Immunity
Commentary

How Do Adjuvants Work? Important Considerations for New Generation Adjuvants

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In this Commentary, McKee et al. highlight the properties of extrinsic vaccine adjuvants that must be considered to achieve the most protective immune response, as occurs naturally with many intrinsic pathogen-derived adjuvants.

The idea that some materials could improve immune responses was recognized many years ago with the work of William Coley, who used bacterial products to treat cancer patients, and Ramon and Glenny, who used unexpected reagents such as tapioca and aluminum hydroxide to improve the responses of horses or guinea pigs to diphtheria and tetanus toxoids (Coley, 1893; Ramon, 1925; Glenny et al., 1926).

Adjuvants can act in several nonmutually exclusive ways to augment the adaptive immune response and to generate effective immunological memory. Many of their effects seem to be on antigen-presenting cells such as dendritic cells (DCs). Thereby adjuvants can affect the migration, maturation, antigen presentation, and expression of costimulatory molecules by DCs, and these events in turn improve the responses to antigen of T and B cells. Adjuvants, apparently via DCs, can also affect the nature of CD4 T helper (Th), CD8 T cell, and B cell responses, with some adjuvants promoting Th1-related responses and others preferentially inducing Th2-biased effects. Furthermore, some adjuvants enhance crosspresentation by DCs of MHC I-restricted antigens to $CD8^+$ T cells. Adjuvants may also act directly on T or B cells, improving their proliferation and/or conversion into memory cells that are essential for the success of vaccines.

Many vaccines were developed in the 19^{th} and 20^{th} centuries against diseases such as measles, smallpox, and yellow fever, diseases that are now controlled to a large extent in developed countries by vaccine-induced antibody. However, despite the increase in our knowledge of the immune system and micro-organisms, we still lack effective vaccines for many diseases. This may be because the invader is too clever for us; an effective vaccine may simply not be possible. On the other hand, the vaccine design may not have been optimal. For example, some infections may be better dealt with by a particular antibody isotype, and the vaccine used may not have induced the correct type of antibody. Also, antibodies are not protective against some intracellular pathogens, and T cell responses may be more effective, so the recent emphasis on T cell-stimulating rather than the antibody-inducing vaccines that have been used in the past may be really helpful in such cases. Because the choice of adjuvant can affect the isotype of antibody and the nature of the T cells produced, new strategies with these points in mind require careful choice of adjuvant.

Different Infections Are Best Dealt With by Different Types of Immunity

The vast majority of viruses are controlled well by the current vaccines that act by inducing antibodies. Even for the successful vaccines of this type there may, however, be room for improvement. For example, the subunit influenza vaccine given in the USA currently includes no adjuvant, according to the manufacturers. One would predict that influenza infection would best be prevented with a vaccine containing an adjuvant that induces IgA and IgG2a responses, and efforts to create such a vaccine are underway. In this case the adjuvant may act on the B cells themselves, but, more likely, would act on DCs, which, in turn, modulate the type of $CD4^+$ helper T cell produced.

For some intracellular pathogens, such as *M. tuberculosis* and *L. major* (Foulds et al., 2006; Kaufmann, 2006), $CD4^+$ T cells that have differentiated into Th1 cells are most protective. Th1 cells mediate direct effects on such pathogens via production of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) and also by promoting the effector functions and survival of $CD8^+$ T cells. Conversely, Th2 $CD4^+$ cells, secreting interleukin (IL)-4, IL-5, and IL-13, protect against helminth infections (Stetson et al., 2004), and Th17 cells, secreting primarily IL-17, may participate in promoting innate responses against extracellular bacteria (Stockinger and Veldhoen, 2007).

What Adjuvants Are Currently Available for Use in Vaccines and How Do They Function?

Many of the vaccines currently used in man contain adjuvants that are intrinsic to the immunogen. For example, vaccines that contain attenuated live or heat-killed viruses or bacteria include components that can engage Toll-like receptors (TLRs) (Tables 1 and 2). These components therefore act as natural adjuvants because TLR

Table 1. Some Products of Invading Organisms that Engage Innate Receptors

Material	Source	Innate Receptor
Peptidyl glycans	bacteria	TLR1,2,6
Lipoproteins	bacteria	TLR1,2,6
GPI	trypanosomes/plasmodia	TLR1,2,6
dsRNA	viruses	TLR3
Lipid A	bacteria	TLR4
Flagellin	bacteria	TLR5
ssRNA	viruses	TLR7,8
Unmethylated CpG	bacteria/viruses	TLR9
Hemozoin pigment	malaria	TLR9
Profilin	protozoa	TLR11 (mouse only)
Peptidoglycan	bacteria	Nod1
Muramyl dipeptide	bacteria	Nod2
Anthrax lethal toxin	*B. anthracis*	NALP1b
Uric acid crystals	endogenous	NALP3, Cryopyrin
Calcium pyrophosphate	endogenous	NALP3, Cryopyrin
Lipopolysaccharide	bacteria	NALP3, Cryopyrin
dsRNA	viruses	NALP3, Cryopyrin
Flagellin	*S. typhimurium*	Ipaf
Flagellin	*L. pneumophila*	NALP5, Ipaf
dsDNA	viruses	RIG-I, MAVS
dsRNA	viruses	RIG-I, MAVS
Viral glycoproteins	HIV	DC-SIGN
Mannose oligosaccharides	protozoa/fungi/bacteria/ viruses	mannose binding lectin
Oligosaccharides	bacteria/viruses	surfactant A, D
Zymosan	yeast	Complement, Dectin-1

Abbreviations: GPI, glycophosphatidylinositol; ssRNA, single stranded RNA; dsDNA, double stranded DNA; TLR, toll-like receptor; Nod, nucleotide-binding oligomerization domain protein 1; NALP, NACHT- and LRR-containing protein; Ipaf, Nod-like receptor family member containing ICE protease activating factor; RIG-1, retinoic acid inducible protein; MAVS, mitochondrial antiviral signaling protein; DC-SIGN, dendritic cell-specific ICAM3 grabbing nonintegrin.

signaling has many of the effects on DC antigen presentation that one would wish for an adjuvant: improvement in antigen presentation and increases in costimulatory molecules and cytokine production, leading usually to improved Th1-related responses. Such responses are well suited to defend against the organisms involved, probably because TLRs have been designed through evolution to respond in exactly the appropriate way to these infections and their attendant, intrinsic adjuvants.

Ironically, because TLR ligands usually work very well and induce excessive and toxic inflammation, they are not approved additives to subunit vaccines. For example, the hepatitis B vaccine contains protein subunits but not intrinsic TLR ligands that are components of the whole virus. Therefore, there has been a flurry of research into separating the unwanted toxic side effects of TLR ligands from their ability to promote cellular immunity. For example, lipopolysaccharide (LPS) is a powerful adjuvant but is too toxic to be safely used in human vaccines. Hydrolysis of the bioactive lipid A in LPS results in a molecule called monophosphoryl lipid A (MPL). This adjuvant has reduced toxicity compared with lipid A but still engages TLR4 and Toll-IL-1 receptor (TIR) domain-containing adaptor inducing interferon β adaptor protein that contains a TIR domain (TRIF)-mediated signaling pathways to enhance cellular immunity (Mata-Haro et al., 2007).

More problematic are the vaccines that depend on adjuvants that are not characteristic of the targeted organism. Here evolutionary selection has not had a chance to play its part, and the artificially included adjuvant may not induce the appropriate response. Of the artificially added materials, by far the most widely used are the particulate adjuvants based on aluminum salt precipitates, called herein alum (Table 2). Alum activates innate responses in vivo and promotes a Th2-biased response and elevated titers of the Th2-dependent antibody isotypes IgG1 and IgE.

Despite its long use, we still do not know exactly how alum mediates its adjuvant effects. One hypothesis is that alum, because it adsorbs antigens, serves as a depot, releasing the antigen slowly into the body, thereby allowing antigen-specific lymphocytes to be exposed to antigen for a longer time. Although alum certainly extends the half-life of antigen in vivo, whether the depot theory accounts for its adjuvanticity has been challenged in several studies. For example, removal of the alum depot at the site of injection 1 week after immunization had no effect on the antibody response that developed against the coinjected antigen (Holt, 1950), and in some cases antigens are rapidly released from the supposed alum depot. Moreover, stable adsorption of an antigen to alum is not necessary for alum's ability to potentiate antibody responses (Iyer et al., 2003).

Alum is certainly recognized by the body, as shown by the fact that it causes rapid influxes of neutrophils and eosinophils at its site of injection (Walls, 1977), induces the appearance of $Gr1^+$, IL-4-secreting cells in the spleen (Jordan et al., 2004), and markedly biases responses toward a Th2

Table 2. Adjuvants Routinely Used in Vaccines

Vaccine (Past and Present)	Added Adjuvant
Diphtheria, Pertussis, Tetanus	Alum
Hepatitis A	Alum
Hepatitis B	Alum
Haemophilus influenzae polysaccharides	Alum
Meningococcal polysaccharides	Alum
Pneumococcal polysaccharides	Alum
Vaccine (Past and Present)	**Intrinsic Adjuvant**
Rabies	ssRNA, dsRNA, CpG
Polio	ssRNA, dsRNA, CpG
Measles, Mumps, Rubella	ssRNA, dsRNA, CpG
Varicella	ssRNA, dsRNA, CpG
Vaccinia	ssRNA, dsRNA, CpG
Yellow fever virus	ssRNA, dsRNA, CpG
Typhoid	Peptidoglycan, LPS, CpG, Flagellin
Cholera	Peptidoglycan, LPS, CpG, Flagellin
B. Calmette Guerin (for TB and leprosy)	Peptidoglycan, LPS, CpG, Flagellin
Anthrax	Peptidoglycan, LPS, CpG, Flagellin
Vaccines in Development	**Combination Adjuvant**
P. falciparum Circumzoite protein	MPL (TLR4), QS21, MF59 (AS02) + HBV virion particles
Papilloma capsid	Alum + MPL (AS04) + virion particles

Abbreviations: ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; TB, tuberculosis; LPS, lipopolysaccharide; *P. falciparum*, *Plasmodium falciparum*; MPL, monophophoryl lipid; TLR, toll-like receptor; HBV, hepatitis B virus.

phenotype. Alum is not recognized by TLRs. In fact, TLR agonists override the Th2-biasing effects of alum. However, other innate receptors may be involved: for example, NALP3, which activates the inflammasome and caspase-1 in response to other kinds of crystals such as those of uric acid (Martinon et al., 2006). Clues to receptors may also come from materials that, like alum, induce eosinophilic exudates and Th2-biased immune responses. These materials include helminth eggs, chitin polymers, and many other types of particles (Reese et al., 2007; Sabin et al., 1996).

Alum fixes complement, and a recent paper showed that complement component 3 (C3)-deficient mice make poorer immune responses than do wild-type animals to antigen plus alum (Yalcindag et al., 2006). However, C3-deficient animals make poor immune responses to antigens regardless of the adjuvant (Pepys, 1974). Thus, the need for complement is not particular to alum. Foreign body reactions are driven by fibrinogen deposition and breakdown on implants, so the clotting cascade may be involved in alum recognition, an idea that needs further investigation in vivo.

An obvious thought is that alum's ability to induce T cells with a Th2 phenotype depends on its effects on DCs. Like other Th2-inducing DCs, DCs exposed directly to alum do not fully upregulate costimulatory molecules and do not produce Th1-driving cytokines, the canonical changes to DC induced by TLR signals (Sokolovska et al., 2007). Rather, alum enhances the secretion of IL-1β and IL-18 by DCs by activating caspase-1 in a myeloid differentiation factor 88 (MyD88)-independent manner (Li et al., 2007). However, IL-1r1-deficient mice sensitized with alum and ovalbumin have asthma-related Th2 responses and pathology similar to those of wild-type mice (Schmitz et al., 2003) and IL-18 plays only a partial role in T cell responses to alum in vivo (Pollock et al., 2003). Likewise, T cells generated with alum-exposed DC in vitro have an unbiased cytokine phenotype (Sokolovska et al., 2007). Thus, alum may not bias T cell responses via DCs.

The Th2-biasing properties of alum may instead be based on its effects on other cells, and in fact, the bias may not be due to induction of Th2 response but rather suppression of Th1 response. Thus, administration of antigen plus alum in the absence of IL-4 allows good T cell responses, which are now unbiased, with T cells secreting both IFN-γ and IL-4 and both IgG1 and IgG2a isotypes (Brewer et al., 1996). The source of the relevant IL-4 remains to be determined, because multiple cell types can produce this cytokine.

Emulsion adjuvants are also often used in experimental animals and, increasingly, in man. Such adjuvants are classified as being either water-in-oil or oil-in-water formulations, and some contain other immunostimulatory substances. The original water-in-oil adjuvants developed by Freund induce adverse toxicity and are relatively unstable, qualities that make them unacceptable for use in human vaccines. The oil-in-water adjuvant MF59 is licensed in Europe and promotes protective antibody responses that are Th2 in nature (Ott et al., 1995). This emulsion-based vaccine forms smaller droplets than Freund adjuvants, is more stable over time, and promotes fewer adverse side effects than do water-in-oil adjuvants. It is not known how MF59 and related adjuvants function. Because they are particles, their modus operandi may be related to that of alum.

Other effective adjuvants include QuilA, a soluble extract from the bark of the *Quillaja saponaria* tree, which contains multiple saponins. Although QuilA is highly effective, it is also too toxic for use in humans, causing severe local reactions, granulomas, and hemolysis. QS21 (Quillaja saponaria

fraction 21) is a less toxic fraction purified from QuilA. Although it promotes Th1 responses against antigens with which it is coinjected (Wong et al., 1999), its effects on Th1 responses and on $CD8^+$ T cell responses are more successful when this adjuvant is combined with others such as the AS02 adjuvant formulation that contains a combination of the oil-in-water emulsion MF59, QS21, and monophosphoryl lipid (see below).

Intrinsic microbial ligands for TLRs are likely to be present in several human vaccines (Tables 1 and 2). Several combinations of the adjuvants discussed above are promising for improving vaccine efficacy against pathogens that so far have eluded vaccinologists. Two such adjuvants are AS04 and AS02, which have been tested in multiple clinical trials. AS04 is a combination of alum and the LPS derivative MPL (see above and Table 2). AS02 is a combination adjuvant of the oil-in-water emulsion MF59 with MPL and the saponin fraction QS21 (see above and Table 2). It is clear that development of a successful new generation of vaccines will require careful consideration of the appropriate adjuvant combination in addition to the consideration of the correct protective epitopes. Below we discuss two examples of how these adjuvants are being applied to human vaccines.

There is increasing interest in vaccines containing more than one adjuvant. For example, a recent promising malarial vaccine candidate, RTS,S, is formulated with the AS02 combination adjuvant mentioned above. As antigens, the vaccine includes a malarial pre-erythrocytic antigen linked to hepatitis B surface antigen. The vaccine forms virus-like particles, which may also increase its immunogenicity, as they do for the recently introduced papilloma virus vaccine (Pinder et al., 2004). Immunization of children with this vaccine resulted in 30% decrease of infections and a 58% drop in the number of infected individuals that developed severe disease (Alonso et al., 2004). The children developed protective immunity against hepatitis B virus as well.

In summary, many vaccines include, by their very nature, adjuvants that are naturally suited to induce an immune response of optimal type. Other vaccines contain artificially added adjuvants, and the task of the vaccinologist now is to pick the adjuvants that induce the appropriate immune response, without damaging the host. Our better understanding of how immune responses work and how different organisms can be attacked offers hope for the future in this regard.

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