A Novel Emulsion Adjuvant Increases the Immunogenic Responses and Protective Efficacy of an Inactivated Influenza Vaccine in BALB/c Mice

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Key Words

Oil emulsion • APR8 vaccine • MF59 • WAF3 • Adjuvant

Abstract

Objective: The research of adjuvants is a hot topic in vaccinology. In this paper, we were seeking a concise method to produce a stable and effective emulsion adjuvant. **Methods:** A novel emulsion [Well Adjuvant Formulation 3 (WAF3)] was produced by adding additional glycerol and using a timely cooling process. Surface morphology analysis of the novel emulsion was performed by atomic force microscopy. Animal experimentation was used to evaluate the efficacy of the novel adjuvant. **Results:** Surface morphology analysis of the novel emulsion showed a homogeneous distribution of the antigen protein. We also confirmed the morphological changes of the WAF3-carrying antigen. The WAF3-adjuvanted vaccine engendered higher antibody responses 128-fold compared to that of a naked antigen. Furthermore, in the presence of the WAF3 adjuvant, the influenza virus antigen was able to reduce the titer disparities of IgG2a and IgG1. The WAF3-adjuvanted influenza vaccine was able to provide full protection and alleviation of infective symptoms as other clinically adjuvanted vaccines. **Conclusion:** The WAF3 adjuvant is a promising candidate to be further investigated in the development of emulsion adjuvants.

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Introduction

The idea of using certain materials to enhance immune responses has been recognized for many years [1]. Since the discovery of aluminum hydroxide as an adjuvant for tetanus toxoids, a lot of components were investigated for their adjuvanticity [2]. Preclinical and clinical studies have confirmed that adjuvants can offer the opportunity to enhance vaccine efficacy and overcome impaired immune responses induced by conventional protein vaccines [3, 4].

Emulsion adjuvants are important parts of adjuvant categories [5]. Emulsions are known as effective adjuvants, able to generate high and durable antibody responses for protein vaccines [6, 7]. In 1916, oil emulsion was used in vaccine formulation by Moignic and Pinoy [8]. After decades, Freund et al. developed Freund's incomplete and complete adjuvants [9]. Freund's complete adjuvant cannot be used for humans because of severe side effects, including pain, abscess formation and fever. Subsequent reports explained that these side effects were related to the bacterial component of Freund's complete adjuvant [5]. Freund's incomplete adjuvant lacks the bacterial component and has been applied in several animal vaccines. However, as the mineral oil used in it can cause tumors in mice, Freund's incomplete adjuvant cannot be used for human vaccines. In the 1980s, a potent delivery

system (Syntex adjuvant formulation) was developed using a biodegradable oil (squalene), whose side effects were acceptable for human use [6]. However, because of the addition of a muramyl dipeptide derivative and pluronic polymer surfactant (L121), the Syntex adjuvant formulation was not used in humans [10].

Subsequently, the Chiron Corporation developed a squalene emulsion (MF59) without additional immunostimulators. MF59 emulsion is a typical oil-in-water emulsion system, which contains 5% squalene, 0.5% Tween 80, and 0.5% Span 85, with a homogeneous droplet size and low viscosity [11]. It has been proven that MF59 emulsion is a potent adjuvant with an optimal safety profile for various vaccines [12]. MF59 enhances the immunogenicity of influenza vaccines and has been shown to be a more potent adjuvant than alum for hepatitis B vaccines in baboons [3]. To date, the MF59-adjuvanted influenza vaccine is licensed in more than 20 countries [13]. The vaccination population of the MF59-adjuvanted flu vaccine consists of more than 30 million subjects [14].

Nevertheless, there are some questions which limit this promising potentiator. First, a homogenizer and microfluidizer are always required during the preparation of MF59. In other words, the preparation method is very tedious. Moreover, large droplets should be removed by a 0.22- μ m filter [15, 16]. In addition, MF59 emulsion is a Th2-directing adjuvant for protein vaccine. It feebly augments Th1 immune responses [15, 17]. Therefore, it is much more valuable to develop concise preparation methods and emulsion systems which have the potential to be used with a variety of protein antigens.

In this paper, we prepared a novel emulsion adjuvant according to a much more concise method, named 'Well Adjuvant Formulation 3' (WAF3). Then, we analyzed the surface morphology of WAF3 microspheres by atomic force microscopy (AFM). We also compared the immunogenic responses of an inactivated influenza vaccine in combination with a WAF3 adjuvant and evaluated the protective efficacy of WAF3 emulsion in mice.

Materials and Methods

Mice

Inbred female BALB/c mice, 6–8 weeks old, were purchased from the Laboratory Animal Center, Hubei Academy of Medical Sciences, and raised in specific pathogen-free living quarters of laboratory animals at the College of Life Science, Hunan Normal University.

Preparation of the WAF3 Emulsion

The WAF3 emulsion was prepared as follows. Glycerol (20%, v/v), squalene (5%, v/v), Tween 80 (0.2%, v/v) and Span 85 (1%, v/v) were emulsified by a vortex agitator for 5 min. Then, the coarse emulsion was submerged in liquid nitrogen for 30 s and dissolved in an ice bath. The dissolved mixture was mixed with a syringe. Meanwhile, phosphate-buffered saline (PBS) including the inactivated antigen was added as an aqueous phase. Finally, the oil phase and aqueous phase were mixed slightly to produce the stable emulsion.

The Surface Morphology of WAF3 Droplets

Surface topography of emulsion droplets (i.e., roughness) can also affect the type, quantity and conformation of an adsorbed protein layer. In order to investigate the surface roughness of the new emulsion surfaces, AFM was used. WAF3 microspheres were prepared as specified above and examined by an atomic force microscope (SAP3800N, SPI, Seiko Corporation, Tokyo, Japan) in the tapping mode. The topography images were further processed by second-order line flattening and the images were exported to ImageJ (NIH software, USA).

Preparation of the MF59 Emulsion

The MF59 emulsion was prepared according to Ott et al. [15]. Briefly, Tween 80 was dissolved in a PBS buffer solution. Separately, Span 85 was dissolved in squalene. These 2 solutions were mixed and processed to obtain a coarse emulsion. Then, this coarse emulsion was passed through a microfluidizer to yield the stable emulsion. Finally, the bulk emulsion was filtered through a 0.22- μ m filter to remove large droplets.

Immunization and Specimens

An inactivated influenza virus A/PR/8/34 (H1N1) vaccine was prepared by the Shanghai Institute of Biological Products and the concentration was checked by a BCA kit (Pierce, Thermo Fisher Scientific Inc., Portsmouth, N.H., USA). The MF59-adjuvanted vaccine was prepared by mixing MF59 1:1 with an inactivated antigen. Each mouse dose contained a 0.015- μ g antigenic protein. The inactivated virus was adsorbed to aluminum hydroxide at 4°. Each mouse dose contained 100 μ g of alum. Animals were immunized i.p. twice, 3 weeks apart, at a volume of 200 μ l. The control mice were injected i.p. with 200 μ l PBS. Three weeks after primary immunization and 1 week after secondary immunization, blood samples were collected from the tail vein of the immunized mice.

ELISA for Serum Antibodies

The titers of IgG and IgG isotypes were measured against the inactivated influenza virus PR8 vaccine by ELISA, as previously described [18]. ELISA was performed sequentially from the solid phase polystyrene plate with a ladder of reagents consisting of: 1st, an inactivated influenza virus PR8 vaccine; 2nd, 2-fold diluted serum; 3rd, biotin-labeled goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, Ala., USA); 4th, streptavidin conjugated with alkaline phosphatase (Southern Biotechnology Associates Inc.); and finally, *p*-nitrophenyl-phosphate. The chromogen count was measured for absorbance at 414 and 405 nm in an autoreader (Labsystems Multiskan Ascent, Thermo Fisher Scientific Inc., Portsmouth, N.H., USA).

Fig. 1. AFM topography of various WAF3 emulsion surfaces.

Table 1. Root mean square roughness values ($\pm$ SD) obtained from the average of 3 images of WAF3 emulsion surfaces

WAF3 emulsions	Root mean square roughness
Blank WAF3 emulsion	109.34 $\pm$ 10.88
Antigen-carrying emulsion	127.41 $\pm$ 15.12
Free antigen	127.59 $\pm$ 17.03
Antigen + MF59 emulsion	130.09 $\pm$ 18.66

Virus Infection

Two weeks after the 2nd immunization, each mouse was anesthetized and challenged with 20 μ l of homologous A/PR/8/34 (H1N1) virus suspension containing a 40 $\times$ 50% lethal dose by intranasal drip. Changes of body weight and the survival rate of the infected mice were observed to evaluate the protective efficacy of these adjuvanted vaccines.

Results

Surface Characteristics of the WAF3 Emulsion

The WAF3-adjuvanted vaccine was analyzed by means of AFM to determine the characteristics of the

WAF3 emulsion as well as the interplay between antigens and emulsion microspheres. To characterize changes in surface roughness, AFM images were taken of a blank WAF3 emulsion, an antigen-coated emulsion and a free antigen. Images were processed using ImageJ (NIH Software), which provided a flattened topographic image and a 3-dimensional image. Figure 1 shows the captured images of the emulsion and antigen samples. ImageJ was also used to generate root mean square roughness. Table 1 lists the average root mean square roughness values obtained for the various WAF3 emulsion surfaces.

Immunogenic Responses to WAF3-Adjuvanted Vaccine

To investigate the suitability of the antigen-loaded WAF3 emulsion, we compared the serum responses of mice after 2 i.p. vaccinations with various vaccines. After the booster immunization, the WAF3-adjuvanted vaccine generated stronger serum IgG titers than the other groups ($p < 0.05$). We also evaluated the blood samples collected before the secondary immunization. Interestingly, although the WAF3 emulsion enhanced the magnitude of the humoral responses at the early stage, there were no significant differences between the WAF3 group

Fig. 2. Protection from adjuvanted vaccines against the lethal challenge infection in BALB/c mice. **a** Loss of body weight after lethal challenge. After the 2nd immunization, naïve or immunized mice were intranasally infected with a 40 × 50% lethal dose of homologous virus. Mice were monitored for changes in body weight daily for 25 days. **b** Survival rates; all mice in the PBS group died by day 10, postchallenge.

and the MF59 group. In other words, the excellent potency of the WAF3-adjuvanted vaccine required secondary inoculation. Furthermore, we described the IgG subtype profiles of the vaccinated groups (table 2). A booster vaccination with the WAF3-adjuvanted vaccine induced stronger IgG2a or IgG1 titers as compared to other groups. Results also confirmed MF59 emulsion as a Th2-type adjuvant. Both the WAF3 and the MF59 emulsions can induce higher IgG1 titers as compared to the naked in-

Table 2. Influenza-specific serum immunoglobulin G (IgG) and IgG subtype antibody titers induced after 1 or 2 doses of the A/PR/8/34 (H1N1) vaccine¹

Formulations ²	Dose of vaccine µg/dose	Serum IgG/IgG2a/IgG1 titers; ELISA(2 ⁿ) ³			
		primary	secondary		
		IgG	IgG	IgG2a	IgG1
WAF3	0.015	15.0 ± 1.0	20.7 ± 1.3*	18.0 ± 0.9*	18.3 ± 0.6*
MF59	0.015	14.0 ± 1.0	17.3 ± 0.5	14.7 ± 0.8	16.7 ± 1.0
Al(OH) ₃	0.015	13.7 ± 0.9	15.7 ± 0.6	12.3 ± 1.0	10.7 ± 0.6
PR8	0.015	11.7 ± 0.6	13.7 ± 1.0	11.3 ± 1.5	9.0 ± 1.5
PBS	–	0	0	0	0

* $p < 0.05$ between the MF59 group and the WAF3 group.

¹ Female mice were immunized twice, 3 weeks apart, with various adjuvanted whole virus vaccines. Serum samples from the animals were collected 3 weeks after primary immunization and 1 week after the booster. Serum antibody titers were measured by ELISA.

² PR8 + WAF3 = inactivated influenza PR8 virus + WAF3 emulsion; PR8 + MF59 = inactivated influenza PR8 virus + MF59 emulsion; PR8 + Al(OH)₃ = inactivated influenza PR8 virus + Al(OH)₃; PR8 = immunization with inactivated influenza PR8 virus alone.

³ Serum samples were diluted 2-fold serially, and 'n' represents the dilution factor. Values represent mean ± SD for each group.

activated vaccine. Another investigation by our group showed that all the adjuvants can reduce the IgG2a/IgG1 ratio of the whole virus vaccine. The IgG2a/IgG1 ratio decreased in the order of alum-absorbed vaccine, MF59-adjuvanted vaccine and WAF3-adjuvanted vaccine (data not shown).

Protective Quality of the WAF3-Adjuvanted Vaccine

To evaluate the protective quality of the WAF3-adjuvanted vaccine against the lethal challenge in the mouse model, body weight changes and survival rates were monitored after the homologous virus challenge (fig. 2a, b). The blank group (injected with PBS) with the challenge infection showed a significant and increased loss in body weight and symptoms of severe influenza virus infection, such as ruffled fur and shiver. In the end, all mice in the blank group lost 35% of their body weight and died. The body weight loss of the antigen-alone group was much less severe than that of the blank group. However, the survival rate of the antigen-alone group was 67.7%. In contrast, there were no symptoms detected in the adjuvanted groups.

Discussion

In the present study, we investigated a method of preparing emulsion adjuvant and prepared a novel emulsion adjuvant (WAF3). Results demonstrate that the WAF3 emulsion holds great promise as a delivery system for protein vaccines. Measurement results obtained by AFM showed that the roughness of the blank WAF3 emulsion microspheres was very indistinct. Cavities and pores were not detected on the surface of the emulsion droplets, which are often seen on the surface of polymer microspheres [19]. In contrast, the surface of the load-carrying WAF3 emulsion microspheres was rather rough. Clearly, there were lots of flossy proteins adsorbed on the surface of the WAF3 emulsion microspheres. Otherwise, the adsorbed antigen protein is much less compact than unrestricted proteins dissolved in PBS (fig. 1). Moreover, conformational changes were detected around the adsorbed antigens. This is likely to be important for their excellent adjuvanticity. First of all, these adsorbed formulations can help protein vaccines to flow into a targeting position with a higher local concentration where the macrophages are enriched by the emulsion adjuvant [20]. Secondly, the surface restructuring of the adsorbed antigens is necessary for their recognition by receptor cells [21, 22]. The apparent relationship between the exposed area of a protein and antigenic reactivity has been previously confirmed. Moreover, the adsorption of an antigen could induce a surface rearrangement of the protein, resulting in the unmasking of cryptic epitopes, which is important for the specificity of immune responses [23].

Immunogenic analysis of the WAF3-adjuvanted vaccine indicated that a booster inoculation of the WAF3-

adjuvanted vaccine can induce stronger humoral immune responses than the immunization with antigen alone. However, the superior adjuvanticity of the WAF3 emulsion seems to depend on a repeated injection. We surmised that the booster-requiring characteristic is allied to the adsorbed formulations and the incompact state of the adjuvanted antigens, but this remains to be proven. IgG subtype profiles of the WAF3-adjuvanted vaccine indicated that the WAF3 emulsion induced higher IgG2a/IgG1 titers than other groups. It is well known that IgG2a and IgG1 antibodies perform different obligations in mice [24]. IgG2a is accomplished in complement activation and antibody-dependent cellular immunity. Thus, IgG2a is more effective in preventing intracellular virus replication. On the other hand, IgG1 plays a role in neutralizing viruses and in reducing the release of a mature virus [25]. WAF3 emulsion did not change the subtype profiles of the protein vaccine, but was able to markedly enhance both the IgG2a and IgG1 production. This is the groundwork of the excellent protective quality of the adjuvanted vaccine. The WAF3-adjuvanted group consistently attained 100% survival rates with lower loss of body weight. This may be related to higher IgG2a titers, which participates in virus clearance via antibody-mediated cellular immunity.

In conclusion, the present study demonstrates that the WAF3 emulsion is a potent 'ready to formulate' adjuvant formulation for the inactivated flu vaccine. It was manufactured in a simple protocol. With a much lower concentration of antigens, the WAF3-adjuvanted flu vaccine can provide optimal protection against lethal virus infection in mouse model. Studies to evaluate these WAF3 emulsions with other antigens are currently underway.

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