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SPECIAL ARTICLE

Adjuvants and autoimmunity

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Some adjuvants may exert adverse effects upon injection or, on the other hand, may not trigger a full immunological reaction. The mechanisms underlying adjuvant adverse effects are under renewed scrutiny because of the enormous implications for vaccine development. In the search for new and safer adjuvants, several new adjuvants were developed by pharmaceutical companies utilizing new immunological and chemical innovations. The ability of the immune system to recognize molecules that are broadly shared by pathogens is, in part, due to the presence of special immune receptors called toll-like receptors (TLRs) that are expressed on leukocyte membranes. 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. Alongside their supportive role, adjuvants were found to inflict by themselves an illness of autoimmune nature, defined as 'the adjuvant diseases'. The debatable question of silicone as an adjuvant and connective tissue diseases, as well as the Gulf War syndrome and macrophagic myofaciitis which followed multiple injections of aluminium-based vaccines, are presented here. Owing to the adverse effects exerted by adjuvants, there is no doubt that safer adjuvants need to be developed and incorporated into future vaccines. Other needs in light of new vaccine technologies are adjuvants suitable for use with mucosally delivered vaccines, DNA vaccines, cancer and autoimmunity vaccines. In particular, there is demand for safe and non-toxic adjuvants able to stimulate cellular (Th1) immunity. More adjuvants were approved to date besides alum for human vaccines, including 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. Perhaps future adjuvants occupying other 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. *Lupus* (2009) **18,** 1217–1225.

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Introduction

Since 1789, when Edward Jenner inoculated his son with swine pox, vaccines have produced the best return to humanity from the medical and economical point of view. Commonly used vaccines are a cost-effective and preventive way of promoting health, compared with the treatment of acute or chronic disease. For example, in the US during the year 2001, routine immunizations saved over US $40 billion per birth-year.

However, not all vaccines are efficient and easy to administer, such as the vaccine against small pox (Vaccinia). Usually, upon injection of a pure antigen, the antigen is not taken up at the injection site, and an immunological reaction fails to be launched. In order to help the immune system to recognize the antigen adjuvants are added to the antigens in the process of developing and producing vaccines.

Definition of adjuvant

The word 'adjuvant' comes from the Latin word *adjuvare*, meaning to help or aid. An immunologic

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adjuvant is defined as 'any substance that acts to accelerate, prolong, or enhance antigen-specific immune responses'. In immunology, an adjuvant, as defined by the National Cancer Institute, is an agent that may stimulate the immune system and increase the response to a vaccine, without having any specific antigenic effect in itself.

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 below.

Aluminium salts

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

Oil-based adjuvants

Oil-based adjuvants (i.e. Freund's adjuvant, pristane, etc.) are commonly found in some formulations of veterinary vaccines. Incomplete Freund's adjuvant (IFA) contains water in oil emulsion, and complete Freund's adjuvant (CFA) also contains killed mycobacteria. The mycobacteria, added to the adjuvant, attracts macrophages and other cells to the injection site, which enhances the immune response, and 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, including MF59 (Novartis), AS03 (GlaxoSmithKline [GSK]), Advax (Vaxine Pty) and QS-21/ISCOMs (as described in the following).

Virosomes

During the last two decades, a variety of technologies have been investigated to improve the widely used alum adjuvants,[1] which have been known to induce local inflammation. Therefore, other novel adjuvants, which can also be used as antigen carrier systems, were developed: the virosomes. Virosomes contain a membrane-bound hemagglutinin and neuraminidase derived from the influenza virus, which both facilitates the uptake into antigen presenting cells (APCs) and mimics the natural immune response.[2]

Novel and experimental adjuvants

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

Toll like receptor-related adjuvants

IC31 is a two-component synthetic adjuvant, signaling through toll-like receptor 9 (TLR-9). This novel adjuvant has been tested as of 2008 in an influenza vaccine combination.[3] Four others, ASO4, ASO2A, CPG 7907 and GM-CSF, have been investigated for highly relevant vaccines such as those against papilloma virus, hepatitis B and malaria.[4] Other TLR-dependent adjuvant candidates are only in clinical development, including RC-529 and ISS, flagellin and TLR agonists.

AS02 and AS04 are proprietary adjuvants of GSK. AS02 contains MPL^{TM} (4′-Mono-Phosphoryl-Lipid A) and QS-21 in an oil-in-water emulsion. AS04 combines MPL with alum.

Unmethylated CpG dinucleotides are the reason that bacterial DNA, but not vertebrate DNA, is immunostimulatory. CPG7909, an adjuvant developed by Coley Pharmaceuticals, has been tested in a few vaccines directed at infectious agents (i.e. hepatitis B), allergens[5] and tumor cells.[6,7]

New formulated adjuvants

MF59 is a sub-micrometer oil-in-water emulsion of a squalene, polyoxyethylene sorbitan monooleate (TweenTM 80) and sorbitan trioleate. MF59 was approved in Europe and is found in several vaccines, such as the influenza vaccine. Other oil-in-water emulsions include Montanide (Seppic), Adjuvant 65 (in use since the 1960s), and Lipovant.

QS-21 is a natural product of the bark of the *Quillaja saponaria* tree species, which is native to Chile and Argentina. This adjuvant is currently under investigation.[8]

ISCOMs is an acronym for immune stimulating complexes. ISCOMs are honeycomb-like structures composed mainly of Quillaja saponins, cholesterol, phospholipid and antigen.

ADVAX is 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.

Table 1 Adjuvants exert their immunological effect by different modes of action[15]

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 which grants the antigen a prolonged delivery and exposure to the antigen for a longer duration.	Upregulating the production of B- and T-cells needed for greater immunological memory in the adaptive immune response.
3	Increase the 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	Induce the release of inflammatory cytokines.	Recruit B- and T-cells at sites of infection and increasing transcriptional events leading to a net increase of immune cells as a whole.
5	Interacting with pattern recognition receptors (PRRs), specifically toll-like receptors (TLRs), on accessory cells.	Increase the innate immune response to antigen.

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 lipopolysaccharides (LPSs).[9] Similarly, the xenobiotic muramyl-dipeptide shown to be the smallest peptidic moiety of bacteria cell walls can replace mycobacteria in CFA.[10]

Studies on bacterial DNA have shown that unmethylated CpG motifs are recognized by, and can activate, cells of the immune system.[11] Such motifs allow the immune system to discriminate between pathogen-derived foreign DNA and self-DNA. CpG motifs were found to activate APCs leading to upregulation of major histocompatibility complex (MHC) and co-stimulatory molecules, and to the secretion of proinflammatory cytokines (i.e. TNF-α, IFN-γ, IL-1, IL-6, IL-12 and IL-18) and switching on T-helper 1 (Th1) immunity.[12–14]

Mechanisms of adjuvancy

Adjuvants accomplish this task by mimicking specific sets of evolutionarily conserved molecules which include liposomes, LPSs, 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. As 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 were thought to pose little or no independent threat to a host organism. However, is this really the case? Adjuvants may exert their immune-enhancing effects according to five immune-functional activities as summarized in Table 1.[15]

Adjuvants and the adaptive and innate immune response

In order to understand the links between the innate immune response and the adaptive immune response 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.[16] In the lymph nodes, DCs mince the engulfed pathogen and then express the pathogen clippings as antigens on their cell surface by coupling them to a special receptor known as a MHC. T-cells can then recognize these clippings and undergo a cellular transformation resulting in its own activation.[17] Macrophages can also activate T-cells using a similar approach. 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 to and seal off the site of infection to allow immune cells of both systems to clear the area of pathogens. In addition, mast cells also release chemokines which result in a positive chemotaxis of other immune cells of both the innate and adaptive immune responses to the infected area.[18] Owing to the variety of mechanisms and links between the innate and adaptive immune response, 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 is, in part, due 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.[19] In fact, some studies have shown that in the absence of TLRs, leukocytes become unresponsive to some microbial components such as LPSs.[20] There are at least 13 different forms of TLRs 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, dsRNA, 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.[21] 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. These proteins are known as MyD88, Trif, Tram and Tirap (also called Mal).[22] These recruited proteins are then responsible for the subsequent activation of other downstream proteins, including protein kinases (IKKi, IRAK1, IRAK4 and TBK1) that 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 for 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.

Mechanism of adjuvants effects

The mechanisms underlying adjuvant adverse effects are under renewed scrutiny because of the enormous implications for vaccine development. In addition, new low-toxicity adjuvants are sought to enhance vaccine formulations. Muramyl dipeptide (MDP) is a component of the peptidoglycan polymer and was 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.*[23] tested the adjuvant properties of the MDP–Nod2 pathway. They found that MDP, compared with 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.*[24] 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 aluminium phosphate and aluminium hydroxide adjuvants do not promote a strong commitment to the Th2 pathway when they are co-administered with some

Th1 adjuvants. Iglesias *et al*.[25] have shown that subcutaneous immunization, in aluminium 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 or aluminium is known to induce the production of proinflammatory cytokines *in vitro*, it has been repeatedly demonstrated that aluminium does not require intact TLR signaling to activate the immune system. This fact was suggested by Gavin *et al*.[26] 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*.[27] show that aluminium adjuvants activate the intracellular innate immune response system, the Nalp3 (also known as cryopyrin, CIAS1 or NLRP3) inflammasome. Production of the proinflammatory cytokines interleukin-1 and interleukin-18 by macrophages in response to aluminium *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 caspase-1 failed to mount a significant antibody response to an antigen administered with aluminium adjuvants, whereas the response to CFA remained intact. The authors identified the Nalp3 inflammasome as a crucial element in the adjuvant effect of aluminium adjuvants; in addition, they showed that the innate inflammasome pathway can direct a humoral adaptive immune response.

Recently, Kool *et al*.[28] succeeded in exposing an angle of its mysterious mechanism. The authors 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 was previously seen in response to the injection of uric acid into mice. In mice injected with antigens, OVA-peptide, mixed with alum, uric acid levels increased within hours. The uric acid might be released by the cells lining the body's cavities that 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 lymph nodes via the flowing lymph. The resident node DCs, however, did not process the alum-free antigens efficiently 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 those injected with enzymes that degrade uric acid. These findings suggest that alum is immunogenic by exploiting 'nature's adjuvant', the inflammatory DC through induction of the endogenous danger signal: uric acid.

In another study Kool *et al*.[29] showed that alum adjuvant induces the release of IL-1β 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 OVA in alum. The early production of IL-1β and the influx of inflammatory cells into the peritoneal cavity are strongly reduced in NALP3-deficient mice. The activation of adaptive cellular immunity to OVA-alum is initiated by monocytic dendritic cell precursors that induce the expansion of Ag-specific T-cells in a NALP3-dependent way. The authors propose that, in addition to TLR stimulators, agonists of the NALP3 inflammasome should also be considered as vaccine adjuvants.

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.[30] Some researchers consider adjuvants as environmental factors involved in autoimmune diseases. Several laboratories are pursuing the molecular identification of endogenous adjuvants. Sodium monourate and the high mobility group B1 protein (HMGB1) are, among those identified so far, well known to rheumatologists. However, even the complementation of apoptotic cells with potent adjuvant signals fail to cause clinical autoimmunity in most strains: the autoantibodies generated are transient, do not

undergo to epitope/spreading and do not cause disease.

Lastly, as vaccines may protect or cure autoimmune diseases, adjuvants may play also a double role in the mechanisms of these diseases. Myasthenia gravis (MG) and its animal model, experimental autoimmune myasthenia 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, 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 anti-idiotypic/clonotypic antibodies (Ab) and lowering levels of AChR Ab. These studies employed keyhole limpet hemocyanin (KLH) as a carrier and CFA. In advance of a clinical trial, McAnally *et al.*[31] tested the efficacy of RhCA 611-001 when combined with different adjuvants that are approved for use in humans. Adjuvants chosen for comparison were IFA and aluminium hydroxide (alum). 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 anti-peptide Ab response. This work demonstrate 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 were found to inflict an illness of autoimmune nature, defined as 'the adjuvant diseases'.[32]

Mineral oils as a cause of autoimmunity

Mineral oils are generally considered 'non-toxic' and have been used extensively in food, cosmetics, medicines and other products. Subcutaneous injections of mineral oil induce sclerosing lipogranulomas, a chronic local inflammatory reaction.[33] 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 (i.p.) 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.[34] 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.[35,36] Satoh, Reeves and colleagues reported that, in addition to pristane,[37,38] IFA and squalene, but not medicinal mineral oils, can induce lupus-related anti-nRNP/Sm and -Su autoantibodies in non-autoimmune-prone strains of mice. These data suggest that hydrocarbons can have a variety of immune effects. Kuroda *et al.*[39] 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 as non-toxic also may have pathogenetic implications in human autoimmune diseases. Moreover, Kuroda *et al.*[40] have reported that a single i.p. injection of the adjuvant oils pristane, IFA or squalene induces lupus-related autoantibodies to nRNP/Sm and -Su in non-autoimmune BALB/c mice. Induction of these autoantibodies appeared to be associated with the hydrocarbon's ability to induce IL-12, IL-6 and TNF-α, suggesting a relationship with hydrocarbon's adjuvanticity. Whether this is relevant in human vaccination is a difficult issue 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 basic research (Table 2).

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

Spiera *et al.*[41] reviewed the literature examining the association of silicone-gel-filled implants and connective tissue disease. The authors state that silicones are not biologically inert. Injectable as well as 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* is compatible with the hypothesis that they may contribute to the development of connective tissue disease. The association seems most likely with scleroderma; however, there is as yet

Table 2 Adjuvants involvement in autoimmune manifestation

Adjuvant involved	*Manifestations/disease/Ab*	*Species*	*References*
MDP;LPS;Gram+ CoxackieB3,IL1β,TNFά	Experimental thyroiditis; Myocarditis	Mice	Rose[48]
Mineral oils	Sclerosing lipogranulomas	Mice human?	Di Benedetto[33]
Pristane, mineral oils	Plasmacytomas	Mice	Anderson and Potter[34]
Pristane, squalene, IFA	Chronic arthritis	Mice, rats	Cannon *et al*.[35] Carlson *et al*.[49]
Pristane, squalene, IFA	Lupus-related anti nRNP/Sm/Su antibodies	Mice	Satoh *et al*.[37,38]
Pristane, squalene, IFA, mineral oils	Anticytoplasmic Ab, anti ssDNA/chromatin Ab	Mice	Kuroda *et al*.[39]
Pristane, squalene, IFA	Lupus-related anti nRNP/Sm/Su antibodies	Mice	Kuroda *et al*.[39,40]
Silicone	Human adjuvant disease connective tissue diseases	Human	Hennekens *et al*.[50]
Silicone	Scleroderma, SLE, RA	Human	Spiera *et al*.[41]
Alum in vaccines (HBV, HAV, tetanus)	MS, Chronic fatigue syndrome, polymyalgia rheumatica	Human	Gherardi[44]
Aluminium hydroxide, squalene	Gulf War syndrome, antibodies to squalene	Human	Asa *et al*.[45]

inadequate epidemiological data to definitively establish causality. Janowsky *et al*.[42] 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 summary 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*.[43] propose a definition of silicone-related disorder, by major and minor criteria including, tenderness, capsule formation, change in shape or position, and/or rapture of envelope, chronic fatigue lasting 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 is a condition first reported in 1998, the cause of which remained obscure until 2001.[44] The condition manifests by diffuse myalgias and chronic fatigue, forming a syndrome that meets both Center for Disease Control 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 is due to persistence for years at the site of injection of an aluminum adjuvant used in vaccines against HBV, hepatitis A virus, and tetanus toxoid. Aluminum hydroxide is known to potently stimulate the immune system and to shift immune responses towards 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 Gulf War syndrome (GWS). Results concerning macrophagic myofasciitis may well open new avenues for etiologic investigation of this syndrome. Indeed, both type and 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 aluminium hydroxide and, possibly, squalene, another Th-2 adjuvant. Asa *et al*.[45] 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 deploy, had antibodies to squalene. In contrast, none of the deployed Persian Gulf veterans not showing signs and symptoms of GWS have antibodies to squalene. Neither patients with idiopathic autoimmune disease nor healthy controls had detectable serum antibodies to squalene. If safety concerns about long-term effects of aluminium hydroxide are confirmed it will become mandatory to propose novel and alternative vaccine adjuvants to rescue vaccine-based strategies and the enormous benefit for public health they provide worldwide.

Conclusions and future goals

Owing to the adverse effects exerted by adjuvants, there is no controversy that safer adjuvants are

needed to be developed and incorporated into future vaccines.

The problem with 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 in these vaccines.[46] 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 non-toxic adjuvants able to stimulate cellular (Th1) immunity. However, a few more adjuvants were approved to date besides alum for human vaccines, among them are 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 (Table3).[47] 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.

Table 3 Adjuvants in human vaccines (according to Reed *et al*.[47])

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

Abbreviations: DPT, Diphtheria–Pertussis–Tetanus; DT, Diphtheria–Tetanus; HAV, Hepatitis A Virus; HBV, Hepatitis B Virus; HIV, Human Immunodeficiency Virus; HPV, Human Papilloma Virus.

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 non-immune 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. 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 the door to 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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