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Review

Immunopotentiation and Delivery Systems for Antigens for Single-Step Immunization: Recent Trends and Progress

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The use of adjuvants for immunopotentiation has been investigated since the 1920s and a number of comprehensive reviews and monographs have been published on this subject. A recent trend in immunopotentiation has been the use of delivery systems which allow for sustained or controlled release of antigens and which induce prolonged immunity following a single dose. This concept has been termed either single-step or single-shot immunization. The delivery system has been modulated to potentiate the immune response either by delivering the antigen (and perhaps an adjuvant or adjuvants) either over a prolonged period of time or in a predetermined sequence or by incorporating substances with immunoadjuvant properties (e.g., lecithin and certain biodegradable polymers) as carriers within the delivery system. This Review focuses on the progress made in the design of delivery systems for immunopotentiation. Particular emphasis is given to delivery systems designed to achieve single-step immunization.

KEY WORDS: single-shot immunization; controlled-release vaccine; sustained antigen delivery; protein delivery; adjuvant; novel vaccine; immune response; antibody response

INTRODUCTION

The dosage form, dose, and frequency of administration of an antigen play important roles in stimulating an effective immune response (1–5). In conventional immunization procedures, multiple-dose administration of the antigen is usually required to evoke lasting immunity with killed, purified, and subunit vaccines. A single dose of the antigen is first delivered to induce a primary response, followed by repeated injections at intervals to evoke secondary, tertiary, and quaternary immune responses to the antigen (6). Small multiple doses of antigen have proven effective in achieving protective immunity (7–9). The need for multiple injections of antigens limits the practical value of some immunization programs. The time intervals between injections can be prolonged by using oil-based media such as Freund's complete adjuvant (FCA)[5] to deliver antigens, but more than one injection is still required. Moreover, adjuvants such as FCA and indeed other oil emulsions are too toxic to be used in humans: they elicit severe granulomatous reactions because of the presence of poorly degradable mineral oil, and in addition, some of them contain toxic biological substances (e.g., killed mycobacteria in FCA). The feasibility of using metabolizable lipid emulsions composed of biodegradable materials such as glycerol and lecithin has also been studied, but reports on their efficacies conflict (10,11). Alternatively, delivery systems capable of releasing the antigen in a sustained or controlled manner (e.g., microspheres, nanoparticles, subcutaneous implants) and/or capable of potentiating the immune system (e.g., liposomes) have been investigated to achieve single-step immunization.

Recent reviews have dealt with the use of adjuvants (12–14) and different antigen delivery systems, e.g., liposomes (15–17) and implants (6), for immunopotentiation; here, however, we provide a collective description of various antigen delivery systems which are aimed at achieving prolonged or lasting immunity. The primary objective of this review is to focus on the recent trends and progress in the design of delivery systems for immunopotentiation, with particular emphasis on those having the potential for use in single-step immunization.

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[5] Abbreviations used: BGG, bovine γ-globulin; BSA, bovine serum albumin; CTTH, *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine hexyl ester; DL-PLG, poly(D,L-lactide-co-glycolide); DT, diphtheria toxoid; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; HSA, human serum albumin; MDP, muramyl dipeptide; OVA, ovalbumin; PBS, phosphate-buffered saline; RES, reticuloendothelial system; RSA, rabbit serum albumin; sc, subcutaneous; SEB, staphylococcal enterotoxin B; TNP-HEA, trinitrophenyl-hen egg albumin; TNP-KHL, trinitrophenyl-keyhole limpet hemocyanin; T_t, phase transition temperature; VM, viomycin; w/o, water-in-oil.

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IMMUNOADJUVANTS AND CARRIERS OF ANTIGENS WITH ADJUVANT PROPERTIES

Immunoadjuvants—A Brief Overview

Adjuvants are defined as substances that enhance the immune response to an antigen (17). The role of an antigen in a vaccine is to elicit a specific immune response; however, an adjuvant is usually required to stimulate an optimal response to an antigen, particularly when nonliving vaccines are administered. Some killed bacteria may have self-adjuvanting properties because they contain immunostimulatory molecules such as muramyl dipeptide (MDP) in addition to the epitopes of interest; however, synthetic and purified peptide and protein antigens are almost invariably poorly immunogenic and require the presence of an immunological adjuvant to stimulate an adequate response (18,19).

Since their discovery in the 1920s by Ramon (20), a variety of substances has been identified as immunological adjuvants. Many of these are biological substances (e.g., mycobacteria, mycobacterial fragments) and their synthetic analogues (e.g., synthetic MDP, lauroyltetrapeptide). Some chemical substances (e.g., synthetic polymers, dextran sulfate) are also known to possess adjuvant properties. The substances that possess adjuvant properties are diverse, and hence their mode of action is not singular. The mode of action of these adjuvants is not discussed here in detail since this review was aimed to concentrate mainly on delivery systems designed to work as adjuvants. As a generalization, the adjuvants may act either on the antigen or on the host cells involved in the immune process or on both. Adjuvants can induce conformational changes of the antigen molecules or alter the net polar charges of the molecules, each of which plays an important role in their immunogenic action. Some adjuvants either denature the antigen molecules or convert them to particular substances, thus facilitating easier recognition by the macrophages and other cells that present antigen to the lymphocytes. In the host adjuvants can bind antigens to cell membranes, activate the cells, and modify the synthesis or release of cytokines capable of influencing the immune process. Certain adjuvants have the ability to retain the antigen at its site of deposition and release it slowly and continuously over a prolonged period of time and/or deliver the antigen specifically and directly to the cells of the reticuloendothelial system (RES) or attract the antigen presenting cells, lymphocytes, and other related cells to the site of their administration to form a granuloma (13). An individual substance cannot perform all these functions and may require the assistance of a pharmaceutical dosage form which, according to the definition of an adjuvant, also serves as an adjuvant.

Antigen Delivery Systems with Adjuvant Properties

Delivery systems such as emulsions, liposomes, nanoparticles, and microspheres are widely regarded as carriers with immunoadjuvant properties. A comparison between these delivery systems is shown in Table I. Most of these dosage forms exhibit adjuvant properties due to their ability to release the antigen over a longer period than when the antigen is delivered in free form, while others are capable of modulating the immune system in addition to their sustained- or controlled-release properties.

Emulsions

Antigens either absorbed onto a mineral gel (e.g., aluminium hydroxide or phosphate) or solubilized in water and then emulsified in an oil phase as water-in-oil (w/o) emulsions possess enhanced immunological effects. Typical examples of emulsions with adjuvant properties are FCA and Freund's incomplete adjuvant (FIA). FCA is a w/o emulsion containing killed *Myobacterium tuberculosis* in the oil phase and the antigen in the water phase. Mycobacteria present in FCA play a key role in potentiating the immune response to the antigen, although they are not the sole immunomodulating factor. In the absence of mycobacteria, the oily phase alone also acts to a lesser extent as an adjuvant and is known as FIA. FCA has been recognized as the most potent adjuvant available due to its ability to enhance strongly both humoral and cellular immunity (14,21); however, because of the reaction at the site of injection and sensitisation to *Mycobacterium tuberculosis,* this adjuvant has an application limited to research. The mechanism responsible for the adjuvant effect of emulsions is poorly understood, but the general hypothesis is that emulsions retain the antigen at the site of injection performing a depot function, protect the antigen from rapid destruction, facilitate phagocytosis, and stimulate the cells of the immune system. The rate of release of the antigen from the injection sites (granulomas) appears to be a crucial factor in causing the adjuvant effect. About one-third of a dose of ^{125}I-labeled ovalbumin (OVA) (emulsified in FIA) injected in flank folds of sheep was present in the granuloma 20 weeks after injection, while only 0.18–0.22% of the antigen was present in the regional prefemoral lymph node (22); an insufficient rate of release of the antigen from the granuloma was found inadequate to sustain the maximum circulating antibody response in the host animals.

In a study with a viomycin (VM)-protein conjugate, Hu *et al.* (4) compared the role of FCA and FIA in inducing primary and secondary antibody responses in mice and demonstrated that FCA played a dominant role as an adjuvant during the primary response, while FIA was more effective during booster injections.

Selective induction of the appropriate isotype or subclass of antibody response is an important consideration in providing protection against an infection using a vaccine. In studies on the adjuvant activity of nonionic block copolymer surfactants in mice immunized with trinitrophenyl-hen egg albumin (TNP-HEA) in 2% squalane-in-water emulsions, Hunter *et al.* (23) demonstrated that these copolymers affected the isotype and subclass of the antibody response as a function of the molecular weights (chain lengths) of their hydrophobes.

Aluminium-based suspensions emulsified in mineral oils are also used as adjuvants. The aluminium-based suspensions, such as precipitated alum and preformed aluminium hydroxide and aluminium phosphate gels, are the only adjuvants approved for human use. The adsorptive behavior of antigens to aluminium hydroxide and aluminum phosphate gels is influenced by, among other factors, the surface charge of the antigen and presence of any buffer ions in the system (19).

Table I. Comparison of Various Antigen Delivery Systems with Adjuvant Properties

Antigen delivery system	Factor influencing the immune response	Antigen(s)/animal used in the experiment	Remarks	Ref. No.
Emulsions FCA & FIA	Emulsification of the antigen in the oil	VM–protein conjugate/mice	Emulsification of the antigen in the oil resulted in a significantly higher level of anti-VM antibodies compared to the antigen mixed with FCA or dissolved in saline and delivered through miniosmotic pumps continuously over a month.	5
	The release of antigen and the presence of lymph nodes	OVA and ferritin in FIA/sheep	The release of the antigen from granulomas was more important than the number of granulomas in inducing antibodies.	22
	Production of granulomas	Antigens of cyst fluid *Taenia multiceps* in FCA/rabbits	Increasing the number of injection sites and hence granulomas induced higher levels of antibodies against the antigen.	61
Squalane-in-water	Distribution of the antigen (in either aqueous or oil phase)	TNP-HEA/mice	Antigen incorporated in the oil phase stimulated significantly higher levels of antibodies compared to antigen in the aqueous phase of identical formulations.	23
Liposomes	Types (unilamellar or multilamellar) of liposomes	BSA/mice	Unilamellar vesicles were more potent in inducing antibodies than multilamellar vesicles.	62, 63
	Phospholipid-to-antigen ratio	Tetanus toxoid/mice	A very high phospholipid–antigen ratio can suppress the immune response.	64
	Net charge of liposomal bilayers	Diphtheria toxoid (DT)/mice	Negatively charged and neutral liposomes induced higher levels of antibodies than positively charged liposomes.	24
		BSA/mice	Positively and negatively charged liposomes worked equally well.	65
		HSA, BGG/rabbits	Positively and negatively charged and neutral liposomes worked equally well.	66
		Artemisia scoparia pollen extract/mice	Negatively charged liposomes induced higher levels (statistically insignificant) of antibodies than positively charged liposomes.	26
	Position of the antigen in liposomes (entrapped within or adsorbed on the surface)	Egg albumin/mice	Surface-bound antigen was more immunogenic.	67
		BSA/mice	Surface-bound antigen was less immunogenic.	68
		Tetanus toxoid/mice	Surface-bound and encapsulated antigens were indifferent.	64
	T_t of the phospholipid(s)	Tetanus toxoid/mice	Phospholipids with a low T_t ($\leq$41.5°C) were more potent than those with a high T_t (54°C).	64
	Suitability for oral immunization	BSA/rats	Oral immunization produced enhanced salivary IgA antibodies compared to sc administration of the antigen in liposomes.	27
	Suitability for immunotherapy of allergic diseases	*Artemisia scoparia* pollen extract/mice	Reduced allergenicity compared to the allergen not entrapped in liposomes.	26
Nanoparticles and microspheres	Encapsulation of the antigen	SEB/mice	Induced IgG antitoxin antibodies 500 times higher than nonencapsulated SEB	21
		TNP-KLH/mice	Stimulated enhanced production of anti-TNP-KLH antibodies	21
		OVA/mice	Induced a significantly higher amount of IgG antibodies during primary immunization than did OVA in FCA for up to 10 weeks	32
		HSA/mice	Elicited strong HSA-specific plaque forming cell responses, in contrast to HSA delivered either in free form or together with empty microspheres	34, 69
		DT/mice	Microspheres containing the antigen induced antibodies comparable to those induced by three injections of the antigen with calcium phosphate as an adjuvant.	33

Table I. Continued

Antigen delivery system	Factor influencing the immune response	Antigen(s)/animal used in the experiment	Remarks	Ref. No.
	Size of microspheres	SEB/mice	Microspheres with a diameter of 1 to 10 μm exhibited stronger adjuvant activity than those with a diameter >10 μm.	21
	Size of microspheres and suitability for oral immunization	SEB/mice	Microspheres of 1–10 μm were suitable for oral immunization since they could target the Peyer's patches specifically as opposed to microspheres of >10 μm. Three peroral immunizations with the 1- to 10-μm microspheres induced both secretory (mucosal IgA) and circulating (IgM, IgG, & IgA) antitoxin antibodies, in contrast to free antigen administered perorally, which did not stimulate any antibodies.	51, 53

Liposomes

Following the first report by Allison and Gregoriadis (24) on the adjuvant activity of liposomes, considerable attention has been focused on their possible utility as antigen carriers. The literature contains encouraging experimental results on liposomes as adjuvants, although no liposomal vaccine preparation is available on the market for commercial use in humans or animals.

"Liposomes serve as carriers of antigens and adjuvants, as depots for slow releasing antigen, and as targeting agents for delivery of novel antigens and adjuvants to antigen presenting cells" (17). It was observed that some antigens with no immunogenic properties became highly immunogenic when combined with either FCA or aluminium hydroxide and delivered through liposome preparations (17). Liposomes are also suitable for use in the immunotherapy of allergic diseases, because allergens entrapped in liposomes do not produce anaphylactic reactions in sensitive hosts (25). Arora and Gangal (26) demonstrated that allergen (*Artemisia scoparia* pollen extract) entrapped in liposomes reduced allergenicity in terms of a decrease in serum-specific IgE in mice which received repeated injections compared to mice treated with unentrapped allergen; the immunogenic response to the allergen, measured in terms of IgG antibodies, was higher in the mice treated with liposome-entrapped allergen after a third booster injection. Liposomes also have the potential to be used for oral immunization to protect against infection of mucous membranes which are bathed by IgA (27).

Several factors affect the adjuvant properties of liposomes (Table I). These include the net charges of the liposomal bilayers and the antigen to be delivered, the position of the antigen either entrapped within the liposome or adsorbed on the surface, the type and fluidity of liposomal lipids and their phase transition temperature (T_t), the type of liposomes (unilamellar or multilamellar) and their sizes, and the phospholipid-to-antigen ratios. Several authors have comprehensively reviewed these factors (16,28–30).

Booster doses of antigens in liposomes after a priming dose with the liposome-associated antigen do not show any adjuvant effect. Rabbits primed with liposome-associated human serum albumin (HSA) injected intravenously were reported not to have an enhanced secondary immune response after booster injection compared to that when the booster dose of HSA was administered (intravenously) free in solution (31).

Nanoparticles and Microspheres

Particulate delivery systems such as nanoparticles and microspheres also possess adjuvant activity when used as vehicles for antigens (see Table I). The adjuvant effect of microspheres made of poly(D,L-lactide-co-glycolide) (DL-PLG) copolymer containing staphylococcal enterotoxin B (SEB) and subcutaneously (sc) injected into mice was comparable to that of FCA, but in contrast to FCA, the microspheres did not induce inflammation and granulomata in the mice. OVA, a poor immunogen, entrapped in 5.34-μm DL-PLG microparticles, induced a significantly higher amount of IgG antibodies in mice during primary immunization than did OVA in FCA for up to 10 weeks. After booster doses of OVA, delivered from the microparticles, the IgG antibody response to OVA was also higher than that induced by OVA in FCA, but the difference was not significant (32).

As with other peptide and macromolecular drugs, the rate of release of antigens from biodegradable microspheres was shown to depend mainly on degradation of the microsphere matrix (33). The adjuvant activity of biodegradable microspheres during primary responses is explained by their rapid uptake by the mononuclear cells in the RES following intravenous injection, resulting in high local concentrations of the antigens in these cells, and also by their ability to be degraded rapidly in the lysosomal milieu of the macrophages. During booster injections, the antigens exposed on the surfaces of the biodegradable microspheres are considered more important for their adjuvant action, because of their ready availability to bind to antigen-specific antibodies in the circulation (34). The size of the microspheres also plays a role in their adjuvant activity (see Table I).

Protein Polymer Beads

Several workers have reported on the adjuvant properties of serum albumin polymer beads when used as biode-

gradable carriers for bacterial and viral antigens (18,35). Virus particles cross-linked into rabbit serum albumin (RSA) beads injected into rabbits induced the formation of anti-virus IgG antibodies comparable to those obtained when the virus was emulsified in FCA (35). In contrast to FCA, the RSA beads did not cause inflammation or necrosis in the recipient rabbits. The adjuvant effect of the RSA beads was postulated as being due to their slow-release properties.

ANTIGEN DELIVERY SYSTEMS WITH POTENTIAL FOR SINGLE-STEP IMMUNIZATION

The adjuvants and the delivery systems with adjuvant properties described previously either facilitate modification of antigen by the cells of the immune system or rely on a short-term "simple" depot effect to exert their immunomodulatory effect. Although antibody titers can be significantly improved using these adjuvants, they fall short of levels required for lasting protective immunity following a single-dose administration. In addition, some of these devices (e.g., liposomes) suffer serious stability problems during storage and in biological fluids. Terminology such as "single-step" or "single-shot" immunization came into existence in the literature in the mid-1970s upon the introduction of long-acting devices used by Langer and his co-workers to deliver antigens (36,37). Table II summarizes the recent trends in designing antigen delivery systems for achieving single-step immunization and the progress made.

Table II. Comparison of Various Antigen Delivery Systems with Potential for Single-Step Immunization

Antigen delivery system	Factor apparently responsible for long-term immune response	Antigen(s)/ host used in the experiment	Period of immune response obtained after single-dose administration	Remarks	Ref. No.
Polymeric implants					
Nonbiodegradable	Antigen delivered in an initial pulse release followed by a trickle of continuous delivery	BSA, HSA, BGG/mice	Over 10 (HSA, BGG, & BSA) and 25 (BSA) weeks	BSA delivered from implants prepared from ethylene–vinyl acetate copolymer induced antibody responses comparable to those with two injections of the antigen in FCA.	36
Biodegradable	Long-term sustained delivery of the antigen and adjuvant activity of a degraded product of the polymer used	BSA/mice	Over 56 weeks	Implants prepared from CTTH-iminocarbonate polymer showed an adjuvant effect in inducing antibody responses. The adjuvant effect of a degraded product (CTTH) of the polymer was as potent as that of FCA and MDP.	39, 40
Lipid implants	Antigen delivered in an initial pulse release followed by a trickle of continuous delivery	BSA/mice	Over 43 weeks	Implants prepared from cholesterol alone or cholesterol-lecithin as carriers induced antibody responses comparable to or higher than those with three injections of the antigen in PBS.	44
Liposomes					
Containing monophosphoryl lipid A and adsorbed to $Al(OH)_3$	The adjuvant effects of the delivery system and monophosphoryl lipid A	Recombinant malarial antigen ($R32NS1_{81}$)/ humans	Sole single-dose effect not studied	Antigens encapsulated in liposomes containing the adjuvant and adsorbed to $Al(OH)_3$ induced much higher antibodies than antigens adsorbed to $Al(OH)_3$ without encapsulation.	47
Encapsulated in microspheres	Depot effect of the microsphere-encapsulated liposomes	BSA/rats	Over 21 weeks	Antigen delivered through the microsphere-encapsulated liposomes elicited 2- to 3-fold stronger antibody responses than antigen delivered from either liposomes (nonencapsulated) or saline or FCA.	49
A combination of microspheres of two sizes	Multiphasic pulse release of the antigen due to differences in size of the microspheres	SEB/mice	Both primary and secondary responses	Smaller microspheres (1–10 μm) released the antigen at an accelerated rate, resulting in the primary response, while larger microspheres (20–125 μm) released antigen slowly, causing secondary responses.	51

Polymeric Implants

Langer and Folkman (36) found that various macromolecular drugs could be delivered in a sustained-release manner for periods exceeding 100 days from implants prepared from a nonbiodegradable ethylene–vinyl acetate copolymer. Preis and Langer (37) later described the use of this system for delivery of antigens of different molecular weights: ribonuclease (MW 14,000), bovine serum albumin (BSA) (MW 68,000), and bovine γ-globulin (BGG) (MW 158,000). They demonstrated that delivery of these antigens from the implants elicited prolonged immune responses following a single-dose administration. BSA delivered through these implants induced IgG antibodies comparable to those induced by two injections of the antigen in FCA for over 25 weeks. The release of antigens from these pellets was shown to occur mainly through diffusion, with a delivery profile consisting of an initial burst effect followed by a trickle continuous delivery (38). Factors such as antigen particle size and loading doses of the antigens influenced their release profiles; the larger the particle size and the higher the loading dose, the greater was the rate of release of the antigen. The diffusion occurred through a series of interconnecting microchannels in the polymer matrix caused by the incorporation of either sold or liquid during preparation of the implants. Increasing the particle size of antigens and/or increasing their loading dose increased the size of the channels and resulted in increased release rates. The aqueous solubility of the antigen also played a determining role in the release process. A high solution concentration within the matrix channels caused a high driving force for diffusion and hence faster release rates.

The advantage of single-dose administration of these implants is minimized by the fact that the spent device must be removed surgically, requiring a second visit to health personnel in an actual immunization program. In addition, the manufacturing technique of these implants requires heat and organic solvent treatment of the antigen. Above all, the safety of using nonbiodegradable polymers such as ethylene–vinyl acetate copolymer in humans is still questionable, and the device can cause general discomfort and predispose to infection at the site of implantation.

An implant prepared from a biodegradable polymer has also proven effective for sustained delivery of antigens and for inducing antibodies for a prolonged period of time following a single-dose administration. BSA delivered from *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine hexyl ester (CTTH)-iminocarbonate polymeric implants induced significant anti-BSA antibodies for over 56 weeks following single-dose administration in mice (39,40). A biologically degraded product of the polymer, CTTH, was found to act as an adjuvant which was as potent as FCA and MDP in enhancing the immune response.

The technique for manufacturing these implants also requires that the antigen be treated with an organic solvent, which might limit their practical application in delivering protein antigens. Biodegradable polymers are a major focus in implant technology, although there are not many reports about their use (as implants) for the delivery of antigen and the safety of biodegradable polymers such as CTTH-iminocarbonate for use in humans is not yet well established. Nonetheless, the concept of using polymers as sustained- or controlled-release carriers which degrade into adjuvants such as CTTH is certainly attractive and may stimulate the discovery of other similar substances.

Lipid Implants

Despite the significant use of subcutaneous implants prepared from lipids and other waxy materials to deliver various drugs, their utility has not been well investigated for the delivery of antigens. In 1990, however, Beck (41) noted in a patent the feasibility of using fatty acid esters such as glycerol stearates to prepare implants for delivering antigens to obtain prolonged immunity. Recently, we investigated the use of subcutaneous implants prepared from cholesterol alone (42–44) and from cholesterol and hydrogenated egg lecithin (43,44) for sustained delivery of antigen. Our studies, using BSA as a model antigen, confirmed that these implants can induce and maintain an immune response in mice for at least 10 months following a single-dose administration of the antigen (Fig. 1). The implants containing BSA induced either equal or higher levels of anti-BSA antibodies as did the same total dose of the antigen given in the form of three injections (14 days apart) in phosphate-buffered saline (PBS). The implants did not induce abnormalities at the sites of implantation and some formulation were bioerodible (44). Diffusional release of BSA from the implants was demonstrated both *in vitro* (43) and *in vivo* (42,44). A pulsatile delivery (within 2–5 days) followed by trickle delivery of the antigen from the implants over up to 7 months was observed in mice (44). A significant correlation existed between the release of BSA

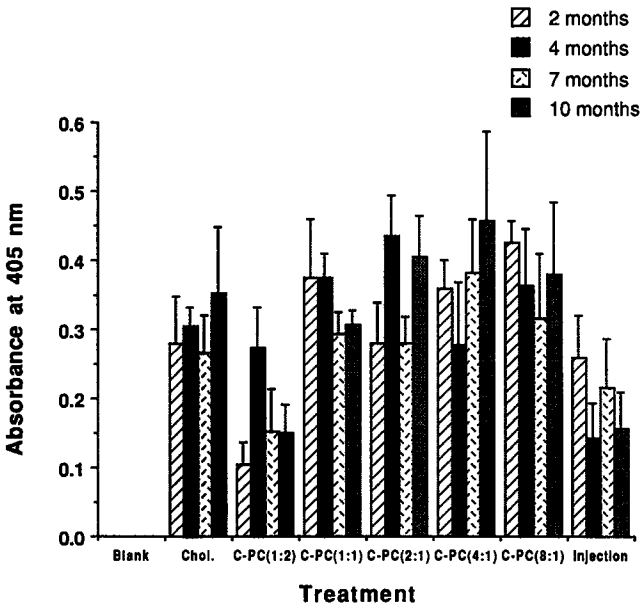

Fig. 1. Anti-BSA antibody levels in sera of mice following administration of BSA by single-dose implants prepared from cholesterol alone (Chol.) and from cholesterol and lecithin (C-PC) and by three injections (Injection). The cholesterol–lecithin implants were formulated at various cholesterol–lecithin ratios, which are indicated in parentheses. Control mice received blank implants (without BSA) with a cholesterol–lecithin ratio of 8:1 (Blank). Vertical bars indicate SE of the means (n = 5 or 6). [Reproduced from Khan *et al.* (44)].

and the erosion of the cholesterol–lecithin pellets during *in vitro* studies under active hydrodynamic conditions, and increasing the lecithin content of the implants enhanced the release rate. Among other factors, the hydrodynamic conditions of the dissolution medium and the method used to mix cholesterol and lecithin in preparing the implants greatly influenced the release process (43).

Previous studies with cholesterol implants suggested that variable release of peptides and proteins occurred from these implants and the device was not biodegradable (45,46). However, results of separate *in vivo* studies carried out in our laboratories demonstrated that the release profile of BSA from the cholesterol implant was reproducible (42,44). Perhaps the different manufacturing technique adopted in previous studies was responsible for the variable release rates. The main drawback of the cholesterol system, however, is the persistence of the implants, although the addition of lecithin to the system makes the implants biodegradable (43,44). The technique for preparing these implants is simple compare to that required to prepare polymeric implants and the antigen is not subjected to either heat or organic solvent treatment. These implants can also be used as carriers for adjuvants such as saponin, MDP, and perhaps cytokines, in addition to their use to deliver antigen. However, the efficacy of these implants in delivering actual vaccine antigens, the isotype of antibody responses induced, the pharmacokinetics of the release of antigen, and the techniques for sterilizing the implants require further investigation.

Heterogeneous Delivery Systems

Other approaches to enhancing the potentiation power of adjuvants include the use of a combination of two or more adjuvants in the same system to achieve a synergistic action and a modification of the delivery system to extend the duration of antigen delivery. For example, a recombinant malarial antigen, *Plasmodium falciparum*, $R32NS1_{81}$, was found to be poorly immunogenic when adsorbed with $Al(OH)_3$. The antigen encapsulated in liposomes containing monophosphoryl lipid A and then adsorbed to $Al(OH)_3$ induced much higher antibodies in humans than those induced by the antigen adsorbed to $Al(OH)_3$ without liposomal encapsulation (47). The presence of monophosphoryl lipid A in the liposomes was found to play a dominant role in enhancing the antibody levels. Similar results were obtained with another recombinant malarial antigen—*Plasmodium falciparum*, $R32tet_{32}$ (48). Cohen *et al.* (49) studied the feasibility of creating a subcutaneous depot of liposomes to obtain long-term delivery of antigens; they encapsulated the liposomes containing the antigen into alginate-poly(L-lysine) microcapsules and demonstrated that the depot effect of the liposomes could be extended substantially in rats by this method. The antibody responses induced by the alginate-poly(L-lysine) microcapsules were two- to threefold higher than those induced by the antigen delivered from either liposomes (without encapsulation) or saline or FCA, and the high antibody levels were maintained for over 150 days after a single-dose injection. The authors were able to recover 30% of the total ^{125}I-labeled antigen delivered through these microencapsulated liposomes from the injection sites 80 days after injection. In contrast, no radioactivity was detected in rats injected with the antigen in saline, liposomes, or FCA 50 days after injection.

Chang (50) described microencapsulation of antigens within a biodegradable polylactic acid membrane and suggested that a dosage form consisting of a vaccine antigen partly in solution form and partly in microencapsulated form in various types of microcapsules could be used for single-shot immunization; microcapsules prepared from different polymers would be degraded at various times so as to release the antigen at predetermined intervals.

Eldridge *et al.* (51) reported that a combination of two microspheric preparations could release a vaccine antigen at different times, providing discrete primary and booster doses following a single injection. A mixture of 1- to 10- and 20- to 125-μm microspheres, made of copolymer DL-PLG (50:50) containing SEB toxoid, induced both a primary and an anamnestic anti-SEB response following single-dose injection in mice (see Fig. 2). Microspheres <10 μm in diameter were phagocytosed and released the antigen at a substantially accelerated rate compared to the other microspheres contributing to the primary response, while the microspheres with larger dimensions, being slowly phagocytosed, released the antigen at a slower rate and resulted in stimulating the secondary response in the mice. A similar heterogeneous microspheric delivery system with multiphasic controlled-release properties for antigens was also described by Silvestri and Pyle (52). As mentioned earlier, the rate of release of antigen from biodegradable microspheres depends on the degradation of the matrix. The rate of degradation of the matrix can be manipulated by varying factors such as the size of microspheres, type of polymer, or ratio of various monomers used in a copolymer (51). DL-PLG (50:50) microspheres (1–10 μm) first exposed to 0.1 *M* HCl for 2 hr and then kept in a neutral solution for about 18 days released 30% of the antigen very rapidly within about 2–4 days and retained the remaining 70% until the experiment was terminated (53). DL-PLG (85:15) microspheres (45–250 μm) administered intramuscularly or sc into mice did not start to biodegrade until about days 60 to 90 and were completely resorbed by 180 days (53).

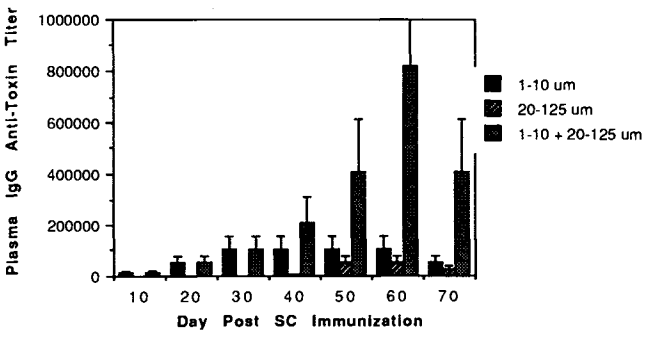

Fig. 2. Controlled vaccine release through alteration in microsphere size. Anti-SEB toxin antibodies of the IgG isotype in the plasma of mice immunized with SEB toxoid in 50:50 DL–PLG microspheres 1–10 μm in diameter or 20–125 μm in diameter or in a mixture of microspheres 1–10 and 20–125 μm in diameter. Antibodies were measured by end-point titration. [Reprinted from *Mol. Immunol.* 28, J. H. Eldridge *et al.*, Biodegradable microspheres as a vaccine delivery system, 287–294 (1991), with permission from Pergamon Press Ltd., Headington Hill Hall, Oxford OX3 0BW, UK.]

In a patent Beck (41) described techniques to incorporate antigens into microparticles (shaped matrix) prepared from different biocompatible materials (e.g., biodegradable polymers, fatty acid esters) to be blended together and placed in an appropriate liquid vehicle to form a single delivery system for the antigen. The delivery system administered in the form of an injection would release the antigen in a controlled manner over a period of time as required to induce prolonged immunity. The author also recommended simultaneous administration of an antigen through an implant (shaped matrix) and in free form (as an injection) to achieve pulsatile and sustained delivery of the antigen suitable for inducing prolonged immunity.

Most of the heterogeneous delivery systems described above use biodegradable and biocompatible polymers; a polymer such as DL-PLG is known for its safety for use in humans (51). However, some of these delivery systems require organic solvents to manufacture them, which may denaturalize the antigen. The experimental results reported in some of these studies with vaccine antigens are promising but further clinical studies are required to establish their efficacy.

OPTIMUM ANTIGEN DELIVERY PROFILES FOR PROLONGED IMMUNITY

Little is understood of what constitutes an optimal release profile for the delivery of antigen. It has been demonstrated with conventional liquid dosage forms that several small doses of antigen are more effective than a single inoculum or a few large doses of the antigen in stimulating a protective immune response (7,8) and that the primary dosage of antigen should be greater than the secondary and subsequent doses; protein concentrations as low as 0.001 μg constitute a sufficient stimulus for a secondary response (54). A state of immunological unresponsiveness can be induced by high and low doses of antigen (55,56) and by antigen injected daily for several weeks (57). Further, particular immunization regimens have been shown to induce IgE antibodies in mice (58), which may result in hypersensitivity.

Most of the studies on controlled- or sustained-delivery devices for antigens reported in the literature (as reviewed above) focus on successful induction of prolonged immune responses. The *in vivo* delivery pattern of the antigen from these devices has not been well investigated and studies have not been carried out to establish the effects of altering the delivery profiles of antigens on the immune response. The results of our work on lipid implants (42–44) and other reports on polymeric implants (37–40) suggest that an initial pulsatile delivery of the antigen followed by a trickle of continuous delivery should work equally well in inducing protective lasting immunity. Further work carried out in one of our laboratories demonstrated that continuous delivery of BSA through a miniosmotic pump (zero-order release rate) for up to 3 months was as effective as either two or three injections of the antigen over the same period (59). It was also observed that a priming dose was not an essential requirement for induction of an immune response. Moreover, the continuous delivery of the antigen (0.1 μg/hr) over 3 months did not induce significantly greater levels of IgE antibodies than did antigen delivered by injections, and there was no evidence of immunological tolerance (59). However, partial or total suppression of the formation of IgG plaque forming cells has been reported following continuous delivery of high (1-mg) and low (0.01-mg) doses of 2,4,6-trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) to mice by an osmotic pump for 7 days (60).

The dearth of information on what constitutes an ideal release profile for antigens to induce high levels of prolonged immunity indicates that more work is required on the effects of zero-order release kinetics and other delivery profiles on immune responses before a particular system can be adopted for use in designing delivery systems for single-step immunization.

CONCLUSIONS

Recombinant, subunit, synthetic, and purified antigens are poorly immunogenic and invariably require the presence of an immunomodulator to induce immunity. The use of immunoadjuvants can potentiate the immune response to varying degrees, but the few adjuvants acceptable for use in humans and animals often have undesirable side effects and may not provide immunomodulation of sufficient duration for lasting protective immunity. Additional enhancement of the immune response can be achieved by designing appropriate dosage forms which would modulate the immune system by delivering the antigen and perhaps the adjuvant in a profile appropriate for maximizing the immune response; some of these dosage forms (delivery systems) may also enhance the immune response in their own right. The induction of IgG antibodies following a pulsatile plus trickle delivery of the antigen from nonbiodegradable polymeric implants (37,38) demonstrates that a secondary response can be obtained with this delivery profile of antigen without a secondary burst delivery effect. IgG antibodies were also induced by microencapsulated liposomes with similar release characteristics (49). Tolerance was not reported as a consequence of slow and continuous delivery of antigen (33,59). These facts and the prolonged high antibody titers obtained by delivering antigen from sustained (37–40,49)- and controlled (51)-release devices suggest that single-step immunization is a feasible proposition. The current trend in designing these dosage forms and the experimental results obtained so far are encouraging. Vaccine preparations for single-step immunization of animals and perhaps humans are likely to be available on the market in the near-future if this trend continues.

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