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Minireview

Recent Advances in Vaccine Adjuvants

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New generation vaccines, particularly those based on recombinant proteins and DNA, are likely to be less reactogenic than traditional vaccines but are also less immunogenic. Therefore, there is an urgent need for the development of new and improved vaccine adjuvants. Adjuvants can be broadly separated into two classes based on their principal mechanisms of action: vaccine delivery systems and immunostimulatory adjuvants. Vaccine-delivery systems generally are particulate (e.g., emulsions, microparticles, iscoms, and liposomes)and function mainly to target associated antigens into antigen-resenting cells. In contrast, immunostimulatory adjuvants are derived predominantly from pathogens and often represent pathogen-ssociated molecular patterns (e.g., lipopolysaccaride, monophosphoryl lipid A, CpG DNA), which activate cells of the innate immune system. Recent progress in innate immunity is beginning to yield insight into the initiation of immune responses and the ways in which immunostimulatory adjuvants may enhance this process. The discovery of more potent adjuvants may allow the development of prophylactic and therapeutic vaccines against cancers and chronic infectious diseases. In addition, new adjuvants may also allow vaccines to be delivered mucosally.

KEY WORDS: vaccine adjuvants; immunostimulators; vaccine delivery systems; microparticles; emulsions.

INTRODUCTION

Traditional vaccines have mainly consisted of live attenuated pathogens, whole inactivated organisms, or inactivated bacterial toxins. Generally, these approaches have been successful for vaccine development as a result of the induction of antibodies, which neutralize viruses or bacterial toxins, inhibit the binding of microorganisms to cells, or promote their uptake by phagocytes. However, to develop vaccines against more challenging and difficult pathogens that often establish chronic infections, e.g., HIV, hepatitis C virus (HCV), tuberculosis, and malaria, the induction of potent and focused cell-mediated immunity (CMI) will be necessary and may require the induction of cytotoxic T lymphocytes (CTL), which kill host cells infected with intracellular organisms. Unfortunately, non-living vaccines generally have proven ineffective at inducing potent CMI responses, particularly of the Th1 type. T helper cells can be classified into Th2 and Th1 subtypes, mainly based on their production of cytokines. Th1 responses are characterized by the production of γ interferon. In addition, although live vaccines can induce CTL, live attenuated vaccines may cause disease in immunosuppressed individuals, and some pathogens are difficult or impossible to grow in culture (e.g., HCV), making the development of inactivated vaccines impossible. In addition, many traditional inactivated vaccines based on whole cells often contain components that can cause side effects and safety problems, e.g., lipopolysaccharides (LPS). As a result of these limitations, several new approaches to vaccine development have emerged that may have significant advantages over more traditional approaches. These approaches include 1) recombinant protein subunits; 2) synthetic peptides; 3) protein polysaccharide conjugates; and 4) plasmid DNA. Although these new approaches may offer some advantages, a general problem is that the vaccines alone are often poorly immunogenic. Traditional vaccines often contain many components that can elicit additional T cell help or function as adjuvants, e.g., bacterial DNA or LPS in whole cell vaccines. However, these components have been eliminated from new generation vaccines, which, therefore, need potent adjuvants. In the very recent past, there has been great interest in DNA vaccines because they appear to offer significant potential for the induction of potent CTL responses (1). Nevertheless, the potency of DNA vaccines in humans has so far been disappointing, particularly in relation to their ability to induce antibody responses (2,3). This has prompted investigators to work on adjuvants and delivery systems for DNA vaccines and also to use DNA in a prime/boost setting with alternative modalities, e.g., live viruses (4–6).

Immunological adjuvants were described originally by Ramon (7) as "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone." This broad definition encompasses a very wide range of materials (8). However, despite extensive evaluation of a large number of candidates over many years, the only adjuvants currently approved by the US Food and Drug Administration are aluminum-based mineral salts (generically called alum). Alum has a good safety record, but comparative studies show that it is a weak adjuvant for anti-

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body induction to recombinant protein vaccines and induces a Th2, rather than a Th1 response (9). In addition, Alum is not effective for the induction of mucosal IgA antibody responses. Moreover, alum adjuvants can induce IgE antibody responses and have been associated with allergic reactions in some subjects (9,10). Although Alum has been used as an adjuvant for many years, its mechanism of action remains poorly defined. It was originally thought to provide a "depot" effect, resulting in the persistence of antigen at the injection site. However, more recent studies involving radiolabeled antigens suggest that this is not the case (11). Recent work has indicated that Alum upregulates costimulatory signals on human monocytes and promotes the release of interleukin (IL)-4 (12). Alum adsorption may also contribute to a reduction in toxicity for some vaccines because of the adsorption of contaminating LPS (13).

A key issue in adjuvant development is toxicity because safety concerns have restricted the development of adjuvants since Alum was first introduced more than 50 years ago (14). Many experimental adjuvants have advanced to clinical trials and some have demonstrated high potency, but most have proven too toxic for routine clinical use. For standard prophylactic immunization in healthy individuals, only adjuvants that induce minimal adverse effects will prove acceptable. Additional practical issues that are important for adjuvant development include biodegradability, stability, the ease of manufacture, cost, and applicability to a wide range of vaccines. Examples of different classes of adjuvants that have been evaluated for vaccines against infectious diseases are shown in Table I.

Table I. Selective List of Different Classes of Adjuvants That Have Been Evaluated for Enhancing Immune Responses to Vaccines

Mineral salts Aluminum phosphate* Calcium phosphate*	Aluminum hydroxide*
Immunostimulatory adjuvants Saponins e.g., QS21 MDP derivatives Bacterial DNA (CpG oligos) LPS MPL and synthetic derivatives Lipopeptides	Cytokines e.g., IL-2, IL-12, GM-CSF
Lipid particles Liposomes Virosomes* Iscoms Cochleates	Emulsions e.g., Freund's, SAF, MF59*
Particulate adjuvants Poloxamer particles Virus-like particles	PLG microparticles
Mucosal adjuvants Cholera toxin (CT) Mutant toxins e.g., LTK63 and LTR72 Microparticles Polymerized liposomes Chitosan	Heat labile enterotoxin (LT)

Note: With the exception of cochleates and polymerized liposomes, all of these adjuvants have been evaluated in clinical trials. However, only those marked* are currently included as adjuvants in approved vaccine products.

THE ROLE OF ADJUVANTS IN VACCINE DEVELOPMENT

Adjuvants can be used to improve the immune response to vaccine antigens in several different ways, including 1) increasing the immunogenicity of weak antigens; 2) enhancing the speed and duration of the immune response; 3) modulating antibody avidity, specificity, isotype, or subclass distribution; 4) stimulating CTL; 5) promoting the induction of mucosal immunity; 6) enhancing immune responses in immunologically immature or senescent individuals; 7) decreasing the dose of antigen in the vaccine to reduce costs; or 8) helping to overcome antigen competition in combination vaccines.

The mechanisms of action of most adjuvants still remain only poorly understood because immunization often activates a complex cascade of responses and the primary effect of the adjuvant is often difficult to clearly discern. However, if one accepts the geographical concept of immune reactivity, in which antigens that do not reach the local lymph nodes do not induce responses (15), it becomes easier to propose mechanistic interpretations for some adjuvants, particularly those based on a "delivery" mechanism. If antigens, which do not reach lymph nodes, do not induce responses, then any adjuvant that enhances the delivery of antigen into the cells that traffic to the lymph node may enhance the response. A subset of dendritic cells (DCs) are thought to be the key cells that circulate in peripheral tissues and act as "sentinels," being responsible for the uptake of antigens and their transfer to lymph nodes, where they are then presented to T cells. Circulating immature DCs are efficient for antigen uptake, whereas mature DCs are efficient at antigen presentation to T cells. Hence, promoting antigen uptake into DC, traficking to lymph nodes, and DC maturation are thought to be key components to the generation of potent immune responses. DCs are thought to be the most effective antigen-presenting cells (APCs), although macrophages can also function in this role.

The dominant paradigm in immunology for several decades was that the immune system evolved to discriminate self from nonself (16). This hypothesis resulted in significant progress in understanding the clonal recognition of antigenic epitopes mediated by B and T lymphocytes. However, the self/nonself framework offers little insight into why some nonself antigens are found to be poorly immunogenic. In the last decade, alternative models of immunity have been established that emphasize the selective pressures on the host to induce a pro-inflammatory innate immune response after exposure to pathogen-associated molecular patterns (17,18) and tissue damage (19–21). These responses are not antigen-specific and are mediated by the innate immune system, which is the first line of immune defense and is highly conserved throughout many species. s are perceived as "danger signals" after binding to toll-like receptors (TLRs) on phagocytic APCs and induce the release of pro-inflammatory cytokines, which stimulate and focus the adaptive immune response (22,23). In this new model of immunity, vaccines will elicit a potent immune response only when the nonself antigens mimic key aspects of infectious agents or cause some degree of localized tissue damage.

Traditional vaccines such as bacterial toxoids and attenu-

ated viral vaccines often contain most of the features of real pathogens and, therefore, are sufficiently potent to induce protective immune responses. In contrast, recombinant vaccines are highly purified, lack many of the features of the original pathogen, and do not evoke strong immune responses. Hence, it can be argued that the role of adjuvants for recombinant vaccines is to ensure that the vaccine resembles infection closely enough to initiate a potent immune response (17,22). In addition, the innate immune system directs the balance of humoral and CMI (23), and adjuvants can control the type of acquired immune response induced (24). Adjuvants can be divided into different broad groups based on their principal modes of action, depending on whether or not they have direct immunostimulatory effects on APC or function as antigen delivery systems. However, any classification of adjuvants is difficult and many examples resist easy definitions.

IMMUNOSTIMULATORY ADJUVANTS

Monophosphoryl lipid A (MPL) is derived from LPS of *Salmonella minnesota*, a gram-negative bacteria and, therefore, is classified as a PAMP. Like LPS, MPL is thought to interact with TLR4 on APCs, resulting in the release of pro-inflammatory cytokines. In a number of preclinical studies, MPL has been shown to induce the synthesis and release of IL-2 and interferon (IFN)-γ, which promote the generation of Th1 responses (25,26). has been formulated into emulsions to enhance its potency (27). Clinically, MPL has often been used in complex formulations, including liposomes and emulsions, and has also been used in adjuvant combinations with alum and QS21. For example, MPL showed good tolerability and an adjuvant effect in a limited number of volunteers in combination with alum (28). Overall, MPL has been extensively evaluated in the clinic, with >10,000 subjects immunized (T. Ulrich, personal communication) for cancer (melanoma and breast), infectious disease vaccines (genital herpes, HBV, malaria, and HPV), and for allergies, with an acceptable profile of adverse effects. Recently, a vaccine containing MPL was approved in Canada for use against melanoma. In addition, MPL has been approved in Europe for use in combination with allergy vaccines (29). Structure–function studies of MPL allowed identification of a new generation of synthetic adjuvants based on aminoalkyl glucosamine phosphate compounds (30), the lead candidate of which (RC-529), is currently being evaluated in a clinical trial with a recombinant hepatitis B surface antigen (HbsAg). In addition, several synthetic mimetics of MPL are available from alternative sources, which have yet to be evaluated in human subjects (31). It has also been claimed that MPL may be used as an adjuvant for DNA vaccines (32), although these data have been difficult to reproduce, and for mucosal delivery of vaccines (33).

In the last few years, a whole new class of adjuvant active compounds have been identified following the demonstration that bacterial DNA, but not vertebrate DNA, had direct immunostimulatory effects on immune cells *in vitro* (34,35). The immunostimulatory effect was due to the presence of unmethylated CpG dinucleotides (36), which are under-represented, and methylated in vertebrate DNA. Unmethylated CpG in the context of selective flanking sequences is thought to be recognized by cells of the innate immune system to allow discrimination of pathogen-derived DNA from self DNA (37). It has recently been shown that cellular responses to CpG DNA are mediated by binding to TLR9 (38). Previously, it was reported that CpG are taken up by non-specific endocytosis and that endosomal maturation is necessary for the cell activation and the release of pro-inflammatory cytokines (39). The Th1 adjuvant effect of CpG appears to be maximized by their conjugation to protein antigens (40) or their formulation with delivery systems (Fig. 1) (41). Importantly, CpGs also appear to have potential for the modulation of existing immune responses, which may be useful in various clinical settings, including allergies (42). Although, CpG have mainly been evaluated in rodent models, recent articles have described sequences that are active in primates, including humans (43). In addition, preliminary studies have shown a potent adjuvant effect when CpG was used in combination with HbsAg in human subjects.

A third group of immunostimulatory adjuvants are the triterpenoid glycosides, or saponins, derived from the bark of a Chilean tree, Quillaja saponaria. Saponins appear to function mainly through the induction of cytokines. Saponins have been widely used as adjuvants for many years and have been included in several veterinary vaccines. QS21, which is a highly purified fraction from Quil A, has been shown to be a potent adjuvant for Th1 cytokines (IL-2 and IFN-γ) and antibodies of the IgG2a isotype, which indicates a Th1 response in mice (44). Saponins have been shown to intercalate into cell membranes through interaction with structurally similar cholesterol, forming "holes" or pores (45). It is currently unknown whether the adjuvant effect of saponins is related to pore formation; this may allow antigens to gain access to the endogenous pathway of antigen presentation, promoting a CTL response. A number of clinical trials have been per-

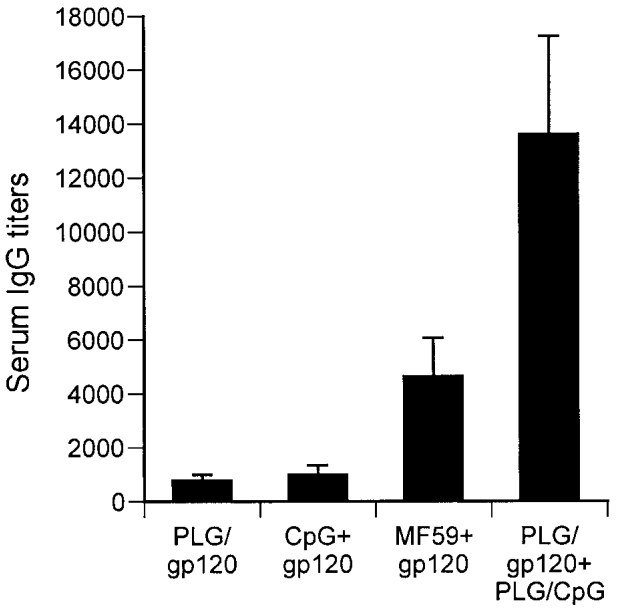

Fig. 1. Antibody responses after two intramuscular immunizations 4 weeks apart in mice with CpG adjuvant adsorbed to cationic PLG microparticles co-administered with HIV-1 env gp120 recombinant protein adsorbed onto anionic PLG microparticles. For comparison, we evaluated PLG with gp120 adsorbed and CpG with gp120. In addition, the responses induced were compared to gp120 in MF59. Geometric mean titer ± SE represented for each group.

formed, using QS21 as an adjuvant, initially for cancer vaccines (melanoma, breast, and prostate), and subsequently for infectious diseases, including HIV-1, influenza, herpes, malaria, and hepatitis B (46), and more than 3,500 people have been immunized with QS21. Doses of 200 μg or higher of QS21 have been associated with significant local reactions (46), but lower doses appear to be better tolerated. In a recent clinical trial with HIV-1 env antigen, QS21 was able to allow a significant dose reduction for the antigen and also enhanced proliferative T cell responses but not CTL (47). However, pain on injection was a common problem for many vaccine recipients. Hence, the balance of potency vs. adverse events is key for this adjuvant, and an effective adjuvant dose that is tolerable needs to be established in humans for each vaccine indication. A recent study showed that pain at the injection site could be reduced by reformulation of the adjuvant (48). QS21 has also been purported to perform as an adjuvant for DNA vaccines after both systemic and mucosal administration (49). QS21 has also shown enhanced potency in combination with additional adjuvants, to include , CpG DNA, and alternative Quil fractions.

As an alternative to the use of cytokine inducing adjuvants, cytokines may also be used directly. Most cytokines have the ability to modify and redirect the immune response. The cytokines that have been evaluated most extensively as adjuvants include IL-1, IL-2, IFN-γ, IL-12, and GM-CSF (50). However, all of these molecules exhibit dose-related toxicity. In addition, because they are proteins, they have stability problems, a short *in vivo* half-life, and are relatively expensive. Therefore, it is unlikely that cytokines will prove acceptable for use as adjuvants in vaccines designed to protect against infectious diseases. Nevertheless, considerable progress has been made in the use of cytokines for the immunotherapy of cancer (51).

PARTICULATE ANTIGEN DELIVERY SYSTEMS

The use of particulate adjuvants, or antigen delivery systems, as alternatives to immunostimulatory adjuvants has been evaluated by several groups. Particulate adjuvants (e.g., emulsions, microparticles, iscoms, liposomes, virosomes, and virus-like particles) have comparable dimensions to the pathogens that the immune system evolved to combat. Immunostimulatory adjuvants may also be included in particulate delivery systems to enhance the level of response or to focus the response through a desired pathway, e.g., Th1. In addition, formulating potent immunostimulatory adjuvants into delivery systems may limit adverse events, through restricting the systemic circulation of the adjuvant.

Lipid Particles as Adjuvants

A potent oil-in-water (o/w) adjuvant, the syntex adjuvant formulation (52) was developed using a biodegradable oil (squalane) in the 1980s as a replacement for Freund's adjuvants. Freund's adjuvants are strong adjuvants comprised of a water-in-oil emulsion with or without killed mycobacteria (53). However, syntex adjuvant formulation contained a bacterial cell wall-based synthetic adjuvant, threonyl muramyl dipeptide (MDP), and a non-ionic surfactant, poloxamer L121, and proved too toxic for widespread use in humans (14). Therefore, a squalene o/w emulsion was developed (MF59) without the presence of additional immunostimulatory adjuvants, which proved to be a potent adjuvant with an acceptable safety profile (54). MF59 enhanced the immunogenicity of influenza vaccine in small animal models (55–57) and was shown to be a more potent adjuvant than alum for hepatitis B vaccine (HBV) in baboons (58). Subsequently, the the safety and immunogenicity of MF59 adjuvanted influenza vaccine (FLUAD™) was confirmed in elderly subjects in clinical trials (59,60) and these data allowed the approval of this product for licensure in 1997. A recent study has shown that the potency of MF59 as an adjuvant for influenza vaccines might be particularly advantageous to protect against potential pandemic strains of virus (61). The potency of MF59 for HBV has also been confirmed in a human clinical trial, in which MF59 was shown to be 100-fold more potent than the commercial Alum adjuvanted vaccine (Fig. 2) (62). In addition, MF59 has also been shown to be an effective adjuvant for a protein/polysaccharide conjugate vaccines in infant baboons (63). Experience in the clinic (>18,000 subjects immunized in Chiron controlled clinical trials) with HIV, HSV, CMV, HBV, and influenza has shown that MF59 is safe and well-tolerated in humans (64–67) In addition, MF59 was shown to be safe and well-tolerated in newborn infants in a HIV vaccine trial (68). MF59 may also be used with recombinant proteins as an effective booster vaccine after immunization with live viruses (69) or DNA (70) vaccines. In summary, MF59 is a safe and well-tolerated vaccine adjuvant in humans and is effective for the induction of potent antibody responses.

In many studies, emulsions have also been used as delivery systems for immunostimulatory adjuvants, including MPL and QS21. This approach allows immunostimulatory adjuvants to be targeted for enhanced uptake by APC. An o/w emulsion containing MPL and QS21 induced protection in a

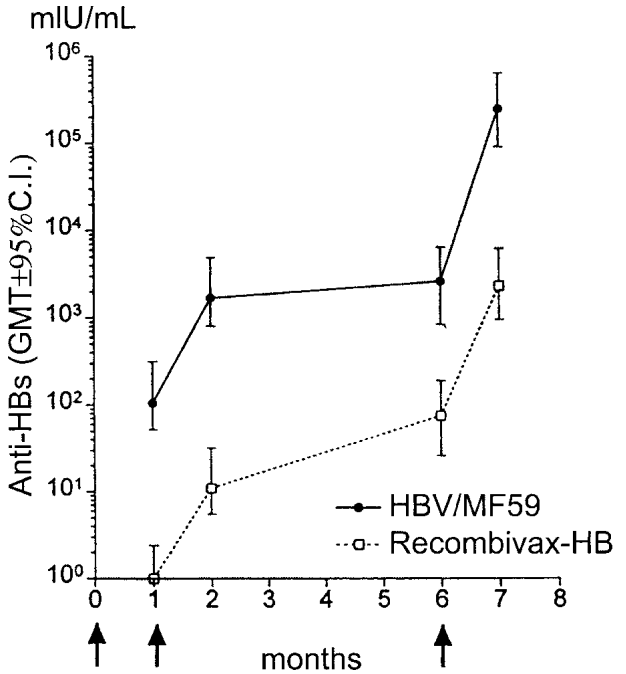

Fig. 2. Two injections of MF59 adjuvant in combination with HBV induced significantly higher antibody responses in humans than the commercially available Alum-adsorbed vaccine (Recombivax).

mouse model of malaria that was comparable or better than the levels of protection induced with the vaccine in Freund's complete adjuvant (71). The adjuvant formulation (SBAS-2) subsequently showed protective efficacy against an experimental challenge in human volunteers exposed to infected mosquitoes, although protection was of short duration (72). In a subsequent trial with HIV-1 env, SBAS-2 induced high titers and proliferative T cell responses but did not induce CTL or primary isolate-neutralizing antibodies (73). In addition, the formulation was associated with a significant number of adverse events, and the reactogenicity profile observed appeared to preclude its use for most if not all prophylactic vaccines. An alternative emulsion-based approach involves the use of the Montanide series of adjuvants, which can be formulated as water in oil, o/w, or water in o/w emulsions (74,75). The water in mineral oil (Drakeol) adjuvant (ISA-51) has been evaluated as an immunotherapeutic vaccine in HIV infected individuals (76). However, becaues of significant adverse effects, mineral oil adjuvants are unlikely to be considered as acceptable for prophylactic vaccines, although they might be appropriate for some therapeutic vaccines. An alternative approach, comprising water in squalene emulsion (ISA-720), has also been evaluated in a malaria vaccine trial (74). However, although potent, this adjuvant induced severe local reactions in some volunteers and may not prove acceptable for routine clinical use in prophylactic vaccines (77,78).

Liposomes are phospholipid vesicles that have been evaluated both as adjuvants and as delivery systems for antigens and adjuvants (79,80). Liposomes have been commonly used in complex formulations, often including MPL, which makes it difficult to determine the contribution of the liposome to the overall adjuvant effect. Nevertheless, several liposomal vaccines based on viral membrane proteins (virosomes) without additional immunostimulators have been extensively evaluated in the clinic and are approved as products in Europe for hepatitis A and influenza (81). Immunopotentiating reconstituted influenza virosomes are unilamellar liposomes composed of mainly phosphatidylcholine, with influenza haemagglutinin intercalated into the membrane. The use of viral membrane proteins in the formation of virosomes offers the opportunity to exploit the targeting and fusogenic properties of the native viral membrane proteins, perhaps resulting in effective delivery of entrapped antigens into the cytosol for CTL induction (82). An alternative approach to vaccine delivery that may have some advantages over traditional liposomes has been described using "archaeosomes," which are vesicles prepared from the polar lipids of *Archaobacteria* (83). In some studies, archaosomes have been shown to be more potent than liposomes (83,84). Cationic lipid vesicles have also been described recently, which comprise cationic cholesterol derivatives with or without neutral phospholipids (85). The best results were obtained with cationic vesicles to which antigen were bound to the surface, which greatly out-performed neutral liposomes, which did not bind antigen (85). O/w liposomal formulations recently were described in which mineral oil was emulsified in the presence of liposomes, which donated phospholipids as stabilizers (86). However, this is a complex formulation, which would need to show a dramatic improvement over alternative approaches before it can be accepted as a significant advance in the field. Modified liposomal structures termed "cochleates" are also being evaluated as systemic and mucosal adjuvants in small animal models (87). In addition, the development of polymerized liposomes, which show enhanced stability in the gut, also offers potential for the development of mucosal vaccines (88).

The immunostimulatory fractions from Quillaja saponaria (Quil A) have been incorporated into lipid particles containing cholesterol, phospholipids, and cell membrane antigens, which are called iscoms (89). In a study in macaques, an influenza iscom vaccine was shown to be more immunogenic than a classical subunit vaccine and induced enhanced protective efficacy (90). A similar formulation has been evaluated in human clinical trials and has been shown to induce CTL responses (91). The principal advantage of the preparation of iscoms is to allow a reduction in the dose of the hemolytic Quil A adjuvant and to target the formulation directly to APCs. In addition, within the Iscom structure, the Quil A is bound to cholesterol and is not free to interact with cell membranes. Therefore, the hemolytic activity of the saponins is significantly reduced (89,92). It is well established that Iscoms induce cytokine production in a range of mouse strains and a recent study has indicated that the induction of IL-12 is key to the adjuvant effect of iscoms (93), In previous studies, strong IFN-γ responses were also described (94). In a study in rhesus macaques, iscoms induced potent Th1 responses against HIV-1 env, whereas MF59 induced a Th2 response, although both vaccines offered a significant degree of protection against viral challenge (95). Although not evaluated in this study, iscoms are generally considered to be the most potent adjuvant for the induction of CTL responses with recombinant proteins in pre-clinical models. For example, in a recent study, we demonstrated the induction of potent long lasting CTL responses in rhesus macaques immunized with a recombinant core antigen from hepatitis C virus adsorbed to a novel iscom formulation (96). In addition, potent T cell proliferative responses have been induced in primates with iscom vaccines containing CMV, flu, HIV, HCV, and EBV antigens (89,97). However, the efficacy for CTL induction, and the safety profile of iscoms needs to be further established in human subjects, although initial studies are encouraging (98). Iscoms are also being evaluated as cancer vaccines and initial results are promising. A potential problem with iscom's is that inclusion of antigens into the adjuvant is often difficult, and may require extensive antigen modification (99). Nevertheless, recent work has identified novel ways by which some antigens can be effectively associated with iscoms, without significant formulation difficulties (96). Iscoms can also be used for intranasal delivery of vaccines, including influenza virus (97).

An alternative approach involving lipid vesicles has also been described involving non-ionic surfactant vesicle, or "niosomes," which have induced potent responses in small animal models (100). In addition, it has been suggested that an important component of the adjuvant effect of synthetic lipopeptide antigens is their ability to aggregate into particulate structures (101), although interaction with Toll-like receptors is also important. In addition, we have shown that the potency of lipopeptides can be enhanced by their formulation into particulate delivery systems (102).

Microparticles as Adjuvants

Antigen uptake by APCs is enhanced by the association of antigen with polymeric microparticles or by the use of

polymers or proteins that self-assemble into particles. The biodegradable and biocompatible polyesters, the polylactide-co-glycolides (PLGs), are the primary candidates for the development of microparticles as adjuvants because they have been used in humans for many years as suture material and as controlled-release drug-delivery systems (103,104). The adjuvant effect achieved through the encapsulation of antigens into PLG microparticles was first demonstrated by several groups in the early 1990s (105–108). In contrast to alum, PLG microparticles have been shown to be effective for the induction of CTL responses in rodents (102,109,110). The adjuvant effect of microparticles seems to be largely a consequence of their uptake into APC. Microparticles also appear to have significant potential as an adjuvant for DNA vaccines (111,112). We have recently described a novel approach in which cationic microparticles with adsorbed plasmids were used to dramatically enhance the potency of DNA vaccines (112). Importantly, the cationic microparticles enhanced responses in a range of animal models, including non-human primates (Table II). They efficiently adsorbed DNA and delivered several plasmids simultaneously on the same formulation at a range of different loading levels (113,114) The microparticles appeared to be effective as a consequence of efficient delivery of the adsorbed plasmids into DCs, the most important APC for presentation of antigen to naive T cells (115). In addition, cationic microparticles can be used as delivery systems for adjuvant active molecules, including CpG DNA (41). Similar anionic microparticles can also be used for delivery of adsorbed proteins and are effective for CTL induction in mice (116). In a recent study with HIV-1 vaccines, the potency of microparticles as an adjuvant was significantly enhanced by their formulation into MF59 (117). A particularly attractive feature of microparticles is their ability to control the rate of release of entrapped antigens (118,119). Controlled release of antigen may allow the development of single-dose vaccines, which would result in improved vaccine compliance, particularly in the developing world. However, much work is needed to ensure the stability of antigens entrapped in microparticles. It has been shown on several occasions that controlled-release microparticles work optimally for bacterial toxoid based vaccines when they are combined with traditional Alum adjuvants (119). Recent pronouncements suggest that this approach will be evaluated in the clinic in the near future (120).

Polymers that self-assemble into particulates (poloxamers) (121) or soluble polymers (polyphosphazenes) (122) may also be used as adjuvants, but the safety and tolerability of these approaches remains to be further evaluated.

Recombinant proteins that naturally self assemble into particles can also be used to enhance delivery of antigens to DCs. The first recombinant protein vaccine that was developed, HbsAg, was expressed in yeast as a particulate protein (123). Recombinant HBsAg is potently immunogenic and can be used to prime CTL responses *in vivo* (124). HBsAg and other virus-like particles (VLPs) can also be used as adjuvants for co-expressed proteins (125). For example, recombinant Ty VLPs from *Saccharomyces cerevisiae* carrying a string of up to 15 CTL epitopes from *Plasmodium* species have been shown to prime protective CTL responses in mice after a single immunization (126). In addition, Ty VLPs have also been shown to induce CTL activity in macaques against co-expressed SIV p27 (127). Clinical trials of Ty VLPs have shown them to be safe and immunogenic in humans (128).

Table II. Levels of Enhancement of Antibody Responses Achieved with Cationic PLG/DNA Microparticles in Comparison to Naked DNA (HIV-1 gag) after Two Intramuscular Immunizations 4 weeks Apart in Various Animal Models

Species	DNA dose (μg)	Geometric mean titer serum IgG		Fold increase over naked DNA
		Naked DNA	PLG/CTAB /DNA	
Mice	1 μg	22	7664	>300
Guinea pigs	100 μg	868	12882	>15
Rabbits	250 μg	644	8778	>12
Rhesus macaques	500 μg	190	10,220	>200

ALTERNATIVE ROUTES OF IMMUNIZATION

Although most vaccines traditionally have been administered by intramuscular or subcutaneous injection, mucosal administration of vaccines offers a number of important advantages, including easier administration, reduced adverse effects, and the potential for frequent boosting. In addition, local immunization induces mucosal immunity at the sites where many pathogens initially establish infection of hosts. In general, systemic immunization has failed to induce mucosal IgA antibody responses. Oral immunization would be particularly advantageous in isolated communities, where access to health care professionals is difficult. Moreover, mucosal immunization would avoid the potential problem of infection resulting from the re-use of needles. Several orally administered vaccines are commercially available that are based on live-attenuated organisms, including vaccines against polio virus, Vibrio cholerae, and Salmonella typhi. In addition, a wide range of approaches are currently being evaluated for mucosal delivery of vaccines (129), including many approaches involving non-living adjuvants and delivery systems.

The most attractive route for mucosal immunization is oral because of the ease and acceptability of administration through this route. However, as a result of the presence of acidity in the stomach, an extensive range of digestive enzymes in the intestine and a protective coating of mucus that limits access to the mucosal epithelium, oral immunization has proven extremely difficult with non-living antigens. However, novel delivery systems and adjuvants may be used to significantly enhance the responses following oral immunization.

Mucosal Immunization with Microparticles

In mice, oral immunization with PLG microparticles has been shown to induce potent mucosal and systemic immunity to entrapped antigens (130–133). In addition, mucosal immunization with microparticles induced protection against challenge with *Bordetella pertussis* (134–137), *Chlamydia trachomatis* (138), and *Salmonella typhimurium* (139). In primates, mucosal immunization with inactivated SIV in microparticles induced protective immunity against intravaginal challenge (140). Also in primates, mucosal immunization with microparticles induced protection against aerosol challenge with

staphylococcal enterotoxin B (141). Comparative studies have indicated that microparticles are one of the most potent adjuvants available for mucosal delivery of vaccines (142). In recent studies, microparticles have also shown some promise for the mucosal delivery of DNA (143,144). The ability of microparticles to perform as effective adjuvants after mucosal administration is largely a consequence of their uptake into the specialized mucosal-associated lymphoid tissue (145). Although most of this work has described particle uptake after oral delivery, a recent paper described the uptake of microparticles into mice following intranasal delivery (146). The potential of microparticles and other polymeric systems for mucosal delivery of vaccines was recently reviewed (147), as was the use of a broader range of antigen delivery systems (148). Although microparticles have significant potential for mucosal delivery of vaccines, their potency may be improved by their use in combination with additional adjuvants. This is likely to be a pre-requisite for the development of effective oral vaccines, since the challenges should not be under estimated. Accumulated experimental evidence suggests that simple encapsulation of vaccines into microparticles is unlikely to result in the successful development of oral vaccines and improvements in the current technology are clearly needed (149).

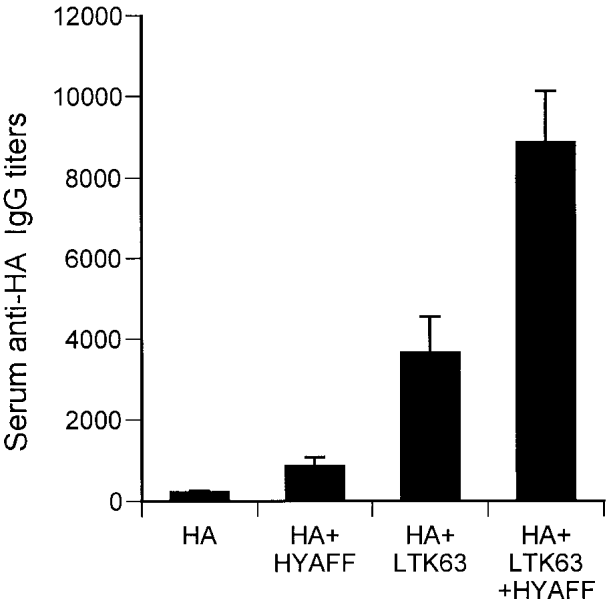

Fig. 3. After two intranasal immunizations 4 weeks apart in mice, enhanced serum antibody responses were obtained with influenza vaccine (HA) and mucosal adjuvant LTK63 in combination with bioadhesive HYAFF microspheres (HA + LTK63 + HYAFF). For comparison, mice were also immunized with antigen alone (HA), antigen and microspheres (HA + HYAFF) or antigen plus adjuvant (HA + LTK63). Geometric mean titer ± SE represented for each group.

Adjuvants for Mucosal Immunization

The most potent mucosal adjuvants currently available are the bacterial toxins from *Vibrio cholerae* and *Escherichia coli,* cholera toxin (CT), and heat-labile enterotoxin (LT), respectively. However, because CT and LT are the causes of cholera and travellers diarrhoea, they are generally considered too toxic for use in humans. Therefore, they have been genetically manipulated to reduce toxicity (150–152). Single amino acid substitutions in the enzymatic A subunit of LT allowed the development of mutant toxins that retained potent adjuvant activity, but showed negligible or dramatically reduced toxicity (153–155). LT mutants have been used by the oral route to induce protective immunity in mice against *H. pylori* challenge (156). In addition, LT mutants have been shown to be potent oral adjuvants for influenza vaccine (157) and model antigens (158).

Nevertheless, because of the significant challenges associated with oral immunization, various alternative routes of immunization have been evaluated with LT mutants, including nasal, intravaginal, and intra-rectal. Of these, intranasal immunization offers the most promise, both because of the potent responses induced by this route and the easy access and simple administration devices that already exist. On many occasions, the ability of LT mutants to induce potent antibody responses after intranasal immunization has been demonstrated (159). In recent studies, LT mutants have shown protection against challenge with *B. pertussis* (160), *S. pneumoniae* (161), and herpes simplex virus (162) after intranasal immunization and the induction of potent CTL responses (163,164). In addition, we recently showed that the potency of LT mutants may be enhanced by their formulation into a novel bioadhesive microsphere delivery system (Fig. 3) (165). In addition, the potency of LT mutants was not affected by the presence of pre-existing immunity to the adjuvant (166). A virosomal influenza vaccine with low-dose LT wild type has been evaluated in human clinical trials and showed potent responses while appearing to be safe (167,168). The apparent safety of this approach in humans using wild type LT is strongly supportive of the approach using genetically detoxified LT mutants.

Although the mechanisms of action of CT and LT remain to be fully defined, it appears that there are important contributions to the adjuvant effect from the B subunit binding domain, the presence of an intact A subunit, which interacts with regulatory proteins inside cells, and also the enzymatic activity of the A1 subunit (159). Recently, an enzymatically inactive recombinant CT mutant has been proposed to directly activate APC and T cells (169). In addition, the ability of CT to induce the activation and maturation of human DC has been reported (170).

Recent studies have indicated that potent mucosal adjuvants such as CT may also allow vaccination after topical application to the skin (171) and that this approach may be applicable to humans (172). In addition, epidermal immunization may be achieved using needle-free devices, which use helium gas to deposit powdered vaccine into the epidermis (173). An alternative approach to the development of mucosal adjuvants involves the use of plant lectins (174). Furthermore, oral immunization may also be achieved through the ingestion of transgenic plants expressing antigens and adjuvants (175,176).

ADJUVANTS FOR THERAPEUTIC VACCINES

It seems increasingly likely that novel adjuvants may prove sufficiently potent to allow the development of therapeutic vaccines. Rather than prevent infection, therapeutic vaccines would be designed to eliminate or ameliorate existing diseases, including 1) chronic infectious diseases, e.g.,

those caused by HSV, HIV, HCV, HBV, HPV, or *H. pylori*; 2) tumors, e.g., melanoma, breast, or colon cancer; and 3) allergic or autoimmune disorders, e.g., multiple sclerosis, Type I diabetes, and rheumatoid arthritis. For example, a preliminary clinical study in subjects infected with HSV-2 showed a therapeutic benefit following vaccination with an adjuvanted recombinant vaccine (177).

The level of toxicity acceptable for an adjuvant to be used in a therapeutic situation is likely to be higher than for a prophylactic vaccine designed for use in healthy individuals, particularly if the vaccine is designed to treat cancer, or the life-threatening consequences of an infectious disease. However, the acceptable safety profile for any new vaccine/adjuvant combination needs to be established in the clinic. Many adjuvants, including (178), QS21 (28), and cytokines (179) have been evaluated for the development of cancer vaccines and recent data has been encouraging.

Therapeutic vaccines may also be developed for mucosal administration. For example, an LT mutant has been used to eradicate an established infection with *H. pylori* in mice (180). In addition, preliminary studies offered some encouragement that oral administration of antigens can result in the amelioration of autoimmune diseases, including diabetes (181).

FUTURE DEVELOPMENTS IN VACCINE ADJUVANTS

Several recent issues have served to highlight the urgent need for the development of new and improved vaccines. These problems have included 1) the inability of traditional approaches to develop successful vaccines against "difficult" organisms such as HIV and HCV; 2) the emergence of new diseases, for instance, Ebola, West Nile, and nvCJD; 3) the re-emergence of "old" infections like tuberculosis; 4) the continuing spread of antibiotic-resistant bacteria; and 5) the potential use of microorganisms for bioterrorism. In this review, we have suggested that the adjuvants to be used in these vaccines may have to closely mimic an infection and/or induce localized tissue damage to elicit protective immunity. This may be achieved through the use of particulate delivery systems, which have similar dimensions to pathogens and are able to target antigens to macrophages and DCs. In addition, it may also be necessary to deliver one or more adjuvant active , which will more fully activate the innate response and may result in the desired type of adaptive response. If this hypothesis is correct, it suggests that a delicate balance must be maintained between the desired initiation of immune responses and avoidance of the problems potentially associated with a robust response, e.g., local tissue damage and systemic cytokine release. Many of these new generation vaccines will require the induction of potent CMI, including CTL responses. Accumulated research shows that induction of CTL is difficult with proteins and may require much stronger stimulation of the immune system than is normally required for a humoral response. Therefore, DNA remains an attractive approach for many pathogens but needs to be delivered more effectively to improve its potency in humans. In addition, live virus booster immunizations may also be required for optimal induction of CTL.

Targeted delivery of adjuvants and vaccines to specific cell types or tissues may reduce potential toxic effects, or help to achieve a specific desired response. Targeting may be achieved at several different levels, to include tissue specific delivery to local lymph nodes, cell specific targeting to APC, or targeting to subcellular compartments e.g., the proteasome to promote Class I presentation and CTL, or the nucleus for DNA vaccines. However, "active" targeting may also be achieved through the use of ligands designed to specifically interact with preferred cell types, including the non-clonal receptors on APC, which evolved to recognize various components of bacteria and viruses, including TLR. An alternative target is the mannose receptor, which has been used to target liposomes to APCs (182). Lectins have already been successfully used to target antigens (183), liposomes (184), and microparticles (185) to the M cells of mucosal-associated lymphoid tissue after mucosal delivery. In addition, lectin targeting has also been used to enhance the extent of uptake of microparticles following oral delivery (186). However, the use of targeting ligands on particulate systems requires the construction of a highly sophisticated delivery system, which will be required to show dramatic improvements over non-targeted systems to justify commercialization. Further developments in the delivery of adjuvants may be achieved through the identification of specific receptors on APC, which might be extra- or intracellular. If intracellular, then a means to promote uptake of the delivery system by the relevant cells may also be required for optimal efficacy. An interesting approach to targeting APCs has been described that involves co-expression of two linked proteins, with a targeting component and an adjuvant signal (187–189). An alternative approach to vaccine targeting for CTL induction has also been described using a fusion protein with a bacterial toxin to deliver the antigen specifically to the Class I processing pathway (190,191)

Future developments in adjuvants are likely to include the development of more site-specific delivery systems for both mucosal and systemic administration. In addition, the identification of specific receptors on APCs is likely to allow targeting of adjuvants for the optimal induction of potent and specific immune responses. However, further developments in novel adjuvants will likely be driven by a better understanding of the mechanism of action of currently available adjuvants and this is an area of research that requires additional work.

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