The Development and Use of Vaccine Adjuvants

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Abstract

Interest in vaccine adjuvants is intense and growing, because many of the new subunit vaccine candidates lack sufficient immunogenicity to be clinically useful. In this review, I have emphasized modern vaccine adjuvants injected parenterally, or administered orally, intranasally, or transcutaneously with licensed or experimental vaccines in humans. Every adjuvant has a complex and often multi-factorial immunological mechanism, usually poorly understood in vivo. Many determinants of adjuvanticity exist, and each adjuvanted vaccine is unique. Adjuvant safety is critical and can enhance, retard, or stop development of an adjuvanted vaccine. The choice of an adjuvant often depends upon expensive experimental trial and error, upon cost, and upon commercial availability. Extensive regulatory and administrative support is required to conduct clinical trials of adjuvanted vaccines. Finally, comparative adjuvant trials where one antigen is formulated with different adjuvants and administered by a common protocol to animals and humans can accelerate vaccine development.

Index Entries: Vaccine adjuvants; Phase I and II clinical trials; adjuvant mechanisms; adjuvant safety.

1. Introduction

Adjuvants have been used to augment the immune response to antigens for more than 70 years. Ramon first demonstrated that it was possible to increase levels of diphtheria or tetanus antitoxin by the addition of bread crumbs, agar, tapioca, starch oil, lecithin, or saponin to the vaccines (1). In this review, I will provide an overview of how modern vaccine adjuvants are developed and used. First, a general discussion of adjuvants will include definitions of commonly used terms, mechanisms of action, safety, characteristics of an ideal adjuvant, impediments to development, and preclinical and clinical regulatory issues. Finally, I will provide examples of experimental adjuvants that have entered clinical trial to enhance a variety of licensed and experimental vaccines in humans. For additional expositions on this complex subject and for a historical perspective, the reader is referred to recent textbooks on vaccine adjuvants (2–4) and a selection of useful review articles published over the past 21 years (5–14).

Interest in vaccine adjuvants is growing rapidly for several reasons. First, dozens of new vaccine candidates have emerged over the past decade to prevent or treat infectious diseases, cancer, fertility, allergic, and autoimmune diseases. Many of these candidates require adjuvants. Second, vaccines have become commercially more profitable in the past few years. Third, the Children's Vaccine Initiative (CVI) initiated in 1990 (15), and the Global Alliance for Vaccines initiated in 1999 (16), have helped to energize political and public health interest in vaccine adjuvants by establishing ambitious goals for enhancing present vaccines and for developing new ones. Fourth, refinements in the fields of analytical biochemistry, macromolecular purification, recombinant technology, and improved understanding of immunological mechanisms and disease pathogenesis have helped to improve the technical basis for adjuvant development and application. Finally, the development of experimental adjuvants has been driven by the failure of aluminum

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compounds to enhance many vaccines in humans, to enhance many subunit vaccine antigens in animals, or to stimulate cytotoxic T-cell responses.

2. Definitions

The discussion of vaccine adjuvants will be facilitated by a definition of terms.

2.1. Adjuvant

The term "adjuvant" (from the latin, adjuvare = help) was first coined by Ramon in 1926 for a substance used in combination with a specific antigen that produces more immunity than the antigen used alone (17). The enormous diversity of compounds that increase specific immune responses to an antigen and thus function as vaccine adjuvants makes any classification system somewhat arbitrary. Adjuvants in Table 1 are grouped according to origin rather than according to mechanism of action, because the mechanism for most adjuvants are incompletely understood. By contrast, Cox and Coulter (11) have classified adjuvants into two broad groups, particulate or non-particulate. A third classification scheme, modified from Audibert and Lise (18), identifies at least four main sources of adjuvants, as follows: (1) botanical, e.g, saponin or glucan extract; (2) bacterial, e.g., muramyl dipeptides, monophosphoryl lipid A, cholera toxin, and CpG oligodeoxynucleotides; (3) chemical, e.g., aluminum salts, pluronic block polymers, lactide and glycolide, and polyphosphazenes; (4) cytokines and hormones, e.g., interleukin-2, granulocyte-macrophage colony stimulating factor, and dehydroepiandrosterone.

2.2. Carriers, Vehicles, and Adjuvant Formulations

Several terms used in **Table 1** need to be defined. A "carrier" has several meanings. It is an immunogenic protein bound to a hapten or a weakly immunogenic antigen (19). Carriers increase the immune response by providing T cell help to the hapten or antigen (20,21). Alternatively, a carrier may also be a living organism (or vector) bearing genes for expression of the foreign hapten or antigen (22–27). A DNA vaccine is a carrier in the sense that, like some living vectors, it carries a plasmid-based DNA vector encoding the production of the protein antigen upon inoculation into the host (28,29).

A "vehicle" provides a substrate for the adjuvant, the antigen, or the antigen–carrier complex. Unlike carriers, vehicles are not immunogenic. Some vehicles provide a fairly consistent adjuvant effect (30,31), while others do not (32). The immunostimulatory effects of vehicles are often augmented by the addition of conventional adjuvants to constitute "adjuvant formulations," as discussed below.

Examples of adjuvant formulations tested in humans with a variety of antigens (and with variable success) include: monophosphoryl lipid A and cell wall skeleton of Mycobacterium phlei adjuvants in a squalane-in-water emulsion vehicle (33), monophosphoryl lipid A adjuvant in a liposome vehicle (34), threonyl-muramyl dipeptide adjuvant and Pluronic L-121 block polymer adjuvant in a vehicle emulsion of squalane and Tween 80 (35), muramyl tripeptide-dipalmitoyl phosphatidylethanolamine adjuvant in a squalene-in-water emulsion vehicle (36), and monophosphoryl lipid A and QS-21 adjuvants in a proprietary oil-in-water emulsion (37).

3. Examples of Modern Vaccine Adjuvants Used in Animals and Humans

3.1. Adjuvants for Parenteral Vaccines

Agents listed in **Table 1** are examples of the many varieties of immunopotentiators used during the past 30 years. The majority have been injected intramuscularly or parenterally. The majority are being developed and tested by industry. The list of adjuvants is incomplete, because I have not conducted an exhaustive literature search, because the results have appeared in abstracts in non-indexed publications, and because many studies are proprietary. The adjuvants marked by an asterisk in Table 1 have completed trial in humans, or they are now undergoing clinical trial. Promising adjuvants not yet tested in humans are also listed. In some instances, adju-

Table 1
Classes of Modern Vaccine Adjuvants

1. Mineral Salts	2. Surface-active agents and Microparticles	3. Bacterial Products	4. Cytokines and Hormones	5. Unique Antigen Constructs
Aluminum ("Alum") Aluminum hydroxide * Aluminum phosphate * Calcium phosphate *	Nonionic block polymer surfactants * Virosomes * Ty-virus-like particles * Saponin (QS-21) * Meningococcal outer membrane proteins (Proteosomes) * Immune stimulating complexes (ISCOMs) * Cochleates Dimethyl dioctadecyl ammonium bromide (DDA) Avridine (CP20,961) Vitamin A Vitamin E	Cell wall skeleton of *Mycobacterium phlei* (Detox^{R}) * Muramyl dipeptides and tripeptides Threonyl MDP (SAF-1) * Butyl-ester MDP (Murabutide^{R}) * Dipalmitoyl phosphatidylethanolamine MTP * Monophosphoryl lipid A * *Klebsiella* pneumonia glycoprotein * *Bordetella* pertussis * Bacillus Calmette-Guérin * *V. cholerae* and *E. coli* heat labile enterotoxin * CpG oligodeoxynucleotides * Trehalose dimycolate	Interleukin-2 * Interleukin-12 * Interferon-alpha * Interferon-gamma * Granulocyte-macrophage colony stimulating factor * Dehydroepiandrosterone * Flt3 ligand * 1,25-dihydroxy vitamin D_3 Interleukin-1 Interleukin-6 Human growth hormone 2-microglobulin Lymphotactin	Multiple peptide antigens attached to lysine or polyoxime core (MAP) * CTL epitope linked to universal helper T cell epitope and palmitoylated at the N terminus (Theradigm-HBV) *

6. Polyanions	7. Polyacrylics	8. Miscellaneous	9. Carriers	10. Living Vectors	11. Vehicles
Dextran Double-stranded polynucleotides	Polymethylmethacrylate Acrylic acid cross-linked with allyl sucrose (Carbopol 934P)	N-acetyl-glucosamine-3yl-acetyl-L-alanyl-D-isoglutamine (CGP-11637) * Gamma inulin + aluminum hydroxide (Algammulin) * Transgenic plants * Human dendritic cells * Lysophosphatidyl glycerol Stearyl tyrosine Tripalmitoyl pentapeptide	Tetanus toxoid * Diphtheria toxoid * Meningococcal B outer membrane protein (proteosomes) * *Pseudomonas* exotoxin A * Cholera toxin B subunit * Mutant heat labile enterotoxin of enterotoxigenic *E. coli* * Hepatitis B virus core * CpG dinucleotides * Cholera toxin A fusion proteins Heat shock proteins Fatty acids	Vaccinia virus * Canarypox virus * Adenovirus * Yellow fever vaccine virus * Attenuated *Salmonella typhi* * Attenuated *Shigella* * Bacillus Calmette-Guérin * *Steptococcus gordonni* * Herpes simplex virus Polio vaccine virus rhinovirus Venezuelan equine encephalitis virus Sindbis virus *Yersinia enterocolitica* *Listeria monocytogenes* *Bordetella pertussis* *Saccharomyces cerevisiae*	Water-in-oil emulsions Mineral oil (Freund's incomplete) * Vegetable oil (peanut oil) * Squalene and squalane * Oil-in-water emulsions Squalene + Tween 80 + Span 85 (MF59) * Liposomes * Biodegradable polymer microspheres Lactide and glycolide * Polyphosphazenes * Beta-glucan Proteinoids

* Identifies adjuvants administered to humans. Of these, only aluminum salts, virosomes, and MF59 are adjuvants approved as licensed vaccine formulations in the United States

vants have been combined in an adjuvant formulation hoping to gain a synergistic or additive effect.

3.2. Vaccine Adjuvants versus Non-specific Enhancers of Immunity

Agents listed in **Table 1** enhance specific antigens and are administered concurrently with the antigen. Adjuvants not administered in a single dose at or near the time of antigen inoculation and into the same injection site as the antigen, are not listed. Thus, adjuvants administered repeatedly as non-specific enhancers of immune response are largely excluded. Immunopotentiating agents administered to humans separately in time or location from the vaccine may be impractical for vaccinating large numbers of persons, and are potentially unsafe because of their physiological effects on the entire body. They may have a role, however, in immunizing a small number of high risk, immuno-incompetent individuals, such as renal dialysis patients at risk for hepatitis B or the very elderly at risk of influenza. Examples of such "whole body" adjuvants used in humans to augment vaccines include Na diethyldithiocarbamate (38), thymosin alpha one (39), loxoribine (40), granulocyte-macrophage stimulating factor (41), cimetidine (42), and dehydroepiandrosterone sulfate (43). The results of such trials to date have been disappointing.

3.3. Adjuvants for Mucosal Vaccines

Recent advances in vaccinology have created an array of vaccines that can be delivered to mucosal surfaces of the respiratory, gastrointestinal, and genitourinary tracts using intranasal, oral, and vaginal routes (44). The development of mucosal vaccines has come at a time when the use of the syringe and needle for parenteral vaccination is losing favor. There are several reasons for this. First, the contamination of reused needles and syringes with HIV, hepatitis B, and hepatitis C viruses is a growing hazard, particularly in developing countries of Africa and Asia. Second, the number of marketed, parenteral pediatric vaccines are increasing worldwide. Currently, 20 separate vaccine injections are administered to U.S. infants over the first 18 months of life. Parents and physicians are demanding fewer injections. Third, vaccines administered mucosally, compared to vaccines administered parenterally, may provide better protection against the numerous respiratory, gastrointestinal, and genital pathogens that infect and proliferate at mucosal surfaces. Well-tolerated adjuvants that enhance such vaccines will play an important role in mucosal immunization. Some of the more promising adjuvants completed or near clinical trial include microspheres composed of copolymers of lactic and glycolic acids (45,46); proteosomes (47,48), liposomes (49), CpG DNA (50), cochleates (51), and virus-like particles (52).

Cholera toxin (CT) and the closely related, heat-labile enterotoxin of enterotoxigenic *Escherichia coli* (LT) are powerful adjuvants that augment the local and systemic serum antibody response to coadministered antigens, particularly when delivered by the mucosal route (53–60). Mutant CT and LT molecules have been engineered to reduce toxicity but to retain sufficient adjuvanticity to enhance local IgA, systemic IgG, and cellular immune responses to co-administered vaccine antigens (61–64). Clinical trials using mutant LT toxins as adjuvants of nonliving vaccine antigens are in progress (13). Recent safety concerns, engendered by passage of CT and LT into the olfactory bulb of Balb/C mice after intranasal instillation, must be resolved before clinical evaluation of these powerful adjuvants as intranasal adjuvants can proceed (65).

Attenuated recombinant bacteria (26,66,67) and viruses (22), administered orally as live vectors of cloned genes encoding protective antigens of other pathogens, have undergone phase I trials to stimulate immune effector responses. Most of these early attempts to stimulate mucosal immune responses in volunteers using live vectors have only been marginally successful. The first attempts to immunize volunteers against LT and Norwalk virus antigen encoded in a transgenic potato and administered as edible vaccines were more successful (68,69). It remains to be seen if

other protein antigens (e.g., HBsAg) when given via transgenic plants will be immunogenic or will instead induce tolerance to the antigen.

3.4. Adjuvants for Transcutaneous Vaccines

Another needle-free method of immunization is via the transcutaneous route (70—72). The skin is a robust immunological organ heavily populated with Langerhans' antigen-presenting cells (73). Transcutaneous vaccination involves topical application of antigens and a variety of adjuvants to intact skin using a simple occlusive patch (72). The ability of skin to process foreign antigens has been exploited by other vaccine strategies, e.g., immunization with the "gene gun," which injects plasmid DNA through the stratum corneum (74), smallpox vaccine via scarification, and BCG vaccine via intradermal injection (27). By utilizing the proper adjuvant in mice, it is possible to induce both systemic and mucosal immunity via the skin (75,76). In humans, CT applied to the skin with tetanus toxoid induced systemic immunity to the toxin (70), and a systemic immune response was engendered when LT was co-administered with a pilus protein of *E. coli* as a prototype traveler's diarrhea vaccine (77). Mucosal responses were not measured. This novel approach to vaccine delivery, if found to be safe and non-reactogenic in continuing studies, will be aggressively developed for a variety of preventive and therapeutic vaccines.

4. Mechanisms of Adjuvant Action

To date, most subunit vaccines are poor antigens, be they natural products, recombinant products, or synthetic peptides. Subunit antigens fail for a variety of reasons, such as incorrect processing by the immune system, rapid clearance, stimulation of inappropriate immune response, and lack of critical B-cell or T-cell epitopes. Potentially, some of these failures can be overcome by administering subunit antigens with adjuvants. It should be remembered, however, that the best adjuvant will never correct the choice of the wrong (non-protective) epitope.

Traditional live vaccines or whole-cell inactivated microbial vaccines are generally better immunogens than subunit vaccines. Live and inactivated whole organisms are structurally more complex than subunit vaccines, and so contain many redundant epitopes, which offer more opportunity to bypass genetic restriction of the vaccinee. Such vaccines also provide a larger antigen mass than subunit vaccines, particularly if they replicate in vivo. Their antigens are larger molecules, portions of which may serve as carrier proteins and thus function as intrinsic adjuvants to enhance immunogenicity by providing T cell help. Finally, bacterial DNA may directly stimulate the host's immune system due to it large content of unmethylated CpG dinucleotides (78), and whole bacterial vaccines may contain CpG DNA.

4.1. Specific Immune Mechanisms

Some mechanisms of adjuvant action are discussed below and are summarized in **Table 2**. Vaccine adjuvants can (1) increase the potency of small, antigenically weak synthetic or recombinant peptides. (2) They can enhance the speed, vigor, and persistence of the immune response to stronger antigens. For example, aluminum adjuvants used with licensed pediatric vaccines (e.g., DTP) elicit early and higher antibody response after primary immunization than do unadjuvanted preparations. (3) Adjuvants can increase the immune response to vaccines in immunologically immature, immunosuppressed, or senescent individuals. (4) Adjuvants can select for or modulate humeral or cell-mediated immunity, and they can do this in several ways. First, antigen processing can be modulated, leading to vaccines which can elicit both helper T cells and cytotoxic lymphocytes (CTL) (reviewed in 8,79). Second, depending upon the adjuvant, the immune response can be modulated in favor of MHC class I or MHC class II response (8,79). For example, the QS-21 adjuvant can elicit MHC class I CTL responses when mixed with protein antigens, peptides, or inactivated viruses (80,81). Aluminum adjuvants, among others, elicit principally MHC class II antibody responses when combined with protein antigens or inactivated organisms (79,82). Third, adjuvants can modulate the immune response by preferentially stimulating Th1 or Th2

Table 2
Some Mechanisms of Adjuvant Action

- Stabilizes epitope conformation
- Generates a depot at the site of inoculation with slow release of antigen
- Targets the antigen to antigen-presenting cells (APCs) by formation of multimolecular aggregates, or by binding antigen to a cell-surface receptor on APCs.
- Directs antigen presentation by major histocompatibility complex (MHC) class I or MHC class II pathways, by means of fusion or disruption of cell membranes, or by direct peptide exchange on surface MHC molecules.
- Preferentially stimulates Th1 or Th2 CD4+ T-helper cells or CD8+ cytotoxic T-lymphocytes, by modulation of the cytokine network in the local microenvironment.

Table 3
Beneficial Effects of Vaccine Adjuvants

- Increase the potency of antigenically weak peptides
- Enhance the speed, vigor, and persistence of the immune response to stronger antigens
- Modulate antibody avidity, specificity, quantity, isotype, and subclass
- Select for or enhance the cytotoxic T cell response
- Increase the immune response to vaccines in immunologically immature, suppressed, or senescent individuals
- Decrease the amount of antigen required, thus reducing the cost and the likelihood of antigen competition in combination vaccines

CD4+ T-helper cells (83). The Th1 response is accompanied by secretion of interleukin-2 (IL-2), interferon-gamma (IFN-γ), and TNF-beta leading to a CMI response, including activation of macrophages and CTL and high levels of IgG2a antibodies in mice. The Th2 response is modulated by secretion of IL-4, IL-5, IL-6, and IL-10, which provide better help for B cell responses, including those of IgG1, IgE and IgA isotypes in mice. Aluminum salts principally stimulate the Th2 response (84), while the Th1 response is stimulated by manyadjuvants, such as muramyl dipeptide, monophosphoryl lipid A, and QS-21 (8,35,85,86). (5) Vaccine adjuvants can modulate antibody avidity, specificity, quantity, isotype, and subclass against epitopes on complex immunogens (9,87,88). For example, only certain adjuvants, vehicles, and adjuvant formulations can induce the development of the protective IgG2a antibody isotype against *Plasmodium yoelii* (9). (6) Vaccine adjuvants can decrease the amount of antigens in combination vaccines, thus reducing the likelihood of antigen competition and carrier-specific epitope suppression. In addition, by reducing the quantity of antigen needed to protect, adjuvants can decrease the cost and increase the availability of vaccines. On the other hand, the high cost of some modern adjuvants may offset the savings realized by the reduced antigen requirement, thereby paradoxically driving up vaccine cost overall.

One must remember that in vivo, most adjuvants have complex and multifactorial immunological mechanisms, often poorly understood. The immunological mechanisms utilized by many adjuvants are under investigation. Such investigations will provide answers to some of the following questions. Does the adjuvant induce cell mediated (Th1) immunity, humoral (Th2) immunity, or a balance of Th1 and Th2? Which IG isotypes dominate? Which cytokines are induced? Are CD4+ T-helper cells or CD8+ cytotoxic T-lymphocytes induced? The list of such questions is extensive, and grows in proportion to our understanding of immunological mechanisms in general.

5. Advantages of Adjuvants

Vaccine adjuvants influence the immune response to our benefit in one or more ways (**Table 3**). The ability of adjuvants to influence so

Table 4
Modulators of Vaccine Adjuvant Effects

- Route
- Timing
- Dose
- Adjuvant formulation
- Antigen construct
- Host dpecies
- Intra-species genetic variation
- Immune status of the host

Table 5
Real and Theoretical Risks of Vaccine Adjuvants

1. Local acute or chronic inflammation with formation of painful abscess, persistent nodules, ulcers, or draining lymphadenopathy
2. Influenza-like illness with fever.
3. IgE-type immediate hypersensitivity to vaccine antigen, including anaphylaxis.
4. Chemical toxicity to tissues or organs.
5. Induction of hypersensitivity to host tissue, producing autoimmune arthritis, amyloidosis, anterior uveitis.
6. Cross-reactions with human tissue antigens, causing glomerulonephritis or meningoencephalitis.
7. Immune suppression or oral tolerance
8. Carcinogenesis
9. Teratogenesis or abortogenesis
10. Spread of a live vectored vaccine to the environment

many parameters of the immune response greatly complicates the process of finding an effective adjuvant. This is because our knowledge of how any one adjuvant operates on a cellular level is insufficient to support a completely rational approach for matching the vaccine antigen with the proper adjuvant. Consequently, many investigators advocate an empirical approach for antigen selection based on the balance among toxicity, adjuvanticity in animals, and whether one wishes to stimulate a Th1 response, a Th2 response, or a balance of the two responses. Finally and importantly, one must remember that the advantages of adjuvants are modulated strongly by the immunization schedule and route, by the antigen and adjuvant formulation, and by the host (**Table 4**).

6. Safety

The most important attribute of any adjuvanted vaccine is that it is more efficacious than the aqueous vaccine, and that this benefit outweighs its risk. During the past 70 years many adjuvants have been developed, but they were never accepted for routine vaccination because of their immediate toxicity and fear of delayed side effects. The current attitude regarding risk-benefits of vaccination in our Western society favors safety over efficacy when a vaccine is given to a healthy population of children and adults. In high risk groups, including patients with cancer and AIDS, and for therapeutic vaccines, an additional level of toxicity may be acceptable if the benefit of the vaccine was substantial.

Unfortunately, the absolute safety of adjuvanted vaccines, or any vaccine, cannot be guaranteed, so we must minimize the risks. The concern about adjuvant safety has encouraged continued use of aluminum adjuvants because of their long record of relative safety in children. Safety concerns have helped justify the development of unique synthetic antigen constructs and DNA vaccines not dependent on adjuvants. For example, large polymerized monomers of haptens and peptides have been linked together in a multimeric form designed to increase intrinsic adjuvanticity (multiple antigen peptide systems [MAPs]) (89,90). The first phase 1 trials of DNA-based vaccines showed them to be safe (28,29,74). It remains to be seen if MAPs, DNA vaccines, and other unique antigen constructs will retain enough inherent adjuvanticity to avoid the risk of administering them with extraneous chemical or biological adjuvants to humans. The fact remains that, in general, the inflammatory reaction induced by most adjuvants seems to enhance adjuvanticity, so the more robust the adjuvanticity, the more robust the reactogenicity to that adjuvant. The clinical goal is to arrive at an acceptable balance between adjuvanticiy and reactogenicity.

The real or theoretical risks of administering vaccine adjuvants have been discussed in detail (5,6,91,92) and are summarized in **Table 5**.

Undesirable reactions can be grouped as either local or systemic.

6.1. Local Reactions

The most frequent adverse side effect associated with adjuvanted vaccines is the formation of local inflammation with signs of swelling and erythema, and symptoms of tenderness to touch and pain on movement. Such reactions occur more frequently in preimmune individuals, or after repeated immunization (33,93,94). The inflammation is thought to be the result of formation of inflammatory immune complexes at the inoculation site by combination of the vaccine antigen with preexisting antibodies and complement, resulting in an Arthus-type reaction. Such reactions tend to occur more frequently after adjuvanted vaccines than after aqueous vaccines because of the high antibody titers induced by adjuvants. In addition, inflammatory cytokines released by many adjuvants contribute to both the local inflammation and systemic flu-like symptoms.

Painful abscesses and nodules at the inoculum site are seen, but far less frequently (reviewed in 5). Possible mechanisms for such local reactions include: (1) contamination of the vaccine at the time of formulation with reactogenic chemicals and microbial products; (2) instability of the vaccine on storage with breakdown into reactogenic side products; and (3) poor biodegradability of the adjuvanted vaccine resulting in prolonged persistence in the tissues and reactive granuloma formation. Such local reactions are of special concern for depot-type adjuvants, such as aluminum salts, liposomes, biodegradable polymer microspheres, and, especially, oil emulsions. Severe local reactions in humans have followed injections of vaccines adjuvanted with IFA (incomplete Freund's adjuvant) (reviewed in 5), DETOX™ (monophosphoryl lipid A + cell wall skeleton of *M. phlei* + squalane oil vehicle + Tween 20 emulsifier) (33,95), muramyl tripeptide covalently linked to dipalmitoyl phosphatidylethanolamine (MTP-PE) in a squalene-in-water emulsion (96), and the squalene oil adjuvant, Montanide ISA 720 (97).

We have noted development of local ulceration for as long as 70 d after intradermal inoculation of volunteers with a recombinant BCG-OspA Lyme disease vaccine; the open sores drained viable rBCG-OspA before they spontaneously healed (27). Development of similar draining sores occur commonly in adults after intradermal inoculation with standard BCG vaccine (98,99). We and others have observed a "recall reaction," characterized by immediate swelling, hives, and intense pruritus at the skin site of a previous antigen injection within 5–20 min after reexposure to that antigen at a remote site (100–102). The reaction seem to be associated with circulating IgE antibody or high-titered serum antibody of yet unknown isotype.

Severe local pain has occurred immediately after intramuscular injection of 15 of 108 volunteers administered a recombinant HIV protein formulated with QS-21 (103). Although the pain lasted from several minutes to several hours and was associated with several vasovagal reactions in several volunteers, no long-lasting side effects were reported. Addition of excipients, such as Triton X-100, to QS-21 formulations has eliminated severe painful injections without affecting adjuvanticity (C.R. Kensil, personal communication).

Finally, 14 of 19 volunteers immunized transdermally with LT and an *E. coli* pilus antigen developed a localized, pruritic, contact-dematitis-like rash at the vaccination site. The rash began 1 to 2 d after the second or third application and lasted 5–7 d. A skin biopsy was compatible with cutaneous delayed type sensitivity (77). Such local reactions may impede the development of transcutaneous immunization.

6.2. Systemic Reactions

Anterior chamber uveitis has been reported with MDP and several MDP analogs in rabbits (104) and monkeys (105). Anterior uveitis has been systematically sought in at least one adjuvant vaccine study involving 110 volunteers, but it was not found (106). A slit lamp examination of volunteers to detect subclinical uveitis is not commonly performed. Adjuvant-associated arthritis (107–109) has not been reported in humans, even after long-term followup (110–113). More theo-

Table 6
Characteristics of the Ideal Adjuvant

1. It must be safe, including freedom from immediate and long-term side effects.
2. It should be biodegradable or easily removed from the body after its adjuvant effect is exhausted to decrease the risk of late adverse effects.
3. It should elicit a more robust protective or therapeutic immune response combined with the antigen than when the antigen is administered alone.
4. It must be defined chemically and biologically, so that there is no lot-to-lot variation in the manufactured product, thereby ensuring consistent responses in vaccinees between studies and over time.
5. Efficacy should be achieved using fewer doses and/or lower concentrations of the antigen.
6. It should be stable on the shelf to be commercially and clinically useful.
7. The adjuvant should be affordable.

retical risks include the induction of autoimmunity or cancer. Fortunately, in 10 and 18 year followup studies, the incidence of cancer, autoimmune, and collagen disorders in 18,000 persons who received oil-emulsion influenza vaccine in the early 1950s was not different from that in persons given aqueous vaccines (30,112). A 35 yr follow up of these vaccinees again failed to demonstrate higher mortality associated with a variety of chronic diseases (113). It requires decades of expensive and time-consuming follow up to identify low-incidence reactions, and at present a mechanism for the systematic, active follow-up of vaccinees given experimental adjuvants is not available (114).

To date, the largest and most systematic published investigation of the safety of vaccine adjuvants in humans involves HIV-negative, healthy volunteers followed on average for 2.4 yr as part of the NIAID-sponsored AIDS Vaccine Evaluation Group trials (115). This informative report includes safety data from 1398 volunteers immunized with seven recombinant, two synthetic peptide, and two live poxvirus-vectored HIV-1 vaccines in 25 randomized, double-blind studies conducted between 1988 and 1997. The adjuvants tested by themselves or in combination included several aluminum preparations, deoxycholate, MF-59, QS-21, monophosphoryl lipid A, liposomes, muramyl tripeptide-PE, muramyl dipeptide, SAF/2, and recombinant vaccinia and canarypox. Safety data were compiled for 1711 person-years of follow-up among vaccine recipients, and 308 person-years among placebo recipients. The mean duration of protocols was 1.5 yr, and the mean number of immunizations was 3.5. The candidate vaccines without adjuvant were generally well tolerated. The only adverse effects clearly related to vaccination were associated with moderate to severe local pain or inflammation, self-limited in nature, that were associated with the adjuvants, particularly alum plus deoxycholate, MTP-PE, and QS-21. MTP-PE was also associated with severe, self-limited febrile reactions similar to that reported for MTP-PE and influenza virus vaccine (96). No serious adverse laboratory toxicities and no evidence of significant immunosuppressive events occurred after immunization. A few volunteers experienced rash, hemolytic anemia, or arthralgia that might relate to an underlying immunopathologic mechanism, but such reactions were mild and quite infrequent. Eleven volunteers were diagnosed with malignancies, which was within the 95% confidence interval of the number of cases predicted by the National Cancer Institute for the general population (115).

7. Characteristics of an Ideal Adjuvant

It is likely that the "ideal" adjuvant does not and will not exist, because each adjuvant and its targeted antigen will have their unique requirements. Nevertheless, the generic characteristics summarized in **Table 6** would be desirable. To date, no adjuvant meets all of these goals.

8. Impediments to Rational Development

As already discussed, safety of new adjuvants is a major concern, particularly of those rare reac-

tions that occur once in several thousand doses and that may not be detected until late in the development program (114). But other impediments exist that retard orderly development of adjuvants; those impediments proposed by Gupta and Siber are discussed below (7).

8.1. Limited Adjuvanticity

Most adjuvants are effective with some antigens but not others. For example, aluminum compounds failed to augment vaccines against whooping cough (116), typhoid fever (117), trachoma (118), adenovirus hexon antigens (119), influenza hemagglutinin (120), and *H. influenzae* type b capsular polysaccharide conjugated to tetanus toxoid (121). It is not always possible to predict compatible and incompatible adjuvant–vaccine combinations early in development, before the late stages of preclinical or early clinical development. This situation is especially common when there are no reliable animal models. Although ovalbumin is often used as a "model antigen" for preliminary screening, doses used are often too high to discriminate between small differences among adjuvant formulations (122), and no functional antibody assays are available for this non-pathogenic antigen. If possible, initial preclinical studies should be done with the antigen destined for clinical studies at minimal threshold concentrations for preliminary evaluation of adjuvants (7,91).

8.2. Suboptimal Use of Aluminum Adjuvants

Aluminum salts have become the reference preparations for evaluation of new adjuvants for human vaccines. Therefore, it is important that aluminum adjuvants be used optimally to allow correct evaluation of the experimental adjuvant (5,7,123). Aluminum adjuvants are difficult to manufacture in a physicochemically reproducible way, and this failure affects immunogenicity. Thus, during the adsorption of antigens on aluminum adjuvants, attention must be paid to the chemicof aluminum adjuvant, conditions of adsorption, and concentration of adjuvant (7,123–125). Although these adjuvants are commonly called "alum" in the literature, referring to all aluminum adjuvants as "alum" is misleading. Alum is $Al(SO_4)2{\cdot}12H_2O$, and not all aluminum salts labeled "alum" are equally effective. For instance, aluminum hydroxide is more potent than aluminum phosphate (123). To minimize the variations and to avoid nonreproducible results due to use of different preparations of aluminum compounds, it has been recommended that a specific preparation of aluminum hydroxide such as Alhydrogel from a single manufacturer be chosen as a scientific standard for evaluation of new adjuvant formulations (126).

8.3. Animal Models

Different animal species, and different strains within a species, may behave differently to the same adjuvant. Intraspecies variation in immune response to adjuvants and vaccines is particularly true among mouse strains (7,127). For this reason, preclinical studies in one strain of a single animal species should be interpreted with caution. Again and again, we have discovered that biological differences between animal models and humans have led to the failure of formulations in clinical trials after showing great promise in preclinical studies.

Guinea pigs have been used widely for vaccine quality control, and guinea pigs may be the animal of choice for evaluating adjuvant formulations (126), although the absence of reagents to analyze guinea pig cytokines and IgG subclasses may impede full utilization. A useful rabbit model has been described by FDA and NIH investigators to evaluate the toxicity and adjuvanticity of adjuvant formulations (91). The rabbit model provides a new and much needed standard protocol linking preclinical assessment of adjuvant formulations with phase I trials. The wide availability of murine cytokine and Ig subclass reagents, low husbandry costs, and ease of handling will still ensure the continued use of mice despite their inconsistent responses to adjuvants. It is recommended that at least two strains of mice with different haplotypes be utilized, in addition to rabbits or guinea pigs. Vaccine alone, adjuvant alone, and vaccine–adjuvant combinations should be studied for toxicity and immunogenicity, and their concentrations should mimic and exceed human doses (7,91).

8.4. Immunoassays

In addition to measuring antibodies by ELISA or other antigen–antibody binding assays, one should measure antibody function by neutralization, opsonophagocytic, or bacteriocidal assays if available. However, the most decisive test is protection against experimental challenge. For example, many adjuvant formulations induced high-titer antibody against malarial (9) and SIV antigens (128), but antibody titers were not sufficient to predict protection even when the antigen contained protective epitopes and protection was mediated by antibody. The induction of protective immunity depended upon the quality rather the quantity of antibody, that is, induction of antibody of the appropriate isotype and fine-epitope specificity. This induction was dependent upon unique, poorly understood interactions between the adjuvant, the antigen, and the host. The conclusions from such experience suggests that the search for an effective vaccine must involve both antigens and adjuvants from the start of preclinical development, and that no adjuvant can be considered a gold standard (9).

9. Selection of Vaccine/Adjuvant Candidates for Clinical Trial

The decision to begin human trials of vaccines and adjuvanted vaccines is complex and depends on a number of criteria (129):

1. The vaccine/adjuvant candidate must address a public health need, and it must be a logical means to prevent or treat the disease of interest.
2. The vaccine/adjuvant must have been designed with a sound scientific rationale.
3. There must be an expectation of safety, as discussed in the section above on safety.
4. There must be animal studies demonstrating the immunogenicity of the product when given in the appropriate dose and route. If an appropriate animal model exists, it should be used to demonstrate protective or therapeutic efficacy against challenge with the virulent organism.
5. The vaccine/adjuvant should be prepared in a practical formulation for phase 1 studies, if possible. Response to a pilot vaccine adjuvant formulation can change after manufacturing scale up or after a more practical formulation is introduced.
6. Unless subsidized by government, clinical development of a new vaccine/adjuvant formulation must attract industrial funding. A company is unlikely to enter into expensive commercial development unless the vaccine/adjuvant formulation is protected by worldwide patent or commercial license.

10. Preclinical and Phase I Clinical Trial Design Issues

10.1. U.S. Food and Drug Administration Regulations

No detailed or specific guidelines exist in the United States for assessing the safety of adjuvant preparations for use in humans. Only two guidelines refer to adjuvants. The first guideline formally issued by the FDA, which includes adjuvanted vaccines (130), refers to tests of t he final container lot of all biological products. These FDA standards are paraphrased in **Table 7** for ease of understanding. It is unclear if adjuvants, such as QS-21 that are added to the vaccine immediately before inoculation, are subject to the final container assay.

The second FDA regulation simply states that, "An adjuvant shall not be introduced into a product unless there is satisfactory evidence that it does not affect adversely the safety or potency of the product" (Code of Federal Regulations, 21 CFR, Part 610.15). Because the definition of "satisfactory evidence" is rather vague, investigators should interact with the professional staff of the Center for Biologics Evaluation and Research, FDA, in order to reach a consensus definition. Incidentally, aluminum compounds alone are not licensed. Aluminum compounds are not considered to be "investigational adjuvants" because they are components in already licensed vaccines. Thus, antigen–adjuvant formulations are licensed for clinical use, but adjuvants alone are not (91).

10.2. Center for Biologics Evaluation and Research (CBER), FDA

The CBER, FDA, Rockville, MD is responsible for regulating vaccines and other biologics in the

Table 7
Standards Used to Test Clinical Lots of Biological Products (21 CFR 610.11)

1.	Safety:	Contains no extraneous toxic contaminants causing unexpected, unacceptable biological activity (no weight loss over seven days in two mice and two guinea pigs).
2.	Sterility:	Contains no contaminating bacteria or yeast (sterile aerobic and anaerobic cultures).
3.	Purity:	Contains no extraneous matter, such as pyrogens or chemicals (negative pyrogenicity assay in eight rabbits).
4.	Potency:	The biological can do what is claimed for it (measure by laboratory or clinical tests).
5.	Identity:	The biological is what you say it is (characterize by physical or chemical tests, microscopy, culture, or by immune assay).

United States. In addition to meeting the general standards before public release (**Table 7**), each vaccine and adjuvant is tested for safety on a case by case basis, preferably with the help and guidance of the CBER as noted before. Such guidance, informal in nature but quite helpful, was published in 1993 in response to the needs of HIV-1 vaccine development (91). The principles laid down by that publication can be adapted to the needs of other vaccines. I recommend that as a general principle, all novel (non-aluminum) vaccine/adjuvant formulations be discussed earlier rather than later in preclinical development with the staff of the CBER. The principles are summarized in the next few paragraphs. These and other preclinical and clinical trial study design issues have been discussed in some detail (91,92).

1. Extensive experience with aluminum compounds have shown them to be safe. Therefore, for vaccines with aluminum adjuvants, postinjection observation of the animal and injection site is generally adequate for preclinical safety without the need for formal toxicology study of the combined product, unless there is some special concern about the antigen.
2. For other adjuvants, additional tests are necessary. These include reactogenicity and toxicology tests of the adjuvant alone and the antigen-adjuvant combination in a manner that is relevant to the intended clinical use, including route of administration, injection volume and clinical formulation. A standard safety assessment protocol in rabbits should be utilized, but only if the rabbit is thought to be sensitive to the biological effects of the vaccine. This standard safety assessment protocol provides a bridging study that links preclinical and clinical development.
3. Early in clinical development the FDA recommends inclusion of a control group of volunteers given antigen alone and/or antigen adsorbed to aluminum as comparison groups. Results of the immunological assessments obtained from such early phase 1 studies should be combined with the safety profile to help define the risk/benefit of proceeding to further clinical studies.

10.3. Clinical Framework Required for Trials of Vaccines and Vaccine/Adjuvant Formulations

The successful clinical development of a vaccine depends upon an number of clinical components or principles (129,131). Most of these principles are shared by vaccine–adjuvant formulations. They include (1) phase 1, 2, 3, and 4 studies; (2) inpatient and outpatient facilities for testing vaccines in volunteers; (3) good clinical practice (GCP, the name given by pharmaceutical companies to the set of federal regulations and guidelines for conducting clinical trials designed to support an application for licensure of a biological or drug); (4) investigational new drug application (IND); (5) institutional review board (IRB); (6) product license application (PLA) and establishment license application (ELA). Laboratory-based investigators concerned with preclinical development should be familiar with these components of clinical development. The steps along the clinical development route leading to the use of a licensed vaccine by the public have been nicely summarized by Davenport (131).

11. Comparative Vaccine Adjuvant Trials

11.1. Animal Studies

Modern studies have compared up to 24 investigational adjuvants individually mixed with one antigen in a single protocol [reviewed by Edelman (132)]. The single protocol controls for confounding test variables, such as antigen, dose, schedule, animal species, and immunological assays. These variables make comparison between two or more separately conducted studies difficult, if not impossible. Concurrent study of several adjuvants formulated with the same antigen and administered by the same dose and schedule in the same animal are more informative. For example, comparative adjuvant studies in mice designed to test concurrently two human cancer antigens (133) or one Neisseria meningitides serogroup B multiple antigen peptide (134) have helped to identify several experimental adjuvants for inclusion in future human Phase I trials. When adjuvants provide equally good immunogenicity in such comparison trials, adjuvant choice may depend upon other factors. These include cost, commercial availability, reactogenicity, mode of action, and induction of the desired arm of the immune response.

Nevertheless, results of comparative trials may fail to identify the best adjuvant or adjuvants. For example, two comparative trials of simian immunodeficiency virus (SIV) vaccines combined with different adjuvants were conducted in macaques (128,135). The results were disappointing in that the mechanism of immunity could not be clearly delineated, and the large number of primates (80 and 98 animals) was still insufficient to allow meaningful statistical comparison of protection between all adjuvant groups.

11.2. Studies in Humans

To date, the largest number of comparative adjuvant trials in volunteers have focused on HIV vaccine candidates. Several Phase I and Phase II clinical trials have compared in the same study two or more adjuvants combined with HIV vaccines in healthy young adult volunteers (46,103,106,115,136–138). These trials illustrate the useful results that can be obtained from comparative adjuvanted vaccine trials using identical clinical protocols by two or more investigators. For example, in a Phase 1, double-blind, randomized, placebo-controlled trial in healthy adults, 50 μg of HIV gp120 was combined with one of seven adjuvants (106). The systemic side effects caused by these vaccine formulations and additional HIV vaccines using similar protocols (115) were discussed in **Subheading 6.2**. Each adjuvanted vaccine was injected into 15 persons at 0, 2, 6, and 18 mo. The adjuvants included aluminum hydroxide, MPL™, liposome-encapsulated MPL™ with aluminum, MF59, MF59/MTP-PE, SAF, and SAF/threonyl-MDP. The group that received SAF/threonyl-MDP was significantly more likely to experience moderate or severe local and systemic reactions compared to all other groups combined, but this group and the SAF/threonyl-MDP group developed the highest geometric mean HIV-1 neutralizing antibody titers. All adjuvant groups except MPL™ induced neutralizing antibody in 80% or more of volunteers after the third dose. The aluminum group had the lowest geometric mean antibody titers. CD8+ CTL responses were not measured (115). The results illustrate the common association of high reactogenicity and high adjuvanticity observed in many adjuvant trials.

All malaria vaccine candidates have been adjuvanted before Phase II trials. Adjuvanted peptides of the circumsporozoite protein (CSP) and several blood stage antigens of Plasmodium falciparum were employed in attempts to protect vaccinees against experimental or natural malaria challenge. Adjuvants used included aluminum (139–142), aluminum plus Pseudomonas aeruginosa detoxified toxin A carrier (143,144), aluminum plus fusion protein of HBsAg and MPL (37,145,146), fusion protein of HBsAg in a proprietary oil-in-water emulsion (37), aluminum plus liposomes and MPL (147), Detox™ (MPL, cell wall skeleton of mycobacteria, and squalane) (33), Montanide ISA 720 (sqalene, squalane, and a mannide mono-oleate emulsifier) (148), and recombinant vaccinia virus (23). Attempts to protect the majority of vaccinees were unsuccessful until Stout et al, using three adjuvant formulations

developed over a decade of trial and error, protected 6 (86%) of 7 volunteers with one of them (37). The successful formulation was composed of CSP fused to a HBsAg peptide and adjuvanted with an oil-in-water proprietary emulsion (SmithKline Beecham Biologicals) plus monophosphoryl lipid A (MPLA) and QS-21. The vaccine formulation was administered repeatedly at 0, 4, and 24–28 wk. The two less protective formulations were composed of CSP-HBsAg in the oil-in-water emulsion, and CSP-HBsAg in a formulation containing alum and MPLA. Of note, the protection was short-lived (145), and expanded trials using two or three spaced vaccinations of three different vaccine doses protected only 15 (48%) of 31 vaccinees (149). The results of these malaria trials demonstrated that strong, complex adjuvant formulations were required, that a protective adjuvant formulation cannot be deduced from animal studies, that the more robust adjuvants produced more severe local and systemic reactions, that antibody alone was insufficient to confer protection, and that suitable numbers of volunteers are necessary to achieve realistic estimates of protective efficacy. The most protective first-generation adjuvanted malaria vaccine was developed because U.S. Army investigators and SmithKline Beecham were committed in partnership to expend therequired time, money, and effort (149). Without such long-term committement, future vaccine development efforts against malaria or any other disease will not likely succeed.

References

1. Ramon, G. (1925) Sur l'augmentation anormale de l'antitoxine chez les chevaux producteurs de serum antidipherique. Bull. Soc. Centr. Med. Vet. **101,** 227–234.
2. Spriggs, D. R., and Koff, W. C. Topics in Vaccine Adjuvant Research. Boca Raton, FL.
3. Powell, M. F. and Newman, M. J. (1995) Vaccine Design: The Subunit and Adjuvant Approach. New York, NY: Plenum Press.
4. O'Hagan, D. T., .ed. (2000) Vaccine Adjuvants. Preparation Methods and Research Protocols. Humana Press, Totowa, NJ.
5. Edelman, R. (1980) Vaccine adjuvants. Rev. Infect. Dis. **2(3),** 370–383.
6. Gupta, R. K., Relyveld, E. H., Lindblad, E. B., Bizzini, B., Ben-Efraim, S., and Gupta, C. K. (1993) Adjuvants—a balance between toxicity and adjuvanticity. Vaccine **11(3),** 293–306.
7. Gupta, R. K. and Siber, G. R. (1995) Adjuvants for human vaccines—current status, problems and future prospects. Vaccine **13(14),**1263–1276.
8. Cooper, P. D. (1994) The selective induction of different immune responses by vaccine adjuvants, in Strategies in Vaccine Design (Ada, G. L., ed.), Landes, Austin, TX, pp. 125–158.
9. Hunter, R. L. and Lal, A. A. (1994) Copolymer adjuvants in malaria vaccine development. Am. J. Trop. Med. Hyg. **50(4 Suppl),** 52–58.
10. Lin, R., Tarr, P. E., and Jones, T. C. (1995) Present status of the use of cytokines as adjuvants with vaccines to protect against infectious diseases. Clin. Infect. Dis. **21(6),** 1439–1449.
11. Cox, J.C. and Coulter, A.R. (1997) Adjuvants—a classification and review of their modes of action. Vaccine **15**(3), 248–256.
12. O'Hagan, D. T. (1998) Recent advances in vaccine adjuvants for systemic and mucosal administration. J. Pharm. Pharmacol. **50(1),** 1–10.
13. Aguado, T., Engers, H., Pang, T., and Pink, R. (1999) Novel adjuvants currently in clinical testing November 2–4,1998, Fondation Merieux, Annecy, France: a meeting sponsored by the World Health Organization. Vaccine **17(19),** 2321–2328.
14. Aucouturier, J., Dupuis, L., and Ganne, V. (2001) Adjuvants designed for veterinary and human vaccines. Vaccine **19(17–19),** 2666–2672.
15. Douglas, R. G., Jr. (1993) The children's vaccine initiative—will it work? J. Infect. Dis. **168,** 269–274.
16. Wittet, S. (2000) Introducing GAVI and the Global Fund for Children's Vaccines. Vaccine **19(4-5),** 385–386.
17. Ramon, G. (1926) Procedes pour accroitre la production des antitoxines. Ann. Inst. Pasteur **40,** 1–10.
18. Audibert, F. M. and Lise, L. D. (1993) Adjuvants: current status, clinical perspectives and future prospects. Immunol Today **14(6),** 281–284.
19. Edelman, R. and Tacket, C. O. (1990) Adjuvants. Int. Rev. Immunol. **7(1),** 51–66.
20. Granoff, D. M., Holmes, S. J., Osterholm, M. T., et al. (1993) Induction of immunologic memory in infants primed with Haemophilus influenzae type b conjugate vaccines. J. Infect. Dis. **168(3),** 663–671.
21. Kayhty, H., Ahman, H., Ronnberg, P. R., Tillikainen, R., and Eskola, J. (1995) Pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine is immunogenic in infants and children. J. Infect. Dis. **172(5)**, 1273–1278.
22. Tacket, C. O., Losonsky, G., Lubeck, M. D., et al. (1992) Initial safety and immunogenicity studies of an oral recombinant adenohepatitis B vaccine. Vaccine **10(10),** 673–676.

23. Ockenhouse, C. F., Sun, P. F., Lanar, D. E., et al. (1998) Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for Plasmodium falciparum malaria. J. Infect. Dis. **177(6),** 1664–1673.
24. Stover, C. K., Bansal, G. P., Hanson, M. S., et al. (1993) Protective immunity elicited by recombinant bacille Calmette-Guerin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate Lyme disease vaccine. J. Exp. Med. **178(1),** 197–209.
25. Evans, T. G., Keefer, M. C., Weinhold, K. J., et al. (1999) A canarypox vaccine expressing multiple human immunodeficiency virus type 1 genes given alone or with rgp120 elicits broad and durable CD8+ cytotoxic T lymphocyte responses in seronegative volunteers. J. Infect. Dis. **180(2),** 290–298.
26. Gonzalez, C., Hone, D., Noriega, F. R., et al. (1994) Salmonella typhi vaccine strain CVD 908 expressing the circumsporozoite protein of Plasmodium falciparum: strain construction and safety and immunogenicity in humans. J. Infect. Dis. **169(4),** 927–931.
27. Edelman, R., Palmer, K., Russ, K. D., et al. (1998) Safety and immunogenicity of recombinant Bacille Calmette-Guerin (rBCG) expressing Borrelia burgdorferi outer surface protein A (OspA) lipoprotein in adult volunteers: A candidate Lyme disease Vaccine. Vaccine **17(7–8),** 904–914.
28. Wang, R., Doolan, D. L., Le, T. P., et al. (1998) Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science **282(5388),** 476–480.
29. MacGregor, R. R., Boyer, J. D., et al. (1998) First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response [In Process Citation]. J. Infect. Dis. **178(1),** 92–100.
30. Davenport, F. M. (1968) Seventeen years' experience with mineral oil adjuvant influenza virus vaccines. Ann. Allergy **26(6),** 288–292.
31. Podda, A. (2001) The adjuvanted influenza vaccines with novel adjuvants: experience with the MF59-adjuvanted vaccine. Vaccine **19(17-19),** 2673–2680..
32. Saul, A., Lawrence, G., Smillie, A., et al. (1999) Human phase I vaccine trials of 3 recombinant asexual stage malaria antigens with Montanide ISA720 adjuvant. Vaccine **17(23–24),** 3145–3159.
33. Hoffman, S. L., Edelman, R., Bryan, J. P., et al. (1994) Safety, immunogenicity, and efficacy of a malaria sporozoite vaccine administered with monophosphoryl lipid A, cell wall skeleton of mycobacteria, and squalane as adjuvant. Am. J. Trop. Med. Hyg. **51(5),** 603–612.
34. Fries, L. F., Gordon, D. M., Richards, R. L., et al. (1992) Liposomal malaria vaccine in humans: a safe and potent adjuvant strategy. Proc. Natl. Acad. Sci. USA **89(1),** 358–362.
35. Lidgate, D. M. and Byars, N. E. (1995) Development of an emulsion-based muramyl dipeptide adjuvant formulation for vaccines, in Vaccine Design: The Subunit and Adjuvant Approach (Powell, M. F. and Newman, M. J., ed.), New York, NY, Plenum, 313–324.
36. Wintsch, J., Chaignat, C. L., Braun, D. G., et al. (1991) Safety and immunogenicity of a genetically engineered human immunodeficiency virus vaccine. J. Infect. Dis. **163(2),** 219–225.
37. Stoute, J. A., Slaoui, M., Heppner, D. G., et al. (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group [see comments]. N. Engl. J. Med. **336(2),** 86–91.
38. Lesourd, B. M., Vincent-Falquet, J. C., Deslandes, D., Musset, M., and Moulias, R. (1988) Influenza vaccination in the elderly: improved antibody response with Imuthiol (Na diethyldithiocarbamate) adjuvant therapy. Int. J. Immunopharmacol. **10(2),** 135–143.
39. Gravenstein, S., Duthie, E. H., Miller, B. A., et al. (1989) Augmentation of influenza antibody response in elderly men by thymosin alpha one. A double-blind placebo-controlled clinical study. J. Am. Geriatr. Soc. **37(1),** 1–8.
40. Goodman, M. G. (1995) A new approach to vaccine adjuvants: Immunopotentiation by intracellular T-helper-like signals transmitted by loxoribine, in Vaccine Design: The Subunit and Adjuvant Approach (Powell, M. F., Newman, M. J., eds.), Plenum, New York, 581–610.
41. Hess, G., Kreiter, F., Kosters, W., and Deusch, K. (1996) The effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on hepatitis B vaccination in haemodialysis patients. J. Viral Hepat. **3(3),** 149–153.
42. Drabick, J. J., Tang, D. B., Moran, E. E., Trofa, A. F., Foster, J. S., and Zollinger, W. D. (1997) A randomized, placebo-controlled study of oral cimetidine as an immunopotentiator of parenteral immunization with a group B meningococcal vaccine. Vaccine **15(10),** 1144–1148.
43. Evans, T. G., Judd, M. E., Dowell, T., Poe, S., Daynes, R. A., and Araneo, B. A. (1996) The use of oral dehydroepiandrosterone sulfate as an adjuvant in tetanus and influenza vaccination of the elderly. Vaccine **14(16),** 1531–1537.
44. Levine, M. M. and Dougan, G. (1998) Optimism over vaccines administered via mucosal surfaces. Lancet **351(9113),** 1375–1376.
45. Tacket, C. O., Reid, R. H., Boedeker, E. C., et al. (1994) Enteral immunization and challenge of volunteers given enterotoxigenic E. coli CFA/II encapsulated in biodegradable microspheres. Vaccine **12(14),** 1270–1274.

46. Lambert, J. S., Keefer, M., Mulligan, M. J., et al. (2001) A Phase I safety and immunogenicity trial of UBI(R) microparticulate monovalent HIV-1 MN oral peptide immunogen with parenteral boost in HIV- 1 seronegative human subjects. Vaccine **19**(23–24), 3033–3042.
47. Lowell, G. H. (1997) Proteosomes for Improved Nasal, Oral, or Injectable Vaccines, in New Generation Vaccines, (Levine, M. M., Woodrow, G. C., Kaper, J. B., and Cobon, G. S., ed.), Marcel Dekker, New York, Inc., 193–206.
48. Fries, L. F., Montemarano, A. D., Mallett, C. P., Taylor, D. N., Hale, T. L., and Lowell, G. H. (2001) Safety and Immunogenicity of a Proteosome-Shigella flexneri 2a Lipopolysaccharide Vaccine Administered Intranasally to Healthy Adults. Infect. Immun. **69(7),** 4545–4553.
49. Chaicumpa, W., Chongsa-nguan, M., Kalambaheti, T., et al. (1998) Immunogenicity of liposome-associated and refined antigen oral cholera vaccines in Thai volunteers. Vaccine **16**(7), 678–684.
50. Moldoveanu, Z., Love-Homan, L., Huang, W. Q., and Krieg, A. M. (1998) CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus Vaccine **16(11–12),** 1216–1224.
51. Gould-Fogerite, S., Edghill-Smith, Y., Kheiri, M., et al. (1994) Lipid matrix-based subunit vaccines: a structure-function approach to oral and parenteral immunization. AIDS Res. Hum. Retroviruses **10**(Suppl 2), S99–103:S99–103.
52. Cryz, S. J., Jr. and Gluck, R. (1998) Immunopotentiating reconstituted influenza virosomes as a novel antigen delivery system. Dev. Biol. Stand. **92,** 219–23:219–223.
53. Spangler, B. D. (1992) Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. Microbiol. Rev. **56(4),** 622–647.
54. Holmgren, J., Czerkinsky, C., Lycke, N., and Svennerholm, A. M. (1994) Strategies for the induction of immune responses at mucosal surfaces making use of cholera toxin B subunit as immunogen, carrier, and adjuvant. Am. J. Trop. Med. Hyg. **50(5 Suppl),** 42–54.
55. Russell, M. W., Moldoveanu, Z., White, P. L., Sibert, G. J., Mestecky, J., and Michalek, S. M. (1996) Salivary, nasal, genital, and systemic antibody responses in monkeys immunized intranasally with a bacterial protein antigen and the Cholera toxin B subunit. Infect. Immun. **64(4),** 1272–1283.
56. Saldinger, P. F., Blum, A. L., and Corthesy-Theulaz, I. E. (1997) Perspectives of anti-H. pylori vaccination. J. Physiol. Pharmacol. **48** Suppl 4:59-65:59-65.
57. Rudin, A., Riise, G. C., and Holmgren, J. (1999) Antibody responses in the lower respiratory tract and male urogenital tract in humans after nasal and oral vaccination with cholera toxin B subunit. Infect. Immun. **67(6),** 2884–2890.
58. Michetti, P., Kreiss, C., Kotloff, K. L., et al. (1999) Oral immunization with urease and Escherichia coli heat-labile enterotoxin is safe and immunogenic in Helicobacter pylori-infected adults. Gastroenterology **116(4),** 804–812.
59. Hall, E. R., Wierzba, T. F., Ahren, C., et al. (2001) Induction of systemic antifimbria and antitoxin antibody responses in Egyptian children and adults by an oral, killed enterotoxigenic Escherichia coli plus cholera toxin B subunit vaccine. Infect. Immun. **69(5),** 2853–2857.
60. Kotloff, K. L., Sztein, M. B., Wasserman, S. S., Losonsky, G. A., DiLorenzo, S. C., and Walker, R. I. (2001) Safety and immunogenicity of oral inactivated whole-cell Helicobacter pylori vaccine with adjuvant among volunteers with or without subclinical infection. Infect. Immun. **69(6),** 3581–3590.
61. Douce, G., Turcotte, C., Cropley, I., et al. (1995) Mutants of Escherichia coli heat-labile toxin lacking ADP- ribosyltransferase activity act as nontoxic, mucosal adjuvants. Proc. Natl. Acad. Sci. USA **92(5),** 1644–1648.
62. Douce, G., Fontana, M., Pizza, M., Rappuoli, R., and Dougan, G. (1997) Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin. Infect. Immun. **65(7),** 2821–2828.
63. Simmons, C.P., Mastroeni, P., Fowler, R., et al. (1999) MHC class I-restricted cytotoxic lymphocyte responses induced by enterotoxin-based mucosal adjuvants. J. Immunol. **163(12),** 6502–6510.
64. Bowman, C. C. and Clements, J. D. (2001) Differential biological and adjuvant activities of cholera toxin and Escherichia coli heat-labile enterotoxin hybrids. Infect. Immun. **69(3),** 1528–1535.
65. Bourguignon, P., Bisteau, M., Veenstra, S., et al. (2001) Reactogenicity and passage into the brains of enterotoxins and cpg-oligonucleotides administered intranasally to mice. Final Program and Abstracts of The Fourth Annual Conference on Vaccine Research, April 23–25, 2001, Hyatt Regency Crystal City Hotel, Arlington, VA.
66. Tacket, C. O., Kelly, S. M., Schodel, F., et al. (1997) Safety and immunogenicity in humans of an attenuated Salmonella typhi vaccine vector strain expressing plasmid-encoded hepatitis B antigens stabilized by the Asd-balanced lethal vector system [published erratum appears in Infect. Immun. Sep;65(9), 3981]. Infect. Immun. **65(8),** 3381–3385.
67. Tacket, C. O., Galen, J., Sztein, M. B., et al. (2000) Safety and immune responses to attenuated Salmonella enterica serovar typhi oral live vector vaccines expressing tetanus toxin fragment C. Clin. Immunol. **97(2),** 146–153.

68. Tacket, C. O., Mason, H. S., Losonsky, G., Clements, J. D., Levine, M. M., and Arntzen, C. J. (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nature **4,** 607–609.
69. Tacket, C. O., Mason, H. S., Losonsky, G., Estes, M. K., Levine, M. M., and Arntzen, C. J. (2000) Human immune responses to a novel norwalk virus vaccine delivered in transgenic potatoes. J. Infect. Dis. **182(1),** 302–305.
70. Glenn, G. M., Rao, M., Matyas, G. R., and Alving, C. R. (1998) Skin immunization made possible by cholera toxin [letter]. Nature **391**(6670), 851.
71. Glenn, G. M., Taylor, D. N., Li, X., Frankel, S., Montemarano, A., and Alving, C. R. (2000) Transcutaneous immunization: a human vaccine delivery strategy using a patch. Nat. Med. **6(12),** 1403–1406.
72. Scharton-Kersten, T., Yu, J., Vassell, R., O'Hagan, D., Alving, C. R., and Glenn, G. M . (2000) Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants. Infect. Immun. **68(9),** 5306–5313.
73. Zierhut, M., Bieber, T., Brocker, E. B., Forrester, J. V., Foster, C.S., and Streilein, J.W. (1996) Immunology of the skin and the eye. Immunol. Today **17(10),** 448–450.
74. Tacket, C. O., Roy, M. J., Widera, G., Swain, W. F., Broome, S., and Edelman, R. (1999) Phase 1 safety and immune response studies of a DNA vaccine encoding hepatitis B surface antigen delivered by a gene delivery device. Vaccine **17(22),** 2826–2829.
75. Enioutina, E. Y., Visic, D., and Daynes, R. A. (2000) The induction of systemic and mucosal immune responses to antigen- adjuvant compositions administered into the skin: alterations in the migratory properties of dendritic cells appears to be important for stimulating mucosal immunity. Vaccine **18(24),** 2753–2767.
76. Glenn, G. M., Scharton-Kersten, T., Vassell, R., Matyas, G. R., Alving, C. R. (1999) Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants. Infect. Immun. **67(3),** 1100–1106.
77. Guerna, F., Taylor, D. N., Hall, E. R., et al. (2001) Safety and immunogenicity of a transcutaneously administered prototype traveler's diarrhea vaccine consisting of heat-labile toxin (LT) and CS6 colonization factor of enterotoxigenic Escherichia coli (ETEC). Final Program and Abstracts of The Fourth Annual Conference on Vaccine Research, April 23–25, 2001, Hyatt Regency Crystal City Hotel, Arlington, VA.
78. Krieg, A. M. (1996) An innate immune defense mechanism based on the recognition of CpG motifs in microbial DNA. J. Lab. Clin. Med. **128(2),** 128–133.
79. Newman, M. J. and Powell, M. F. (1995) Immunological and formulation design consideratins for subunit vaccines. In Vaccine Design: The Subunit and Adjuvant Approach (Powell M. F. and Newman, M. J., eds.), Plenum, New York, pp. 1–42.
80. Newman, M. J., Wu, J. Y., Gardner, B. H., et al. (1992) Saponin adjuvant induction of ovalbumin-specific CD8+ cytotoxic T lymphocyte responses. J. Immunol. **148(8),** 2357–2362.
81. Newman, M. J., Munroe, K. J., Anderson, C. A., et al. (1994) Induction of antigen-specific killer T lymphocyte responses using subunit SIVmac251 gag and env vaccines containing QS-21 saponin adjuvant. AIDS Res. Hum. Retroviruses **10(7),** 853–861.
82. Ulanova, M., Tarkowski, A., Hahn-Zoric, M., and Hanson, L. A. (2001) The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infect. Immun. **69(2),** 1151–1159.
83. Yip, H. C., Karulin, A. Y., Tary-Lehmann, M., et al. (1999) Adjuvant-guided type-1 and type-2 immunity: infectious/noninfectious dichotomy defines the class of response. J. Immunol. **162(7),** 3942–3949.
84. Gupta, R. K. and Siber, G. R. (1994) Comparison of adjuvant activities of aluminium phosphate, calcium phosphate and stearyl tyrosine for tetanus toxoid. Biologicals **22(1),** 53–63.
85. Kensil, C. R., Wu, J. Y., and Soltysik, S. (1995) Structural and immunological characterization of the vaccine adjuvant QS-21. Pharm. Biotechnol. **6,** 525–541.
86. Ulrich, J. T. and Myers, K. R. (1995) Monophosphoryl lipid A as an adjuvant: Past experiences and new directions, in The Subunit and Adjuvant Approach (Powell, M. F. and Newman, M. J., ed.). Plenum, New York, pp. 495–524.
87. Hui, G. S., Chang, S. P., Gibson, H., et al. (1991) Influence of adjuvants on the antibody specificity to the Plasmodium falciparum major merozoite surface protein, gp195. J. Immunol. **147(11),** 3935–3941.
88. Kenney, J. S., Hughes, B. W., Masada, M. P., and Allison, A. C. (1989) Influence of adjuvants on the quantity, affinity, isotype and epitope specificity of murine antibodies. J. Immunol. Methods **121(2),** 157–166.
89. Tam, J. P. (1988) Synthetic peptide vaccine design: synthesis and properties of a high- density multiple antigenic peptide system. Proc. Natl. Acad. Sci. USA **85(15),** 5409–5413.
90. Nardin, E. H., Oliveira, G. A., Calvo-Calle, J. M., et al. (2000) Synthetic malaria peptide vaccine elicits high levels of antibodies in vaccinees of defined HLA genotypes. J. Infect. Dis. **182(5),** 1486–1496.
91. Goldenthal, K. L., Cavagnaro, J. A., Alving, C. R., and Vogel, F.R. (1993) NCVDG working groups: Safety evaluation of vaccine adjuvants: National cooperative vaccine development meeting working group. AIDS Res. Hum. Retrovirus **9,** S47–S51.

92. Bussiere, J. L., McCormick, G. C., and Green, J. D. (1995) Preclinical safety assessment considerations in vaccine development, in Vaccine Design: The Subunit and Adjuvant Approach (Powell, M.F. and Newman, M.J., ed.). Plenum, New York, NY, pp. 61–79.
93. Kaji, M., Kaji, Y., Kaji, M., et al. (1992) Phase 1 clinical tests of influenza MDP-virosome vaccine (KD-5382). Vaccine **10(10),** 663–667.
94. Jacobson, R. M., Swan, A., Adegbenro, A., Ludington, S. L., Wollan, P. C., and Poland, G. A. (2001) Making vaccines more acceptable—methods to prevent and minimize pain and other common adverse events associated with vaccines. Vaccine **19(17-19),** 2418–2427.
95. Schultz, N., Oratz, R., Chen, D., Zeleniuch-Jacquotte, A. Abeles, G., and Bystryn, J. C. (1995) Effect of DETOX as an adjuvant for melanoma vaccine. Vaccine 13(5), 503–508.
96. Keitel, W., Couch, R., Bond, N., Adair, S., Van Nest, G., and Dekker, C. (1993) Pilot evaluation of influenza virus vaccine (IVV) combined with adjuvant. Vaccine **11(9),** 909–913.
97. Duarte Cano, C. A. (1999) The multi-epitope polypeptide approach in HIV-1 vaccine development. Genetic Analysis:Biomolecular Engineering **15,** 149–153.
98. Brewer, M. A., Edwards, K. M., Palmer, P. S., and Hinson, H. P. (1994) Bacille Calmette-Guerin immunization in normal healthy adults. J. Infect. Dis. **170(2),** 476–479.
99. Kemp, E. B., Belshe, R. B., and Hoft, D. F. (1996) Immune responses stimulated by percutaneous and intradermal bacille Calmette-Guerin. (J. Infect. Dis.) **174(1),** 113–119.
100. Rinn, K., Schiffman, K., Otero, H. O., and Disis, M. L. (1999) Antigen-specific recall urticaria to a peptide-based vaccine. J. Allergy Clin. Immunol. **104(1),** 240–242.
101. Gordon, D. M., Duffy, P. E., Heppner, D. G., et al. Phase I safety and immunogenicity testing of clinical lots of the synthetic Plasmodium falciparum vaccine SPf66 produced under good manufacturing procedure conditions in the United States. Am. J. Trop. Med. Hyg. (1996) **55(1),** 63–68.
102. Amador, R., Moreno, A., Murillo, L. A., et al. (1992) Safety and immunogenicity of the synthetic malaria vaccine SPf66 in a large field trial. J. Infect. Dis. **166(1),** 139–144.
103. Evans, T. G., McElrath, M. J., Matthews, T., et al. (2001) QS-21 promotes an adjuvant effect allowing for reduced antigen dose during HIV-1 envelope subunit immunization in humans. Vaccine **19(15–16),** 2080–2091.
104. Waters, R. V., Terrell, T. G., Jones, G. H. (1986) Uveitis induction in the rabbit by muramyl dipeptides. Infect. Immun. **51(3),** 816–825.
105. Allison, A. C. and Byars, N. E. (1991) Immunological adjuvants: desirable properties and side-effects. Mol. Immunol. **28(3),** 279–284.
106. McElrath, M. J. (1994) Adjuvant effects on human immune responses in recipients of candidate HIV vaccines. IBC Conference, "Novel Vacccine Stategies for Mucosal Immunization, Genetic Approaches and Adjuvants," Rockville, MD, pp. 24–26.
107. Pearson, C. M. (1963) Experimental joint disease: Observations on adjuvant-induced arthritis. J. Chronic. Dis. **16,** 863–874.
108. Kleinau, S., Erlandsson, H., Holmdahl, R., and Klareskog, L. (1991) Adjuvant oils induce arthritis in the DA rat. I. Characterization of the disease and evidence for an immunological involvement. J. Autoimmun. **4(6),** 871–880.
109. Murray, R., Cohen, P., and Hardegree, M. C. (1972) Mineral oil adjuvants: biological and chemical studies. Ann. Allergy **30(3),** 146-151.
110. Salk, J. and Salk, D. (1977) Control of influenza and poliomyelitis with killed virus vaccines. Science **195(4281),** 834–847.
111. Stuart-Harris, C. H. (1969) Adjuvant influenza vaccines. Bull WHO **41,** 617–621.
112. Beebe, G. W., Simon, A. H., and Vivona, S. (1972) Long-term mortality follow-up of Army recruits who received adjuvant influenza virus vaccine in 1951-1953. Am. J. Epidemiol. **95(4),** 337–346.
113. Page, W. F., Norman, J. E., and Benenson, A. S. (1993) Long-Term Follow-up of Army Recruits Immunized with Freund's Incomplete Adjuvanted Vaccine. Vaccine Res. **2(3),** 141–149.
114. Jacobson, R. M., Adegbenro, A., Pankratz, V. S., and Poland, G. A. (2001) Adverse events and vaccination-the lack of power and predictability of infrequent events in pre-licensure study. Vaccine **19(17–19),** 2428–2433.
115. Keefer, M. C., Wolff, M., Gorse, G. J., et al. (1997) Safety profile of phase I and II preventive HIV type 1 envelope vaccination: experience of the NIAID AIDS Vaccine Evaluation Group. AIDS Res. Hum. Retroviruses **13(14),** 1163–1177.
116. Butler, N. R., Feng, S., Benson, P. F., et al. (1962) Response of infants to pertussis vaccine at one week and to poliomyelitis, diptheria, and tetanus vaccine at six months. Lancet **2,** 112–114.
117. Cvjetanovic, B. and Uemura, K. (1965) The present status of field and laboratory studies of typhoid and paratyphoid vaccines with special reference to studies sponsored by the World Health Organization. Bull WHO **32,** 29–36.
118. Woolridge, R. L., Grayston, J. T., Chang, I. A., et al. (1967) Long-term follow-up of the initial (1959-1960) trachoma vaccine field on Taiwan. Am. J. Ophthalmol. **63,** 1650–1653.
119. Kasel, J. A., Couch, R. B., Douglas, R. G., Jr. (1971) Antigenicity of alum and aqueous adenovirus hexon antigen vaccines in man. J. Immunol. **107(3),** 916–919.
120. Davenport F. M., Hennessy, A. V., and Askin, F. B. (1968) Lack of adjuvant effect of AIPO4 on purified

influenza virus haemagglutinins in man. J. Immunol. **100,** 1139–1140.
121. Claesson, B. A., Trollfors, B., Lagergard, T., (1988) Clinical and immunologic responses to the capsular polysaccharide of Haemophilus influenzae type b alone or conjugated to tetanus toxoid in 8- to 23-month-old children. J. Pediatr. **112(5),** 695–702.
122. O'Hagan, D. T., Jeffery, H., Davis, S. S. (1993) Long-term antibody responses in mice following subcutaneous immunization with ovalbumin entrapped in biodegradable microparticles. Vaccine **11(9),** 965–969.
123. Gupta, R. K., Rost, B. E., Relyveld, E., and Siber, G. R. (1995) Adjuvant properties of aluminum and calcium compounds, in Vaccine Design: The Subunit and Adjuvant Approach (Powell, M. F. and Newman, M. J., ed.), Plenum, New York, pp. 229–248.
124. World Health Organization. (1977) Manual for the Production and Control of Vaccines – Tetanus Toxoid. World Health Organization.
125. Bumford, R. (1989) Aluminum salts: prospectives in their use as adjuvants, in Immunological Adjuvants and Vaccines (Gregoriadis, G., Allison, A.C., Poste, G., ed.) Plenum, New York, pp. 35–41.
126. Stewart-Tull, D. E. S. (1989) Recommendations for the assessment of adjuvants (immunopotentiators), in Immunological Adjuvants and Vaccines (Gregoriadis, G., Allison, A.C., and Poste, G., ed.). Plenum, New York, pp. 213–226.
127. Hardegree, M. C., Pittman, M.,and Maloney, C. J. (1972) Influence of mouse strain on the assayed potency (unitage) of tetanus toxoid. Appl. Microbiol. **24(1),** 120–126.
128. Murphey-Corb, M., Ohkawa, S., Martin, L., et al. (1993) Comparative efficacy of a whole killed SIV vaccine in combination with various adjuvants. Sixth Annual Meeting of the National Cooperative Vaccine Development Group for AIDS.
129. Tacket, C. O., Rennels, M. B., and Mattheis, M. J. (1997) Initial clinical evaluation of new vaccine candidates: Phase 1 and 2 clinical trials of safety, immunogenicity, and preliminary efficacy, in new generation vaccines (Levine, M. M., Woodrow, G. C., Kaper, J. B., and Cobon, G., eds.), Marcel Dekker, New York, 35–45.
130. U.S.Food and Drug Administration. (1991) General biological products standards. Anonymous code of federal regulations. Department of Health and Human Services, Bethesda, MD, pp. 43–53.
131. Davenport, L. W. (1995) Regulatory considerations in vaccine design, in Vaccine Design, in The Subunit and Adjuvant Approach (Powell, M. F. and Newman, M. J., eds.), Plenum, New York, pp. 81–96.
132. Edelman, R. (1997) Adjuvants for the Future, in New Generation Vaccines (Levine, M. M., Woodrow, G. C., Kaper, J.B., and Cobon, G.S., ed.). Marcel Dekker, New York, pp. 173–192.
133. Kim, S. K., Ragupathi, G., Musselli, C., Choi, S. J., Park, Y.S., and Livingston, P.O. (1999) Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines. Vaccine **18(7–8),** 597–603.
134. Christodoulides, M., Rattue, E., and Heckels, J. E. (1999) Effect of adjuvant composition on immune response to a multiple antigen peptide (MAP) containing a protective epitope from Neisseria meningitidis class 1 porin. Vaccine **18(1–2),** 131–139.
135. Hunsmann, G. (1995) Protection of macaques against simian immunodeficiency virus infection with inactivated vaccines: comparison of adjuvants, doses and challenge viruses. Vaccine **13,** 295–300.
136. Keefer, M. C., Graham, B. S., and McElrath, M. J. (1996) Safety and immunogenicity of Env 2-3, a human immunodeficiency virus type 1 candidate vaccine, in combination with a novel adjuvant, MTP- PE/MF59. NIAID AIDS Vaccine Evaluation Group. AIDS Res. Hum. Retroviruses **12(8),** 683–693.
137. Graham, B. S., Keefer, M. C., McElrath, M. J., et al. (1996) (2000) Safety and immunogenicity of a candidate HIV-1 vaccine in healthy adults: recombinant glycoprotein (rgp) 120. A randomized, double-blind trial. NIAID AIDS Vaccine Evaluation Group. Ann. Intern. Med. **125(4),** 270–279.
138. McCormack, S., Tilzey, A., Carmichael, A., et al. A phase I trial in HIV negative healthy volunteers evaluating the effect of potent adjuvants on immunogenicity of a recombinant gp120W61D derived from dual tropic R5X4 HIV-1ACH320. Vaccine 18(13), 1166–1177.
139. Ballou, W. R., Hoffman, S. L., Sherwood, J. A., et al. (1987) Safety and efficacy of a recombinant DNA Plasmodium falciparum sporozoite vaccine. Lancet **1(8545),** 1277–1281.
140. Herrington, D. A., Clyde, D. F., Losonsky, G., et al. (1987) Safety and immunogenicity in man of a synthetic peptide malaria vaccine against Plasmodium falciparum sporozoites. Nature **328(6127),** 257–259.
141. Patarroyo, M. E., Amador, R., Clavijo, P., et al. (1988) A synthetic vaccine protects humans against challenge with asexual blood stages of Plasmodium falciparum malaria. Nature **332**(6160), 158–161.
142. Alonso, P. L., Smith, T., Schellenberg, J. R., et al. (1994) Randomised trial of efficacy of SPf66 vaccine against Plasmodium falciparum malaria in children in southern Tanzania [see comments]. Lancet **344(8931),** 1175–1181.
143. Fries, L. F., Gordon, D. M., Schneider, I., et al. (1992) Safety, immunogenicity, and efficacy of a Plasmodium falciparum vaccine comprising a circumsporozoite protein repeat region peptide conjugated to Pseudomonas aeruginosa toxin A. Infect. Immun. **60(5),** 1834–1839.

144. Brown, A. E., Singharaj, P., Webster, H. K., et al. (1994) Safety, immunogenicity and limited efficacy study of a recombinant Plasmodium falciparum circumsporozoite vaccine in Thai soldiers. Vaccine **12**(2), 102–108.
145. Stoute, J. A., Kester, K. E., Krzych, U., et al. (1998) Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine. J. Infect. Dis. **178(4),** 1139–1144.
146. Gordon, D. M., McGovern, T. W., Krzych, U., et al. (1995) Safety, immunogenicity, and efficacy of a recombinantly produced Plasmodium falciparum circumsporozoite protein-hepatitis B surface antigen subunit vaccine. J. Infect. Dis. **171(6),** 1576–1585.
147. Heppner, D. G., Gordon, D. M., Gross, M., et al. (1996) Safety, immunogenicity, and efficacy of Plasmodium falciparum repeatless circumsporozoite protein vaccine encapsulated in liposomes. J. Infect. Dis. **174(2),** 361–366.
148. Lawrence, G., Cheng, Q. Q., Reed, C., et al. (2000) Effect of vaccination with 3 recombinant asexual-stage malaria antigens on initial growth rates of Plasmodium falciparum in non-immune volunteers. Vaccine **18(18),** 1925–1931.
149. Kester, K. E., McKinney, D. A., Tornieporth, N., et al. (2001) Efficacy of Recombinant Circumsporozoite Protein Vaccine Regimens against Experimental Plasmodium falciparum Malaria. J. Infect. Dis. **183(4),** 640–647.