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Particulate delivery systems for vaccines: what can we expect?

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Abstract

In our attempts to thwart the unwanted attentions of microbes by prophylactic and therapeutic vaccination, the knowledge of interactions at the molecular level may prove to be an invaluable asset. This article examines how particulate delivery systems such as liposomes and polymer microspheres can be applied in the light of recent advances in immunological understanding. Some of the biological interactions of these delivery systems are discussed with relevance for antigen trafficking and molecular pathways of immunogenicity and emphasis on the possible interaction of liposomal components. In particular, traditional concepts such as antigen protection, delivery to antigen presenting cells and depot formation remain important aspects, whilst the inclusion of selected co-adjuvants and enhanced delivery of these moieties in conjunction with antigen now has a firm rationale.

Introduction

The elucidation of mechanisms of microbial recognition and the complex interactions of cells likely to first encounter antigen, such as macrophages, dendritic cells (DC) and epithelial cells (e.g. in the gut), have begun to offer real opportunities for the rational design of vaccines. Additionally other strategies, although not discussed in detail here, such as the application of molecular biology techniques – including reverse vaccinology and other associated technologies – offer significant and complementary potential by, for example, the definition of protective antigens.

In the evaluation of candidate vaccine formulations it is increasingly possible to define components based on their molecular immunological interactions. The adjuvant and subunit approach has traversed an immunological revolution that justifies a full re-characterization or re-definition of vaccine adjuvants. We can now evaluate biological interactions at the molecular level with much greater significance for their real immunological potential. What then are the implications of this for particulate delivery systems such as liposomes and polymer microspheres?

This short review highlights some of the immunological interactions of specifically acting adjuvants and relates this to the potential for their use in particulate vaccine delivery systems. Co-delivery of associated or entrapped adjuvant and antigen is proposed as a possible path forward in enhancing the potential of particle-based delivery systems, with the main emphasis on improving liposomal and biodegradable polymer-based vehicles.

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Overview of particulate antigen delivery systems

It is largely believed that the manner in which antigen reaches lymph organs is fundamental in the induction of an immune response. Antigen-presenting cells are of critical importance for the transport of antigen from the periphery to local organized lymphoid tissue. If antigen does not reach lymphoid organs, however, it is ignored by immune cells (Zinkernagel et al 1997) and this is essentially why antigen in solution generally fails to provide an effective immune response. The role played by particulate antigen delivery is very much dependent on the mediation of effective uptake by antigen presenting cells (APCs). Following this, antigen trafficking to lymph nodes and other lymphoid organs is able to facilitate effective

immune responses (Ludewig et al 2000; Eyles et al 2001, 2003a). This facet has also been shown to be potentially important for DNA vaccines (Perrie et al 2001). Previous literature has defined delivery system technology and differentiated between immunological adjuvants and delivery systems (Bramwell & Perrie 2005a; Gander 2005). Some adjuvants have been shown subsequently to have a particulate format and many candidate vaccines have intrinsic particulate characteristics, such as dimethyldioctadecylammonium (DDA) and virus-like particles, respectively (Zhou et al 1991; Rose et al 1993; Davidsen et al 2005).

Recent work has shown that antigen (recombinant *Bacillus anthracis* protective antigen) in poly(-lactic acid) (PLA) microspheres was able to activate DC, as measured by the increase in expression of cell surface markers indicative of increased activation (Westwood et al 2005). Conversely, soluble antigen was ineffective in DC activation. Although blank microspheres alone showed some level of ability to activate DC, interestingly this appeared to be increased in the presence of the recombinant antigen. This offers some support to previous attempts to elucidate the mechanisms of adjuvant action for similar polymeric carriers where the authors concluded that the mechanism of action is likely to be distinct from non-specific effects caused by components of the delivery vehicle itself (Eyles et al 2003b). Sun et al (2003) had previously seen increased antigen presentation using poly (lactide-co-glycolide) (PLGA) and non-ionic surfactant vesicles (NISV) for antigen delivery to bone marrow-derived DC. However, they failed to see an increase in DC activation using a different antigen (ovalbumin) to Westwood et al (2005).

The trend in new generation vaccine development is undoubtedly towards clearly defined systems that can also increase the immunogenicity of purified subunit antigens. Aluminium hydroxide or aluminium phosphate (alum) may work well as an adjuvant where the required immune response is biased towards antibody-mediated responses and the antigen is highly amenable to the generation of effective immune responses (e.g. tetanus and diphtheria toxoids). However, the adjuvant effect mediated by alum will not suffice for many potential vaccines. Additionally, whilst sharing some particulate characteristics, stability concerns raised by alum adsorbed and emulsion-based vaccines are issues that could be ameliorated by the use of appropriate delivery system technology. The potential for reduction in the number of doses, supply of a more stable product (e.g. by manufacture of a freeze-dried product (Mohammed et al in press)), mucosal administration and induction of an appropriate or enhanced immune response can all be supported by the use of a particulate delivery system such as liposomes or biodegradable polymer microspheres (Friede & Aguado 2005). Such multiple-step formulation procedures can have difficulties in maintaining good manufacturing practice (GMP) conditions and some systems may adversely affect antigen stability. The sterility of liposomal vaccine formulations can be effectively mediated by gamma irradiation in order to provide a pharmaceutically acceptable product without protein degradation (Mohammed et al in press), and in polymer microparticles components can be chosen in order to avoid polymer-mediated acid hydrolysis. For example, poly (caprolactone) (PCL) degrades more slowly than PLGA and therefore does not generate an unfavourable low pH micro-environment for antigens (Singh et al 2006). However, chemical and physical antigen stability does need to be assessed for these systems; see Jiang et al (2005) for an interesting and informative review on this aspect. The requirements for each system will vary: whilst native or tertiary protein structure will be important for conformationally dependent neutralizing antibody responses, linear or cytotoxic T lymphocyte (CTL) epitopes are not dependent on protein conformation but will be affected by excessive protein degradation.

Currently, liposome-based vaccine products are available as virosome vaccines against hepatitis A and influenza (Epaxal and Inflexal V, respectively), whilst liposomal delivery of a MUC-1 peptide (MUC-1 is a potential cancer antigen) has entered phase II/III clinical trials (Felnerova et al 2004). In terms of a biodegradable polymer-based vaccine, reports of protection following a single administration (Elvin et al in press) and longevity of immune responses (in particular against PLGA entrapped antigen) continue to demonstrate impressive results (Jiang et al 2005) for many candidate vaccine delivery systems.

Applications and opportunities

Enhancing innate immunity

Recent research suggests that cross-talk between cytosolic nucleotide-binding oligomerization domain (NOD)1 and membrane-bound Toll-like receptors (TLR) can enhance responses to the multiple antigens simultaneously presented by a microbe (van Heel et al 2005). Also, pathogens may contain several TLR agonists and in human and mouse DC, TLR3 and TLR4 have been shown to act in synergy with TLR7, TLR8 and TLR9. Results potentially identify a 'combinatorial code' by which DC discriminate pathogens and suggest new strategies for promoting T helper type 1 responses (Napolitani et al 2005). Added to this, the observation that heterodimers of some TLR molecules are able to recognize other moieties gives a real possibility that the number of microbial elements that can be recognized by these innate mechanisms may be further expanded in the future. Clearly opportunities exist for the co-delivery of selected TLR and/or NOD ligands for the enhancement of immune responses. This can provide the basis for the use of particulate delivery systems. The delivery of TLR ligands will likely facilitate efficient induction of specific immune responses where such ligands can be effectively co-delivered to cells of the immune system in conjunction with antigen. Interestingly, the downstream effects of targeting such receptors is likely to be highly dependent on the cell type; for example, a lack of inflammation in human

oral epithelial cells has been noted following stimulation of peptidoglycan recognition proteins despite a marked activation of nuclear factor kappa B (NF-κB) (Uehara et al 2005). In the context of particulate vaccine delivery systems, the further facet of co-delivery to the same cell/s, and to APC in particular, could be a highly effective way of targeting TLRs recognizing nucleic acid derivatives and the growing family of NOD-like receptors, localized in intracellular compartments such as the endosomal membrane (TLRs 7, 8 and 9) (Dunne & O'Neill 2005) and the cytosol (NOD family proteins), and harnessing these potentially powerful immunostimulatory pathways. We may expect that in addition to the co-delivery of adjuvants assessed in a number of studies to date, a clear rationale may be provided for the mechanisms of action regarding these systems. In addition, the increasing knowledge of immunological interaction in terms of pathogen recognition may provide the basis for the rational application of specifically acting adjuvant moieties.

Mucosal immune responses

In the mucosal environment, unlike any parenteral route, a diverse microbial flora makes up a significant part of the local milieu. The ability of innate mechanisms to distinguish between pathogens and commensal bacteria is not clear cut (Kelly & Conway 2005). Immunologically, interactions at the mucosal interface are probably the most complex and interesting, and current opinion suggests that synergistic as well as antagonistic bacterial and host-derived elements contribute to the generation or amelioration of host immune responses, in particular innate immune activation and interaction. Innate immunity (conceivably) uses the integration of recognition events for the generation of a more specific signal (Kelly & Conway 2005). Interestingly, in a detailed review of CpG-containing bacterial DNA and its immunophysiological impact on the intestine, the authors concluded that widespread expression of TLR9 (the receptor for CpG DNA) throughout the gastrointestinal tract implies a capability to detect and respond to bacterial DNA. Furthermore, this response might be expected to be pro-inflammatory owing to the primarily Th1-biased cytokine production observed with CpG-stimulated cells. However, paradoxically, systemic and oral administration of immunostimulatory synthetic CpG motifs or probiotic bacterial DNA reduces the severity of experimental inflammatory colitis (Watson & McKay 2006).

In such an environment, it is therefore clear that in addition to the physiological barriers traditionally associated with mucosal administration of vaccines there are also complex immunological considerations. There is the potential for providing a set of characteristics that may define features desirable for effective vaccines, although this comparatively complex environment remains poorly understood at the molecular level in terms of benefits for vaccine design. It may be expected that co-delivery of specifically acting adjuvants could yield surprising results following mucosal administration and also that these investigations will aid the understanding of interactions at mucosal surfaces. It is encouraging that so many reports have outlined the facilitation of effective immune responses using liposomal or polymer particulate delivery systems, and some of these are outlined further below.

Vaccine delivery format: a biological perspective

Many successful vaccines have been based on attenuated organisms. In the context of viral vaccines, the traditional approach to attenuation is the repeated passage of the virus in semi-permissive cells or altered conditions, such as lower temperature. The ability of the virus to cause disease in its original host is then compromised but ideally they retain immunogenicity and evoke a protective immune response against the wild type virus. Potential problems, however, may relate to the viral lifecycle. In mammalian cells, replication of DNA is highly conserved due to proof-reading by DNA polymerase enzymes, and the replication of an RNA genome such as that of poliovirus is inherently inaccurate due to the lack of any such proof-reading. In fact, it is unlikely that any copy of a viral RNA genome is exactly the same as the template from which it was copied. This relatively high mutation rate can be circumvented during propagation of the virus by the cloning of the whole genome of the seed strains into the producing cells as complementary DNA, as has been achieved for poliovirus. This process is referred to as genetic stabilization but the problem of reversion to virulence after administration still remains for poliovirus and other RNA viruses. Host pathogen interactions are reviewed in more detail elsewhere (Alpar & Bramwell 2002). The move away from live organisms as vaccines represents a potential move towards increased safety.

Recently, the prospective licensure of two new vaccines against human papillomavirus (HPV) has aroused significant public and scientific interest (Bramwell & Perrie 2005b; Cohen 2005). The vaccines – one from Merck and one from GSK – are effectively vaccines against cervical cancer and exploit the link between this common virus, which rarely causes serious disease in itself, and cervical cancer. The production of both vaccines (the Merck vaccine produced in yeast administered with an aluminium adjuvant and the GSK vaccine in baculovirus with the adjuvant AS04; aluminium and bacterial lipid already approved for use in Europe) is based on morphological and immunological similarities to native HPV virions from the production and self-assembly of virus-like particles of the HPV outer L1 and L2 coat proteins initially described in the early 1990s (Zhou et al 1991; Rose et al 1993). Native HPV particles are approximately 55 nm in diameter.

Many viruses owe their discrete tissue tropism to targeting at the receptor level, although there is much diversity that might be exploited for vaccine delivery. For example, it is thought that poxvirus tropism at the cellular level is regulated by intracellular events downstream of

virus binding and entry, rather than at the level of specific host receptors (McFadden 2005). In another scenario, cell-specific gene expression is achievable for the delivery of genes encoding potential antigens using (for example) adenovirus vectors otherwise more promiscuous in gene expression (Sadeghi & Hitt 2005). Using the natural tropism of adenovirus for mucosal surfaces, this platform has shown much potential for use in the delivery of adenoviral expression vectors as well as recombinant vaccines for delivery of heterologous antigens (Rim Seong et al 1998; Santosuosso et al 2005).

In the same way, the diversity of strategies incorporating and exploiting both the natural and contrived characteristics of bacterial invasion and their diverse and tractable genetic features offers much potential in the generation of a vaccine delivery platform. In similar fashion to the delivery of eukaryotic expression vectors as DNA vaccines (notably with the potential for oral delivery), the use of attenuated *Salmonella* for delivery of a recombinant plasmid encoding for the non-toxic B subunit of *E. coli* LT was shown in-vivo for the purposes of vaccination by Clements et al (1986). Oral immunization of mice showed progressively increasing mucosal and serum antibody responses to both the *E. coli* LT subunit and the lipopolysaccharide (LPS) of the vaccine strain, together with the mucosal production of neutralizing IgA. Similar early studies also showed positive results for this strategy; Fairweather et al (1990) used a plasmid-encoding tetanus toxin fragment C. Work building on these results using expression of tetanus toxin fragment C driven by the anaerobically inducible nirB promoter in *S. typhimurium* as a live oral vaccine in BALB/c mice was shown to protect against a lethal tetanus toxin challenge (Chatfield et al 1992; Alpar & Bramwell 2002).

Thus, many strategies for vaccine delivery have used biological processes and discreet traits of pathogenic organisms in order to realize effective antigen delivery systems.

Formulation of particulate delivery systems and co-adjuvants in vaccine delivery

Liposomes and liposome-based delivery systems

Initially described as adjuvants more than 30 years ago (Allison & Gregoriadis 1974), liposomal systems have received much attention for their potential biocompatibility as well as their significant versatility, showing potential for the delivery of protein as well as DNA vaccines (Gregoriadis et al 1999; Perrie et al 2003). The composition of liposomes will undoubtedly have effects on their interaction with cells of the immune system, such as dendritic cells (Foged et al 2004). The use of specific components will also have effects on targeting, uptake and biological response, although interactions may be complex. For example, whilst the incorporation of phosphatidylserine (PS) may facilitate the targeting of APC through recognition by PS receptors, PS-containing liposomes have been found to specifically inhibit immune responses, giving reduced numbers of total leukocytes and antigen-specific CD4+ T cells in mice (Hoffmann et al 2005). This could be a result of the natural function of PS, which promotes the uptake of apoptotic cells and induces anti-inflammatory responses in phagocytes, although interpretation of the data intimates at other complex biological interactions. Mannose-targeted PorA (a major antigen of *Neisseria meningitidis*) liposomes were shown to have promise in terms of targeting of DC and subsequent IL-12 production. However, complement-mediated bactericidal serum activity was similar for all formulations tested following in-vivo administration in mice (targeted and non-targeted liposomes) (Arigita et al 2003). Interestingly, exploitation of mannose receptor uptake by *Mycobacterium tuberculosis* is probably a key event in enhancing the survival of this important pathogen following phagocytosis by human macrophages by limiting phagosome lysosome fusion (Kang et al 2005). This was a function of *M. tuberculosis* derived mannose capped lipoarabinomannan rather than a function of mannose receptor targeting itself, which may be a useful vaccine delivery strategy, corroborated by work that has shown that mannan-coated liposomes enhance immune responses (Toda et al 1997). The idea of targeted delivery is one that has attracted much research and interest for liposome-based systems. Work outlining the potential for the delivery of liposomal agents targeted by the use of specific immunoglobulins generated much excitement (reviewed by Toonen & Crommelin 1983). Even today, improved biodistribution and ligand conjugation (amongst other strategies) offer a lot of potential for the treatment of cancer (Park et al 2004).

The co-delivery of other adjuvants is an area that has shown much promise for liposomal formulations in vaccine delivery. A subsequent study using PorA with different liposomally delivered LPS derivatives showed significant promise for a mutant *N. meningitidis* LPS (*lpxL*) (Arigita et al 2005). The altered LPS is produced in *N. meningitides lpxL* mutants and has 100-fold reduced toxicity compared to LPS (van der Ley et al 2001; Arigita et al 2005). Table 1 shows some strategies and applications for liposomal delivery systems with different compositional elements or co-adjuvants.

The immunostimulatory properties of a *M. bovis* BCG lipid extract have recently been tested for their adjuvant activity by administration of the lipids in dimethyl dioctadecyl ammonium bromide-based cationic lipid vesicles. It was found that this 'mycosome' formulation induced a powerful Th1 response (Rosenkrands et al 2005). The adjuvant properties of the mycobacterial cell wall have been exploited for many decades in Freund's complete adjuvant. The authors (Rosenkrands et al 2005) cite the recent elucidation of the underlying mechanisms behind this adjuvant activity as confirming the potential of these elements as adjuvants for inclusion in their particulate vesicle delivery system.

Interestingly, with regard to delivery of proteins for intracellular processing and exploitation of innate

Table 1 Liposomal delivery systems incorporating co-adjuvants or comparing compositional elements

Liposomal formulation	Antigen/s or disease-causing agent	Relevant points
DSPC DPPC DMPC	*Leishmania donovani*	A strong DTH was observed with antigen in DSPC liposomes which corresponded with 95% protection, compared to almost no protectivity with antigen in DPPC and DMPC liposomes (Mazumdar et al 2005)
Liposome/ polycation/ DNA particles	HPV 16 E7 protein	Induced strong cellular and antibody mediated immune responses and tumour regression in mice (cervical cancer model). Carrier/adjuvant particles may offer potential for other antigens (Cui & Huang 2005)
Liposomes with Pam3CSS lipopeptide adjuvant and a CTL and Th epitope	ErbB2-expressing murine renal carcinoma	Liposomal co-delivery of a CTL epitope from human ErbB2 (HER2), and a Th epitope from influenza haemagglutinin. Peptides were conjugated to the surface of liposomes via a Pam3CSS (synthetic lipopeptide and potent adjuvant) anchor. Showed combination of CTL and Th peptide antigens with lipopeptide adjuvants represent promising synthetic delivery systems for the design of specific antitumour vaccines (Roth et al 2005)
CpG-ODN encapsulated in cationic liposomes	Ovalbumin; model antigen	Enhancement of many immunologically relevant markers in comparison with unmodified CpG-ODN. Notable in stimulating Th1 immunity in mice; possibly exploiting the intracellular location of TLR9 by effective liposomal delivery to relevant cells of the immune system (Suzuki et al 2004)
Liposomes containing lipid A	Oligomeric HIV-1 gp140	Liposomes containing monophosphoryl lipid A and other LPS derivatives have been used extensively in the analysis of liposomal vaccine delivery systems and highlight the potential for the incorporation of lipophilic and amphiphilic adjuvants in liposomes. The cited study emphasized the importance of a stable oligomer/liposome emulsion for the generation of CTL responses and hypothesized that this might be due to the depot effect from a stable emulsion (Richards et al 2004)
DOTAP/cholesterol liposomes	Human sperm surface antigen conjugated to DT	The CD52 human sperm surface antigen core peptide elicited IgA and IgG antibodies in sera and vaginal secretions after intranasal immunization (Hasegawa et al 2002) Other studies have shown cationic lipids to have good adjuvant activity following parenteral administration; for example DC-Chol for hepatitis B surface antigen (Brunel et al 1999) and DDA for multiple tuberculosis antigens (Andersen 1994) and neutral liposomes (DSPC and cholesterol) have also been shown to have adjuvant capability following intranasal (and oral) delivery, but to a lesser extent than parenteral administration (Alpar et al 1992)

DSPC, distearoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; DTH, delayed type hypersensitivity; HPV, human papillomavirus; CTL, cytotoxic T lymphocyte; Th, T-helper; CpG-ODN, unmethylated cytosine-phosphorothioate-guanine oligodeoxynucleotides; DOTAP, dioleoyl trimethylammonium propane; DT, diphtheria toxoid; DC-Chol, 3β[*N*-(*n*′,*N*′-dimethylamionethane)-carbamoyl] cholesterol; DDA, dimethyl dioctadecyl ammonium bromide.

targets, cytosolic delivery of protein antigens for MHC class I and II presentation may be achieved by virosomes. Virosomes can be engineered to bind to sialic acid (the cellular receptor for influenza hemagglutinin) on APCs such as DC, initiating receptor-mediated endocytosis. Fusion of the virosomal and endosomal membrane is triggered by conformational changes in the hemagglutinin at the lower pH of the endosome and encapsulated protein can be released into the cytosol (Daemen et al 2005). The protein can then be processed by proteasomes or remaining protein degraded in the endosome with subsequent presentation on MHC class I and MHC class II molecules respectively.

Polymer-based systems

The adsorption of antigens to polymer-based particulate carriers is attracting increasing interest. Adsorption helps to avoid the exposure of the antigen to organic solvents, high shear stresses and to the low pH caused by polymer degradation. The adsorption of influenza antigens to the surface of polymeric lamellar substrate particles (PLSP) that were produced by a simple precipitation of PLA was almost five times as effective as that obtained using influenza virus adsorbed to submicron PLGA microspheres. Immunogenicity was measured in terms of mean antibody-mediated haemagglutination inhibition by sera samples following intramuscular injection in mice (Coombes et al 1998). The angular lamellar morphology of PLSP is highly unusual, but this facet is thought to contribute to the observed immunogenicity in comparison to plain or spherical PLA particles. The use of modified polymers may also offer potential; the enhancement of immunogenicity mediated by polymer systems has included the development of acid-degradable protein-loaded polymer particles by the use of a more hydrophilic acid-degradable cross-linker, facilitating enhanced survival of tumour-challenged mice (Standley et al 2004). The capability for altering degradation rates by the use of different polymer ratios and polymer molecular weights is well known. Additionally, factors such as size, antigen load and dose are important. A study using a PLGA-encapsulated diphtheria vaccine showed that microspheres could be detected at the injection site for up to 4 weeks post-immunization, supporting the concept of a formulation depot with a prolonged controlled antigen release (Peyre et al 2004). Also of interest from this study were the observations of longer term trafficking of the particles inside phagocytic cells from the injection site to lymphoid organs and that the internalized particles were determined to be less than 5 μm in diameter.

In terms of co-administration of adjuvants, it has been shown that the length of immunostimulatory sequences (ISS) containing CpG motifs can be reduced from 12 bases to 5–7 bases when adsorbed to cationic PLGA microparticles. The adjuvant activity of these shorter ISS is apparently mediated by exploiting the plasmacytoid dendritic cell selectivity of this formulation (as opposed to mediating enhanced adjuvant activity via B cells normally associated with ISS) (Fearon et al 2003). TLR9-mediated signal transduction appears to require internalization of CpG DNA. Inhibitors of endocytosis and endosomal maturation, as well as inhibition of endosome trafficking, block the cell-signalling cascades normally elicited by CpG ODN stimulation (Ahmad-Nejad et al 2002). Intranasal administration of diphtheria toxoid (DT) encapsulated in PLA microspheres formulated incorporating chitosan have been shown to enhance serum DT specific antibody titres. Entrapment of DT in PLA microspheres conferred superior antigen specific antibody titres and this was further enhanced by the use of chitosan (Alpar et al 2001). One study demonstrated prolonged immune responses to a single intramuscular administration of DT encapsulated in PLA particles and alum that was comparable with immunization from two separate doses of alum adsorbed tetanus toxoid in rats (Katare et al 2005). Improved immune responses have been seen in mice following intranasal delivery DT encapsulated in PCL microparticles. The immune response was further enhanced by vitamin E TPGS, a water-soluble derivative of vitamin E (Somavarapu et al 2005). Some of the other relevant polymer-based delivery systems incorporating co-adjuvants are summarized in Table 2.

The work by Westwood et al (2006) outlined in Table 2 may be of particular interest as they directly compare the soluble adjuvant and antigen combination to the co-microencapsulation of adjuvant and antigen, showing a marked difference between these groups. This innovative approach is based on the possible enhancement of immunogenicity by delivery of adjuvant (in conjunction with antigen) to the endosomally located TLR, which would probably be inaccessible to soluble adjuvant of this type.

NISV, immunostimulating complexes and chitosan

NISV were first described 20 years ago (Azmin et al 1985), although the ability of non-ionic surfactants to form lamellar vesicles with cholesterol and dicetyl phosphate had been reported earlier by these researchers. The most common composition of NISV that has been investigated for the formulation of antigenic components is 1-monopalmitoyl glycerol, cholesterol and dicetyl phosphate. The use of the charged moiety dicetyl phosphate in these initial formulations has persisted in NISV-based delivery systems, despite the misleading 'non-ionic' insinuation. Initial investigations showed that NISV were generally better stimulators of IgG2a than the potent adjuvant Freund's complete adjuvant. Antigen-specific IgG1 responses were less pronounced and the adjuvant activity of NISV was dependent on the antigen being entrapped within preformed vesicles; mixing free antigen with vesicles was not effective (Brewer & Alexander 1992).

Originally described by Morein et al (1984), immunostimulating complexes (ISCOMs) are a pioneering example of a highly immunostimulatory delivery system for

Table 2 Polymer delivery systems incorporating co-adjuvants

Polymer formulation	Antigen/s or disease-causing agent	Relevant points
PLLA with single-stranded RNA Double emulsion with DOTAP in the secondary aqueous phase	Model antigen: ovalbumin (OVA)	Interesting results following the evaluation of co-encapsulated antigen with single-stranded RNA (ssRNA). The authors (Westwood et al 2006) showed that co-microencapsulated OVA with ssRNA was markedly better than administration of soluble antigen with ssRNA in terms of production of OVA specific antibody responses and IFN-γ from OVA stimulated spleen cells. C57/BL6 mice received three subcutaneous doses at 14-day intervals
PLLA double emulsion, co-encapsulating IL-4, IL-6 or IFN-γ PVA emulsification agent	Recombinant V antigen (rV) of *Yersinia pestis*	All microencapsulated formulations stimulated strong systemic antigen-specific IgG1 titres, or IFN-γ with lower titres of IgG2a and IgG2b. Co-encapsulation with IFN-γ reduced rV-specific IgG1 and IgG2a titres. The level of cytokine in vaccine preparations may have been a factor as it is critical to the adjuvant effect. Formulations based on rV antigen alone or rV co-encapsulated with IL-6 provided complete protection against systemic challenge with virulent *Y. pestis*. Protective efficacy was impaired by co-encapsulating either IFN-γ or IL-4 with rV (Griffin et al 2002). BALB/c mice received two intranasal doses, on days 0 and 55.
PLGA double emulsion, co-administered or compared with various adjuvants: GM-CSF, MPL, IFA	Her-2/neu p369–377 peptide (KIFGSLAFL) The Her-2/neu oncogene is over-expressed in 30–40% of breast and ovarian cancers	GM-CSF augmented the strength of the CTL response elicited by the Her-2/neu microspheres at an early time point, but microspheres alone were comparable 25 days after immunization. The magnitude of the CTL responses following administration of Her-2/neu microspheres admixed with MPL was greater than that elicited by the mice receiving Her-2/neu microspheres alone (Mossman et al 2005). HLA A*0201/Kb transgenic (A2Tg) mice were immunized subcutaneously. Immunization with peptide alone or peptide formulated in IFA failed to elicit CTL responses
PLGA double emulsion, with DSS or SDS in the external aqueous phase Antigen was adsorbed to anionic microparticles	*E. coli*-derived recombinant *N. meningitides* type B antigens	The addition of the immunostimulatory oligonucleotide CpG further enhanced the responses seen with these formulations. The choice of surfactant was found not to be critical, however the unadsorbed formulation was not as good. This emphasises the need for the protein to be adsorbed onto microparticles for generating higher appropriate correlates of immunity (in this case SBA titres) (Singh et al 2004). Mice were immunized via the intraperitoneal route. Surfactant concentrations in the vaccine formulations were low and DSS is used as a formulation component in licensed products for human administration
Adsorption of HA and LT mutants onto HYAFF microspheres	Influenza HA	In pigs the serum immune responses induced following intranasal immunization with the bioadhesive HYAFF-HA-LTK63 formulation were significantly more potent than those induced by intramuscular immunization with the same vaccine dose (Singh et al 2001). Intranasal immunization with soluble HA+LTK63 was comparable with the intramuscular route but reduced in comparison to the HYAFF-HA-LTK63 formulation

DOTAP, dioleoyl trimethylammonium propane; PLLA, poly-L-lactide; PVA, polyvinyl alcohol; GM-CSF, granulocyte–macrophage colony stimulating factor; CTL, cytotoxic T lymphocyte; MPL, monophosphoryl lipid A; IFA, incomplete Freund's adjuvant; DSS, dioctyl sodium sulfosuccinate; SDS, sodium dodecyl sulphate; SBA, serum bactericidal activity; HA, influenza hemagglutinin; LT, heat labile enterotoxin from *Escherichia coli*; HYAFF, esterified hyaluronic acid; LTK63, mutant non-toxic derivative of LT.

antigen delivery. The adjuvant QS21, an integral component of later ISCOM formulations, has been associated with injection pain in phase I human trials but, encouragingly, it was possible to improve the acceptability of QS21-containing formulations through reformulation with certain excipients (Waite et al 2001). Local reactions including pain, erythema and induration, which are thought to be most likely due to the haemolytic nature of the free saponin, are reported to be ameliorated by the incorporation of the saponin into an ISCOMATRIX adjuvant that essentially removes the haemolytic activity and as a consequence the associated severe local reactions (Pearse & Drane 2005).

The use of chitosan has received much attention for mucosal administration and recent developments are outlined elsewhere (Bramwell & Perrie 2005a). The adjuvant action of muramyl dipeptide (MDP) was facilitated by co-administration of chitosan nasally in mice (Moschos et al 2004) – MDP is an adjuvant not normally effective mucosally and the potential for chitosan in combination with particulate delivery systems for mucosal administration has already been outlined.

Case study: expanding on the biological interactions of liposomal components

As hinted at above for phosphatidylserine, many delivery system components can have marked biological interactions. This may be especially so for liposomal components and a number of cationic lipids have been found to have profound cellular effects. Stearylamine has been found to initiate apoptosis in what was thought to be a caspase-dependent process (Hung et al 1999). In addition to this, a number of cationic lipids have been shown to inhibit protein kinase C (PKC) activity (Farhood et al 1992; Filion & Phillips 1997), although this is also dependent on the other lipids present in the liposomal formulation. However, which isoform of PKC is affected is unclear. This may be important, as PKC has recently been shown to have a contrasting regulatory role on apoptosis according to isotype (Gutcher et al 2003), exerting both inhibitory and stimulatory influences. There is much evidence that apoptosis may be intimately involved with the generation of immune responses and be able to facilitate marked enhancement of immunogenicity (Winau et al 2004; Casares et al 2005). Apoptosis has been proposed as a beneficial gateway to promote protective immunity against intracellular bacteria, mycobacteria and salmonella (Winau et al 2004), and offers an important link for enhancement of specific cytotoxic T lymphocyte (CTL) responses.

It is possible that long-chain bases may activate apoptosis by inhibiting PKC. Interestingly, however, the pretreatment of cells with a PKC activator was unable to rescue cells from apoptosis triggered by long-chain bases (Hung et al 1999). Subsequent study found that a caspase inhibitor was able to block apoptosis initiated by long-chain bases and it was concluded that the observed apoptosis was caspase dependent. Recent observations that pretreatment of cells with a reactive oxygen species scavenger conferred resistance to the induction of the membrane depolarization, cytochrome C release and caspase-3 activation by cationic liposomes led to the conclusion that this apoptosis was initiated through the mitochondrial pathway and was dependent on reactive oxygen species (Aramaki et al 2001). That the effects on PKC and apoptosis are separate were speculated by earlier observations on the synthesis of phosphatidlyserine induced by stearylamine (Aussel et al 1995). Some PKC isoforms may also have a role in the activation of nuclear factor-κB (NF-κB) (Moscat et al 2003). NF-κB has been shown to be important for the generation of pro-inflammatory cytokines, enhancing transcription of TNF-α, IL-1, IL-6 and IL-8, and it is certainly very much involved in TLR-mediated immune interaction. The relationship between inhibition of PKC and reduction in inflammation is substantiated by the inhibition of nitric oxide and TNF-α in the macrophage mediated by cationic lipids (Filion & Phillips 1997). However, there may be many different cellular effects as well as contrasting observations. Caspase 1 is known to activate IL-1 and production of IL-6 may be independent of PKC in macrophages (Filion & Phillips 1997). Such contrasting effects and cell specificity make it difficult to extrapolate some results and offer a rationale for further detailed investigation. With reference to neutral lipid components, fragmented phospholipids formed by oxidation of phosphatidylcholine can have a number of biological implications, being pro-inflammatory agonists promoting chronic inflammation in atherosclerosis and activating monocytes and platelets (Kern et al 1998). However, oxidation products can inhibit expression of inflammatory adhesion molecules and blocking the interaction of LPS with LPS-binding protein and CD14 can inhibit LPS-induced (but not TNF-α- or IL-1β-induced) NF-κB upregulation of inflammatory genes (Bochkov et al 2002). Lysolecithin can contribute to PKC activation depending on the composition of the lipid membrane, although higher concentrations were found to be inhibitory (Sando & Chertihin 1996); elevated levels may impair endothelial barrier function and contribute to inflammatory responses (Huang et al 2005). It is clear that many potential vaccine delivery system components have the potential to elicit profound biological effects that may contribute to their observed immunogenicity. In addition, the characterization of such cellular effects may be important in the determination of the mechanisms of adjuvant action and such elucidation may certainly contribute to the rational design of a vaccine with relevance to desirable immunological correlates.

Conclusions

There is much experimental evidence to support the view that microencapsulation serves to modify the uptake, trafficking and processing of antigen in addition to the adjuvant effect mediated by the inclusion of any specifically acting adjuvants for polymer, liposome and other

particulate-based vaccine delivery systems (Perrie et al 2001; Eyles et al 2001, 2003a; Sun et al 2003).

Synthetic modification of biological delivery systems such as viruses by complexation of cationic liposomes can shield against neutralising antibody (Steel et al 2005) and entrapment in cationic liposomes can also facilitate enhanced delivery and transgene expression of adeno-associated virus (AAV) (Mizuno & Yoshida 1998) – both of these cited studies were based on in-vitro data and while liposomes enhanced AAV delivery, there was a trade-off in adenoviral-mediated transgene expression following complexation with liposomes. In an interesting study, Fisher et al (2001) incorporated targeting ligands, fibroblast growth factor and vascular endothelial growth factor, onto polymer-coated (poly-[N-(2-hydroxypropyl)-methacrylamide]) adenovirus with good results both in-vitro and in-vivo.

Delivery systems with potential application for vaccines comprise an extremely wide diversity of strategies. The best candidates depend on individual scenarios – we recently concluded that technological developments in rational vaccine design will find their best application in our most significant of challenges (Bramwell & Perrie 2005b), i.e. where a critical need exists and vaccine development has been difficult or elusive, our most innovative strategies will be developed and may find potential. Specifically acting powerful adjuvants and synthetic delivery system technology are not exclusive and multicomponent delivery systems offer much potential.

Overwhelmingly, effective vaccine delivery systems, whether biological or synthetic, have particulate characteristics. Increased knowledge of the molecular mechanisms of the interaction of biological systems with cells of the immune system and the elucidation of more precise mechanisms of adjuvant action have transformed how we think of vaccine adjuvants. The use of synthetic carrier systems has already benefited from better prediction and understanding of potentially advantageous adjuvant and co-adjuvant combinations. In this environment of increased awareness, there is a need to more thoroughly examine the biological effects of synthetic systems such as liposomes and polymer microspheres. Whilst direct receptor recognition and specific molecular interaction have not been identified for most liposomal and polymer synthetic systems, undoubtedly observations such as those outlined in this review will impact on gene expression in ways that relate to TLR signalling. In addition to pharmaceutical characterization, increasingly we should see the routine biological characterization of multicomponent vaccine delivery systems in terms of their effects on molecular signalling and gene expression in innate immune responses. This should lead to a more complete critical evaluation of the mechanisms of adjuvant action of synthetic particulate delivery systems. Facets previously ascribed as important for adjuvant action, including uptake, trafficking and processing of antigen, depot effect and co-delivery of adjuvants, need to be assessed in conjunction with the impact of these systems on the molecular mechanisms of immune recognition and responsiveness.

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