Edited by
Yehuda Shoenfeld, Nancy Agmon-Levin
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Vaccines and Autoimmunity

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Introduction

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Vaccines and Autoimmunity is a result of decades of experience in vaccinology, immunology, and autoimmunity, and of a review of the vast literature in this field. The book has three parts. Part I deals with general mechanisms of vaccine- and adjuvant-induced autoimmunity. In Parts II and III, we have asked the different authors to summarize, on one hand, individual vaccines and which common autoimmune diseases they may trigger in susceptible individuals (Part III), and on the other, the common autoimmune diseases and identified vaccines which may trigger their emergence (Part III).

The editors of this book are quite confident that vaccinations represent one of the most remarkable revolutions in medicine. Indeed, vaccines have been used for over 300 years and are probably one of the most effective strategies for preventing the morbidity and mortality associated with infections. Like other drugs, vaccines can cause adverse events, but unlike conventional drugs, which are prescribed to people who are ill, vaccines are administered to healthy individuals, which increases the concern over adverse reactions. Most side effects attributed to vaccines are mild, acute, and transient. Nonetheless, rare reactions, such as hypersensitivity and induction of autoimmunity, do occur, and can be severe and even fatal. In this regard, the fact that vaccines are delivered to billions of people without preliminary screening for underlying susceptibilities is thus of concern (Bijl *et al.*, 2012; Tomljenovic and Shaw, 2012; Soriano *et al.*, 2014).

Indeed, it is naive to believe that all humans are alike. Notably, autoimmune diseases have been increasingly recognized as having a genetic basis, mediated by HLA subtypes. For instance, celiac disease has been strongly associated with HLA haplotype DR3-DQ2 or DR4-DQ8 (Liu *et al.*, 2014), multiple sclerosis with HLA-DRB1 (Yates *et al.*, 2014), rheumatoid arthritis with HLA-DR4 and HLA-DQ8 (Vassallo *et al.*, 2014), and type I diabetes with HLA-DR3/4 (Steck *et al.*, 2014). Thus, certain HLA genes create a genetic predisposition toward development of autoimmune disease, typically requiring some environmental trigger to evolve into a full-blown disease state (Luckey *et al.*, 2011). One such environmental trigger which is commonly associated with development of autoimmunity is viral (Epstein Barr virus, cytomegalovirus, and hepatitis C virus) or bacterial (*Heliobacter pylori*) challenge (Rose, 2010; Magen and Delgado, 2014).

The multifacet associations between infectious agents and subsequent development of autoimmune or autoinflammatory conditions have been well established, and a number of mechanisms by which infectious agents can bring about such responses have been identified (molecular mimicry, epitope spreading, polyclonal activation, and others) (Molina and Shoenfeld, 2005; Kivity *et al.*, 2009; Shoenfeld, 2009; Rose, 2010).

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Recently, we and others have suggested another mechanism, namely the adjuvant effect, by which infections may relate to autoimmunity in a broader sense (Rose, 2010; Rosenblum *et al.*, 2011; Shoenfeld and Agmon-Levin, 2011; Zivkovic *et al.*, 2012; Perricone *et al.*, 2013). Adjuvants are substances which enhance the immune response. For this purpose, they are routinely included in vaccine formulations, the most common of which are aluminum compounds (alum hydroxide and phosphate). Although the mechanisms of adjuvancy are not fully elucidated, adjuvants seem to modulate a common set of genes, promote antigen-presenting cell recruitment, and mimic specific sets of conserved molecules, such as bacteria components, thus increasing the innate and adaptive immune responses to the injected antigen (Agmon-Levin *et al.*, 2009; Israeli *et al.*, 2009; McKee *et al.*, 2009; Exley *et al.*, 2010; Perricone *et al.*, 2013).

Although the activation of autoimmune mechanisms by both infectious agents and substances with adjuvant properties (such as those found in vaccines) is common, the appearance of an autoimmune disease is not as widespread and apparently not always agent-specific. The adjuvant effect of microbial particles, namely the nonantigenic activation of the innate and regulatory immunity, as well as the expression of various regulatory cytokines, may determine if an autoimmune response remains limited and harmless or evolves into a full-blown disease. Additionally, as already mentioned, the genetic background of an individual may determine the magnitude of adverse manifestations. For example, it has been shown that the vaccine for Lyme disease is capable of triggering arthritis in genetically susceptible hamsters and that, when the adjuvant aluminum hydroxide is added to the vaccine, 100% of the hamsters develop arthritis (Croke *et al.*, 2000). Other studies have shown that the development of inflammatory joint disease and rheumatoid arthritis in adults in response to the HepA and HepB vaccines, respectively, is correlated to the HLA subtype of the vaccinated individual (Ferrazzi *et al.*, 1997; Pope *et al.*, 1998). Given that aluminum works as an adjuvant by increasing expression of MHC (Ulanova *et al.*, 2001), it perhaps should not be surprising that in individuals susceptible to autoimmune disease on the basis of the MHC, HLA subtype might be adversely affected by the use of aluminum hydroxide in vaccines. In addition to aluminum, the vaccine preservative thimerosal has also been demonstrated to induce a systematic autoimmune syndrome in transgenic HLA-DR4 mice (Havarinasab *et al.*, 2004), while mice with a genetic susceptibility for autoimmune disease show profound behavioral and neuropathological disturbances. These results are not observed in strains of mice without autoimmune sensitivity.

We have recently reported a new syndrome: "autoimmune/inflammatory syndrome induced by adjuvants" (ASIA), which encompasses a spectrum of immune-mediated diseases triggered by an adjuvant stimulus such as chronic exposure to silicone, tetramethylpentadecane, pristane, aluminum, and other adjuvants, as well as infectious components, which may also have an adjuvant effect. All these environmental factors have been found to induce autoimmunity and inflammatory manifestations by themselves, both in animal models and in humans (Israeli *et al.*, 2009; Shaw and Petrik, 2009; Shoenfeld and Agmon-Levin, 2011; Gherardi and Authier, 2012; Israeli, 2012; Cruz-Tapias *et al.*, 2013; Lujan *et al.*, 2013; Perricone *et al.*, 2013).

The definition of the ASIA syndrome thus helps to detect those subjects who have developed autoimmune phenomena upon exposure to adjuvants from different sources. For example, the use of medical adjuvants has become common practice, and substances such as aluminum adjuvant are added to most human and animal vaccines, while the adjuvant silicone is extensively used for breast implants and cosmetic procedures (Kaiser *et al.*, 1990; Molina and Shoenfeld, 2005; Israeli *et al.*, 2009; Shoenfeld and Agmon-Levin, 2011; Cohen Tervaert and Kappel, 2013). Furthermore, "hidden adjuvants" such as infectious material and house molds have also been associated with different immune-mediated conditions associated with the so-called "sick-building syndrome" (Israeli and Pardo, 2010; Perricone *et al.*, 2013).

Although ASIA may be labeled a "new syndrome," in reality it reflects old truths given a formal label (Meroni, 2010). Notably, in 1982, compelling evidence from epidemiological, clinical, and animal research emerged to show that Guillain-Barre syndrome and other demyelinating autoimmune neuropathies (i.e., acute disseminated encephalomyelitis and multiple sclerosis) could occur up to 10 months following vaccination (Poser and Behan, 1982). In such cases, the disease would first manifest with vague symptoms (arthralgia, myalgia, paraesthesia, weakness; all of which are typical ASIA symptoms), which were frequently deemed insignificant and thus ignored by the treating physicians. However, these

symptoms would progress slowly and insidiously until the patient was exposed to a secondary immune stimulus (in the form of either infection or vaccination). This would then trigger the rapid and acute clinical manifestation of the disease (Poser and Behan, 1982). In other words, it was the secondary anamnestic response that would bring about the acute overt manifestation of an already present subclinical long-term persisting disease.

Thus, it was already recognized in the early 1980s that vaccine-related manifestations often presented themselves as unspecific, yet clinically relevant symptoms (termed "bridging symptoms" Poser and Behan (1982) or "nonspecific ASIA symptoms" by us (Shoenfeld and Agmon-Levin, 2011)). These manifestations pointed to a subclinical, slowly evolving disease. Whether this disease would eventually progress to its full-blown clinically apparent form depended on whether the individual was further exposed to noxious immune stimuli, including subsequent vaccinations. As a case in point, we recently described six cases of systemic lupus following HPV vaccination (Gatto *et al.*, 2013). In all six cases, several common features were observed; namely, a personal or familial susceptibility to autoimmunity and an adverse response to a prior dose of the vaccine, both of which were associated with a higher risk of post-vaccination full-blown autoimmunity. Similarly, in an analysis of 93 cases of autoimmunity following hepatitis B vaccination (Zafrir *et al.*, 2012), we identified two major susceptibility factors: (i) exacerbation of adverse symptoms following additional doses of the vaccine (47% of patients); and (ii) personal and familial history of autoimmunity (21%).

It should further be noted that some individuals who are adversely afflicted through exposure to adjuvants do not satisfy all of the criteria that are necessary to diagnose a full-blown and clinically apparent autoimmune disease (Perricone *et al.*, 2013). Nonetheless, these individuals are at higher risk of developing full-blown autoimmunity following subsequent adjuvant exposure, whether that be via infections or vaccinations (Poser and Behan, 1982; Zafrir *et al.*, 2012; Gatto *et al.*, 2013).

A casual glance at the US Centers for Disease Control and Prevention (CDC, 2013)_immunization schedule for infants shows that according to the US prescribed guidelines, children receive up to 19 vaccinations during infancy, many of which are multivalent in the first 6 months of their life (Table I.1).

The various vaccines given to children, as well as adults, may contain either whole weakened infectious agents or synthetic peptides and genetically engineered antigens of infectious agents and adjuvants (typically aluminum). In addition, they also contain diluents, preservatives (thimerosal, formaldehyde), detergents (polysorbate), and residuals of culture growth media (*Saccharomyces cerevisiae*, gelatin, bovine extract, monkey kidney tissue, etc.; Table I.2). The safety of these residuals has not been thoroughly investigated, primarily because they are presumed to be present only in trace amounts following the vaccine manufacture purification process. However, some studies

Table I.1 Typical pediatric vaccine schedule for preschool children currently recommended by the US Centers for Disease Control and Prevention (2013a). Shaded boxes indicate the age range in which the vaccine can be given. Asterisks denote Al-adjuvanted vaccines. Hep A is given in 2 doses spaced at least 6 months apart. According to this schedule, by the time a child is 2 years of age, they would have received 27 vaccinations (3 × HepB, 3 × Rota, 4 × DTaP, 4 × Hib, 4 × PCV, 3 × IPV, 2 × Influenza, 1 × MMR, 1 × Varicella, and 2 × HepA)

Birth	1 month	2 months	4 months	6 months	12 months	15 months	18 months	19–23 months	2–3 years	4–6 years
HepB*	HepB*			HepB*						
	Rota	Rota	Rota							
	DTaP*	DTaP*	DTaP*			DTaP*			DTaP*	
	Hib*	Hib*	Hib*	Hib*						
	PCV*	PCV*	PCV*	PCV*						
	IPV	IPV		IPV						IPV
						Influenza (yearly)				
				MMR					MMR	
				Varicella					Varicella	
						HepA*				

Hep A, hepatitis A; Hep B, hepatitis B; Rota, rotavirus; DTaP, diphtheria-pertussis-tetanus; Hib, *Haemophilus influenzae* type b; PCV, pneumococcal; IPV, inactivated polio; MMR, measles-mumps-rubella

Table I.2 Complete list of vaccine ingredients (i.e., adjuvants and preservatives) and substances used during the manufacture of commonly used vaccines. Adapted from US Centers for Disease Control and Prevention (2013b)

Vaccine	Vaccine excipient and media summary
DT (Sanofi)	aluminum potassium sulfate, peptone, bovine extract, formaldehyde, thimerosal (trace), modified Mueller and Miller medium
DTaP (Daptacel)	aluminum phosphate, formaldehyde, glutaraldehyde, 2-phenoxyethanol, Stainer–Scholte medium, modified Mueller's growth medium, modified Mueller–Miller casamino acid medium (without beef heart infusion)
DTaP (Infanrix)	formaldehyde, glutaraldehyde, aluminum hydroxide, polysorbate 80, Fenton medium (containing bovine extract), modified Latham medium (derived from bovine casein), modified Stainer–Scholte liquid medium
DTaP (Tripedia)	sodium phosphate, peptone, bovine extract (US sourced), formaldehyde, ammonium sulfate, aluminum potassium sulfate, thimerosal (trace), gelatin, polysorbate 80 (Tween 80), modified Mueller and Miller medium, modified Stainer–Scholte medium
DTaP-HepB-IPV (Pediarix)	formaldehyde, gluteraldehyde, aluminum hydroxide, aluminum phosphate, lactalbumin hydrolysate, polysorbate 80, neomycin sulfate, polymyxin B, yeast protein, calf serum, Fenton medium (containing bovine extract), modified Latham medium (derived from bovine casein), modified Stainer-Scholte liquid medium, Vero (monkey kidney) cells
DTaP-IPV/Hib (Pentacel)	aluminum phosphate, polysorbate 80, formaldehyde, gutaraldehyde, bovine serum albumin, 2-phenoxethanol, neomycin, polymyxin B sulfate, Mueller's Growth Medium, Mueller–Miller casamino acid medium (without beef heart infusion), Stainer–Scholte medium (modified by the addition of casamino acids and dimethyl-beta-cyclodextrin), MRC-5 (human diploid) cells, CMRL 1969 medium (supplemented with calf serum)
Hib (ActHIB)	ammonium sulfate, formalin, sucrose, Modified Mueller and Miller medium
Hib (Hiberix)	formaldehyde, lactose
Hib (PedvaxHIB)	aluminum hydroxphosphate sulfate
Hib/Hep B (Comvax)	yeast (vaccine contains no detectable yeast DNA), nicotinamide adenine dinucleotide, hemin chloride, soy peptone, dextrose, mineral salts, amino acids, formaldehyde, potassium aluminum sulfate, amorphous aluminum hydroxyphosphate sulfate, sodium borate
Hep A (Havrix)	aluminum hydroxide, amino acid supplement, polysorbate 20, formalin, neomycin sulfate, MRC-5 cellular proteins
Hep A (Vaqta)	amorphous aluminum hydroxyphosphate sulfate, bovine albumin, formaldehyde, neomycin, sodium borate, MRC-5 (human diploid) cells
Hep B (Engerix-B)	aluminum hydroxide, yeast protein, phosphate buffers
Hep B (Recombivax)	yeast protein, soy peptone, dextrose, amino acids, mineral salts, potassium aluminum sulfate, amorphous aluminum hydroxyphosphate sulfate, formaldehyde
Hep A/Hep B (Twinrix)	formalin, yeast protein, aluminum phosphate, aluminum hydroxide, amino acids, phosphate buffer, polysorbate 20, neomycin sulfate, MRC-5 human diploid cells
Human Papillomavirus (HPV) (Cerverix)	vitamins, amino acids, lipids, mineral salts, aluminum hydroxide, sodium dihydrogen phosphate dehydrate, insect cell and viral protein
Human Papillomavirus (HPV) (Gardasil)	yeast protein, vitamins, amino acids, mineral salts, carbohydrates, amorphous aluminum hydroxyphosphate sulfate, L-histidine, polysorbate 80, sodium borate
Influenza (Afluria)	beta-propiolactone, thimerosol (multi-dose vials only), monobasic sodium phosphate, dibasic sodium phosphate, monobasic potassium phosphate, potassium chloride, calcium chloride, sodium taurodeoxycholate, neomycin sulfate, polymyxin B, egg protein
Influenza (Fluarix)	sodium deoxycholate, formaldehyde, octoxynol-10 (Triton X-100), α-tocopheryl hydrogen succinate, polysorbate 80 (Tween 80), hydrocortisone, gentamicin sulfate, ovalbumin
Influenza (Fluvirin)	nonylphenol ethoxylate, thimerosal (multidose vial–trace only in prefilled syringe), polymyxin, neomycin, beta-propiolactone, egg proteins
Influenza (Flulaval)	thimerosal, α-tocopheryl hydrogen succinate, polysorbate 80, formaldehyde, sodium deoxycholate, ovalbumin
Influenza (Fluzone: standard, high-dose, & intradermal)	formaldehyde, octylphenol ethoxylate (Triton X-100), sodium phosphate, gelatin (standard formulation only), thimerosal (multidose vial only), egg protein
Influenza (FluMist)	ethylene diamine tetraacetic acid (EDTA), monosodium glutamate, hydrolyzed porcine gelatin, arginine, sucrose, dibasic potassium phosphate, monobasic potassium phosphate, gentamicin sulfate, egg protein

Table I.2 (*Continued*)

Vaccine	Vaccine excipient and media summary
Meningococcal (MCV4Menactra)	formaldehyde, phosphate buffers, Mueller Hinton agar, Watson Scherp media, Modified Mueller and Miller medium
Meningococcal (MCV4Menveo)	formaldehyde, amino acids, yeast extract, Franz complete medium
Meningococcal (MPSV4Menomune)	thimerosal (multidose vial only), lactose, Mueller Hinton agar, Watson Scherp media
MMR (MMR-II)	vitamins, amino acids, fetal bovine serum, sucrose, sodium phosphate, glutamate, recombinant human albumin, neomycin, sorbitol, hydrolyzed gelatin, chick embryo cell culture, WI-38 human diploid lung fibroblasts
MMRV (ProQuad)	sucrose, hydrolyzed gelatin, sorbitol, monosodium L-glutamate, sodium phosphate dibasic, human albumin, sodium bicarbonate, potassium phosphate monobasic, potassium chloride, potassium phosphate dibasic, neomycin, bovine calf serum, chick embryo cell culture, WI-38 human diploid lung fibroblasts, MRC-5 cells
Pneumococcal (PCV13 – Prevnar 13)	casamino acids, yeast, ammonium sulfate, Polysorbate 80, succinate buffer, aluminum phosphate
Polio (IPV – Ipol)	2-phenoxyethanol, formaldehyde, neomycin, streptomycin, polymyxin B, monkey kidney cells, Eagle MEM modified medium, calf serum protein
Rabies (Imovax)	albumin, neomycin sulfate, phenol, MRC-5 human diploid cells
Rabies (RabAvert)	β-propiolactone, potassium glutamate, chicken protein, ovalbumin, neomycin, chlortetracycline, amphotericin B, human serum albumin, polygeline (processed bovine 14 gelatin)
Rotavirus (RotaTeq)	sucrose, sodium citrate, sodium phosphate monobasic monohydrate, sodium hydroxide, polysorbate 80, cell culture media, fetal bovine serum, vero cells (DNA from porcine circoviruses (PCV) 1 and 2 has been detected in RotaTeq; PCV-1 and PCV-2 are not known to cause disease in humans)
Rotavirus (Rotarix)	amino acids, dextran, sorbitol, sucrose, calcium carbonate, xanthan, Dulbecco's Modified Eagle Medium (DMEM) (Porcine circovirus type 1 (PCV-1) is present in Rotarix; PCV-1 is not known to cause disease in humans)
Td (Decavac)	aluminum potassium sulfate, peptone, formaldehyde, thimerosal, bovine muscle tissue (US sourced), Mueller and Miller medium
Td (Tenivac)	aluminum phosphate, formaldehyde, modified Mueller–Miller casamino acid medium without beef heart infusion
Td (Mass Biologics)	aluminum phosphate, formaldehyde, thimerosal (trace), ammonium phosphate, modified Mueller's media (containing bovine extracts)
Tdap (Adacel)	aluminum phosphate, formaldehyde, glutaraldehyde, 2-phenoxyethanol, ammonium sulfate, Mueller's growth medium, Mueller–Miller casamino acid medium (without beef heart infusion)
Tdap (Boostrix)	formaldehyde, glutaraldehyde, aluminum hydroxide, polysorbate 80 (Tween 80), Latham medium derived from bovine casein, Fenton medium containing a bovine extract, Stainer–Scholte liquid medium
Typhoid (inactivated – Typhim Vi)	hexadecyltrimethylammonium bromide, phenol, polydimethylsiloxane, disodium phosphate, monosodium phosphate
Typhoid (oral – Ty21a)	yeast extract, casein, dextrose, galactose, sucrose, ascorbic acid, amino acids
Varicella (Varivax)	sucrose, phosphate, glutamate, gelatin, monosodium L-glutamate, sodium phosphate dibasic, potassium phosphate monobasic, potassium chloride, sodium phosphate monobasic, EDTA, residual components of MRC-5 cells including DNA and protein, neomycin, fetal bovine serum, human diploid cell cultures
Yellow Fever (YF-Vax)	sorbitol, gelatin, egg protein
Zoster (Shingles – Zostavax)	sucrose, hydrolyzed porcine gelatin, monosodium L-glutamate, sodium phosphate dibasic, potassium phosphate monobasic, neomycin, potassium chloride, residual components of MRC-5 cells including DNA and protein, bovine calf serum

suggest that even these trace amounts may not be inherently safe, as was previously assumed (Moghaddam *et al.*, 2006; Rinaldi *et al.*, 2013).

What is obvious, nonetheless, is that a typical vaccine formulation contains all the necessary biochemical components to induce autoimmune manifestations. With that in mind, our major aim is to inform the medical community regarding the various autoimmune risks associated with different vaccines. Physicians need to be aware that in certain individuals, vaccinations can trigger serious and potentially disabling and even fatal autoimmune manifestations. This is not to say that we oppose vaccination, as it is indeed an important tool of preventative medicine. However, given the fact that vaccines are predominantly administered to previously healthy individuals, efforts should be made to identify those subjects who may be at more risk of developing adverse autoimmune events following vaccine exposure. In addition, careful assessment should be made regarding further vaccine administration in individuals with previous histories of adverse reactions to vaccinations. The necessity of multiple vaccinations over a short period of time should also be considered, as the enhanced adjuvant-like effect of multiple vaccinations heightens the risk of post-vaccine-associated adverse autoimmune and inflammatory manifestations (Tsumiyama *et al.*, 2009; Lujan *et al.*, 2013). Finally, we wish to encourage efforts toward developing safer vaccines, which should be pursued by the vaccine manufacturing industry.

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I Mosaic of Autoimmunity

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