Choice of Adjuvant in Vaccination Trials

Guidelines

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The art of delivery systems

Numerous tumors and infectious diseases could be eradicated by Adjuvant's ability to intensify and modulate the immune response appropriately. In particular, the capacity of specifically inducing a Th1 type response is decisive for the cure of these diseases. Moreover, the new adjuvants should be as free as possible from side effects in order to be considered for clinical translation.
The purpose of this application note is to present the various adjuvants currently used in vaccinology, by classifying them according to their chemical nature and by exposing the knowledge for each category relating to their activity and toxicity.

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The term "adjuvant" derives from *adjuvare* meaning to assist. Any material that increases the humoral and/or cellular immune response of an antigen is considered as an adjuvant and it is said to be immunogenic. A multitude of adjuvants of extremely diverse origin exist. Two of the most distinguished adjuvant types are the immune-stimulating agents and the "vaccine-carriers". The immune-stimulating agents activate the immune cells directly by binding to different receptors. While the «vaccine-carriers» contain the antigen and are able to incorporate it into the immune system in different manners. However, vehicles often have their own immunostimulatory properties, simply because they constitute foreign bodies.
Although they are tested in the context of certain immunotherapies, adjuvants are mainly used as constituents of vaccines. They are essential for the installation of a protective immune response. Indeed, the immunogenicity of a non-adjuvanted vaccine, especially if inactivated, is usually too weak to perfectly mimic a natural infection. Even if they contain the specific epitopes protectors, an agent capable of amplifying and of directing the specific immune response without being the target is needed; this fact has paved the way of the adjuvant's research and development.

The heterogeneity of potential adjuvants and their mechanisms have given rise to numerous attempts to classify them. Adjuvants can be ordered according to their chemical species and their origin.

Expanding its comprehensive line of delivery systems, OZ Biosciences introduces now vaccine adjuvants -**VaxOZ adjuvants**- as a gold standard references for any immunization experiment:

AlumVax Hydroxide/Phosphate
The two classical aluminum-based adjuvants

CFAVax
The **C**omplete **F**reund's **A**djuvant

IFAVax
The **I**ncomplete **F**reund's **A**djuvant

SqualVax
A nanometric oil-in-water emulsion

CNE-CPO
Cationic **N**ano **E**mulsion Vaccine Adjuvant

CaLiVax-DOTAP
Cationic **Li**posome-DOTAP

PolyVax-CPO
Cationic **PO**lymer-based Vaccine Adjuvant

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ADJUVANTS CLASSIFICATION

Mineral adjuvants

The adjuvants family of aluminum salts, represented by aluminum hydroxide (**AlumVax Hydroxide**) and aluminum phosphate (**AlumVax Phosphate**), are the most widely used compounds in vaccines currently on the market due to their safety of use, low price and adjuvant efficacy in combination with a large number of antigens. Their effects were discovered in 1926 by Glenny but more clearly established in the 1930s. The most commonly used preparation method is adsorption of the antigen to a preformed aluminum gel mainly by electrostatic interactions. The adsorption of antigens to aluminum depends on physical and chemical characteristics of the antigen, type of aluminum adjuvant and conditions of absorption. These conditions must be taken into account because an aluminum adjuvant poorly formulated does not give its optimal adjuvant efficiency.

Freund's adjuvants

Freund's adjuvant is a solution of antigen emulsified in mineral oil and used as an immunopotentiator. The complete form, Complete Freund's Adjuvant (**CFAVax**) is composed of thermally inactivated and dried mycobacteria (usually *M. tuberculosis*). Whereas the incomplete form (**IFAVax**) lacks the myco-bacterial components, hence corresponds to water in oil emulsion. It is named after Jules T. Freund.

Oil-based Nano-emulsion adjuvants

The name "oily adjuvants" groups together the different types of emulsions. An emulsion is a system where two immiscible phases are dispersed together being stabilized by the intervention of surfactants such as for **SqualVax** and **CNE-CPO**. Conventional oily adjuvants are water in oil (w/o) emulsions, where drops (nanometer to millimeter scale) of hydrophilic antigen solution are embedded within a continuous lipophilic phase.

Cationic polymer-based Nano-emulsion adjuvants

These amphipathic polymers are typically constituted of both cationic modified hydrophilic poly(ethylene oxide) block (PEO) and hydrophobic chains of polypropylene oxide (PPO). They are mainly used as surfactants in o/w emulsions (**e.g. CNE-CPO**). They allow the immunogen presentation on the oily droplets surface, both by hydrogen bindings with the PEO, by electrostatic interactions with the cationic part and through hydrophobic interactions with the PPO block.

In vivo grade manufactured and quality controlled, the **VaxOZ adjuvants** are intended for research purposes only.

AlumVax Hydroxide/Phosphate

CFAVax

IFAVax

SqualVax

CNE-CPO

CaLiVax-DOTAP

PolyVax-CPO

ADJUVANTS CLASSIFICATION

Saponins and "ImmunoStimulating COMplexes" (ISCOMs)

Saponins are natural glycosides of steroid or triterpene, which are widely distributed in the plant and exhibit many pharmacological activities (antiallergic, antitumor, antiviral and so on). The most extensively used saponin-based adjuvants are extracted from the bark of *Quillaja saponaria molina*, a tree from South America. Heterogeneous raw extracts with unpredictable effects today give way to standardized preparations such as QuilA®, as well as pure saponins (QS-7, QS-21). Saponins are capable of interacting with cholesterol to form pores in cell membranes. This property confers their toxic effects, but it is also the basis of the development ImmunoStimulating COMplexes (ISCOMs). ISCOMs are cage-like vesicles, which can be easily prepared by mixing Quil-A®, cholesterol and phosphatidylcholine. The Quil-A® and the cholesterol form the structure of the complex, and phosphatidylcholine, less rigid, allows incorporation of an amphipathic antigen.

Lipid-based adjuvants

Vesicular adjuvant represents a lipid bilayer of variable composition. The composition delimits the aqueous microsphere. In addition, the physico-chemicals properties can be modified by the addition of surfactants. They mostly include liposomes such as **CaLiVax-DOTAP**. According to their more or less hydrophobic or hydrophilic features, the immunogen can be adsorbed on their surface, inserted into their bilayer or contained into their aqueous phase.

Polymer-based adjuvants

Polymers are known to enhance the shelf-life of antigens and are capable of inducing long lasting antibody responses as well as cell-mediated immunity. The immunomodulators or ligands targeting specific cells can be easily coupled to polymers (**PolyVax-CPO**), which can easily be developed in the form of micro/nano based particulate adjuvants such as emulsions, microparticles, liposomes.

MODE OF ACTION, EFFECTIVENESS AND TOXICITY OF MAIN ADJUVANTS

An effective adjuvant should not only enhance the immune response, but also orient this response in function of the pathogenesis specific to each infection. Each adjuvant is characterized by its ability to selectively activate helper T lymphocytes (CD4 +) type Th2 or Th1, which corresponds to two major pathways that the immune response can take, namely humoral and cellular, respectively. An adjuvant can also promote more particulars of immune response, such as the delayed type hypersensitivity response (DTH) or the response to cytotoxic T lymphocytes (CTL), which correspond to two parameters of cell-mediated immunity, or the production of IgA production in a Th2 context. Overall, a possible immune response induced by vaccine adjuvants is represented in *Figure 1.*

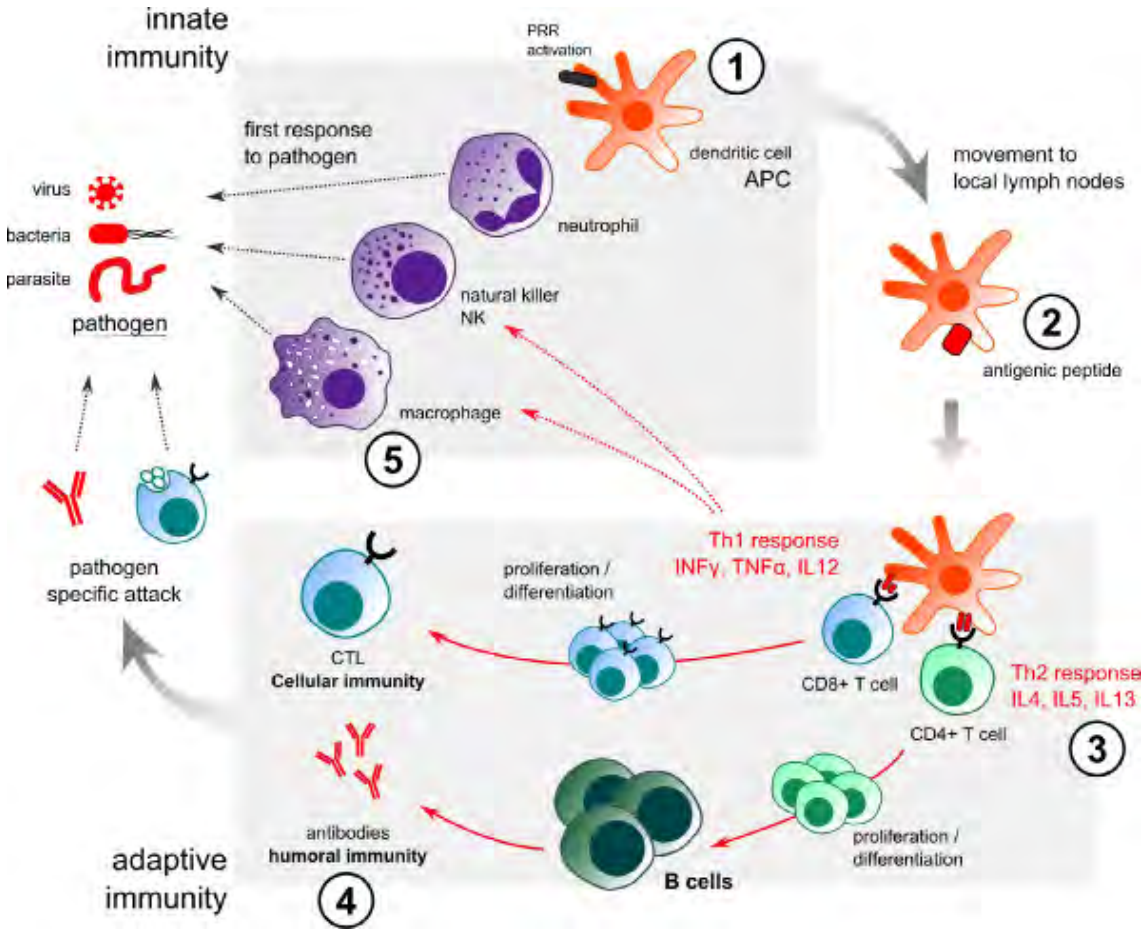

Figure 1. Innate and immune response to pathogen/adjuvant actions. AlumVax/SqualVax/IFAVax: (1) recruitement of APC at the injection site; (2) enhancement of antigen uptake; (3) enhancement of Th2 response; (4) increasing of humoral immune response; (5) AlumVax: improvement of NALP3/inflammasome in macrophages.

This section describes the biologicals mechanisms that govern the adjuvant action, as well as the immune potential of each adjuvant. The toxic effects of these preparations and special methods of use are also briefly introduced. Knowledge of the adjuvant effects is far from complete and some aspects of their activity are still under investigations.
The main features of some commercial adjuvants are summarized in *Table I.*

MODE OF ACTION, EFFECTIVENESS AND TOXICITY OF MAIN ADJUVANTS

Vaccine Category	Vaccine Adjuvants	Vector	Admnistration route	Targeted disease	Action Mode	Benefits	Drawbacks
Minerals salts [1] [2] [3] [4]*	**AlumVax Hydroxide** **AlumVax Phosphate**	DNA/proteins	Intramuscular, intradermal, intramuscular,	Tetanus, Diphteria toxoid, B. Pertussis, Poliomyelitis, Leishmania, Hepatitis A, Hepatitis B,	- Short term depo effect - Induction of cytokine network - Complement activation - Delivery of antigens to different APCs - Strong Th2 response	- Inexpensive - Consider the safest adjuvant - Efficient uptake of alum adsorbed antigens by APCs - Induce potent Th2-type immune response - Long lasting immune response - Antigen slow release	- Not suitable for recombimant proteins and sub unit vaccines - Adverse effects have been reported such as inflammation and stimulation of local production of erythema, granuloma, subcutaneous nodules and Ig E mediated hypersensitivity. - Poor inducer of T -cell response
O/W Emulsion: • **Freund's** [5] [6]*	**CFAVax** **IFAVax**	DNA/proteins	Intramuscular, intraperitoneal, subcutaneous,	*Mycobacterium Tuberculosis, Ebola*, Influenza, Polio-myelitis, antibody production, Cancer	**CFAVax** - Short term depot effect Strong Th1 and Th2 response **IFAVax** - Short term depot Weaker Th1 and and Th2 response	**CFAVax** - Strong immune response **IFAVax** - Less side effect than CFAVax	**CFAVax** - Highly toxic **IFAVax** - Poor immunomodulatory effect Local irritant effect may induce granuloma and cyst formation
• **Nanoemulsion** [7] [8] [9]*	**SqualVax** **CNE-CPO**	DNA/proteins	Intramuscular, intraperitoneal, subcutaneous,	Influenza, Coronavirus, *Malaria*, Hepatitis C, HIV, Cytomegalovirus (CMVs), Cancer	- Inducing local immune stimulatory effect at the site of injection - Regulates cytokines, chemokines - Recruitment of CDB11b+, MHC II + cells and enhance antigen uptake	- Superior immunization than alum adjuvant for influenza - Promote potent Thf response	- Pain at injection site - Reactogenicity - Induces inflammatory arthritis
Delivery Vehicles: • **Lipids** [10] [11]*	**CaliVax-DOTAP**	RNA/DNA/ proteins	Intramuscular, intraperitoneal, intraveinous	Influenza, Hepatisis A, Tuberculosis, Malaria, Leishmaniasis, HIV, Cancer	- Fuse with macrophages membrane enable antigen into the cytoplasma - Enter MHC class I path way - Activate CD8 CTL response	- Strong immune response - Use in large variety of antigens - Favourable biodistribution	- Manufacturing difficulties due to stability - High cost - Severe pain at the site of injections
• **Polymer** [12] [13]*	**PolyVax-CPO**	DNA/proteins	Intramuscular, oral, intranasal	HIV, Rotavirus, Cytomegalovirus (CMVs), Hepatitis, Influenza	- Long term depot effect from weeks to months - Pulsatile release of antigens - Target to APCs	- Mimic the priming and boosting effect of conventional vaccine - Potential for single shot vaccines and can reduces the cost of vaccination - control the way of antigens release (slow or fast)	- Issues on stability of antigens during micro-encapsulation and storage - Issues on dose optimization

Table 1 : Characteristic of vaccine adjuvants

* see references at the end of the document (p8)

AlumVax Hydroxide/Phosphate

CFAVax

IFAVax

SqualVax

CNE-CPO

CaLiVax-DOTAP

PolyVax-CPO

MODE OF ACTION, EFFECTIVENESS AND TOXICITY OF MAIN ADJUVANTS

Mineral adjuvants

AlumVax stimulates the production of antibodies by inducing a Th2-type response (Nicklas, 1992). During mouse animal models, the antibodies produced are essentially of IgG1 type; most of the cytokines associated with this production are IL-4 and IL-5. Significant IgE levels are also observed, mainly due to the IL-5 production. Antigens are adsorbed onto the adjuvant, and the binding of antigen to aluminium salts is believed to be a strong electrostatic interaction that enhances antigen uptake and presentation by anti-gen presenting cells (APCs). AlumVax could stimulate the NLRP3 inflammasome activation and thus stimulating IL-1b and IL-18 productions. **[14-15]** This may explain their ability to induce local inflammation, antigen presenting cell (APC) recruitment, dendritic cell maturation, enhanced antigen uptake, and stimulation and differentiation of T cells. It has been noted that not all proteins are adsorbed efficiently by aluminium salts. **[16]**
Aluminium salts are one of the first adjuvants registered for use in vaccines since the 1930s. They have demonstrated both sufficient efficiency and safety to be used in commercial prophylactic vaccines. Although they are effective against various diseases, the antibody production induced by these adjuvants remains moderate.

Freund's adjuvants

These adjuvants carry molecular patterns specific to bacteria, sometimes called PAMP (Pathogen Associated Molecular Patterns), and are able to activate non-specific defense mechanisms. The CFAVax and its derivatives are powerful inducers of IL-1 production. Their mode of action is based on an interaction of LPS and its derivatives with Toll-Like receptors (TLRs), including in particular TLR-4. **[17]** The CFAVax therefore combines the deposit effect of a water-in-oil emulsion and the non-specific immunity activation from the inactivated bacteria, not to mention the stimulation of specific helper T cells from M. tuberculosis epitopes. These properties make it a powerful activator of both the humoral and cellular responses. The humoral component of the induced response is related to the oil-in-water emulsion, while its cellular component is attributable with mycobacterial extracts. **[18]**
The pro-inflammatory feature of Complete Freund's adjuvant is untowardly at the basis of its many deleterious effects and can cause at the onset abscesses, granulomas, and cutaneous necrosis as well as auto immune reactions. The IFA, (CFA without the mycobacteria) is the best known and most used water-in-oil emulsion. It can induce a better antibody response than alum adjuvant. However, IFA is unable to significantly stimulate cellular responses against tumors and against some viral infections. Even if it doesn't have the toxic effects systems of the CFA, the IFA can cause lesions at injection site.

[14-15] Sun B, Ji Z, Liao Y-P, Wang M, Wang X, Dong J, et al. Engineering an effectiveimmune adjuvant by designed control of shape and crystallinity of aluminumoxyhydroxide nanoparticles. ACS Nano 2013;7:10834–49.

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Nano-emulsion adjuvants

The oil in water (o/w) emulsion favors the collection by APCs. This phenomenon of «targeting» of dendritic cells and macrophages can be explained by different mechanisms: the antigen incorporated by the APCs is focused and protected against degradation, phagocytosis is stimulated by different interactions ligands-receptors and the structures formed can fix the complement component C3b.**[19]** When an o/w emulsion is administered, the aqueous phase continues to disperse quickly. On the other hand, the released oily spherules would transport the antigen directly to the lymph nodes via lymphatic vessels. The targeting and the deposition effect of this type of adjuvant is therefore exerted not only at the injection site but also within the lymphoid tissue. Most of the emulsions used as adjuvant do not induce local reaction. The synthetic copolymers used are not highly inflammatory and the squalene is an abundant constituent of the body such as precursor of cholesterol. In contrary with water-in-oil emulsions, the rapid dispersion of an oil emulsion in water prevents formation of granulomas and abscesses. These emulsions have the consistency of milk and are therefore easy to inject. The delicate incorporation of the antigen into a preformed emulsion also allows to avoid a partial denaturation.

Saponins and «ImmunoStimulating Complexes

The most popular saponin preparations used are the QS-21 and Quil-A®. These adjuvants induce a CTL-type response as well as a significant IgG2a production. The Quil-A®-alum association is found in several veterinary vaccines, for example labelled vaccines inactivated against IBR (Infectious Bovine Rhinotracheitis). ISCOMs are used in veterinary medicine including in a vaccine for equine influenza and rhinopneumonia (Equilis Resequin®), where they are once again combined with aluminium hydroxide. They also induce a mixed Th1/Th2 response, by targeting APCs, and are able in particular to stimulate MHC I (Major Histocompatibility Complex Type I) cytotoxic T lymphocytes. This phenomenon is probably explained by the ability of saponins to interact with cholesterol to generate pores into membrane cells. In this sense, the antigen could be introduced directly into the endogenous track. However, this type of interaction between saponins and membranes can cause hemolysis. In addition to the classic parenteral routes, ISCOMs can be administered orally or nasally.

Lipid-based adjuvants

The immunostimulatory activity of liposomes is based on their ability to present the antigen adequately for the exogenous route of presentation by APCs, and both to be internalized by APCs in order to stimulate endogenous mode of presentation. The virosomes can target very specific APCs, and tend to reproduce the natural mode of presentation of viral antigens. It is by example the case of IRIVs (Immuno-potentiating Reconstituted Influenza Virosomes). In addition, vesicular adjuvants have some deposit effects. The result is characterized by both high antibody levels and by CTL activity. However, these systems are difficult to formulate.

Polymer-based adjuvants

These particulate systems are usually directly taken up by APCs. Unlike conventional adjuvants, these polymeric particles are biocompatible, degradable and are capable of delivering antigens more efficiently. They can release antigen in vivo in a controlled manner and protect the antigens against fast degradation after administration within the organism.

[19] KO, Eun-Ju et KANG, Sang-Moo. Immunology and efficacy of MF59-adjuvanted vaccines. Human vaccines & immunotherapeutics, 2018, vol. 14, no 12, p. 3041-3045.

CONCLUSIONS AND OUTLOOK

A large number of adjuvants and their various modes of action are currently being studied both in y veterinary filed and in clinical applications. To date, the adjuvants for use in commercials vaccines do not induce a Th1-dominant response. To optimize this property could be the key of effective immunization against many tumors or infectious diseases that are nowadays incurable. However, the production of specific antibodies remains essential to obtain protection; this is why the new vaccines will undoubtedly have to combine several adjuvants. This is particularly the case with new experimental vaccines against HIV.

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