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DEVELOPMENT OF VACCINE ADJUVANTS: A HISTORICAL PERSPECTIVE

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1.1 INTRODUCTION

Since the earliest attempts to raise significant immune responses against nonliving agents, investigators have tried to identify useful additives that can be combined with antigens to enhance immune responses. Such immune-enhancing additives are known as *adjuvants*. Virtually all adjuvant systems developed to date have focused on one of two mechanisms: specific immune activation or the delivery–depot effect. Although many adjuvant systems have been developed and tested in preclinical models, few have actually proved useful for human vaccines. The primary limitations for the use of new adjuvant systems with human vaccines revolve around safety issues. Whereas the toxicity of adjuvants has been reduced systematically through research and development efforts over the last 80 years, the safety barriers presented by regulatory and liability issues have continued to increase. Adjuvants to be used with prophylactic vaccines in normal, healthy populations need to have virtually pristine safety profiles. The fact that most vaccines today are given to infants or children heightens the safety concerns of vaccine adjuvants.

In this chapter we review the history of vaccine adjuvant development from the beginning studies of the early twentieth century through to the present day. We recognize four periods of adjuvant development: (1) the initial

development of adjuvants for toxoid vaccines from the 1920s to the 1940s, (2) the broadened use of oils and aluminum adjuvants from the 1940s to the 1970s, (3) the development of synthetic adjuvants and second-generation delivery–depot systems from the 1970s to the 1990s, and (4) the development of rational receptor-associated adjuvants that active the innate immune system from the 1990s until the present day. We provide perspectives in the areas of work in preclinical systems, clinical evaluation and the use of adjuvants, and the interplay between immunology and adjuvant development in each of these periods.

1.2   INITIAL DEVELOPMENT OF ADJUVANTS FOR TOXOID VACCINES: 1920s–1940s

Some of the earliest studies leading to the development of adjuvants for active vaccines involved live [1] or killed bacterial vaccines in which the antigen and immune-stimulating agents were both provided by the bacteria [2,3]. Protection against diphtheria by passive transfer of horse antidiphtheria antiseria was a Nobel Prize–winning advance by von Behring [4]. The concept of an active subunit vaccine was first demonstrated in 1907 by Smith, who demonstrated that administration of toxin/antitoxin in immunoprecipitating ratios could provide protection, and von Behring used this approach in people with some success in the period 1910–1920 [4]. Addition of oil or lanolin with killed salmonella is the first documented study with a delivery–depot substance used with a killed bacterial vaccine [5]. Adjuvant research began in earnest with the development of diphtheria subunit toxoid [6] vaccines due to the weak immunogenicity observed with these vaccines [7–9]. As noted by Freund: "Interest in promoting antibody formation by addition of unrelated substances to antigens has never been lacking" [10]. Substances such as agar, tapioca, lecithin starch oil, saponin, salts of calcium and magnesium, killed *Salmonella typhi*, and even bread crumbs were tested [6,11,12].

The most significant vaccine adjuvants to be developed are the aluminum salt adjuvants: generically, but not correctly, referred to as *alums*. The first alum-adjuvanted vaccine was formulated by coprecipitation of diphtheria toxoid dissolved in carbonate buffer (pH 8.0) with aluminum (a purification trick), resulting in a coprecipitate of aluminum hydroxide and diphtheria toxoid [13,14]. The alum adjuvant was developed on the basis of faster and higher antitoxoid antibody responses in guinea pigs. The results of human trials with diphtheria toxoid precipitated with alum were published as early as 1934 [15]. Coprecipitated alum–toxoid nearly eradicated diphtheria in Canada in the 1920s and 1930s. Successful trials with tetanus toxoid were completed in the same time frame [16]. However, some early alum formulations showed poor reproducibility, and results of failed clinical trials were also published by Volk [17]. The alternative approach of adsorbing antigen to the surface of "naked" alum particles was demonstrated as early as 1931 [18] and later came

into common use. Only occasional and moderate toxicities were reported with these early alum–toxoid vaccines. The levels of toxicity seen were deemed acceptable given the dramatic decreases in diphtheria and tetanus disease resulting from use of the vaccines.

While the low-toxicity depot approach with alum went forward in clinical applications, efforts were made to generate more potent vaccines using several approaches. One such approach was the use of toxin–antitoxin mixtures [19]. Another approach involved work with tuberculosis (TB) vaccines which demonstrated that the inflammation induced by TB could enhance immune responses to other antigens. As early as 1924, Lewis noted that intraperitoneal injection of live TB a few days before immunization with a variety of antigens dramatically increased antibody responses to those antigens [20]. Presentation of antigen at inflammatory TB foci resulted in elevated antibody titers [21]. These observations pushed forward the immunostimulatory adjuvant approach, which in the 1930s meant the generation of inflammation.

The next advance in adjuvant development involved the combination of killed tubercle with oils. Initial combinations of killed tubercle with paraffin oil produced sensitization to TB but no increased protection from disease [22,23]. Freund demonstrated similar increased antibody responses using live TB with oils. Freund made two jumps in the technology in the 1930s with the substitution of killed TB for live TB and the use of a water-in-oil emulsion [24], inspired by repository formulation techniques being used at the time [10]. The water-in-oil emulsion was formed by the mixture of one volume of 10% Arlacel A (mannide monooleate) and 90% mineral oil with one volume of antigen solution. This system became the standard for adjuvant activity when Freund demonstrated that the emulsion without killed TB was almost as potent as the emulsion with killed TB when used as an adjuvant with diphtheria toxoid and far exceeded the potency of an alum–toxoid formulation [10]. These emulsions went on to become the standard potent adjuvant systems used in preclinical settings and became known as *complete Freund's adjuvant* (CFA, with killed TB) and *incomplete Freund's adjuvant* (IFA, without TB). The emulsion adjuvant was shown to have activity with a variety of antigens, including those from Japanese encephalitis and influenza virus being developed in the same period [25,26]. Water-in-oil emulsion without TB was tested in early human trials with influenza vaccine and demonstrated faster and higher antibody responses than those of vaccine alone [27].

By the mid-1940s, two major adjuvant systems had emerged: the low-reactogenic, modestly effective, and difficult-to-reproduce alum systems, and the new, more potent water-in-oil emulsion systems. It was postulated that alum worked by means of a slow-release depot system [14]. Freund attributed the activity of the water-in-oil emulsion in some part to extended antigen presentation [10]. In this era, adjuvant discovery scientists appeared to be closely involved with immunologists of the day, with adjuvant mechanisms contributing to immunological theory.

1.3 BROADENED USE OF ALUMINUM AND OIL ADJUVANTS: 1940s–1970s

Although the definition of any real scientific boundary in adjuvant development in the mid-1940s is somewhat artificial, the period before that time was largely characterized by initial formulation of alum and Freund's systems, while the period from the 1940s through the 1970s can be characterized by extensive efforts to develop these systems for safe, reproducible use in human vaccines and realization of the limitations of their use.

One of the first alum precipitate vaccines [diphtheria, tetanus, pertussis (DTP)] was licensed in 1948 [28], just as the first report of diphtheria vaccine adsorbed to aluminum phosphate was published showing a more controllable composition [29]. A number of studies on the use of alum with pertussis vaccines reported varying success [30–33]. Variability of the potency of the alum-adjuvanted pertussis vaccines seemed to be a common problem. It was demonstrated that alum provided increased antibody titers [34], but another study showed that alum provided no advantage in protection [35]. Whereas the results with alum-adjuvanted pertussis were variable, results with alum-adjvuanted DPT vaccines were more consistent and favorable. It has been suggested that the pertussis component of these combination vaccines actually served as an adjuvant for the diphtheria and tetanus components [31,32]. Alum has continued as the nearly universal adjuvant for DPT vaccines.

Several limitations of alum were becoming clear with continued human use. Alum was observed not to be useful in boosting immunizations with diphtheria and tetanus antigens [36] or influenza hemagglutinin (HA) [37]. Granulomas were often observed at the injection site [38–40]. Occasional erythema was observed [31,32] as well as increases in IgE [41,42]. By the early 1980s, aluminum adjuvants were a major part of human vaccines, but the limited potency, lack of biodegradability, and IgE responses left room for other approaches.

Development of the oil adjuvants continued in the same time period. Freund was demonstrating the wide range of potency of water-in-oil emulsions in the 1940s and 1950s. Use of the original oil formulations containing the commonly available mineral oil Drakeol and the surfactant Arlacel A continued due to the conclusion that nonmetabolizable oil was required for full activity [43]. The use of Freund's adjuvant proceeded in several directions. A number of basic studies (utilizing both IFA and CFA) defined the range of antigens that were made highly immunogenic by presentation with the adjuvant and addressed the mechanism of action [44]. Freund and co-workers demonstrated their usefulness with additional viral antigens, such as rabies and polio [45], as well as sensitization to small molecules (e.g., picryl chloride) [46] and self-antigens [44]. Production of allergic aspermatogenesis, allergic encephalomyelitis, neuritis, and uveitis were described. These studies contributed to fears that the use of potent adjuvants could lead to accidental generation of autoimmunity.

Mechanistic work by McKinney and Davenport [47] demonstrated that the mode of action of mineral oil adjuvants was complex. They concluded that the mechanism involved an initial antibody stimulus resulting from antigen dispersal; the slow release of antigen, which maintains antibody levels; and the inflammatory response, which promotes better utilization of antigen. Studies, including irradiation at periods after vaccination in the presence of adjuvant, excision and reimplanting of granulomas, implantation of virus-saturated cotton plugs, and daily injection experiments, indicated that an early response (<16 days) is critical for the generation of antibody titer. This early response has been linked with both attraction of certain cells to the inoculation site [48] and development of inflammation at the injection site [44]. The long-term maintenance of antibody was correlated with presence of the adjuvant depot for a period of months after injection [47]. These basic precepts of adjuvant activity remained through the 1960s and 1970s.

Whereas the more toxic CFA adjuvant was not deemed appropriate for human use nor was it required for antibody generation, the mycobacterial component had been shown to be necessary for cellular and tubercular sensitization [49]. Attempts to fractionate the active material from killed cells showed that a wax fraction, not the protein fraction, was responsible for the generation of tubercular hypersensitivity [50–52]. This fraction was subsequently shown to be composed of mycolic acid, polysaccharides, and amino acids [53]. This marked the beginning studies of immune agonists that moved beyond the consideration of adjuvant function as an antigen reservoir and granulomatous source of inflammation.

Large-scale testing of IFA for human vaccines was made practical when Salk et al. [48,54] produced highly purified mineral oil and Arlacel A surfactant for use in studies on influenza and polio vaccines. Very large scale evaluation of the adjuvant was done both in the public sector [55] and with the U.S. military in influenza vaccine trials [56]. These studies, as well as studies by Salk on polio [57,58], validated the potency of the adjuvant for enhancement of antibody titers in human subjects. Although failure of efficacy was reported for adenovirus [59], the potency of Freund's adjuvant became established as the "gold standard" for most vaccines. The adjuvant was applied to allergy therapy as well [60], but the special hazards of incompletely controlled exposure of allergic persons to allergen in the presence of adjuvant was unacceptable.

Issues with toxicology made acceptance of IFA controversial in the 1950s and 1960s. Intense inflammation and formation of granulomatous lesions at the injection site were documented [61], but perhaps more alarming was the finding that the emulsion was not entirely retained at the injection site [44,47] and that the poorly metabolized mineral oils might be a risk as carcinogens. The subject of the risk associated with vaccination using the adjuvant and the acceptability of the local reactions was reviewed very extensively by Hilleman [62], who noted that only 109 reactions were reported from 23,917 doses of adjuvanted poliovirus vaccine [63] and commented: "The remaining and most questionable aspects in relation to decision making rest largely on speculative

grounds extrapolated from effects which have been observed in animals in connection with experiments designed for other purposes."

Licensure for IFA did not occur, and a tone of extreme caution with respect to adjuvants extended through the 1970s. While attempts to formulate water-in-oil adjuvants with metabolizable oils had been made [43,64], Hilleman and his Merck collaborators [65–67] introduced an efficient peanut oil–based adjuvant using purified Arlacel A and aluminum stearate as stabilizers. Adjuvant 65, as this formulation was named, was reported to be of similar potency to Freund's in both animal and human vaccination with influenza virus [68–70], although in a British influenza trial it was also reported to be significantly less potent [71]. Despite extensive review of safety over 10 years, data showing induction of tumors in mice by Arlacel A [72] kept this system from achieving licensure. The approach of water-in-oil emulsions for adjuvant purposes was set back severely, but would reappear in the 1990s with the Seppic-produced systems.

Work in the aftermath of the water-in-oil adjuvant experience was marked by extreme caution. A seminal review by Edelman [73] cautioned that adjuvants should not risk induction of autoimmunity or allergy, produce no teratogenic effects, and have a very low incidence of adverse events. Chemical composition should be well defined, demonstrated to be carcinogen-free and biodegradable, and the type of immunity induced should be specific for the vaccine and not generally activating.

The next generation of adjuvants was composed of two classes of agents: small molecules often derived from bacterial fractions shown previously to be stimulatory with water-in-oil emulsion adjuvants, and particulate vehicles of dimensions similar to either bacteria or viruses where the agonists are naturally found. Both of these approaches were encouraged by concurrent advances in immunololgy [74] which indicated that much more complex interactions with a number of cell types, including Langerhans cells, macrophages, and dendritic cells, might be important.

A significant part of the small molecule agonist library was derived from bacterial extracts from *Mycobacterium tuberculosis*, *M. avium*, and saphrophytic strains of mycobacteria. White et al. [75] screened fractions from a variety of bacterial sources that showed activity in Wax D, phosphatide, Wax C, and cord factor fractions of mycobacterial strains, which increased antibody titers. Additional activity was found in DNA and RNA digests [76]. A large body of work was devoted to a peptide-containing fraction isolated from Wax D and characterized in the 1940s [50,52]. The composition of the active fraction appeared to be analogous to that of the water-soluble cell wall peptidoglycan [77], and the major part of the activity was ultimately isolated from the cell wall by lysozyme digestion [78,79]. Although a broad range of bacteria exhibited varying adjuvant activity, suggesting a variety of possible variants [76], structural work on a few key strains, including *M. bovis*, *Nocardia rubra*, and *Listeria monocytogenes*, was accumulated and the minimal active subunit of

the cell wall was defined as *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) [80]. It was noted by both major groups characterizing MDP activity that in vivo activity required administration in water-in-oil emulsions; saline solutions were inactive. In addition, mycobacterial MDP was shown to be pyrogenic [81]. Attempts to optimize activity led to chemical synthesis of novel MDP derivatives [82] for both vaccine application and induction of nonspecific resistance [83]. Structure–function studies were undertaken [84] to reduce toxicity, optimally activate an as yet undiscovered receptor, and create compatibility with a variety of delivery systems to be discussed later.

While additional work with mycobacterial fractions such as the cord factor first described by Bloch [85] and identified as trehalose dimycolate [86] continued to find application in experimental adjuvants [87], activities of agonists from other sources were also being characterized. Antibody-enhancing adjuvant activity of both poly A : U [88] and polyribo I : C was demonstrated with rabies vaccine [89], along with reports of interferon induction in primates by polylysine/carboxymethylcellulose–stabilized poly I : C [90].

The ability of gram-negative bacilli to enhance antibody titer had also long been established [91], and the adjuvant and endotoxic properties of the purified agent endotoxin were characterized [92]. The adjuvant and endotoxic activities were shown to be separable by both acylation [93] and desterification [94] of the liposaccharide mixtures, and detailed structural work was under way in the 1970s [95].

Finally, saponins, first noted as having adjuvant activity by Ramon [8], were rediscovered and found to be useful in foot-and-mouth vaccines [96]. The modern era of saponin use began with the discovery that extracts from Quillaja saponaria are the most adjuvant active of the saponins [97] and that partial purification of the extracts produced the fraction Quil A, which, although still reactogenic, was markedly better than the crude Quil saponin [98].

In addition to the development of small molecule agonists, several new delivery vehicle approaches that targeted phagocytic cells and did not produce granulomas were demonstrated. Liposomes adopted as carriers for a variety of molecules [99,100] were shown to be adjuvants as carriers of antigen and adjuvant agonists [101,102]. An alternative approach to targeting phagocytic cells, use of very slowly biodegradable methacrylate polymer nanospheres, was introduced by Kreuter and co-workers [103,104]. They demonstrated induction of antibody-mediated protection against influenza in mice with antigen either incorporated into the particles or bound to the particle surface [105].

By 1980 the adjuvant field was beginning to recover from the very serious setbacks incurred when water-in-oil emulsions did not achieve licensure with influenza vaccine. Although the dominant correlate for protection by vaccine remained neutralizing titer, many adjuvant approaches moved away from granuloma-inducing depots to targeting of phagocytic cells, emphasizing both chemotaxis and uptake by macrophages. The move toward micro-/nanoparticle delivered agonists and antigen association had begun.

1.4   RATIONAL RECEPTOR-DRIVEN ADJUVANTS THAT ACTIVATE THE INNATE IMMUNE SYSTEM: 1990s–PRESENT

1.4.1   Major Breakthroughs of the Era

The early 1980s brought two major changes to the vaccine and adjuvant world. First, the first recombinant DNA–generated vaccine made against hepatitis B [106] was successfully demonstrated and ultimately achieved commercial licensure. This signaled the beginning of recombinant production of a spectrum of recombinant subunit antigens, many of which, like diphtheria and tetanus toxoids, would prove to be active only with adjuvant. Second, the discovery of the HIV virus responsible for AIDS [107,108] and definition of the gp120/140 and gag antigens from the virus set in motion an unprecedented wave of investigation into adjuvants suitable for protection against AIDS with subunit vaccines.

Caution with respect to toxicity continued to be the major factor in moving materials into clinical trials [109]. Potent adjuvants of low toxicity were developed and achievement of commercial licensure for the MF59-adjuvanted flu vaccine Fluad [110] in the European Economic Community and the IRIV(immunopotentiating reconstituted influenza virosome)-based hepatitis A vaccine [111] in Switzerland finally brought acceptance of post-alum adjuvants.

The fields of adjuvant development and immunology became tightly intertwined as the professional antigen-presenting cells were characterized [112–114], the cytokine profiles responsible for generating Th2 versus Th1 immunity were demonstrated [115], the requirements for MHC (major histocompatability complex) class I versus class II presentation were defined [116], and the relationship between the innate immune system and many of the known adjuvant-active molecules was demonstrated with the characterization of Toll-like receptors (TLRs) responsible for signaling innate immune activity [117].

1.4.2   Historical Progression

Molecular Adjuvants   Two mycobacterial components with a history of adjuvant activity but marginal toxicity profiles received further attention. Trehalose dimycolate (TDM) was investigated further by Masihi et al. [118], and a greater effort was made with muramyl peptides, where less toxic or pyrogenic derivatives were synthesized. The water-soluble Murabutide had no toxicity problems in humans but was not convincingly active in clinical trials with tetanus toxoid [119]. A second water-soluble candidate, threonyl MDP, was nonpyrogenic, did not induce uveitis, and was potent in animal studies [120]. Additional derivatives, including MDP-lys and the lipophilic muramyltripeptide phosphatidylethanolamine (MTP-PE), were tested in human clinical trials for either vaccine adjuvant or chemotherapeutic activities [121,122].

Development of lipid A–related adjuvants was a key activity in this period. While it was shown that there was significant heterogeneity in lipid A components from a variety of gram-negative bacteria [123], significant progress was made in development of adjuvant based on lipid A from *Salmonella minnesota*. Purification and structure were determined by the Ribi group [124], who demonstrated that toxicity could be attenuated dramatically by hydrolysis of the 1-phosphate [125] and 3-hydroxytetradecanoyl groups [126] generating 3D-monophosphoral lipid A (MPL). Data on the safety of MPL in humans was generated quite early in tumor therapy application [127], and the biological activities were shown to include stimulation of synthesis of a number of cytokines, including γ-interferon [128,129]. Both 3D-MPL and later the aminoalkylglucosamine phosphates (e.g., RC529) have further application in humans when combined with particulate delivery systems to be discussed later.

Among the most promising adjuvant actives to be discovered in the post-1980 period are the immunostimulatory DNA sequences comprising an unmethylated CpG. Antitumor activity first demonstrated in bacterial DNA [130] was shown to result from unique palindromic sequences containing unmethylated CG sequences [131] with selected flanking sequences [132] [immunostimulatory sequences (ISSs)]. The activity of the ISS DNA was characterized by induction of interferons, activation of natural killer (NK) cells, production of Th1-biased antibody response [133], and direct activation of B cells [132], murine macrophages [134], and both murine [135] and human plasmacytoid dendritic cells [136]. Plasmid DNA sequences containing certain CpG motifs have been shown to be active as adjuvants for a number of antigen-expressing DNA vaccines [137] as well as protein antigens [133,138]. Use of synthetic phosphorothioate oligonucleotide ISSs [139] as vaccine adjuvants has been investigated for the three classes of immunostimulatory sequences identified [140–142] as well as for other applications. The demonstration of TLR9 as an ISS receptor [143] is allowing studies on TLR distribution and signaling to aid in rational development of ISS-based adjuvants. A spectrum of vaccines utilizing soluble ISSs have been evaluated in preclinical models using protein [144], peptide [145], polysaccharide conjugate [146], and viruslike particles [147]. Vaccines have been administered by mucosal [148,149] as well as intramuscular routes. ISS conjugates of fusion peptides have been employed to generate cytotoxic lymphocytes [150]. Conjugation of ISS to the ragweed protein allergen Amb a 1 has been shown to both increase immunogenicity and decrease allergenicity [151]. ISS oligonucleotides have shown an excellent toxicity profile [152], have been applied to hepatitis B vaccine in human clinical trials both with hepatitis B surface antigen (HBsAg) alone [153,154] and with HBsAg–alum [155]. Additional clinical trials have been performed with soluble ISS in combination with influenza vaccine [155] and the Amb a 1–ISS conjugate (AIC) [156]. Additional work on combination of ISS with delivery systems is discussed later. The use of RNA adjuvant molecules has been difficult despite the advent of stabilized RNA derivatives [157]. However, the activity of the imidazoquinoline derivatives, which also stimulate

RNA receptors TLR7 and TLR8 [158], have shown preclinical potential as Th1-directing adjuvants for herpes simplex vaccines [159–161]. One such product, Imiquimod, has been licensed for topical treatment of herpes simplex [162], but use of imidazoquinoline derivatives as adjuvants remains at preclinical stages. As for the ISS system, antigen conjugates of R848 (another imidazoquinoline derivative) are reported to offer greater activity than that of the soluble mixtures [163]. A number of other TLR7 and TLR8 agonists are under development.

Several nonparticulate adjuvants that have not been identified as TLR agonists have been characterized. Further fractionation of Quil A saponin isolated by Dalsgaard [98] revealed at least 24 peaks [164]. Analysis of adjuvant activity and toxicity revealed the much less toxic compound triterpene glycoside QS-21, which was shown to be an active adjuvant in mice [165], producing Th1 antibody isotypes [166] and CD8 cytotoxic T lympocytes in mice [167]. QS-21 has been used clinically for both cancer immunotherapy [168,169] and prophylactic vaccination against HIV [170] and the malaria peptide SPF66 [171]. However, injection-site pain was a notable problem, making the system unacceptable except in extreme circumstances. Particulate saponin constructs are discussed below.

The mucosal adjuvants cholera enterotoxin (CT) and *Escherichia coli* heat-labile enterotoxin (LT) are potent mucosal adjuvants and have about 80% sequence homology [172]. Their activity has been linked to ADP–ribosyltransferase activity [173]. While CT has been the standard for mucosal adjuvant activity, the toxicity of the A subunit has discouraged clinical use [174]. LT also has toxicity associated with its A subunit, but mutants with significantly lower toxicity have been generated [175,176]. The mutants LTK63 and LTR72 have been shown to have potent activity in the generation of mucosal antibody in preclinical models against a variety of antigens when administered by oral, nasal [177], or transdermal routes [178] and appear ready for clinical testing [177].

The molecular adjuvants thus far described give rise to chemokine and cytokine synthesis. The basic paradigm first described by Mossmann et al. [115] is that two basic types of immune response can be generated. The Th2 response is characterized by a cytokine profile dominated by interleukin-4 and interleukin-5 activates principally B cells. The Th1 response is characterized by γ-interferon, granulocyte monocyte colony stimulating factor, and interleukin-12 activates macrophages and cytotoxic T cells [179]. The direct approach of using cytokines as vaccine adjuvants first concentrated on three cytokines, interleukin-1, interleukin-2, and γ-interferon [180], followed by successful application of interleukin-12 to leishmania vaccine [181] and use of granulocyte monocyte colony-stimulating factor [182] with both peptide and protein tumor antigens. The most extensive efforts on infectious disease vaccines were made with interleukin-2, a T-cell-activating agent that was used alone with rabies vaccines, where it increased protection 25- to 50-fold [183,184] and in combination with several vehicles to be discussed later. The natural activities of cytokines as short-range very low concentration signals between cells are quite different from those of an injection agent in a bolus at high concentrations. The toxicity

of interleukin-2 [185], interleukin-12 [186], and γ-interferon [187], and often a need for complex dosing regimens, has led to restricting the use of cytokines to tumor vaccines [188] and to exploration of DNA vaccines in which antigen and cytokine are coexpressed, such as a herpes simplex virus (HSV)-2gD/interleukin-12 system [189] or an HIV env/interleukin-12 vaccinia system [190].

Particulate Adjuvants The primary delivery systems before 1980 were characterized in large part as depot systems. The aluminum salt adjuvants, until recently the only licensed adjuvants for human use [191], continue to be regarded as safe [192] and are in common use with tetanus, diphtheria, pertussis, and poliomyelitis vaccines as well as more recent use with hepatitis B (HBV), hepatitis A (HAV), and anthrax vaccines [193]. Although these systems are not workable for a number of proteins and peptides [37,194], considerable progress has been made in understanding binding parameters [195]. The limitations of alum are a driving force for research into new adjuvant systems. The characterization of aluminum salt adjuvants as Th2-directing systems that stimulate IgE production and very poor cellular immunity is well documented [196,197]. Thus, alum alone is inappropriate for use against a variety of diseases that require Th1/cellular immune responses. The combination of alum with molecular adjuvants (discussed later) may overcome some of these problems. Alum suffers additionally from some reactogenicity at the injection site, giving rise to swelling and cutaneous nodules [109,198]. Although these effects are tolerable, adjuvants that disperse more quickly or do not give rise to inflammation were desired.

Alternative particulate adjuvants giving an extended presence of antigen have been described. Replacement of aluminum salts with calcium phosphate has long been described [199]. Efforts with calcium adjuvants have continued [200], and work with calcium phosphate nanoparticles has had some preclinical success [201]. Use of stearyl tyrosine has been described for a variety of antigens now in use with aluminum adjuvants, including tetanus toxoid [200], diphtheria toxoid [202], and recombinant hepatitis B [203]. Although residence time is shorter than for aluminum salt systems, the benefits have not yet given rise to clinical trials. Use of tyrosine as an adjuvant in allergy vaccines has a long clinical history and a good record of safety [204].

A number of groups have invested effort in controlled release of antigen by polymeric particles aimed at single-dose vaccines. The first demonstration of a single immunization system [205] with nondegradable ethylvinyl acetate showed six-month antibody maintenance against bovine serum albumin (BSA). While several classes of biodegradable polymers, including polyanhydrides and polyorthoesters, have been described for medical applications [206], more recent efforts have used the well-characterized biodegradable poly(lactide-co-glycolide) polymeric microparticle systems [207,208]. This approach advanced to use of very active sub-10-μm particles taken up by antigen-presenting cells combined with 30- to 100-μm particles giving long-term release of antigen [208] and pulsed release of antigen using mixtures of particles of varying molecular weight and lactide/glycolide ratio [209].

Although the manufacturing hurdles and protein stability issues have been solved for some systems and controlled-release formulations have been licensed [210], they have not been clinically tested for vaccines.

Interesting pre-1980s formulations in development remain the water-in-oil emulsions originated by Freund and Hosmer [24]. Major factors in their potency included a long-term depot effect of a mineral oil bolus, which often resulted in cutaneous nodules along with longer-term immunity. Additionally, these emulsions attracted a variety of immune cells, resulting in a long-term reactive center. Efforts to continue with the water-in-oil emulsion (a particularly effective approach for peptides) have been made by Seppic. The Montanide ISA adjuvants (utilizing mineral oil and mannide monooleate, which emulsifies water with a low energy input) [211] have a substantial record in veterinary applications. More recently, the ISA 720 formulation using vegetable oil has been tested in clinical trials with HIV peptide [212] and a malaria–HBV core antigen [213].

Oil-in-water emulsions judged to be ineffective when using mineral oil [10] were reexamined when Ribi and co-workers found antitumor activity with trehalose dimycolate surfaces on drakeol oil-in-water emulsions [214]. The somewhat-toxic trehalose dimycolate surface was replaced with pluronic polyol block polymer surfactants, and a correlation was established between the hydrophile–lipophile balance (HLB) and activity [215]. Advances in synthetic techniques allowed production of higher-molecular-weight block copolymers. The copolymer CRL 1005 showed good adjuvant activity when mixed directly with inactivated whole virus flu vaccine in mouse studies [216]. Reynolds took a different step from mineral oil–based oil-in-water systems, showing that phospholipid-stabilized lipid emulsions (relatives of nutritional emulsions) had adjuvant activity with viral antigens [217]. Significant progress was made when several groups applied low-HLB surfactants with squalane/squalene oil-in-water emulsions. Ribi and co-workers [118] used squalane–water emulsions with trehalose dimycolate surfaces for veterinary applications. The Syntax adjuvant formulation (SAF) [196] used the potent block copolymer L-121 to generate a squalane-in-water formulation. The SAF M formulation developed for manufacturing was shown to be effective in several primate systems [218–220]. Use of the nontoxic low-HLB spreading agent Span 85 [221] and Tween 80 as stabilizers for a squalene–water emulsion produced the adjuvant MF59 [222,223]. This formulation stimulated neutralizing antibody (but not convincing protection) successfully with recombinant HSV surface antigens in phase III clinical trials [224]. Phase III and IV trials with the commercially available MF59/influenza vaccine Fluad [225,226] showed this vaccine to be particularly effective in the elderly. MF59 also appears to have very good adjuvant activity with H5 influenza vaccines [227].

Polymeric nanoparticles with either encapsulated antigen or protein-binding surfaces were used by Kreuter et al. in the 1970s [103]. Much later work [228] has shown that these easily prepared and well-tolerated poly(methylmethacrylate) nanoparticles are a superior adjuvant to a large array of

particulates when used with HIV-2 split virus. A related set of approaches using poly(lactide-co-glycolide) microparticles (<10 µm) employing either encapsulation of antigen [207,208] or utilization of surface-charged microspheres [229–231] have shown significant promise in preclinical models. This approach has also been applied to delivery of DNA vaccines, with encouraging preclinical results [229].

As noted previously, liposomes adopted as carriers for a variety of molecules [99,100] were shown to be effective as carriers of antigen and adjuvant compounds [101,102]. An extensive amount of work was completed in the 1980s and 1990s in a quest to optimize the adjuvant effects of liposomes. This work has been well reviewed by several authors [232–234]. Efforts to optimize adjuvant efficacy have included comparisons of multilamelllar versus unilamellar systems, variation in size and fluidity of lipids, incorporation of antigen by encapsulation versus surface interaction, and alteration of surface with PEG [poly(ethylene glycol)]-ylated lipids. Preclinical testing has been done with at least 20 antigens. After extensive testing the general conclusion is that liposomal delivery of subunit protein–peptide antigen alone is not a powerful method for enhancement of immunogenicity [235]. Use of liposomes for delivery of DNA vaccines both by encapsulation and by interaction with cationic lipid components has been well studied [236–238]. Development of more effective and less toxic cationic lipids has allowed testing of lipid-adjuvanted DNA vaccines in primate studies [239,240]. However, human trials of DNA vaccines have proceeded with naked DNA and appear to need viral boosts for best [241] effects. It is important to note that liposomal formulations have shown promise in the generation of cellular immunity, particularly cytotoxic lymphocytes, which are suspected to be critical in the protection of a variety of infectious diseases and particularly in cancer therapy. Introduction of pH-sensitive liposomes capable of introducing ovalbumin into the MHC class I pathway in mice and generating cytotoxic T lymphocytes (CTLs) [242,243] marked the beginning of series of CTL-generating formulations using liposome delivery.

The earliest liposome-related vaccines to be licensed, the IRIV and IRIV/hepatitis A vaccines [232], are based on 150-nm unilamellar vesicles created by reconstitution of detergent-extracted influenza surface glycoproteins and phospholipids with egg yolk phosphotidylcholine and phosphatidylethanolamine (PE). For the HepA vaccine (and a number of others in preclinical studies, including tetanus toxoid, poliovirus VP2 peptide, and HBsAg peptide), the PE moiety is cross-linked covalently to the antigen. Cellular entry of the vaccine particle and endosomal fusion are thought to use the neuraminidase (NA)- and HA-mediated entry systems evolved by the influenza virus [244]. The system has intriguing possibilities, but complex composition and formulation issues may limit its application. A second lipid-based particle with extensive application is the ISCOM/ISCOMATRIX system, based on Quillaja saponin, cholesterol, and phospholipids. Dissolution of these components in the presence of the detergent Mega 10 followed by removal of the detergent

by dialysis results in a 30- to 40-nm cagelike structure [245] which will incorporate amphiphilic antigens to produce the original immune-stimulating complex (ISCOM) structure [246]. This structure is an active producer of antibody but is set apart from most of the adjuvant systems developed thus far in that it effectively delivers antigen to the MHC class I pathway [247], although not by the TAP (peptide transporter) pathway. ISCOMs do not interact with any of the known Toll receptors. ISCOMs are effective CTL-generating systems in primate systems, including humans [248]. ISCOMs have been shown to be effective against a broad spectrum of viruses [249] in veterinary applications, with the first commercial use in an equine flu vaccine [250], where continued success has been reported with intranasal boosting [251].

Delivery System/Molecular Adjuvant Combinations   A major tactic from the first days of adjuvant development has been to use delivery systems to protect, deliver, and extend the therapeutic lifetime of molecular adjuvants (the most famous being complete Freund's adjuvant). Use of alternative water-in-oil emulsion systems have been examined with a birth control vaccine based on peptide antigen and a saline/or MDP–squalene emulsion showing clinical promise [252]. A similar approach for veterinary vaccines was taken with Titer Max, where saline was emulsified into squalene with the block copolymer CRL8941 [253]. For the period since 1980, most of the combination adjuvants have been designed with chemically defined Toll agonists delivered with sub-10-μm particulates. Although criticisms of aluminum salt adjuvants are often made, combinations of Toll agonists with aluminum adjuvants continue to be evaluated. Alving began work on vaccines for HIV and malaria using a combination of alum, liposomes, and lipid A [254]. Use of alum–MPL with HSV gD2 showed significantly better performance than that of alum alone in both mice [255] and humans [256], where it remains a possible candidate for future application. An MPL–alum combination has also been tested with hepatitis B vaccine in humans [257,258] and was recently licensed in Europe for use in dialysis patients. A combination of CpG1826 with aluminum salt was shown to be equipotent to IFA with a malarial peptide in mice [259] and may represent an interesting new direction.

A number of groups have used oil-in-water emulsions as carriers or coadjuvants for molecular adjuvants. Extensive work has been done with an SAF squalane/L121 block copolymer system in conjunction with threonyl-MDP pioneered by Allison and Byars [260]. Preclinical efficacy was demonstrated with a spectrum of antigens [261], including HIV in chimpanzees [220]. The squalene–water emulsion MF59 was tested with MTP–PE and HIV vaccines in a phase I clinical trial [262] and with influenza vaccine [263] where reactogenicity in the presence of MTP–PE was unacceptable. Use of catatonically modified MF59 with CpG oligonucleotides has shown preclinical promise without unusual reactogenicity [264]. When tested in cancer patients, use of oil-in-water emulsions with cell wall skeleton (CWS)–MPL in squalane (Ribi Detox), showed no systemic toxicity but some reactogenicity at the injection

site and a few granulomas [265]. Later approaches have omitted the CWS component and used either the monophosphoryl lipid A or later, monophosphoryl lipid A derivative RC529 with the proprietary oil-in-water emulsion SE with HIV peptides in primates [266] or anthrax protective antigen with a squalene–water emulsion in primates showing a strong immune response [267]. The MPL–emulsion approach appears to be a good candidate for next-generation human vaccines [268].

The use of sub-10-μm polymeric particles as a delivery system for adjuvants has been tested using both encapsulation and surface binding of adjuvants. Cationic poly(lactide-co-glycoside) microspheres shown previously to be effective carriers for DNA vaccines [229] have also been shown to enhance the activity of surface-bound CpG-containing oligonucleotides [269]. The alternative approach of encapsulation of molecular adjuvants approximates well-developed drug delivery techniques. This has been shown to potentiate MPL and derivatives [270], and the approach could be used in combination with surface-bound antigen systems [271].

Liposomes are modestly potent delivery systems for antibody induction and have shown potential for CTL generation [242] and as carriers for molecular antigens. The adjuvant delivered may be either encapsulated or surface bound, with results depending on that distribution. Use of liposomes for lipid A and its derivatives has shown potential with malaria vaccine in phase I trials [272] and with malarial peptides in a liposome–alum formulation [273].

Incorporation of cytokines onto liposomes was first demonstrated [274] showing that IL-2 could be incorporated into dehydration and rehydration vesicles, increasing the activity of IL-2. The approach was used in preclinical studies for HSV [275] but was not pursued further. Incorporation of CpG oligonucleotides into cationic liposomal delivery formulations was shown to enhance CpG activity [276]. It has been noted that liposomal delivery of CpG to the endosomal TLR9 receptor may have complex effects [277]. There is indication that CpG liposome formulations can enable CTL generation with HIV antigens [278] or cancer antigens [279]. Liposomal formulations utilizing encapsulated membrane-traversing systems such as listeriolysin O, not usually considered as a molecular adjuvant, can generate anti-ovalbumin CTL [280].

1.5 CONCLUSIONS

Vaccine adjuvant research and development has been an ongoing activity for more that a century. The need for methods to enhance vaccine immunogenicity has been recognized from the days of the very first testing of nonliving vaccines. The development of successful vaccine adjuvants has been a constant balancing act between safety and immunogenicity, delivery and immunostimulation, and simplicity and complexity. The fact that after over 100 years so few adjuvants have been approved for human vaccines attests to the difficulty of this research and development activity. We appear to be at the beginning of a

new era in which a variety of new adjuvants are being approved or are about to be approved for human vaccines. In this chapter we have described the steps involved in the process over the last century that have led to these new vaccine adjuvants.

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