1 Role of Adjuvants in Infection and Autoimmunity

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Introduction

Commonly used vaccines are a cost-effective and preventive way of promoting health, compared to the treatment of acute or chronic disease. However, not all vaccines are as efficient and easy to administer as the vaccine against smallpox (Vaccinia). Usually, upon injection of a pure antigen, the antigen is not taken up at the injection site, and an immunological reaction fails. In order to help the immune system to recognize the antigen, adjuvants are added to the antigens during the process of developing and producing a vaccine. For the last few years, researchers have been striving to elucidate the mechanisms by which adjuvants exert their immunological effects. By deciphering these mechanisms, scientists hope to design more efficient and less harmful adjuvants. As of 2013, the action mechanisms of the most used and "veteran" of adjuvants, alum, are being revealed. It seems that alum acts on multiple pathways, each of which can enhance immunological reactions to antigens independently.

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The different types of adjuvants

Old and novel adjuvants are currently used in human and animal vaccination programs, as well as in experimental models, some of which are listed in this section.

Aluminum salts

Aluminum salt (alum) is an inorganic reagent that carries the potential to augment immunogenicity. Alum salts include alum phosphate and alum hydroxide, which are the most common adjuvants in human vaccines. The organic compound squalene (originally obtained from shark liver oil and a biochemical precursor to steroids) is sometimes added to the preparation.

Oil-based adjuvants

Oil-based adjuvants (e.g., Freund's adjuvant, pristine, etc.) are commonly found in some formulations of veterinary vaccines. Incomplete Freund's adjuvant (IFA) contains water-in-oil emulsion, while complete Freund's adjuvant (CFA) additionally contains killed mycobacteria. The mycobacteria added to the adjuvant attract macrophages and other cells to the injection site, which enhances the immune response. Thus, CFA is usually used for the primary vaccination, while the incomplete version is applied for boosting. Some novel oil-in-water emulsions are being developed by pharmaceutical companies, such as MF59 (Novartis), AS03 (GalxoSmithKline), Advax (Vaxine Pty), and Qs-21/ISCOMs (see further on).

Virosomes

During the last 2 decades, a variety of technologies have been investigated for their ability to

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improve the widely used alum adjuvants (Holzeret *et al.*, 1996), which may induce local inflammation. Thus, other novel adjuvants that can also be used as antigen-carrier systems, the virosomes, have been developed. Virosomes contain a membrane-bound hemagglutinin and neuraminidase derived from the influenza virus, both of which facilitate uptake into antigen-presenting cells (APCs) and mimic the natural immune response (Gluck, 1999).

Novel and experimental adjuvants

In the search for new and safer adjuvants, several new ones have been developed by pharmaceutical companies utilizing new immunological and chemical innovations.

Toll-like receptor-related adjuvants

IC31 is a two-component synthetic adjuvant that signals through toll-like receptor (TLR)-9. This novel adjuvant is tested as of 2008 in influenza vaccine combinations (Riedlet *et al.*, 2008). Four others, ASO4, ASO2A, CPG 7907, and GM-CSF, are investigated for highly relevant vaccines, such as those against papilloma virus, hepatitis B, and malaria (Pichichero, 2008). Other TLR-dependent adjuvant candidates are as yet only in clinical development, such as RC-529 and ISS, Flagellin and TLR-agonists. AS02 and AS04 are proprietary adjuvants of GlaxoSmithKline (GSK). AS02 contains MPL and QS-21 in an oil-in-water emulsion. AS04 combines MPL with alum. MPL is a series of 4′monophosphoryl lipid A that varies in the extent and position of fatty acid substitution. It is prepared from lipopolysaccharide (LPS) of *Salmonella minnesota* R595 by treating the LPS with mild acid and base hydrolysis, followed by purification of the modified LPS. Unmethylated CpG dinucleotides are the reason why bacterial DNA, but not vertebrate DNA, is immunostimulatory. Vertebrate DNA has relatively low amounts of unmethylated CpG compared to bacterial DNA. The adjuvant effect of CpG is enhanced when conjugated to protein antigens. CPG7909, an adjuvant developed by Coley Pharmaceuticals, has been tested in a few vaccines directed at infectious agents (such as Hepatitis B allergen: Creticos *et al.*, 2006) and tumor cells (Alexeevet *et al.*, 2008; Kirkwood *et al.*, 2009).

New formulated adjuvants

MF59 is a submicron oil-in-water emulsion of a squalene, polyoxyethylene sorbitan monooleate (Tween 80), and sorbitan trioleate. MF59 was approved in Europe and is found in several vaccines, including influenza. It has also been licensed to other companies and is being actively tested in vaccine trials. Other oil-in-water emulsions include Montanide (Seppic), adjuvant 65 (in use since the 1960s), and Lipovant. QS-21, a natural product of the bark of the *Quillaja saponaria* tree, which is native to Chile and Argentina, is currently under investigation (Ghochikyan, 2006). Immune-stimulating complexes (ISCOMs) are honeycomb-like structures composed mainly of *Quillaja saponins*, cholesterol, phospholipid, and antigen. Some ISCOMs are formed without antigen and then mixed with antigen, so that the antigen is absorbed on to or conjugated with the ISCOM. Specific isoforms of ADVAX, an adjuvant developed in Australia based on inulin (a natural plant-derived polysaccharide consisting of a chain of fructose molecules ending in a single glucose), are prepared and formulated into compositions suitable for use as adjuvants. A synergistic effect is obtained by combining gamma inulin with an antigen-binding material such as inulin; the product is called Algammulin.

Xenobiotic adjuvants (the natural adjuvants)

Some of the adjuvant properties of the bacterial walls of Gram-negative bacteria have been clearly attributed to the lipid A fraction of LPSs (Ulrich, 1995). Similarly, the xenobioitic muramyl dipeptide, shown to be the smallest peptidic moiety of bacteria cell walls, can replace mycobacteria in CFA (Bahr, 1986).

More recently, interest has been focused on another well-defined natural structure endowed with adjuvanticity: the bacterial DNA. Studies on bacterial DNA have shown that unmethylated CpG motifs displaying 5′ Pu-Pu-CpG-Pyr-Pyr 3′ (Pu: purine, A or G; Pyr: pyrimidine, C or T) nucleotide sequences are recognized by, and can activate, cells of the immune system (Krieget *et al.*, 1995). Such motifs allow the immune system to discriminate pathogen-derived foreign DNA from self-DNA. CpG motifs have been found to activate antigen-presenting cells, leading to upregulation of major histocompatibility complex (MHC) and costimulatory molecules, the secretion of proinflammatory cytokines (TNFα, IFNγ, IL1, IL6, IL12, and IL18), and the switching on of T helper 1 (Th1) immunity (Lipfordet *et al.*, 1997; Millan, 1998; Zimmerman, 1998).

Tuftsin autoadjuvant

Tuftsin is a physiological natural immunostimulating tetrapeptide (Thr-Lys-Pro-Arg), a fraction of the IgG heavy-chain molecule produced by enzymatic cleavage in the spleen. Tuftsin deficiency, either hereditary or following splenectomy, results in increased susceptibility to certain infections caused by capsulated organisms, such as *H. influenza*, *pneumococci*, and *meningococci* and *Salmonella*. Tuftsin, being a self-immunostimulating molecule, can be termed an "autoadjuvant" on the basis of its biological functions, which encompass the following:

1. Binding to receptors on neutrophils and macrophages, to stimulate their phagocytic activity. Tuftsin is able to increase the efficacy of antimicrobial agents. Tuftsin-based therapy was proven successful, by activity of a Gentamicin combined with tuftsin conjugate, in treating experimental keratitis caused by *Pseudomonas aeruginosa* and *Candida peritonis* infections in a murine model. Murine peritoneal macrophages activated by tuftsin killed the intracellular protozoan *Leishmania major*, as well. Moreover, the tuftsin derivative Thr-Lys-Pro-Arg-NH-(CH2)2-NHCOC15H31 protected mice against *Plasmodium berghei* infection. In human studies, tuftsin showed stimulation of the antimicrobial activity of blood monocyte macrophages in leprosy patients.

2. Increasing tumor necrosis factor alpha (TNFα) release from human Kupffer cells.

3. Enhancing secretion of IL1 by activating macrophages (Phillips *et al.*, 1981; Dagan *et al.*, 1987).

4. Interaction with macrophages, resulting in expression of nitric oxide (NO) synthase to produce NO (Dagan *et al.*, 1987).

5. Enhancement of murine natural cell-mediated cytotoxicity (Phillips *et al.*, 1981). Being a natural autoadjuvant small molecule, its implementation may include, in addition to antimicrobial and antifungal activities, the restoration of the innate immune system in immunocompromised hosts, such as AIDS (Fridkin *et al.*, 2005) and cancer (Khan *et al.*, 2007; Yuan *et al.*, 2012) patients. In addition, tuftsin may serve as a good adjuvant for a new generation of vaccines, with minimal or no side effects (Pawan *et al.*, 1994; Gokulan *et al.*, 1999; Wardowska *et al.*, 2009; Liu *et al.*, 2012).

Liu *et al.* (2012) introduced a novel vaccine against influenza A virus, based on a multimer of tuftsin with the extracellular domain of influenza A matrix protein 2 (M2e). Following animal studies, the tuftsin-M2e construct has been proposed as a promising candidate for a universal vaccine against influenza A virus. Assessing malaria vaccine, tuftsin was chemically linked to EENVEHDA and DDEHVEEPTVA repeat sequences of ring-infected erythrocyte surface-antigen protein (an asexual blood-stage antigen) of *Plasmodium falciparum*. Mice immunized with these synthetic constructs had higher antibody titers and better secondary immune responses and antigen-induced T cell proliferation than the peptide dimers alone. Thus, tuftsin-based synthetic conjugates were proposed to be useful for the development of malaria vaccines. In an additional trial, a fusion protein composed of antiidiotypic scFv antibodies mimicking CA125 and tuftsin manifested a number of biological activities, including activation of macrophages and stimulation of the T cell response against cancer (Yuan *et al.*, 2012). Another trial using a chimeric molecule composed of multimeric tuftsin and synthetic peptides of HIV gp41 and gp120 proteins was successful (Gokulan *et al.*, 1999). A significantly stronger immune response was observed in mice immunized with the peptide polytuftsin conjugates than in mice receiving the peptide dimers (peptide–peptide); therefore, this chimeric molecule was proposed as a future candidate for the treatment of AIDS patients.

Tuftsin autoadjuvant is an immunomodulator small molecule in some autoimmune diseases (Lukács *et al.*, 1984; Bhasin *et al.*, 2007; Wu *et al.*, 2012). Tufsin improved the clinical score of naive mice with experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG), a model commonly used for multiple sclerosis. During the progression of EAE, microglia, the immunocompetent cells of the brain, were activated; these accumulated around demyelinated lesions. Microglial activation is mediated by the extracellular protease tissue plasminogen activator (tPA). Successful treatment with tuftsin, a macrophage/microglial activator, revealed that the disease progression could be manipulated favorably in its early stages by altering the timing of microglial activation, which upregulates T helper 2 cells and inhibits disease progression. In systemic lupus erythematosus patients, an impairment in monocyte macrophage chemotaxis can be demonstrated *in vitro* and *in vivo*, in concert with defective phagocytic activity. Exposing defective, lupus-originated monocytes and macrophages *in vitro* to tuftsin resulted in improved chemotaxis similar to that of healthy individuals (Lukács *et al.*, 1984).

Mechanisms of adjuvanticity

Adjuvants accomplish their task by mimicking specific sets of evolutionarily conserved molecules, including liposomes, LPS, molecular cages for antigen, components of bacterial cell walls, and endocytosed nucleic acids, such as double-stranded RNA (dsRNA), single,stranded DNA (ssDNA), and unmethylated CpG dinucleotide-containing DNA. Because immune systems have evolved to recognize these specific antigenic moieties, the presence of adjuvant in conjunction with the vaccine can greatly increase the innate immune response to the antigen by augmenting the activities of dendritic cells (DCs), lymphocytes, and macrophages by mimicking a natural infection. Furthermore, because adjuvants are attenuated beyond any function of virulence, they have been thought to pose little or no independent threat to a host organism. But is this really true? Adjuvants may exert their immune-enhancing effects according to five immune functional activities, summarized in Table 1.1 (Schijns, 2000).

Adjuvants and the adaptive and innate immune response

In order to understand the links between the innate immune response and the adaptive immune response, in order to help substantiate an adjuvant function in enhancing adaptive immune responses to the specific antigen of a vaccine, the following points should be considered: innate immune-response cells such as DCs engulf pathogens through phagocytosis. DCs then migrate to the lymph nodes, where T cells (adaptive immune cells) wait for signals to trigger their activation (Bousso and Robey, 2003). In the lymph nodes, DCs process the engulfed pathogen and then express the pathogen clippings as antigen on their cell surface by coupling them to the MHC. T cells can then recognize these clippings and undergo a cellular transformation, resulting in their own activation (Mempelet *et al.*, 2004). Macrophages can also activate T cells, in a similar manner. This process, carried out by both DCs and macrophages, is termed "antigen presentation" and represents a physical link between the innate and adaptive immune responses. Upon activation, mast cells release heparin and histamine to effectively increase trafficking and seal off the site of infection, allowing immune cells of both systems to clear the area of pathogens. In addition, mast cells also release chemokines, resulting in a positive chemotaxis of other immune cells of both the innate and adaptive immune responses to the infected area (Kashiwakura *et al.*, 2004). Due to the variety of mechanisms and links between the innate and adaptive immune responses, an adjuvant enhanced innate immune response results in an enhanced adaptive immune response.

Adjuvants and TLRs

The ability of the immune system to recognize molecules that are broadly shared by pathogens

Table 1.1 Adjuvants exert their immunological effect by different modes of action. Schijns, V. E. Immunological concepts of vaccine adjuvant activity. *Curr Opin Immunol* 12(4): 456–63. Copyright © 2000, Elsevier

No.	Mode of action	Immunological effect
1	Translocation of antigens to the lymph nodes, where they can be recognized by T cells	Greater T cell activity, heightened clearance of pathogen throughout the organism
2	Protection to antigens, granting a prolonged delivery and longer exposure	Upregulation of the production of the B and T cells necessary for greater immunological memory in the adaptive immune response
3	Increased capacity to cause local reactions at the injection site	Greater release of danger signals by chemokine-releasing cells such as helper T cells and mast cells
4	Induction of the release of inflammatory cytokines	Recruitment of B and T cells at sites of infection and increasing transcriptional events, leading to a net increase of immune cells as a whole
5	Interaction with pattern-recognition receptors (PRRs) (specifically, Toll-like receptors, TLRs) on accessory cells	Increased innate immune response to antigen

is due, in part, to the presence of special immune receptors called TLRs that are expressed on leukocyte membranes. TLRs were first discovered in Drosophila and are membrane-bound pattern-recognition receptors (PRRs) responsible for detecting most (although certainly not all) antigen-mediated infections (Beutler, 2004). In fact, some studies have shown that in the absence of TLRs, leukocytes become unresponsive to some microbial components, such as LPS (Poltoraket *et al.*, 1998). There are at least 13 different forms of TLR, each with its own characteristic ligand. Prevailing TLR ligands described to date (all of which elicit adjuvant effects) include many evolutionarily conserved molecules, such as LPSs, lipoproteins, lipopeptides, flagellin, double-stranded RNA, unmethylated CpG islands, and various other forms of DNA and RNA classically released by bacteria and viruses. The binding of ligand, either in the form of adjuvant used in vaccinations or in the form of invasive moieties during times of natural infection, to the TLR marks the key molecular event that ultimately leads to innate immune responses and the development of antigen-specific acquired immunity (Takeda and Akira, 2005). The very fact that TLR activation leads to adaptive immune responses to foreign entities explains why so many adjuvants used today in vaccinations are developed to mimic TLR ligands.

It is believed that upon activation, TLRs recruit adapter proteins within the cytosol of the immune cell in order to propagate the antigen-induced signal-transduction pathway. To date, four adapter proteins have been well characterized: MyD88, Trif, Tram, and Tirap (also called "Mal") (Shizuo, 2003). These recruited proteins are responsible for the subsequent activation of other downstream proteins, including protein kinases (IKKi, IRAK1, IRAK4, and TBK1), which further amplify the signal and ultimately lead to the upregulation or suppression of genes that orchestrate inflammatory responses and other transcriptional events. Some of these events lead to cytokine production, proliferation, and survival, while others lead to greater adaptive immunity. MyD88 is essential for inflammatory cytokine production in response to all TLR ligands, except the TLR3 ligand. TIRAP/Mal is essential for TLR2- and TLR4-dependent inflammatory cytokine production but is not involved in the MyD88-independent TLR4 signaling pathway. TRIF is essential for TLR3 signaling, as well as the MyD88-independent TLR4 signaling pathway.

Mechanisms of adjuvant adverse effects

The mechanisms underlying adjuvant adverse effects are under renewed scrutiny because of their enormous implications for vaccine development. Additionally, new, low-toxicity adjuvants are being sought, to enhance vaccine formulations. Muramyl dipeptide (MDP) is a component of the peptidoglycan polymer and has been shown to be an active but low-toxicity component of CFA, a powerful adjuvant composed of mycobacteria lysates in an oil emulsion. MDP activates cells primarily via the cytosolic nucleotide binding domain and Leucine-rich repeat-containing family (NLR) member Nod2 (nucleotide binding oligomerization domain containing 2), and is therefore linked to the ability of adjuvants to enhance antibody production. Moreira *et al.* (2008) tested the adjuvant properties of the MDP-Nod2 pathway and found that MDP, compared to the TLR agonist LPS, has minimal adjuvant properties for antibody production under a variety of immunization conditions. They also observed that the oil emulsion IFA supplemented the requirements for the TLR pathway, independent of the antigen. Nod2 was required for an optimal IgG1 and IgG2c response in the absence of exogenous TLR or NLR agonists. By combining microarray and immunofluorescence analysis, Mosca *et al.* (2008) monitored the effects of the adjuvants MF59 oil-in-water emulsion, CpG, and alum in the mouse muscle. MF59 induced a time-dependent change in the expression of 891 genes, whereas CpG and alum regulated 387 and 312 genes, respectively. All adjuvants modulated a common set of 168 genes and promoted antigen-presenting cell recruitment. MF59 was the stronger inducer of cytokines, cytokine receptors, adhesion molecules involved in leukocyte migration, and antigen-presentation genes. In addition, MF59 triggered a more rapid influx of $CD11b^+$ blood cells compared with other adjuvants. The authors proposed that oil-in-water emulsions are the most efficient human vaccine adjuvants, because they induce an early and strong immunocompetent environment at the injection site by targeting muscle cells. Emerging data suggest that alum phosphate and alum hydroxide adjuvants do not promote a strong commitment to the helper T cell type 2 (Th2) pathway when they are coadministered with some Th1 adjuvants. Iglesias *et al.* (2006) have shown that subcutaneous immunization, in alum phosphate, of a mixture comprising three antigens (the surface and core antigens of hepatitis B virus (HBV) and the multiepitopic protein CR3 of human immunodeficiency

virus type 1) elicits a CR3-specific Th1 immune response. Although alum is known to induce the production of proinflammatory cytokines *in vitro*, it has been repeatedly demonstrated that it does not require intact TLR signaling to activate the immune system. This was suggested by Gavin *et al.* (2006), who reported that mice deficient in the critical signaling components for TLR mount robust antibody responses to T cell-dependent antigen given in four typical adjuvants: alum, CFA, IFA, and monophosphoryl lipid A/trehalose dicorynomycolate adjuvant. They concluded that TLR signaling does not account for the action of classical adjuvants and does not fully explain the action of a strong adjuvant containing a TLR ligand. Eisenbarth *et al.* (2008) showed that alum adjuvants activated the intracellular innate immune response system, the Nalp3 (also known as cryopyrin, CIAS1, or NLRP3) inflammasome. Production of the proinflammatory cytokines IL-1 and IL-18 by macrophages in response to alum *in vitro* required intact inflammasome signaling. Furthermore, *in vivo*, mice deficient in Nalp3, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), or caspase1 failed to mount a significant antibody response to an antigen administered with alum adjuvants, whereas the response to CFA remained intact. The authors identified the Nalp3 inflammasome as a crucial element in the adjuvant effect of alum adjuvants; in addition, they showed that the innate inflammasome pathway can direct a humoral adaptive immune response. Recently, Kool *et al.* (2008) succeeded in exposing an angle of its mysterious mechanism: they found that alum activates DCs *in vivo* by provoking the secretion of uric acid, a molecule that is triggered by tissue and cell trauma. The injection of alum induced an influx of neutrophils and inflammatory cytokines and chemokines, a combination that had previously been seen in response to the injection of uric acid into mice. In mice injected with a mixture of antigens, ovalbumin peptide, and alum, uric acid levels increased within hours. The uric acid may have been released by the cells lining the body's cavities, which turn necrotic after contacting the alum. In response to the uric acid, inflammatory monocytes flocked to the injection site, took up the antigens, and broke them down into T cell-stimulating epitopes. The monocytes then migrated to lymph nodes, where they matured into DCs and activated $CD4^+$ T cells. Without alum, the antigens were not taken up at the injection site. Still, they eventually reached the lymph nodes via the flowing lymph. The resident node DCs, however, did not efficiently process the alum-free antigens or express T cell co-stimulating receptors. The resulting subdued immunity was similar to that seen in mice that were depleted of inflammatory monocytes or injected with enzymes that degrade uric acid. These findings suggest that alum is immunogenic through exploitation of "nature's adjuvant," via induction of the endogenous danger signal: **uric acid**. In another study, Kool *et al.* (2008) showed that alum adjuvant induced the release of IL1β from macrophages and DCs, and that this is abrogated in cells lacking various NALP3 inflammasome components. The NALP3 inflammasome is also required *in vivo* for the innate immune response to ovalbumin in alum. The early production of IL1β and the influx of inflammatory cells into the peritoneal cavity is strongly reduced in NALP3-deficient mice. The activation of adaptive cellular immunity to ovalbumin-alum is initiated by monocytic DC precursors, which induce the expansion of antigen-specific T cells in a NALP3-dependent way. The authors proposed that, in addition to TLR stimulators, agonists of the NALP3 inflammasome should also be considered vaccine adjuvants. Flach *et al.* (2011) reported that, independent of inflammasome and membrane proteins, alum binds DC plasma membrane lipids with substantial force. Subsequent lipid sorting activates an abortive phagocytic response, which leads to antigen uptake. Such activated DCs, without further association with alum, show high affinity and stable binding with $CD4^+$ T cells via the adhesion molecules intercellular adhesion molecule 1 (ICAM1) and lymphocyte function-associated antigen 1 (LFA1). The authors proposed that alum triggers DC responses by altering membrane lipid structures. This study therefore suggests an unexpected mechanism for how this crystalline structure interacts with the immune system and how the DC plasma membrane may behave as a general sensor for solid structures. Marichal *et al.* (2011) reported that, in mice, alum caused cell death and the subsequent release of host-cell DNA, which acted as a potent endogenous immunostimulatory signal, mediating alum adjuvant activity. Furthermore, the authors proposed that host DNA signaling differentially regulated IgE and IgG1 production following alum-adjuvanted immunization. They suggested that, on the one hand, host DNA induces primary B cell responses, including IgG1 production, through interferon response factor 3 (Irf3)-independent mechanisms, but that, on the other, host DNA may also stimulate "canonical"

T helper type 2 (Th2) responses, associated with IgE isotype switching and peripheral effector responses, through Irf3-dependent mechanisms. The finding that host DNA released from dying cells acts as a damage-associated molecular pattern that mediates alum adjuvant activity may increase our understanding of the mechanisms of action of current vaccines and help in the design of new adjuvants.

Compiling all the evidence concerning alum's mechanism of action, it seems that alum may play a role in a few parallel and alternative pathways: through the inflammasome, by causing inflammation either directly or by uric acid; by binding DC plasma membrane lipids with substantial force and activating an abortive phagocytic response that leads to antigen uptake; or by causing cell death and the subsequent release of host-cell DNA, which acts as a potent endogenous immunostimulatory signal.

Autoimmunity and environmental/natural adjuvants

Genetic, immunological, hormonal, and environmental factors (i.e., infections, vaccines, xenobiotics, etc.) are considered to be important in the etiology of autoimmunity. Overt autoimmune disease is usually triggered following exposure to such environmental factors, among which infectious agents are considered of great importance (Molina and Shoenfeld, 2005). Some researchers consider adjuvants to be environmental factors involved in autoimmune diseases. Several laboratories are pursuing the molecular identification of endogenous adjuvants. Among those identified so far, sodium monourate and the high-mobility group B1 protein (HMGB1) are well known to rheumatologists. However, even the complementation of apoptotic cells with potent adjuvant signals fails to cause clinical autoimmunity in most strains: autoantibodies generated are transient, do not undergo epitope/spreading, and do not cause disease. Lastly, as vaccines may protect or cure autoimmune diseases, adjuvants may also play a double role in the mechanisms of these diseases. Myasthenia gravis (MG) and its animal model, experimental autoimmune gravis (EAMG), are caused by interference with neuromuscular transmission by autoantibodies against the nicotinic acetylcholine receptor (AChR) on muscle. Two peptides, denoted RhCA 67-16 and RhCA 611-001 and designed to be complementary in structure to the main immunogenic region and the dominant Lewis rat T cell epitope (α-chain residues 100–116) of the AChR, respectively, are effective vaccines that prevent EAMG in rats by inducing antiidiotypic/clonotypic antibodies (Ab) and lowering levels of AChR Ab. Their study employed keyhole limpet hemocyanin (KLH) as a carrier and the CFA. In advance of a clinical trial, McAnally *et al.* (2001) tested the efficacy of RhCA 611-001 when combined with different adjuvants approved for use in humans: IFA and alum hydroxide. As a second goal, the authors evaluated diphtheria toxin (DT) as an alternative carrier protein to KLH. Alum was found to be an effective adjuvant, particularly when used with the peptide conjugated to DT. This combination of carrier and adjuvant provided protection against EAMG comparable with that observed with CFA and KLH. It was found that disease protection is qualitatively, but not quantitatively, related to the antipeptide antibody response. This work demonstrated a vaccine formulation that should be useful in the first soon-to-be-conducted clinical trials of peptide vaccines to specifically correct aberrant T and B cell responses in an autoimmune disease.

Adjuvant-related diseases

Alongside their supportive role, adjuvants have themselves been found to inflict illnesses of autoimmune nature, termed "the adjuvant diseases" (Agmon-Levin, 2008).

Mineral oils as a cause of autoimmunity

Mineral oils are generally considered "nontoxic" and have been used extensively in food, cosmetics, medicines, and other products. Subcutaneous injection of mineral oil induces sclerosing lipogranulomas, a chronic local inflammatory reaction (Di Benedetto *et al.*, 2002). The oil is absorbed through the intestine and distributes throughout the body, causing lipogranulomas in the lymph nodes, liver, and spleen of healthy individuals. Oral or intraperitoneal administration of mineral oil induces similar lesions in laboratory animals. Pristane (2,6,10,14-tetramethylpentadecane) and mineral oil induce plasmacytomas in susceptible strains of mice (Anderson and Potter, 1969). Pristane, IFA, and squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) induce chronic arthritis in mice and rats (Cannon *et al.*, 1993; Carlson *et al.*, 2000). Reeves and colleagues reported that, in addition to pristane (Satoh and Reeves 1994; Satoh *et al.*, 1995), IFA and squalene,

but not medicinal mineral oils, can induce lupus-related anti-nRNP/Sm and Su autoantibodies in nonautoimmune-prone strains of mice. These data suggest that hydrocarbons can have a variety of immune effects. Kuroda *et al.* (2004) investigated whether medicinal mineral oils can induce other types of autoantibodies and whether structural features of hydrocarbons influence autoantibody specificity. Induction of autoantibodies by mineral oils considered nontoxic also may have pathogenetic implications in human autoimmune diseases. Moreover, Kuroda *et al.* (2004) have reported that a single intraperitoneal injection of the adjuvant oils pristane, IFA, or squalene induces lupus-related autoantibodies to nRNP/Sm and Su in nonautoimmune BALB/c mice. Induction of these autoantibodies appears to be associated with the hydrocarbon's ability to induce IL-12, IL-6, and TNFα, suggesting a relationship with hydrocarbon's adjuvanticity. Whether this is relevant in human vaccination is a difficult question, due to the complex effects of vaccines and the fact that immunotoxicological effects vary depending on species, route, dose, and duration of administration. Nevertheless, the potential of adjuvant hydrocarbon oils to induce autoimmunity has implications in the use of oil adjuvants in human and veterinary vaccines, as well as in basic research (Table 1.2).

Human adjuvant disease, silicone as an adjuvant, and connective tissue diseases

Spiera *et al.* (1994) reviewed the literature examining the association of silicone gel-filled implants with connective tissue disease. They stated that silicones are not biologically inert. Injectable and implantable silicones have proven capable of eliciting inflammatory and fibroproliferative responses. The physical and biological properties of silicone gel-filled implants and their behavior *in vivo* are compatible with the hypothesis that they may contribute to the development of connective-tissue disease. The association seems most likely with scleroderma; however, there are currently inadequate epidemiological data to definitively establish causality. Janowsky *et al.* (2000) performed a meta-analysis of the relation between silicone breast implants and the risk of connective tissue diseases. There was no evidence that breast implants were associated with a significant increase in the adjusted relative risk of individual connective tissue diseases. Nor was there evidence of significantly increased risk in the unadjusted analyses or in the analysis restricted to silicone gel-filled implants. Vasey *et al.* (2003) proposed a definition for a silicone-related disorder, by major and minor criteria: tenderness, capsule formation, change in shape or position, and/or rapture of envelope; chronic fatigue lasting

Table 1.2 Adjuvant involvement in autoimmune manifestation

Adjuvant	Manifestations/disease/Ab	Species	References
MDP, LPS, Gram + CoxackieB3,IL1β,TNF	Experimental thyroiditis; Myocarditis	Mice	Rose (2008)
Mineral oils	Sclerosing lipogranulomas	Mice human?	Di Benedetto *et al.* (2002)
Pristane, mineral oils	Plasmacytomas	Mice	Anderson and Potter (1969)
Pristane, squalene, IFA	Chronic arthritis	Mice, rats	Cannon *et al.* (1993), Carlson *et al.* (2000)
Pristane, squalene, IFA	Lupus-related anti-nRNP/Sm /Su antibodies	Mice	Satoh and Reeves (1994), Satoh *et al.* (1995)
Pristane, squalene, IFA, mineral oils	Anticytoplasmic Ab, anti-ssDNA/chromatin Ab	Mice	Kuroda *et al.* (2004)
Pristane, squalene, IFA	Lupus-related anti-nRNP/Sm /Su antibodies	Mice	Kuroda *et al.* (2004)
Silicone	Human adjuvant disease, connective tissue diseases	Human	Hennekens *et al.* (1996)
Silicone	Scleroderma, SLE, RA	Human	Spiera *et al.* (1994)
Alum in vaccines (HBV,HAV, tetanus)	MS, chronic fatigue syndrome, polymyalgia rheumatica	Human	Gherardi (2003)
Aluminum hydroxide, squalene	Gulf War syndrome, antibodies to squalene	Human	Asa *et al.* (2000)

6 months, myalgias with tender muscles; bladder dysfunction, dry eyes or mouth, impaired cognition, and a few more symptoms.

Macrophagic myofasciitis and Gulf War syndrome

Macrophagic myofasciitis was first reported in 1998 but its cause remained obscure until 2001 (Gherardi, 2003). The condition manifests by diffuse myalgias and chronic fatigue, forming a syndrome that meets both Centers for Disease Control and Prevention (CDC) and Oxford criteria for the so-called "chronic fatigue syndrome" in about half of patients. One-third of patients develop an autoimmune disease, such as multiple sclerosis. Electron microscopy, microanalytical studies, experimental procedures, and an epidemiological study recently demonstrated that the lesion results from persistence for years at the site of injection of an alum adjuvant used in vaccines against hepatitis B virus, hepatitis A virus, and tetanus toxoid. Alum hydroxide is known to potently stimulate the immune system and to shift immune responses toward a Th2 profile. Interestingly, special emphasis has been put on Th2-biased immune responses as a possible explanation of chronic fatigue and associated manifestations known as the "Gulf War syndrome" (GWS). Results concerning macrophagic myofasciitis may well open new avenues for etiologic investigation of this syndrome. Indeed, both the type and the structure of symptoms are strikingly similar in Gulf War veterans and patients with macrophagic myofasciitis. Multiple vaccinations performed over a short period of time in the Persian Gulf area have been recognized as the main risk factor for GWS. Moreover, the war vaccine against anthrax, which is administered in a six-shot regimen and seems to be crucially involved, is adjuvanted by alum hydroxide and, possibly, squalene, another Th2 adjuvant. Asa *et al.* (2000) sought to determine whether the presence of antibodies to squalene correlates with the presence of signs and symptoms of GWS. All (100%) GWS patients immunized for service in Desert Shield/Desert Storm who did not deploy but had the same signs and symptoms as those who did had antibodies to squalene. In contrast, no (O%) deployed Persian Gulf veterans not showing signs and symptoms of GWS had antibodies to squalene. Neither patients with idiopathic autoimmune disease nor healthy controls had detectable serum antibodies to squalene. If safety concerns about the long-term effects of alum hydroxide are confirmed, it will become mandatory to propose novel and alternative vaccine adjuvants in order to rescue vaccine-based strategies and the enormous benefit for public health they provide worldwide.

Autoimmune (autoinflammatory) syndrome induced by adjuvants – ASIA

Siliconosis, GWS, macrophagic myofasciitis (MMF) syndrome, and post-vaccination phenomena have all been linked with previous exposure to an adjuvant. Furthermore, these four diseases share a similar complex of signs and symptoms, which further support a common denominator. Shoenfeld and Agmon–Levin (2011) recently suggested that these four somehow enigmatic conditions should be included under a common syndrome entitled the "autoimmune (auto-inflammatory) syndrome induced by adjuvants" (ASIA). The authors further proposed several major and minor criteria, which, although they require further validation, may aid in the diagnosis of this newly defined syndrome. Recently, the sick building syndrome was also suggested as part of ASIA (Israeli and Pardo, 2011). Comparison of the clinical manifestations, symptoms, and signs of the four conditions described by Shoenfeld and Agmon-Levin (2011) with those described for SBS shows that nine out of ten main symptoms are in correlation in all five conditions: namely, myalgia, arthralgias, chronic fatigue, neurological cognitive impairment, fever, gastrointestinal symptoms, respiratory symptoms, skin manifestations, and appearance of autoantibodies. Thus, ASIA may be a common syndrome for all five conditions mentioned. The amassed data regarding each condition may enable a new view of the immune responses to environmental adjuvants, as well as a better definition and diagnosis of these conditions. Moreover, unraveling the pathogenesis of this newly defined syndrome may facilitate the search for preventive and therapeutic interventions.

Conclusions

Due to the adverse effects exerted by adjuvants, there is no controversy over the need for safer adjuvants for incorporation into future vaccines.

The problem with the pure recombinant or synthetic antigens used in modern-day vaccines is that they are generally far less immunogenic than older-style live or killed whole-organism vaccines. This has created a major need for improved and more powerful adjuvants for use

Table 1.3 Adjuvants in human vaccines. Reed, S. G., S. Bertholet, et al. New horizons in adjuvants for vaccine development. *Trends Immunol* 30(1): 23–32. Copyright © 2009, Elsevier

Adjuvants	Human vaccines
Alum	DPT, DT, HBV, HAV, H. influenza B, inactivated polio, strep. pneumonia, HPV, meningococcal
Oil and water-MF59	Herpes simplex, HBV, HIV
MPL AS04/AS01B/AS02A	HBV,HAV,HPV, malaria, tuberculosis, leishmania, HIV, vesicular stomatitis
Virosomes-VLP / IRIV	HBV,HPV/HAV
Cholera toxin B subunit	Cholera

DPT, diphtheria–pertussis–tetanus; DT, diphtheria–tetanus; HAV, hepatitis A; HBV, hepatitis B; HIV, human immunodeficiency virus; HPV, human papilloma virus

Table 1.4 Adjuvants in development

Adjuvants	Formulation	Preclinical or clinical trials
Montanides	Water-in-oil emulsions	Malaria (Phase I), HIV, cancer (Phase I/II)
Saponins (QS-21)	Aqueous	Cancer (Phase II), herpes (Phase I), HIV (Phase I)
SAF	Oil-in-water emulsion containing squalene, Tween 80, Pluronic L121	HIV (Phase I – Chiron)
AS03	Oil-in-water emulsion containing α-tocopherol, squalene, Tween 80	Pandemic flu (GSK)
MTP-PtdEtn	Oil-in-water emulsion	HSV
Exotoxins	*P. aeruginosa*	*P. aeruginosa*, cystic fibrosis (AERUGEN – Crucell/Berna)
	E. coli heat-labile enterotoxin LT	ETEC (Phase II – Iomai Corp.)
ISCOMs	Phospholipids, cholesterol, QS-21	Influenza, HSV, HIV, HBV, malaria, cancer
TLR ligands		
MPL®-SE	Oil-in-water emulsion	Leishmania (Phase I/II – IDRI)
Synthetic Lipid A	Oil-in-water emulsion	Various indications (Avanti/IDRI)
MPL®-AF	Aqueous	Allergy (ATL), cancer (Biomira)
AS01	Liposomal	HIV (Phase I), malaria (ASO1, Phase III, GSK), cancer (Phase II/III, Biomira/MerckKGaA)
AS02	Oil-in-water emulsion containing MPL and QS-21	HPV (Cervarix), HIV, tuberculosis, malaria (Phase III), herpes (GSK)
AS04	Alum + aqueous MPL	HPV, HAV (GSK)
AS15	AS01 + CpG	Cancer therapy (GSK)
RC529	Aqueous	HBV, pneumovax

in these vaccines (Petrovsky and Aguilar, 2004). With few exceptions, alum remains the major adjuvant approved for human use in the majority of countries worldwide. Although alum is able to induce a good antibody (Th2) response, it has little capacity to stimulate cellular (Th1) immune responses, which are so important for protection against many pathogens. In addition, alum has the potential to cause severe local and systemic side effects, including sterile abscesses, eosinophilia, and myofasciitis, although, fortunately, most of the more serious side effects are relatively rare. Consequently, there is a major unmet need for safer and more effective adjuvants suitable for human use. In particular, there is demand for safe and nontoxic adjuvants capable of stimulating cellular (Th1) immunity. Several other adjuvants besides alum have been approved to date for use in human vaccines, among them MF59 in some viral vaccines, MPL, AS04, As01B and AS02A against viral and parasitic infections, virosomes for HBV, HPV, and HAV, and cholera toxin for cholera (Table 1.3) (Reed *et al.*, 2009).

Other needs in light of new vaccine technologies are adjuvants suitable for use with mucosally delivered vaccines, DNA vaccines, cancer, and

autoimmunity vaccines. Each of these areas is highly specialized, with its own unique needs with respect to suitable adjuvant technology.

Although controversial, the high sensitivity of TLR for microbial ligands is what makes adjuvants that mimic TLR ligands such a prime candidate for enhancing the overall effects of antigen-specific vaccinations on immunological memory. The expression of TLRs is vast, as they are found on the cell membranes of innate immune cells (DCs, macrophages, natural killer cells), cells of the adaptive immunity (T and B lymphocytes), and nonimmune cells (epithelial cells). This further substantiates the importance of administering vaccines with adjuvants in the form of TLR ligands, as they will be capable of eliciting their positive effects across the entire spectrum of innate and adaptive immunity. Nevertheless, there are certainly adjuvants whose immune stimulatory function completely bypasses the putative requisite for TLR signaling (Table 1.4). In short, all TLR ligands are adjuvants but not all adjuvants are TLR ligands. We can conclude that there are, in all likelihood, other receptors besides TLRs that have not yet been characterized, opening a field of future research. Perhaps future adjuvants occupying these putative receptors will be employed to bypass the TLR signaling pathway completely, in order to circumvent common side effects of adjuvant-activated TLRs, such as local inflammation and the general malaise felt because of the costly whole-body immune response to antigen. Surely, such issues will be the subject of much debate for future researchers.

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