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Adjuvants designed for veterinary and human vaccines

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Abstract

Adjuvants play an important role in the efficacy of vaccines as the antigens become more and more purified. Indeed recombinant proteins or synthetic peptides are safer than crude inactivated micro-organism, but less immunogenic. This can be balanced by specific adjuvants. But there is no universal adjuvants and their action is not yet clear and rely on different mechanisms. Then, they must be adapted according to several criteria, like the target species, the antigens, the type of immune response, the route of inoculation, or the duration of immunity. For this purpose different type of emulsions have been developed. Water in oil (W/O) emulsions induce a strong and long term immune response. Those based on mineral oils are known to be very efficient but can sometimes induce local reactions with reactive antigens. Non mineral oils are well tolerated but less efficient with poor immunogens. Multiphasic (W/O/W) emulsions can induce short and long term immune responses with various antigens and oil in water (O/W) emulsions are well tolerated and induce a short term immune response. New generation of adjuvants are based on a new concept called 'immunosol' and stem from the association of nanoparticles with a new immunostimulant. They can be used when emulsions are not suitable to obtain a good balance between safety and immunogenicity.

Keywords: Emulsions; Nanoparticles; Adjuvants

1. Introduction

Various adjuvants have been used in order to enhance the immune response against specific antigens since 1925, when Ramon [1] first demonstrated that it was possible to enhance artificially the diphteric and tetanic antitoxin level by the addition of substances such as agar, metallic salts, lecithin or saponin. Adjuvants play an important role in the efficacy of vaccines as the antigens become more and more purified. Indeed, recombinant proteins or synthetic peptides are safer than crude inactivated micro-organism, but less immunogenic. Many molecules have adjuvants properties [2] and can be classified in different ways. Cox and Coulter proposed to separate particular and non-particular adjuvants [3] and Audibert and Lise have identified three main sources of immunoadjuvants [4]. Vegetal, like saponine or glucan extract, bacterial like monophosphoryl lipid A, trehalose dymicolate, choleric toxin or lipopolysaccharides and their derivatives, chemical like aluminium hydroxide, surfactants, emulsions, or micro and nanoparticles. A fourth group containing cytokines like IFNγ or GM-CSF and hormones like Dihydroxyepiandrosterone (DHEA) can be also defined.

Emulsions have been first described 1916 by Le moinic and Pinoy [5] but it is Jules Freund who has developed this concept [6]. However, the initial emulsion was very unstable and viscous, and strong local reactions were observed. Nowadays new generation of oils and surfactants allow the development of stable safe and fluid emulsions [7].

1.1. Definition of emulsions

An emulsion is defined as a dispersion of a liquid called the dispersed phase in a second liquid called the continuous phase with which the first one is not miscible. In vaccine formulations, these phases are water (antigenic media) and oil. In order to stabilise the emulsions, surfactants are added. A surfactant is a compound containing a polar group which is hydrophilic and a non polar group which is hydrophobic

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and often composed of a fatty chain. Surfactants can be defined by their Hydrophilic/Lipohilic Balance (HLB) value which gives an information on their relative affinity for the both phases. According to the HLB value of the surfactant, different kind of emulsions can be achieved [8]. Those having a low HLB value have a high affinity for oily phases and render W/O emulsions. In this case, the antigenic phase is made of droplets dispersed into the continuous oily phase. Those with a high HLB value have a high affinity for the aqueous phase and render O/W emulsions, where the continuous phase is water and the dispersed phase is oil. At last, with certain specific surfactant systems, when the HLB value is intermediate, W/O/W emulsions can be achieved. In this case, the continuous phase is aqueous and the dispersed phase is oil. But inside the oil droplets, an entrapped aqueous phase is found.

1.2. Quality of emulsions

The quality of emulsions is an important parameter as it has a direct impact on the efficacy and the safety of adjuvants. The physicochemical characterisation of an emulsion can be defined by various parameters such as droplet test, conductivity, viscosity, particle size and stability at various temperatures.

Droplet test and conductivity allow the identification of the type of emulsion. A droplet of a W/O emulsion into a beaker containing water will stay at the surface. On the opposite, an O/W emulsion will spread into the water.

The viscosity of the emulsion is closely linked to the surfactant structure and its HLB value [9]. Non optimised HLB renders viscous W/O emulsion, like incomplete Freund adjuvant which have a high viscosity, around 2000 mPa s, rendering it difficult to inject. Fluid W/O emulsions can be achieved with surfactants having an optimised HLB value called required HLB, which depends on the nature of the oil. The viscosity can be then decreased by 10 to reach about 200 mPa s. W/OW emulsions and O/W emulsions have a lower viscosity, respectively around 50 and 10 mPa s. (Table 1). The ratio between the continuous phase and the dispersed phase has a strong influence on the viscosity. An increase of the dispersed phase leads to an increase of the viscosity of the final emulsion, due to droplets close pack network. The continuous phase must increase in order to decrease viscosity. Hence, when water in oil emulsion are achieved with adjuvants adapted for a ratio of 70% of oil and 30% of aqueous phase, the viscosity can decrease to reach 50 mPa s, whereas a similar optimised formula for a ratio 50/50 will have a 4 times higher viscosity (Table 1).

The particle size is also an important parameter influenced by an adequation of the surfactant system and the emulsification process. It is generally recognised that emulsions having a small particle size and a homogeneous distribution are more stable.

Stability is very important and various parameters can have an influence on it. Mineral, vegetal, animal or synthetic oils can be used alone or in combination, but their required HLB are different, hence each oil phase need HLB surfactant adjustment.

The non respect of the ratio defined for initial optimisation between the oil phase and antigenic phase can have a negative effect on the stability of the final emulsion. Moreover, antigenic media often contain proteins which have surfactant properties, as they are constituted of polar and non polar groups. This can modify the global HLB inducing poor stability. In this case, specific HLB adjustments need to be done. At last, the manufacturing process is also important. Indeed, W/O emulsions require high shear homogenisation and then specific device to get stable formulations. The same process applied to make a W/O/W emulsion will render it unstable.

Stability is generally checked at 4°C and at room temperature as it corresponds to the temperatures of storage and use of the vaccine. Trials are also realised at 37°C in order to simulate an accelerate ageing however, the correlation between stability results at 37°C for 1 month and stability results at 4°C for 2 years is not obvious [10].

Table 1
Physicochemical characteristic of W/O, W/O/W, and O/W emulsions and compared to incomplete Freund adjuvant (IFA)

	IFA	Montanide ISA 50	Montanide ISA 70	Montanide ISA 206	Montanide ISA 25
Oil/water ratio	50/50	50/50	70/30	50/50	25/75
Droplet test	W/O	W/O	W/O	W/O/W	O/W
Conductivity	<1 μs	<1 μs	<1 μs	>1 ms	>1 ms
Viscosity	2000 mPa s.	200 mPa s.	50 mPa s.	50 mPa s.	20 mPa s.
Microscopic aspect	1–5 μm	1 μm	1 μm	1 μm	0.5–1 μm
Stability 4°C	Few weeks	>2 years	>2 years	>2 years	>2 years
Stability 20°C	Few weeks	>2 years	>2 years	>6 months	>2 years
Stability 37°C	Few weeks	>3 months	>3 months	>1 week	>2 months

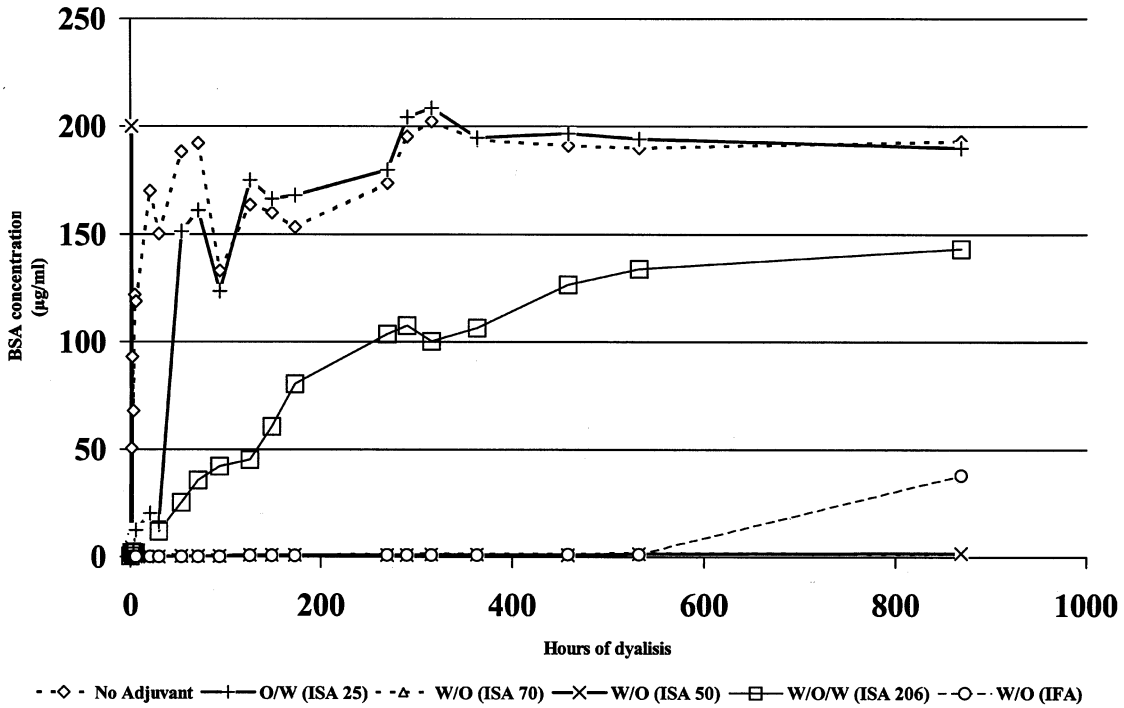

Fig. 1. In vitro kinetic release of Bovine Serum Albumin formulated in various emulsions.

1.3. Mechanism of action

Their action is not yet clear and relies on different mechanisms. The first one is the depot effect and the slow release of the antigen from the injection site. According to the type of emulsion, the kinetic release of the antigen varies. Experimentation where the inverted dialysis tube method is employed [11] to assess the in vitro kinetic release of a protein from an emulsion clearly show differences according to the type of emulsion (Fig. 1). Whereas the protein without adjuvant is immediately released, O/W emulsions allow a slight delay, but the protein is quickly released. W/O emulsions induce no or very low release of antigen. This is correlated with the stability of the emulsion and as soon as the emulsion breaks, large amounts of antigen are released, but slower than O/W emulsions. W/O/W emulsions have an in between behaviour. The depot effect is not the only mechanism as Freund has demonstrated that the excision of the material at the injection site does not suppress the adjuvant effect [12]. Microdiffusion of oil droplets to the draining lymph nodes can partly explain this observation [12]. Emulsions protect also the antigen from a rapid degradation by enzymes and could modify the electric charge of the antigen becoming then, more immunogenic [13]. They create also an inflammation and stimulate the recruitment of antigen presenting cells such as macrophages, but also lymphocytes. They are also able to favour the uptake of antigens by APC [14] and this can be explained by the interactions between the surfactant and the cellular membrane. Lymphocyte trapping is another mechanism of action of oil adjuvants. They stimulate the accumulation of lymphocytes in draining lymph nodes and alter recirculation hence facilitating cell association [15]. Finally, specific cytokines can be induced according to the type of emulsion selected [16].

1.4. Safety

Traditional oil adjuvants can induce local and general reactions, like granuloma, abscesses or fever, but they depend on different parameters. The origin of the oil is important. Indeed, highly purified non mineral oils are well tolerated as they are rapidly metabolised and eliminated from the injection site, inducing a weak and transient inflammation [17]. But this is to the detriment of their efficacy [18,19]. Mineral oils stay at the injection site and are progressively eliminated by competent cells like the macrophages. They can be also partially metabolised in fatty acids, triglicerids, phospholipids or sterols [20]. In fact, a very low level of real hydrocarbons is found outside the injection site. Bollinger et al. demonstrate that 30% of the mineral oil disappear during the first month and majority of the oil found outside the injection site is in the liver and fatty tissues in the form of phospholipids and fatty acids [20]. The mineral oils are a mix of several hydrocarbons with different length of carbon chain. Stewart-Tull et al. [21] clearly show the direct impact of the length on the safety of adjuvants. Small chains are efficient but induce local reactions, whereas longer chains ($>$ C14) are

safer but less efficient. The solubilising and detergent properties of small chains are probably responsible for these local reactions.

The quality of the surfactant is also important. Hardegree et al. [22] demonstrate that the toxic effect of non ionic sugar ester surfactants is correlated with free fatty acid level. Optimisation of specific chemical synthesis now allows a low residual fatty acid level. Checking various parameters, like acid value, saponification value or iodine value, into very narrow range allow the control of the reproducibility and consistency of the surfactants. Their tendency to oxidise can be avoid by an appropriate storage under inert gas and a control of the peroxide index.

The antigen has also a strong influence on the safety of the vaccine formulation. Bacterial crude extracts often induce strong local reactions when administered in emulsion. This can be explained by their structure, which can contain immunostimulating compounds like LPS structures or peptidoglycan fragments, responsible for the induction of secondary reactions [23]. Viral antigens are generally safer but in order to avoid side effects, purified antigens or synthetic peptides can be used. Antigenic concentration and vaccine dose are also important, as side effect can be avoided by decreasing them.

2. Veterinary vaccines

In order to be used in this field, adjuvants must enhance the specific immune response against pathogens and improve protection. They must be stable and safe as secondary effects can have an impact on the growth of the animal, the reproduction rate, the comfort of the animal or cause carcass blemish. Adjuvants must be easy to use. It means that the emulsification manufacturing process as well as the injectability have to be convenient. They must be also cost effective. A good adjuvant can allow a reduction of the dose or of the antigenic concentration, decreasing then the price of the vaccine. Emulsions seem to encounter those criteria as more than 500 million doses of vaccines are used each year for livestock [24].

2.1. Water in oil emulsions

Generally, water in oil emulsions are recommended for bovine, small ruminants, poultry and fishes when long term immunity is required. In the case of foot and mouth disease, mineral oil based emulsions can protect bovine for 1 year with one vaccination whereas formulations based on aluminium hydroxide required two boosts ore more [25]. When more reactive antigens are tested, emulsions based on non-mineral oils can be used but this is to the detriment of the vaccine efficacy. Then, the mix of mineral and non-mineral oil can be a good alternative. Even if some local reactions occur, W/O emulsions can be used when the protection against specific diseases as compared to other formulations or other routes of administration, is enough to justify some side effects. This is the case for fish vaccines against furonculosis, where the procedures can be limited to one injection as the protection is maintained during the all growing period [26].

W/O emulsions allow the reduction of the vaccine dose or the antigen concentration, which is important as vaccines must be cost effective. Chickens, vaccinated against Newcastle disease with a mineral oil based adjuvant are protected against challenge even if only 1/100 of the dose is administered [27]. Non mineral oil adjuvants are less efficient but they can still induce 100% of protection with 1/50 of the dose. W/O formulations can also enhance cellular immune response. Various screening studies in mice with viral, bacterial or parasitic antigens, have shown that water in oil emulsions induce higher IgG2a antibody levels than other type of emulsions (unpublished data). Vaccination of sheep against Heartwater with W/O formulations enhance protection against challenge and are well tolerated. Protection is not correlated with humoral response suggesting that cellular immune response is enhanced [28]. This is confirmed by flow cytometry analysis showing an important increase of the CD8 + cells after immunisation with water in oil formulation and challenge [29]. At last, W/O emulsions are able to induce a protective cytotoxic T cell response [30].

2.2. Water in oil in water emulsion

First, multiphasic emulsions developed by Herbert [31] were made in a two step process, rendering the manufacture of the vaccine formulation difficult. Moreover, the stability of the emulsion was not very good [7]. Now, one step emulsifying process has been developed, giving stable, fluid and easy to use double emulsions. The interests of these emulsions are their low viscosity and their ability to enhance short and long term immune response. The antigen in the external aqueous phase is immediately available to the immune system like aqueous formulations whereas antigen in the internal aqueous phase is slowly released like water in oil emulsions. In foot and mouth disease, such formulation is able to protect swine as well as bovine against the disease only 4 days after vaccination [32] which can be very useful in case of outbreak. But, multiphasic emulsions can also induce long term immunity and protect bovine against haemorragic septicaemia for 1 year after only one vaccination [33]. Those based on mineral oil are recommended for swine, however, with reactive antigens it is preferable to avoid vaccination of fattening pigs as it can cause carcass

blemish. As W/O emulsions, the mix of mineral oil and non mineral oil can be a good alternative. W/O/W emulsions generally enhance humoral immune response. A comparative study where mice were vaccinated with a recombinant adenovirus formulated in various types of emulsions, has shown that W/O/W emulsion based on mineral oil and is the only one able to induce IL6 cytokine and gives the best protection [16].

2.3. Oil in water emulsions

Oil in water emulsions are very fluid, well tolerated and induce strong short term immune responses. The oil phase ratio is very low, between 15 and 25%, and partly explains their safety. The manufacturing process is very convenient as it requires just a low mixing. Emulsions based on mineral oil can be safely used for fattening pigs in order to enhance antibody responses against bacterial [34] or viral infection [35] but also the potency of live vaccine like pseudorabie vaccine [36]. Ganne et al. have also improved the efficacy of a DNA vaccine against pseudorabie in a mice model [37]. Vaccines for pets and horses must not induce any local reactions and then O/W emulsions based on non mineral oil are adapted.

2.4. Nanoparticles

Even if emulsions can be used in numerous vaccines, there is still a need for new generations of adjuvants. Water dispersed liquid nanoparticles combined with an immunostimulating compound are an interesting concept. These nanoparticles have a size, which can vary from 10 to 500 nm. No specific process is required to manufacture the vaccines, consisting in a simple mixing of antigenic medium and nanoparticles solution. Trials in swine against atrophic rhinitis or pleuropneumonia demonstrate that such formulation could enhance immune response without inducing local reactions [38,39] and vaccination of bovine against Anaplasmosis gave 100% of protection [40]. Moreover fish trials confirm their good efficacy and safety and various trials in pets and horses are ongoing.

3. Human vaccines

The only adjuvants authorised in human are aluminium compounds [41]. But many clinical trials are ongoing to assess vaccines containing new adjuvants formulations and recently a new oil in water emulsion has been registered in an influenza vaccine. W/O emulsions are also assessed in human field for therapeutic application such as aids, malaria or cancer immunotherapy. Since 1945, more than 1 million of persons have been vaccinated with mineral oils. First trials reveal some sterile abscesses or cysts [42] but they were related to the quality of the surfactant or the oil. With the development of new grades of surfactants and oils avoiding free fatty acids, and short fatty chain, J. Salk has realised between 1951 and 1953 vaccination trials against flu on 18 000 soldiers with incomplete Freund adjuvant and compared to 22 000 soldiers vaccinated with classical formulation. The follow-up during 35 years reported first by Salk [43], then by Beebe [44,45] and finally by Page [46] demonstrate the safety and non carcinogenicity of these formulations. Today, more than 1000 people have been vaccinated with formulations containing Montanide ISA 51, which is an mineral oil based emulsion and various publications report the well tolerance of this adjuvant [47,48]. Another adjuvant, Montanide ISA 720 [49], based on non mineral oil is also in current human clinical trials and phase I–II demonstrate the well tolerance of the product [50]. Other type of formulations could be interesting for human application such as Montanide ISA 25D or 206D rendering, respectively O/W and W/O/W emulsions. However, even if a large background exists in the veterinary field with these adjuvants, the lack of toxicological data in human field renders reluctant to start preclinical trials.

4. Conclusion

There is no universal adjuvant and they must be adapted according to several criteria in order to have the best balance between safety and efficacy. According to the target species, the choice of the emulsion will vary as some animals are more sensitive than others. Bovine and chickens can be vaccinated with W/O emulsions whereas swine will require well tolerated adjuvant like O/W emulsions. In the case of humans, W/O emulsions are currently in clinical trials for immunotherapy projects but O/W and W/O/W could be interesting to test. The antigen, which can be a crude extract, a purified protein or a synthetic peptide, coming from bacteria, virus or parasite origin should influence the selection. Mineral oils can be used when non reactive antigens like purified proteins or synthetic peptides are used. Non mineral oils are preferable with more reactive antigens. W/O/W and O/W can be used with live or DNA vaccines. Adjuvants must be able to enhance humoral or cellular mediated immune according to the mechanism of protection against the disease. W/O emulsions are able to induce cellular response. W/O/W or O/W enhance humoral response but can be associated with adjuvants able to enhance cellular response. The duration of immunity has also to be considered and the selection of the adjuvant is different if short or long term immunity is required. The route of

inoculation is also important as it can have an influence on the local reactions and on the type of immune response induced. Subcutaneous and intramuscular administration of the same vaccine formulation can give different immune responses [51]. When emulsions are not satisfying according to these criteria, liquid nanoparticles can be a good alternative.

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