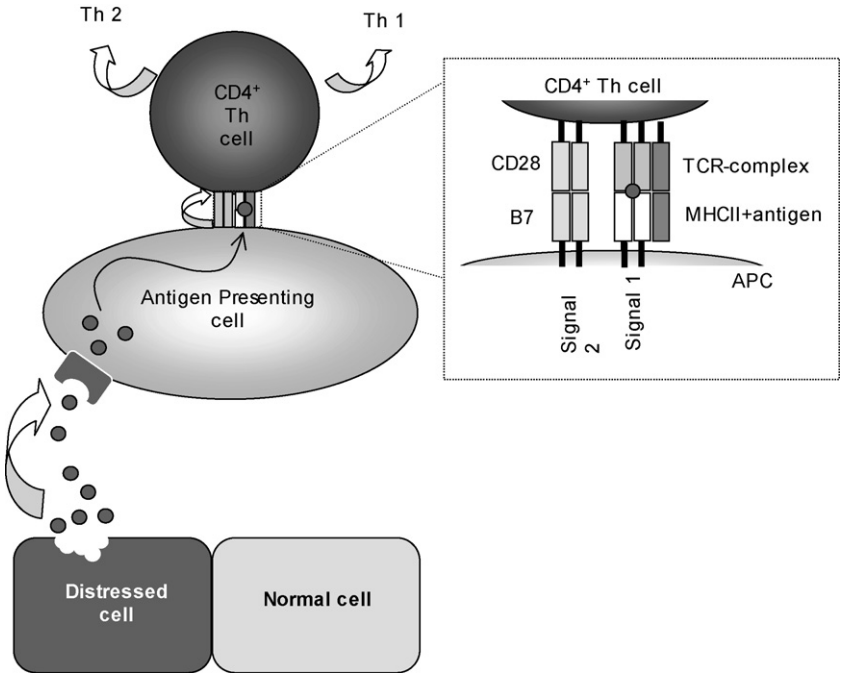


Fig. 2. Antigen uptake and presentation. Immunity is primarily activated by endogenous (e.g. heat-shock proteins, DNA and RNA damage, etc.) or exogenous (e.g. pathogen or adjuvant) danger signals (Signal 0). These signals are typically mediated by Toll-like receptors and enable innate immunity and adaptive immunity though antigen presentation (Signal 1) and concurrent co-stimulation (Signal 2) of CD4-positive T-helper (Th) cells. Activation of these Th cells results in the secretion of cytokines and chemokines, which can directly affect viability and replication of live pathogens, or the Th cells can further stimulate Th1 and Th2 pathways to stimulate cytotoxic T cells and B cells. In the absence of Signal 2 (stimulation of CD28 on T cells by B7 molecules), stimulation of T cells through T-cell receptor complex induces tolerance not immunity. Figure modified from Storni et al. (2005).

and components associated with potentially pathogenic entities is listed in Table 1. Interestingly, the recent identification and interest in TLR function in host immune responses has led to the implication of these mechanisms in pathogen immune subversion such as TLR induced immunosuppression (IL-10 release through TLR2), blockade of TLR recognition, and TLR mediated induction of viral replication (Netea et al., 2004) in much the same way as invading organisms have been shown to subvert other immune response elements that normally protect the host from succumbing to disease. Examples of these are the production of homologous cytokines or cytokine receptors, interfering with antigen presentation, and blocking apoptosis. In the light of recent discoveries involving pathogen associated molecular patterns, at least one of these mechanisms of immune evasion implicates TLR function. Therefore, the discovery and elucidation of TLR function has provided new knowledge on the host pathogen interface, and a more comprehensive view of immunological events and pathways. For example, pathways specifically involved in immune responses against complex pathogens such as *Mycobacterium tuberculosis* are mediated by a number of TLR interactions (Quesniaux et al., 2004). In addition to microbial interaction and intervention, a number of agents that can be used as adjuvants have been associated with specific TLR involvement in the initiation and qualitative direction of immune responses. This is potentially highly important for vaccine design.

Table 1
Selected adjuvants and moieties recognised by TLRs

Adjuvant/moiety	Cellular location	TLR designation
Triacyl lipoproteins	Cell surface	TLR 1
Gram positive peptidoglycan; lipoproteins; lipoteichoic acids; fungi; viral glycoproteins	Cell surface	TLR 2
dsRNA/Poly I:C (synthetic dsRNA analog)	Cell compartment	TLR 3
Monophosphoryl lipid A (MPL)/synthetic lipid A mimetics	Cell surface	TLR 4
Flagellin	Cell surface	TLR 5
Diacyl lipoproteins	Cell surface	TLR 6
ssRNA/guanosine analogue, Loxoribine	Cell compartment	TLR 7
Small synthetic compounds; ssRNA	Cell compartment	TLR 8
Unmethylated CpG DNA	Cell compartment	TLR 9
Unknown	Cell surface	TLR 10
Profilin	Cell surface	TLR 11

Signal transduction events mediated by TLR molecules, share a number of adaptor molecules (e.g. Myeloid differentiation factor 88 primary response gene (MyD88)/MyD88 adapter-like (MAL)/Toll interleukin-1 receptor (TIR) domain-containing adaptor-inducing interferon-β (TRIF)/TRIF-related adaptor molecule (TRAM)) both within the TLR family and with other immunologically important molecules (such as the IL-1 receptor and the tissue necrosis factor receptor superfamily), mediating intracellular signalling, leading to crucial events such as dendritic cell maturation and inflammatory cytokine production (Kobayashi et al., 2004) and implicating activation of NF-κB (Takeuchi and Akira, 2001). It is clear that the recognition of microbial patterns by the immune system through TLR and similar receptors has begun to rationalise what was largely an empirical process of adjuvant discovery in the field of vaccine formulation. Continuing identification of TLR ligands and the involvement of specific moieties in immune activation via TLR induced events has stimulated much research into the development of these moieties and their synthetic analogues as vaccine adjuvants (Baldridge et al., 2004).

4.2. Particulate delivery systems as adjuvants

Interestingly, all the pharmaceutical vaccine adjuvant formulations presently being tested are particulate based. The concept of

particulate adjuvants is clearly derived from nature: all pathogens are particulate and particulates are passively targeted to the antigen-presenting cells within the immune system, and have the ability to provide persistent antigen due to slow degradation. Indeed immunological memory, as already identified as a key attribute of a successful vaccine, depends on the persistence of antigen on dendritic cells in lymphoid follicles (Nossal and Gl, 1971). This is essentially why antigens in solution generally fail to provide an effective immune response, since if an antigen does not reach lymphoid organs it is ignored by immune cells (Zinkernagel et al., 1997).

Synthetic particulate delivery systems for protein sub-unit vaccines (e.g. liposomes or polymer microspheres) have received much interest as potential adjuvants and there is a large body of research investigating them which has been extensively reviewed by many (e.g. Bramwell and Perrie, 2005a,b). When considering the mechanism of adjuvant action of particulate delivery systems, it may be especially important to consider the geographical concepts of antigen distribution. The induction of immune reactivity is thought to depend upon antigen reaching and being available in lymphoid organs in a dose and time dependent manner. It is thought that antigen that does not reach lymphoid organs is ignored by immune cells (Zinkernagel et al., 1997). It has been suggested that antigen kinetics, load and distribution are different for pathogens and model antigens and that this also contributes to the effective immune responses initiated against pathogens in comparison to soluble antigen (Bachmann et al., 1998). Facilitation of effective antigen delivery to draining lymph nodes is therefore a potentially highly desirable facet of candidate vaccine particulate delivery systems. It is likely that biodistribution, including any depot effect, and antigen kinetics mediated by incorporation into a delivery system play a highly important role in the mechanisms of adjuvant activity of particulate delivery systems. At least in part, this role may be assisted by effective uptake of many particulate systems by antigen-presenting cells. The extent and diversity of formulation materials and methods employed experimentally for these systems is truly enormous and even the longest reviews cannot encompass more than the tip of the proverbial iceberg (Bramwell and Perrie, 2005a,b). However, some of the most promising and interesting formulations include carriers that utilise chitosan as an absorption enhancing agent for mucosal delivery, immunostimulating complexes or 'ISCOMs', first described by Morein et al. (1984) and now further refined (Könnings et al., 2002; Demana et al., 2004) with novel cationic systems also being developed (Lendemans et al., 2005) and implant systems (Demana et al., 2005). Virus-like particles and virosomes, as well as cationic lipid vesicles prepared from (e.g.) dimethyl dioctadecyl ammonium (DDA) chloride (e.g. Davidsen et al., 2005) or DC-Cholesterol (e.g. Vangala et al., 2007) have also been investigated. Brewer et al., 2004; Fig. 1 shows examples of particulate delivery systems in vaccine design along with the major mechanisms of adjuvant activity postulated for these delivery systems.

5. Liposomal systems

Systems of specific interest within our laboratories are lipid based formulations as they offer many opportunities to integrate the above formulation and immunological strategies. In particular we have recently focused on the use of cationic surfactant based systems. Initial investigations compared various particulate delivery systems for the delivery of a sub-unit tuberculosis (TB) vaccine. Throughout these investigations, cationic adjuvant liposome formulations consistently initiated an enhanced and diverse immune response as compared to other systems, encompassing both humoural and cell mediated responses, with a particular emphasis on Th1 immunity – and more specifically the key cytokine marker for anti-mycobacterial immunity, IFN-γ – an important facet for TB vaccine efficacy. Indeed, despite initial promising results from microsphere systems incorporating the cationic adjuvant DDA, and subsequent attempts to optimise and improve the immunogenicity of the microsphere formulation through variation of formulation parameters and constituents, liposomes continued to show a greater ability and potential to act as sub-unit vaccine delivery systems (Kirby et al., 2008, in press).

Cationic lipids and cationic liposomes are more frequently reported for their use as gene delivery systems than for applications as vaccine adjuvants. However, considering the possibility of associated toxicity (e.g. Farhood et al., 1992) and their problems with rapid recognition *in vivo*, this is surprising. Early work (Black and Gregoriadis, 1976) using cationic liposomes as drug delivery systems recognised the problems of cationic moieties interacting with serum proteins and this was clearly identified as a problem in the first gene delivery studies using Lipofectin (Felgner et al., 1987) with lipoplex mediated transfection being inhibited by the presence of sera. Therefore, a great deal of effort has been focussed on circumventing these problems such that these systems can have applications in non-viral gene therapy. However, the issues with biological recognition of cationic lipids, whilst a problem in gene therapy could indeed mean they offer a realistic opportunity for vaccine adjuvants (McNeil and Perrie, 2006) – not only for DNA vaccines (e.g. Perrie et al., 2003) but also for protein-based vaccines (e.g. Davidsen et al., 2005; Vangala et al., 2006). Cationic lipids such as DC-Chol and DDA have been effectively used as adjuvants for such systems (Andersen, 1994). For example, it has been claimed that DC-Chol is able to overcome the observed non-responsivness to hepatitis B vaccine: Brunel et al. (1999) have outlined its use in a liposomal adjuvant where, if used in conjunction with genetically engineered Hepatitis B, the levels of specific antibodies (IgG1 and IgG2a) and cell factors can be increased.

An extensively investigated cationic system are the adjuvant vesicles used by Holten-Andersen et al. (2004) for the delivery of tuberculosis sub-unit vaccines. These are formulated using the cationic, micelle-forming surfactant DDA mixed with trehalose 6,6′-dibehenate (TDB). TDB is a synthetic analogue of trehalose 6,6′-dimycolate (TDM, or cord factor), which is an immunostimulatory component of the mycobacterial cell wall (Pimm et al., 1979; Olds et al., 1980). This combination showed an effective adjuvant activity with the ability to raise a high level of protective immunity in comparison to the DDA carrier alone. The adjuvant monophosphoryl lipid A was also shown to improve the effectiveness of the DDA vesicle formulation as an adjuvant for mycobacterial protein antigens in this study (Holten-Andersen et al., 2004) and previously (Brandt et al., 2000). However, neither of these formulations was examined for their physico-chemical characteristics, such as vesicle size, surface charge (zeta) potential, quantification of antigen adsorption, antigen release profiles or morphological characteristics (e.g. using transmission or environmental scanning electron microscopy; Mohammed et al., 2004; Vangala et al., 2006). In this case, the desire to take such a potentially effective vaccine adjuvant system towards clinical analysis and trials has led to extensive non-clinical evaluation in an effort to fulfil the requirements outlined in World Health Organisation (WHO) and European Medicines Agency (EMEA) guidelines concerning vaccine adjuvants and delivery systems. Therefore, subsequent publications outlining the potential of the DDA–TDB combination have directed efforts towards the improvement of stability and generation of a sterile product by freeze drying and γ-irradiation sterilisation, respectively (Mohammed et al., 2006) as well as extensive work towards full characterisation of this adjuvant delivery system (Davidsen

et al., 2005). Partly as a result of these investigations, the utilisation of this system as a potentially robust platform technology for other antigens has emerged. For example Vangala et al. (2007) have recently proposed the use of this system in the search to find a more effective vaccine against hepatitis B. The development of an effective therapeutic vaccine against hepatitis B would have enormous clinical benefit and a proportion of vaccine recipients fail to respond successfully to the current prophylactic immunisation schedule. The cited work (Vangala et al., 2007) presented extensive physico-chemical and pharmaceutical analysis along with strong immunological analysis. The exact mechanism of action of the TDM analogue, or the DDA–TDB combination is as yet unknown. However, as TDM is produced by *M. tuberculosis* there has been ample opportunity for the evolution of discrete and specific innate immunological signalling associated with this moiety. Overall, the DDA–TDB system is a potent vaccine adjuvant that incorporates a very modern adjuvant by traditional definition in combination with specific delivery system characteristics imparted by the DDA carrier.

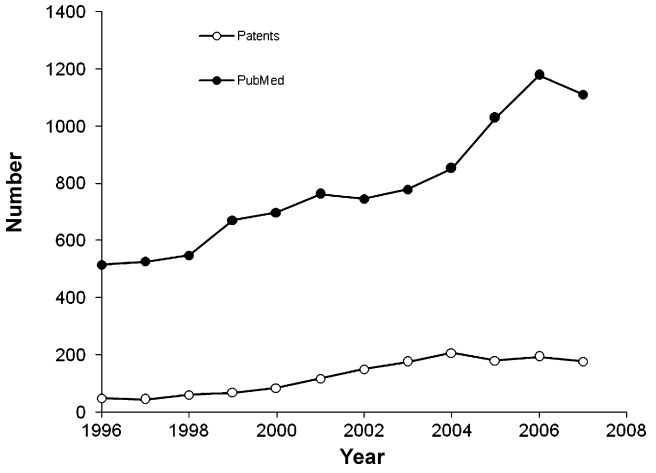


Fig. 3. Comparison of publication and patent applications published using Pubmed and the European Patent office using the search criteria of "vaccine AND adjuvant". Both sites were access 12/01/08.

6. Opportunities for adjuvant combinations

Despite the elucidation of TLR ligands from a diverse range of microbial sources (Storni et al., 2005) observations have been interpreted to argue against TLR involvement in the generation of immune responses by many adjuvants, including PLGA and other particle mediated antigen delivery systems—it is thought that the range of receptors required would be unfeasibly numerous (Sun et al., 2003). However, and quite probably important for the rational design of some vaccines (Bramwell and Perrie, 2005a,b), the use of co-adjuvants in delivery system formulations may facilitate the involvement of these pathways.

Oil emulsions, adjuvant vesicles, liposomes and niosomal delivery systems are all highly amenable to the inclusion of co-adjuvants that could increase mechanisms of adjuvant action to include TLR signalling. Evidence for the potential of this approach is highlighted by improved uptake by target cells facilitating enhanced activity of CpG motifs (that bind with TLR 9) mediated by liposomal entrapment (Suzuki et al., 2004) and a similar approach for enhancing immune responses to intradermally administered dendritic cell targeted peptides (Ludewig et al., 2000). One strategy for the delivery of a co-adjuvant in liposomes involves synthetic lipopeptide analogues of potent lipoprotein immunoadjuvants covalently linked to small peptide epitopes. Exploiting amphipathic properties of the lipopeptide adjuvant moieties for easier incorporation into liposomes, their immunological activity can be improved and, additionally, functionalized lipopeptides can facilitate chemo selective conjugation of peptides to the surface of the vesicles (Roth et al., 2004). Nasal administration of chitosan has been shown to facilitate the adjuvant action of muramyl dipeptide indicating the potential for co-administration of these adjuvants in order to obtain further enhanced immune responses (muramyl dipeptide is an adjuvant not normally effective *via* this route) (Moschos et al., 2004).

7. Future developments in vaccines against diseases, old and new

There is clearly a strong body of literature investigating the various adjuvant systems and their application within vaccines however the progression of these systems from a research project to a viable pharmaceutical product is severely limited. This is demonstrated by the lack of patent applications despite the large volume of published investigations into vaccine adjuvant systems (Fig. 3) suggesting we are not effectively exploiting this knowledge to systematically develop new vaccines.

Indeed, despite all our advances in research we still are without effective vaccines against the three recognised 'global killers'—HIV, TB and Malaria. The problems with developing vaccines for each of these infections are well reported, yet we remain unable to find mechanisms to circumvent these problems. For example, there are several reasons why we need to replace BCG, including manipulation of immune responses (as for virulent TB) (Gagliardi et al., 2004) and variation between propagated strains of BCG. Recent studies (Demissie et al., 2004; Flynn et al., 1993) suggest we need to focus our efforts on designing vaccines which suppress interleukin 4. For HIV, only one HIV candidate vaccine has completed clinical trials phases I, II, and III in more than 20 years of the epidemic. The phase III trial was based on the use of recombinant envelope proteins with the aim of evoking virus neutralizing antibodies. However, results from this were disappointing.

Vaccination remains a key tool in the prevention and treatment of many diseases. In the trade off of improved safety versus efficacy there is clearly an important role for adjuvants, especially particulate delivery systems. However, both the physical attributes and the cellular effects induced by delivery system components are fundamental in the elicitation of immune responses directed against associated or entrapped antigen and the understanding of such interactions are equally important in the design of particulate delivery systems for vaccines.

There is an increasing body of research that supports the tenet that different mechanisms of adjuvant activity can engender additive or even synergistic effects. This includes the combination of delivery system technology with known TLR ligands as well as adjuvants that have other mechanisms of action, such as surface active agents, for example by facilitating the elucidation of adjuvant activity following mucosal administration (e.g. Moschos et al., 2004). Delivery systems function in ways that can be perceived to be distinct from other adjuvants with a defined immunological interaction. Here too there is much scope for the maximisation of adjuvant activity by the alteration of formulation characteristics, for example by the use of polymers with different hydrophobicity for the formulation of microsphere or nanosphere polymer carriers. Recent developments encourage the characterisation of the mechanisms of adjuvant action in order to facilitate the rational design of vaccine formulations suitable for the delivery of protein subunit antigens. Interpretation of the importance of specific adjuvant

mediated events serves to highlight the importance of evaluation of these immunological interactions and pathways as an adjunct to, rather than a replacement of, our present understanding of carrier system technology for vaccine delivery.

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