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Mini review

Progress in understanding adjuvant immunotoxicity mechanisms

Alexander Batista-Duharte[a,*], Erik B. Lindblad[b], Ernesto Oviedo-Orta[c]

[a] Immunotoxicology Laboratory, Toxicology and Biomedicine Center (TOXIMED), Medical Science University, Autopista Nacional Km. 1 1/2 CP 90400, AP 4033 Santiago de Cuba, Cuba
[b] European Adjuvant Advisory Committee (EAAC), Elsenbakken 23, DK-3600 Frederikssund, Denmark
[c] University of Surrey, Faculty of Health and Medical Sciences, Guildford, UK

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ABSTRACT

Over the last twenty years research has provided an important insight into the mechanisms responsible for the immunotoxicity of both local and systemic adverse reactions following the use of immunostimulating drugs and adjuvants. In this article we provide an update of the present knowledge relating to the various parameters and reactants of the immune system at the cellular as well as molecular level that are believed to play a key role in reactogenicity. We discuss evidence obtained from observations *in vitro*, *in vivo* in animal models and from clinical applications, including adjuvants used in large scale vaccination today. The data discussed are mainly taken from animal models following hyperstimulation of the immune system; either by the use of very powerful adjuvants, like Freund's that are too toxic for use in practical vaccination, by deliberate high dose application of adjuvants or by the *in vivo* application of cytokines. Although such hyperstimulating regimens are unlikely to find their way into practical vaccination of humans, this information is of great value as it may facilitate the understanding of the toxicity mechanisms, aid the design of standardised models for the assessment of adjuvant safety and the possible application of new adjuvants in vaccines for humans.

Contents

1. Introduction

Immunological adjuvants (from the latin *adjuvare*, to help) are substances, of highly diverse in their chemical structure, used for enhancing the immune response against a simultaneously administered antigen (Cox and Coulter, 1997). These compounds are

* Corresponding author. Tel.: +53 22 64 3796/3926; fax: +53 22 64 3926.
E-mail address: a.batista@toxi.scu.sld.cu (A. Batista-Duharte).

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routinely used for a variety of purposes including: (a) as part of vaccine formulations with the aim of eliciting the desired immune response with sufficient immunological memory to protect against infectious organisms, to trigger a state of tolerance (e.g. anti-allergic effect) or to break antigen specific tolerance (e.g. anti-tumour therapeutic affect); (b) in biomedical research to obtain higher titres of antibodies with better specificities and responses (e.g. production of polyclonal or monoclonal antibodies); (c) as tools for studying inflammation or autoimmune diseases; (d) in toxicological assays to evaluate hypersensitivity reactions against defined antigens.

A number of new substances with documented adjuvant activity have been reported in the literature over the last twenty years (Cox and Coulter, 1997; Kovarik and Siegrist, 2001; Tritto et al., 2009). However, owing to safety concerns, very few adjuvants have been licensed for use in human vaccines (Tritto et al., 2009). Alum was first applied as an adjuvant more than 80 years ago (Glenny et al., 1926). Since then, aluminum-based adjuvants are the only adjuvants used in vaccines approved for use in humans by the US Food and Drug Administration (FDA) in the form of particulate aluminum salts, such as $Al(OH)_3$ and $AlPO_4$ (Baylor et al., 2002). In other countries, including members of the European Union (EU), additional adjuvants have been approved for human use including: Chiron Microfluidized oil/water emulsion (MF59), which was initially licensed for a flu vaccine formulation (Fluad) in Italy in 1997 (Vesikari et al., 2009). Adjuvant system 03 (AS03), another oil-in-water emulsion, was recently approved as a component of a pre-pandemic H5N1 vaccine (Prepandrix) (Chu et al., 2009; Leroux-Roels, 2009; Schwarz et al., 2009). Finally, a combination of two adjuvants, monophosphoryl lipid A (MPL) and aluminum hydroxide, named adjuvant system 04 (AS04), was approved for use in HBV (Fendrix) and HPV (Cervarix) vaccines (Tritto et al., 2009).

2. Efficacy *vs.* safety

It is generally accepted that for adjuvants, potency and toxicity must be balanced in order to provide maximum immune stimulation with minimal side effects (Gupta et al., 1993). However, this balance is itself controversial, as to some extent the same mechanisms that are responsible for the positive immunostimulating effects, are responsible for the side effects that are acknowledged as adverse in nature.

When choosing an adjuvant one of the main issues to be considered is the species in which the adjuvant is to be used. To some extent, we have an empirical basis for predicting the level of side effects that a preparation may exert. There are examples of acceptance of a certain level of adverse side effects in some veterinary vaccine preparations, which would be unacceptable for humans (Spickler and Roth, 2003).

In preventive vaccination, where a vaccine is administered to healthy individuals, a compromise on efficacy is not unrealistic to avoid adverse side effects. However, in the case of the development of therapeutic vaccines against severe human diseases, such as cancer and AIDS, the criteria may be less rigid. Here, the acceptability of adverse reactions to the vaccine must be balanced against the general prognosis of the disease.

Another important factor to consider is the age of the individual that will receive the adjuvant formulation. Recently there has been an increasing incentive to vaccinate pregnant women against maternally transmitted diseases, forcing scientists to add further considerations with regard to potential teratogenic effects of new adjuvants (Prater et al., 2006). For this reason, embryo/foetal and perinatal toxicity studies are required, in principle, if the vaccine is intended for use in women of reproductive age or during pregnancy.

Table 1
A classification of immunological adjuvants.

Modes of action	Adjuvants
Antigen delivery systems	Aluminum salts, calcium phosphate, and other gels; Montanides, AS03, MF 59 and other emulsions; PLG, liposomes, virosomes, ISCOMs, virus-like particles, cochleates, and other micro or nanoparticles
Immunopotentiators	LPS; MPL and synthetic derivatives; MDP and derivatives; saponins and derivatives, including ISCOMs; CpG oligonucleotides; flagellin; cytokines; dsRNA; resiquimod, imiquimod and other small-molecule immunopotentiators

AS03, adjuvant system 03; PLG, polylactide co-glycolide; ISCOMs, immunostimulating complexes; LPS, lipopolysaccharide; MLP, monophosphoryl lipid A; MDP, muramyl dipeptide; dsRNA, double-stranded RNA.

Bearing this in mind, two main categories of adjuvant applications can be distinguished (Dawson and Taylor, 1995; Degen et al., 2003):

1. Applications for which efficacy has been given priority over the safety aspects (e.g. antibody production in animals, investigation of the immune system in rodent models).
2. Applications where safety is more important than efficacy (e.g. preventive human vaccines).

3. Effects of adjuvants

Many different types of adjuvants have been described, with a wide range of structures though knowledge about the true mechanisms underlying adjuvanticity is still modest and largely empirical. Consequently, it is difficult to predict the exact profile of side effects when an adjuvant is administered as part of a vaccine formulation, therefore adjuvants have been called "the immunologist's dirty little secret" by Charles Janeway (Janeway, 1989). In spite of this, some advances have been achieved due to growing knowledge in basic immunology and as a result the "secret" is slowly being unveiled (Tritto et al., 2009).

It is now accepted that adjuvants provide start signals for immune reactivity and guide the response to an acceptable magnitude, through regulation and facilitation of immune responses by antigen delivery (signal 1), co-stimulation (signal 2) and regulating the quality of immunity (signal 3) (Schijns, 2006). They are often divided into two main types according to their mode of action: (a) antigen delivery systems, which promote antigen uptake by antigen presenting cells (APCs), and (b) immunopotentiators, which activate the APC, mainly through receptors of the innate immune system (O'Hagan, 2006) (Table 1).

The immunological action of adjuvants can be associated with local or systemic side effects that involve various immunological mechanisms although some initial reactions can be non-immunological in nature (Fig. 1 and Table 2): as previously mentioned, it is a balance (often dose dependant) of judgement when a negligible subclinical, local reaction may develop into a significant and clinically unacceptable adverse reaction.

3.1. Local effects

The majority of parenteral adjuvants produce adverse reactions at the inoculation site. The most common reactions are local tenderness and swelling, while the most severe reactions involve the formation of painful abscesses and nodules (Gupta et al., 1993). Local pain is often associated with the use of adjuvants and is a consequence of tissue damage or the formation of a local inflammatory focus at the injection site. The severity of local pain can cause behavioural disturbances and has been observed in animal models

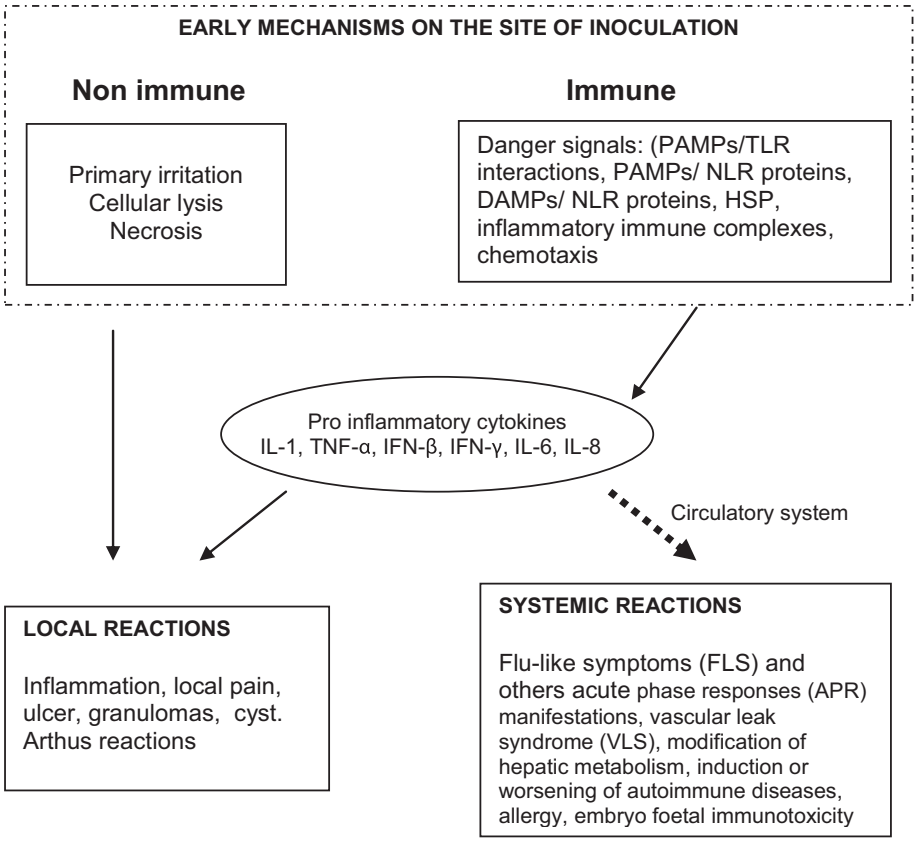

Fig. 1. Schematic representation of the immunotoxicity events that may occur after the use of vaccine adjuvants. After inoculation, adjuvants can produce direct tissue lesions, such as irritation, lysis or necrosis without the intervention of the immune system (non immune toxicity). Thereafter immunological mechanisms can cause inflammation initiated by danger signals produced by the adjuvant itself or endogenous factors released though tissue damage (e.g. PAMPs, DAMPs and HSPs). These early events promote the secretion of pro-inflammatory cytokines by the innate immune system, which consolidates the local reactions, and after subsequent transfer to the circulatory system, produces systemic manifestations of immunotoxicity. *Note*: This figure depicts a theoretical extreme situation and is not representative of any particular adjuvant. PAMPs, pathogen-associated microbial patterns; TLR, toll-like receptors; DAMPs, danger-associated molecular patterns; NLR, NOD-like receptors; HSP, heat shock proteins.

in which loss of weight, piloerection and other multiple manifestations of distress have been recorded (Burstein, 2007; Leenaars et al., 1998).

The appearance of local ulcers and necrosis following administration of mineral oil emulsions can be attributed to primary irritation of the tissue. This is due to the presence of short chain hydrocarbons with detergent-like effects in the formulation, dissolving the lipid bilayer of the cell membrane thus causing cell lysis (Gupta et al., 1993). Mineral oils are a mix of several hydrocarbons with carbon chains of different length. Short chains are efficient, but induce local reactions, whereas longer chains (>C14) are safer, but less efficient (Aucouturier et al., 2002). Mannide monooleate is often used in water/oil emulsion adjuvants as an emulsifier and may give rise to toxic fatty acids, through enzymatic breakdown of native lipid chains causing local inflammatory reactions (Hardegree and Pittman, 1966). Some surface active adjuvants, such as saponins derived from *Quilajja saponaria Molina*, interact with cell membranes leading to cell lysis (Sun et al., 2009). This capability is also used in some *in vitro* assays for quantification of saponins, utilizing their ability to lyse SRBCs and release haemoglobin.

Induction of local delayed hypersensitivity (DTH) is commonly seen after the use of adjuvants (Gupta et al., 1993). This is due to immunocompetent cells, including $CD4^+$ T lymphocytes, $CD8^+$ cytotoxic T lymphocytes (CTLs), macrophages and other cells secreting inflammatory cytokines (e.g. interleukin (IL)-1, IL-2, colony stimulating factors, chemokines, and other mediators) migrating to the inoculation site causing cellular recruitment, oedema and fibroblastic proliferation (Schijns, 2001). The recruitment of innate immune cells (neutrophils, eosinophils, monocytes, and subsequently dendritic cells [DCs]) to the site of injection is a critical function of particulate adjuvants and is a direct consequence of the local production of chemoattractants, such as CCL2, CXCL1 (Haas et al., 2005; Liu et al., 2005).

Granuloma formation, as an extreme manifestation of DTH, has been also reported after the use of Freund's adjuvants, alum and other adjuvants (Leenaars et al., 1994). The process of granuloma formation can be divided into four phases: (a) the initiation phase, characterized by the migration of macrophages to the persistent inflammatory stimulus; (b) the accumulation phase, with accumulation of $CD4^+$ T cells, more macrophages, other T cells and in some cases eosinophils; (c) the effector phase: in which various effectors cells attempt to eliminate the initial stimulus and limit their dissemination and (d) the resolution phase, the final step where once the foreign agent has been reduced or eliminated, the infiltrating cell population is reduced and the formation of scar tissue is induced finishing with local fibrosis (Co et al., 2004).

Granulomas may appear "metastatic" when excessive amounts of oil emulsions are injected at a single site. Emulsions injected subcutaneously into the dorsal region may migrate by fistulous tracts to the skin and more distant sites, such as the ventral region (Hanly et al., 1997). Other lesions reported with the use of Freund's adjuvant include bone lysis, infiltration into the marrow space and spinal canal, causing neurological disturbances such as posterior paresis (Kleinman et al., 1993; Hanly et al., 1997; Hill et al., 2006).

Another potential mechanism linked to local immunological effects of adjuvants is the Arthus reaction, which is characterized by the formation of inflammatory immune complexes (IC) at the inoculation site between the vaccine antigen or the adjuvant and

Table 2
Local and systemic reactions induced by some selected immunoadjuvants.

Manifestation of toxicity	Examples of adjuvant/components	Selected references
Local reaction		
Lysis, ulcer, necrosis	Mineral oil emulsions	Gupta et al. (1993), Aucouturier et al. (2002)
	Saponin from *Q. saponaria*	Sun et al. (2009)
Granuloma	FCA	Leenaars et al. (1994, 1998)
	Aluminum hydroxide	Gupta et al. (1993)
Arthus reactions	Toxoid-containing vaccines	Ponvert (2009)
Macrophagic myofasciitis	Aluminum hydroxide	Lacson et al. (2002), Siegrist (2005), Authier et al. (2006), Lach and Cupler (2008)
Bell palsy (intranasal route)	LTK63	Lewis et al. (2009)
Systemic reactions		
Acute phase response	LPS and derivatives, MDP	Gupta et al. (1993)
Vascular leak syndrome	IL-2	Baluna and Vitetta (1997)
	GM-CSF	Rechner et al. (2003)
Modification of hepatic metabolism	LPS	Yang et al. (2008)
	FCA	Projean et al. (2005)
Induction or worsening of autoimmune	FCA	Pearson (1956)
	Squalene	Carlson et al. (2000)
Allergy	Aluminum hydroxide	Böhler-Sommeregger and Lindemayr (1986)
Embryo foetal immunotoxicity	CPG	Prater et al. (2006)

pre-existing antibodies or complement components. This is a phenomena associated with the high antibody titre induced by the adjuvant, which can be misleadingly diagnosed as DTH (Mowat et al., 1991; Maloy et al., 1994; Furrie et al., 2002). Deposition of IC triggers Fc gamma receptor-dependent inflammation, where migration inhibitory factor (MIF) is released by macrophages upon recognition of IC, leading to tissue damage (Magalhaes et al., 2009; Paiva et al., 2009).

Infiltrations of aluminum containing macrophages gathered around the muscular fibers, in the myofascii, were observed in occasional deltoid muscular biopsies from patients vaccinated i.m. (Siegrist, 2005). This local reaction, described in adults and children (Lacson et al., 2002; Lach and Cupler, 2008) was characterized by the presence of AlOOH-loaded macrophages. This phenomenon was named MMF (macrophagic myofasciitis) and was attributed to the persistence of aluminum hydroxide for years at the site of a previous intramuscular injection (Authier et al., 2006). Attempts have been made to link the presence of such aluminum-containing macrophage manifestations to various clinical conditions, such as myalgia, muscle fatigue and, more controversially, to neurological disorders with no obvious etiological relation to the vaccination (Authier et al., 2001). However, such correlations are associated with statistical problems. There is very high vaccination coverage in Western countries. Hence, it is expected statistically that patients suffering from a wide range of etiologically unrelated diseases would all have been vaccinated with aluminum-containing vaccines at some point in their medical history. Another problem is that adequate statistical control groups of non-vaccinated persons may be hard to find within the same population (Lindblad, 2007).

In a controlled study in primates it was not possible to detect any histological changes after injection of aluminum-adjuvanted vaccine. Apart from the local inflammatory focus itself, no other abnormal clinical signs ascribable to it were found (Verdier et al., 2005), adding further to speculation as to the mechanism of this lesion.

A study using two strains of rats showed that Lewis rats with Th1-biased immunity had significantly smaller lesions than Sprague–Dawley rats with balanced Th1/Th2 immunity. This indicates that genetic determinants of cytotoxic T-cell responses could interfere with the clearance process, causing the persistence of vaccine-induced MMF-lesions (Authier et al., 2006).

Apart from the adjuvants, other factors associated with local reactions include: the antigen itself, contamination of the vaccine formulation with reactogenic chemicals or microbial products (thiomersal, formaldehyde, LPS, peptidoclycans, etc.) and instability of the formulation during storage with breakdown into reactogenic by-products. As a consequence adjuvants have no general approval, but are subject to safety evaluation as part of the complete antigen-containing vaccine formulation.

3.1.1. Role of the "danger signals"

Based on the "danger model", some degree of disruption to tissue integrity is required for the development of optimal immune responses, since primary immune reactions are initiated by signals emitted from damaged or stressed cells. These signals mimic tissue destruction and evoke the expression of co-stimulatory molecules on antigen-presenting cells (APCs), leads to the recruitment, activation and proliferation of lymphocytes (Matzinger, 1994). Based on these elements, the definition of an adjuvant could, in principle, be extended to a preparation capable of inducing a "danger" signal yielding direct/indirect tissue damage, or mimicking bacteria and viruses. Such signals may stimulate immune cells to produce pro-inflammatory cytokines necessary for an adequate immune response (Schijns, 2001), but could also result in local and systemic side effects.

3.1.1.1. Heat shock proteins (HSPs). HSPs are a family of ubiquitous intracellular molecules that function as molecular chaperones in numerous processes (e.g. protein folding and transport) and are induced under stress conditions such as fever, radiation, infections and neoplasia. In addition to maintaining cell homeostasis under physiological and stress conditions, some heat shock proteins (HSPs) are potent inducers of immunity and have even been harnessed as adjuvants in experimental vaccines targeted to cancers and infections (Segal et al., 2006).

HSPs released from necrotic cells owing to the local effects of several adjuvants are recognized by APCs through specific receptors, such as TLRs, scavenger receptors (LOX-1), CD91, CD14 (resulting in increased antigen display by MHC class I and II molecules) and priming T cells. Several HSPs activate the nuclear factor NF-kB pathway and induce the maturation of DCs and the secretion of the proinflammatory cytokines interleukin (IL)-12, tumour necrosis factor (TNF)-α, and chemokines (Srivastava and Amato, 2001; Segal et al., 2006). The migratory and cytolytic activity of natural killer cells can also be activated by HSPs, such as HSP96 and HSP70 (Pilla et al., 2005; Massa et al., 2005).

3.1.1.2. Pathogen-associated molecular patterns (PAMPs). PAMPs are highly conserved structural motifs expressed by microbial pathogens, and include various bacterial cell wall components such as lipopolysaccharides (LPS), peptidoglycans and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA. These structures are recognized by evolutionary conserved receptors, homologues of the *Drosophila* Toll gene called Toll-like Receptors (TLRs), expressed by cells belonging to the innate system and playing a critical role in early innate immunity against invading pathogens by sensing microorganisms (Medzhitov et al., 1997). Taking into account that several recognized adjuvant active substances are obtained from microorganisms bearing PAMPs, TLRs can be considered as adjuvant receptors. Most of the PAMPs used as vaccine adjuvants, such as CpG oligonucleotides and monophos-

phoryl lipid A (MPL) are agonists of TLRs (van Duin et al., 2006). Other components with adjuvant activity, but not used as adjuvants in practical vaccination due to toxicity (e.g. LPS and muramyldipeptides/MDP) have also been shown to be TLR-agonists.

3.1.1.3. Danger-associated molecular patterns (DAMPs). The NOD-like receptors (NLRs) are a family of intracellular sensors of microbial motifs and other signals of cellular stress (low intracellular potassium concentrations, reactive oxygen species (ROS), ATP increase (via P2X7 receptor), monosodium urate crystals and calcium pyrophosphate dihydrate deposition). These receptors have emerged as crucial components of innate immune responses during inflammation (Petrilli et al., 2007a; Martinon et al., 2006). Several NLRs (NALPs and IPAF) form a caspase-1-activating multiprotein complex, termed inflammasome, which processes pro-inflammatory cytokines, such as IL-1β (Petrilli et al., 2007b) and IL-18 (Li et al., 2007). Amongst the various inflammasomes, NALP3 is particularly qualified to sense a plethora of different molecules used as adjuvants, including bacterial peptidoglycan/muramyl dipeptide (Martinon et al., 2004), LPS, bacterial RNA, poly I:C and imidazolequinoline-derived compounds (Kanneganti et al., 2006). Studies in animal models suggest that the direct activation of NOD-like receptor protein 3 (NLRP3) may contribute to the adjuvanticity of particulate adjuvants, such as alum, chitosan and Quil-A (De Gregorio et al., 2009), although further studies are necessary to confirm these results.

Interestingly, phenotypical and functional modifications have also been observed in short-term cultures of macrophages exposed to aluminum hydroxide *in vitro*. They express the classical markers of myeloid dendritic cells (HLA-DR^{high}/$CD86^{high}$/$CD83^{+}$/$CD1a^{-}$/$CD14^{-}$) and display a potent ability to induce MHC-II-restricted antigen-specific memory responses, while retaining a macrophage morphology (Rimaniol et al., 2004).

3.2. Systemic effects

Systemic reactions following the application of immunological adjuvants will generally disqualify them from use in practical vaccination. Consequently, few observations on the systemic side effects of vaccine adjuvants are available. Where systemic side effects are observed, they are considered rare and with the exception of acute phase responses, are regarded as subclinical.

Toxic systemic effects after adjuvant administration are generally the consequence of the hyper-activation of immunological mechanisms induced by the adjuvant formulation. These effects are often mediated by the release of cytokines, such as IL-1β, TNF-α, IFN-β, IFN-γ, IL-6 and others (Li et al., 2007; Sokolovska et al., 2007). Cytokines used directly as immunological adjuvants (Boyaka and McGhee, 2001; Dong et al., 1995) or subsequently released after the application of various immunomodulators are not only essential in the early phase of the immune response, but are directly or indirectly involved in the pathogenesis of immune-mediated disorders described in humans and animals. Therefore, it is not surprising that such disorders develop after the administration of pharmacological doses of these cytokines (Vial and Descotes, 1995; Delavallee et al., 2008).

3.2.1. Acute phase response (APR)

APR defines a transient syndrome characterized by changes in plasma proteins and is associated with fever and other constitutional symptoms (leukocytosis, changes in cell/tissue metabolism and organ function) that accompany typical side effects seen in flu-like reactions, such as fatigue and anorexia (Gribble et al., 2007). Flu-like symptoms (FLS), as part of APR, are one of the main side effects reported to develop after hyperstimulation of the immune system. They are mediated by several cytokines and other humoral factors, usually appearing within hours following administration of an immunostimulating drug or vaccine, and uneventfully recede within a few hours. FLS typically consist of fever, chills, fatigue, myalgia, headache and nausea. Fever is the most common finding in patients with FLS. It can be of variable magnitude, ranging from a moderate increase in body temperature (38–39 °C) to marked hyperpyrexia exceeding 40 °C, in some cases (Descotes and Vial, 2007).

IL-1 released by leukocytes, and other endogenous factors (IL-1β, TNF-α, IFN-β, IFN-γ, IL-6, IL-8) along with macrophage inflammatory protein-1, act as pyrogens (Conti et al., 2004). These circulating cytokines can enter the circumventricular organs through fenestrated capillaries where they induce the production of prostaglandins, such as PGE_2, a centrally controlled mediator of fever. Synthesis of PGE_2 depends on cyclooxygenase (COX) activity; the induction of COX-2 in response to peripheral injection of a fever-inducing dose of LPS was demonstrated in brain endothelial cells, perivascular microglia and meningeal macrophages (Cao et al., 1997; Descotes and Vial, 2007).

A role for the complement anaphylatoxins in APR has been also suggested. It has been shown that intravenous administration of LPS triggers complement cascade activation within 2 min via the alternative pathway, resulting in the production of C4a, C3a and C5a in the blood (Descotes and Vial, 2007). PGE_2 is produced in response to activation via the hydrolysis of membrane-associated phosphoinositide by phosphoinositide-specific phospholipase C, activated by the complement cascade. The anaphylatoxin C5a has been identified as an important mediator in this pathway (Blatteis et al., 2005). However, these are findings from experimental immunology, as vaccines are never administered intravenously.

Some of the main targets of APR are the liver, the hematopoietic system, and the hypothalamic–pituitary–adrenal axis. Hepatocytes respond to these cytokines primarily by altering gene transcription and increasing the production and secretion of acute phase proteins. Several hepatic proteins are elevated in serum including complement factors (C2, C3, C4, C5, C9, C1-inhibitor, C4-binding protein; mannose-binding lectin [MBL], Factor B), coagulation/fibrinolysis factors (fibrinogen, plasminogen, protein S, von Willebrand Factor [VWF], plasminogen activator inhibitor-1 [PAI-1], tissue plasminogen activator [TPA]), C-reactive protein [CRP] and serum amyloid A [SAA]. Several of these, such as CRP and SAA, are currently being used as biomarkers of APR (Gribble et al., 2007). Although these findings have no direct relevance to vaccination they are never the less of interest for the understanding of the toxicity of LPS.

3.2.2. Induction or worsening of autoimmune diseases

Although there are only a few examples of autoimmune effects induced by adjuvants without any joint-specific antigen (Pearson, 1956; Stasiuk et al., 1997; Billiau and Matthys, 2001), this is one of the best examples for the potential of adverse reaction with the combined effect of adjuvant/antigen.

Experimentally, it is rather easy to induce an autoimmune disease in genetically susceptible animals via immunization with a formulation containing a strong adjuvant (e.g. FCA) and an autoantigen (Billiau and Matthys, 2001). In clinical practice however, reports of possible associations between vaccines and autoimmune diseases are rare, relative to the large number of vaccinated human subjects, and epidemiological studies have so far failed to demonstrate any causal relationship between vaccination and autoimmune diseases (Schattner, 2005).

In theory, a classical vaccine formulation contains the necessary elements for a possible induction of autoimmune diseases. Namely, the antigen that may contain mimetic epitopes and the adjuvant for the upregulation of co-stimulatory molecules and other products of

inflammation leading to the polyclonal activation of autoreactive T cells. Furthermore, bystander activation can wake up functionally silenced (anergic) cells with auto-aggressive potential or trigger the expansion of low-affinity autoreactive cells that have escaped negative selection (Christen and von Herrath, 2004). On the other hand, the role of pre-existing risk factors including genetic predisposition and environmental factors are largely accepted as being involved (Vial and Descotes, 2004).

In human populations with a high level of diversity amongst HLA haplotypes, it is not surprising to find differences in susceptibility to developing autoimmune reactions (Theofilopoulos, 1995). It has been suggested that some genetic pattern could be a predisposing factor in determined subjects, in which antigen presentation, influenced by certain HLA haplotypes, can lead to the autoimmune cascade after vaccination (Santoro et al., 2010).

The causative relationship between hyperstimulation of the immune system associated to vaccination and induction of autoimmunity remains unclear. This highlights the need for more research to understand the possible role of adjuvants and vaccines in triggering autoimmunity in clinical practice.

3.2.3. Modification of hepatic metabolism

Administration of immunostimulating drugs, Bacillus Calmette Guerin (BCG) vaccine, or interferons has been shown to affect oxidative drug metabolism by the hepatic cytochrome P 450 (CYP) system. For example, Renton and co-workers (Renton, 2001) reported decreased elimination of theophylline after influenza vaccination, associated with changes in the hepatic metabolism of sufficient magnitude to cause acute theophylline toxicity in humans, despite the use of therapeutic doses. In another report, these authors also demonstrated that the release of cytokines, such as IL-1, IL-2, IL-6, TNF, TGF-β and IFNs is involved in modulating the expression of several P450 isoforms. Reversible changes in the pharmacokinetic parameters of theophylline and decreased expression of CYP1A, 2B1/2, and 3A subfamily have also been reported in rats after intravenous administration of lipopolysaccharide (endotoxin) derived from *Klebsiella pneumoniae* (Yang et al., 2008).

Other studies have shown the rapid decrease in the total CYP450 liver content of FCA-treated rats and the selective down-regulation of specific CYP isoforms through a direct reduction in mRNA levels (CYP2B, CYP2CI1, CYP3A1, and CYP2E1), protein content (CYP2B, CYP2C11, and CYP2E1) and catalytic activity (CYP2C6, CYP2C11, and CYP2E1) (Projean et al., 2005). CYP3A1 mRNA levels were severely decreased by FCA administration, whereas CYP3A2 mRNA and protein levels remained unchanged. These early biochemical and metabolic modifications may have pharmacokinetic and pharmacodynamic consequences when drugs cleared by the liver are administered in conjunction with FCA (Projean et al., 2005) and possibly other potent immunostimulators. Shifts in hepatic CYP expression could also lead to increased exposure to drugs or toxic metabolites. This effect has been considered as a possible cause of hepatic toxicities, as the variable suppression of drug-metabolizing enzymes involved in drug biotransformation and elimination during infection and inflammation could lead to a range of patterns of hepatic damage (Renton, 2001).

3.2.4. Vascular leak syndrome (VLS)

VLS is a serious side effect seen after administration of IL-2 (Vial and Descotes, 1995), IL-1 (Worth et al., 1997), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Emminger et al., 1990), and other cytokines, previously proposed as vaccine adjuvants. VLS is characterized by an increase in vascular permeability, accompanied by extravasation of fluids and proteins resulting in interstitial oedema and organ failure. Manifestations of VLS include increased body weight, hypotension, fluid shifts, peripheral oedema, pleural and pericardial effusions and ascites. In severe cases, VLS-related conditions may progress to pulmonary and cardiovascular failure (Baluna and Vitetta, 1997).

The pathogenesis of endothelial cell damage associated to this syndrome is complex and poorly understood. It may involve the activation or damage of endothelial cells and leukocytes, the release of cytokines and inflammatory mediators (e.g., IL-1, TNF-α), components of the complement cascade, alteration in cell–cell interactions and cell matrix adhesion, as well as alterations in cytoskeleton function resulting in disturbance of vascular integrity (Baluna and Vitetta, 1997). Other studies have connected cytotoxicity of lymphokine-activated killer (LAK) cells on vascular endothelial cells with the development of VLS (Damle and Doyle, 1989) and demonstrated the involvement of perforin, Fas ligand and CD44 through the use of gene-targeted mice (Rafi et al., 1998, 1999). Not surprisingly the observation of VLS led to the disqualification of these cytokines from use as adjuvants in vaccines.

3.2.5. Allergy

The question of allergy to adjuvants is controversial. The general ability of aluminum adjuvants to stimulate the production of IgE as part of the overall Th2 profile and increase eosinophilia is well established although, the underlying mechanism is unknown. However in practical conditions, it has been difficult to demonstrate cases where vaccination with aluminum adjuvants has led to IgE-mediated allergy toward the vaccine antigen (Lindblad, 2004).

An adjuvant-induced increase in IgE levels against the antigen should be considered as a potential concern regarding the development of hypersensitivity reactions.

3.2.6. Embryonic immunotoxicity

Adjuvants could have immunomodulatory effects on a successful embryonic gestation and may also affect the immune system of the developing foetus. The balance of Th1/Th2 responses is important for a successful pregnancy where maternal responses are being biased toward humoral immunity (Th2) and away from cell-mediated immunity (Th1) (Raghupathy, 1997).

Recent reports have revealed that a shift toward a Th1 cytokine profile during pregnancy may increase the risk of foetal morphological defects. The injection of high doses of CpG ODN, a vaccine adjuvant inducing strong Th1 responses, to pregnant C57BL/6 mice resulted in a marked increase in foetal resorption and craniofacial/limb defects, while lower doses had little or no effect. The histological examination of the placentas showed cellular necrosis with mixed inflammation and calcification in the spongiotrophoblast layer and dysregulation of labyrinthine vascular development (Prater et al., 2006).

Hence, any vaccine or adjuvant that skews immune responses toward Th1 could, in theory, have adverse effects on the embryo-foetal development through Th1-type immunity as it appears to pose a risk to successful pregnancy (Raghupathy, 1997). Cytokines, NK cells and gamma delta T cells of maternal origin are all thought to be involved in processes, such as foetal recognition, placental development and regulation of gene expression during organogenesis (Szekeres-Bartho, 2002). Hence any effect of vaccination on these cells and cytokines may potentially affect development of the foetus. Currently there is little information on developmental immunotoxicity available, thus highlighting the importance of carrying out further studies on alterations of the immune system after pre- or post-natal exposure to xenobiotic agents (Kushima et al., 2007). Teratogenic effects that may result from exposure to vaccine adjuvants require a particular attention (Prater et al., 2006).

4. Impact of routes of administration on the effects of adjuvants

The selected route of administration may, to some extent, influence the profile of side effects associated with adjuvants. With the subcutaneous route (s.c.), the vaccine inoculum is introduced into a compartment with numerous sensorial nerve cells. This induction of a local inflammatory response may lead to local irritation, itching (pruritus), erythema and pain. In addition, transient swelling as a consequence of the inflammatory focus, may become easily palpable through the skin (Leenaars et al., 1998). With the intramuscular route (i.m.), swelling even of similar size may be less easily palpable as it is located deeper within the tissue. In addition, the intramuscular compartment is not as innervated with sensory neurons as the skin (Davies, 1986; Spickler and Roth, 2003). On the other hand, lesions in the muscular tissue following i.m. administration may be associated with the release of creatine phosphokinase (CPK) into the circulation, which in some cases is used as an indicator of tissue damage (Patel et al., 2002). Adjuvants can be also administered via the intraperitoneal (i.p.) route to animals, primarily in experimental immunology. Here the inoculum is introduced into the body cavity and instead of forming a localized deposit, it may spread out and induce disseminated reactions, including chemical peritonitis, ascitis and the formation of fibrous adherences between various organs in the body cavity.

Gizurarson et al. (1996) evaluated the local toxicity of several adjuvants after the intranasal vaccination of guinea pigs. They found damage to the mucosal epithelium ascribed to direct toxicity of a given inoculum. This can lead to contact of lymphoid cells with the submucosal tissue or draining lymphatic vessels, causing immunological responses in which antigen uptake is carried out by M cells (Gizurarson et al., 1996). However, a damaged mucosa may lead to the establishment of secondary opportunistic infections due to breach of the mucosal barrier, an important aspect that requires further investigation. Another concern when using the nasal route for immunization is the potentially direct passage of the inoculum into the brain through the olfactory pathways. Noticeably, entry into the central nervous system (CNS) through primary sensory olfactory neurons is an established route for several viruses however, no data is yet available for the entry of bacteria or bacterial-derived toxins through the same route (Illum, 2003). Hence, the potential for neurotoxicity must not be ignored in the use of mucosal adjuvants in humans. The expression of GM1 gangliosides on sensory olfactory neurons in the nasal tract provides a pathway for entry of enterotoxins used as adjuvants into the olfactory bulbs (e.g. CT and LT) following nasal application (Fujihashi et al., 2002). It has been reported that nasally applied LT induced inflammatory responses in the meninges, the olfactory nerve and glomerular layers of the olfactory bulbs (reviewed in Fujihashi et al., 2002) also a link has been suggested between this pathway and diseases, such as Alzheimer's dementia (Weller, 1998). However, to date there is not sufficient evidence to support the hypothesis that intranasal immunization is dangerous for the brain.

An association has been established between facial nerve paralysis (Bell's palsy) and the intranasal administration of an inactivated influenza virosome vaccine containing an LT adjuvant in Switzerland (Mutsch et al., 2004). Further reports have been published in relation to intranasal vaccines containing genetically detoxified mutant of *Escherichia coli* LT (LTK63). The lack of reported facial nerve paralysis following nasal immunization in subjects not receiving LT adjuvants, implies a causal relationship with LT and allows the suggestion that this paralysis may be due to a transient interference with peripheral nerve function. Such transient interference could be due to accumulation of LTK63 molecules, to inflammation arising from immune response to LTK63 following ganglioside binding, retrograde neuronal transport or to other still unknown causes (Lewis et al., 2009). This pathological condition is currently the focus of attention for vaccine regulatory agencies.

5. Present problems and future perspectives

The search for new, more potent and safe adjuvants represents one scientific challenge today. Over the last decades very few adjuvants have been licensed for prophylactic vaccines due to toxic properties detected during pre-clinical or clinical studies of the many new candidates being evaluated. Researchers or companies, sometimes unfairly, refer to a "regulatory barrier" forgetting that as far as preventive formulation is concerned, the primary obligation is safety.

Unfortunately, there are several challenges in the design of adequate safety studies: the lack of suitable experimental models and standardized predictive methods, the difficulties in studying certain adverse effects (e.g. autoimmunity and hypersensitivity reactions) and the clinical hurdle of detecting infrequent effects that can develop in certain specific "at-risk" subpopulations (e.g. those carrying particular HLA phenotypes, or suffering from certain chronic diseases, or exposed to drugs and other environmental factors).

Surveillance systems should be expected to be able to detect adverse effects in these specific situations. Currently however, these are mostly passive surveillance systems collecting reports of events voluntarily submitted by patients who experienced them, caregivers, or others. Passive surveillance systems (such as VAERS) are subject to multiple limitations, including underreporting, reporting of temporal associations or unconfirmed diagnoses, and a lack of denominator data and unbiased comparison groups. Due to these limitations, determining causal associations between vaccines and adverse events from these reports is usually not possible.

Although vaccines are the most successful medical invention of the last century, it is obvious that future vaccines will require adjuvants with predictable activity (Schijns, 2006). For these reasons, the regulatory agencies are very reluctant to approve novel adjuvants, consequently efforts for global harmonization toward improved and consistent standards for nonclinical and clinical evaluation are required to better assess the safety of preventive vaccines and predict their toxicity.

The aim of this paper is to draw attention to the fact that, to a large extent, the very same parameters of the immune system that are essential for the normal function of both the innate and the adaptive immune response, are also key players in the reactions and side effects that we consider adverse in nature. It is a balance and a matter of judgement when for example a normal subclinical local reaction to a vaccination turns from being a temporary cosmetic issue into a clinically unacceptable reaction because of hyperstimulation of the immune system. We have discussed data obtained from practical vaccination, but also from model systems through immunostimulatory regimens that would be considered completely unacceptable for practical vaccination. However, when taken together these data illustrate the dilemma of immunostimulation and demonstrate the variety of factors with the potential to affect the level of immunotoxicity. We believe that fellow scientists should take inspiration from this when they evaluate new potential adjuvants intended for use in vaccines. Even though some progress has been achieved in regulatory policies and guidelines, approaches to improve the safety assessment of preventive vaccines is still evolving and should be promoted by regulatory agencies.

Conflict of interest statement

The authors declare no conflict of interest.

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