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SCIENCE DIRECT
Vaccine 24 (2006) 5400–5405
Vaccine
www.elsevier.com/locate/vaccine

# Structure- and oil type-based efficacy of emulsion adjuvants

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Available online 17 April 2006

**Abstract**

Oil-based emulsions are well-known immunopotentiators for inactivated, "killed" vaccines. We addressed the relationship between emulsion structure and levels of in vivo antibody formation to inactivated New Castle Disease virus (NDV) and Infectious Bronchitis virus (IBV) as antigens in 3-week-old chickens. The use of a polymeric emulsifier allowed for direct comparison of three types of emulsions, water-in-oil (W/O), oil-in-water (O/W) and W/O-in-water (W/O/W), while maintaining an identical content of components for each vehicle. They were prepared with either non-metabolizable, mineral oil or metabolizable, Miglyol 840. In addition, we assessed the inherent release capacity of each emulsion variant in vitro. Remarkably, we noted that W/O-type emulsions induced the best immune responses, while they released no antigen during 3 weeks. In general, mineral oil vaccines showed superior efficacy compared to Miglyol 840-based vaccines.


*Keywords:* Oil emulsions; Vaccine; Antibody responses; Adjuvant; Release

## 1. Introduction

Poorly immunogenic vaccine antigens require an immunopotentiator, or adjuvant, to induce the desired type and magnitude of immune response. W/O emulsions have a long history and are widely used as immunopotentiating delivery systems for veterinary vaccine antigens, notwithstanding their risk for the occurrence of adverse effects at the site of injection [1]. Certain W/O emulsions are currently tested in clinical trials as potentiators of therapeutic anti-tumour vaccines [2]. An oil emulsion was first used in 1916, for an inactivated *Salmonella typhimurium* vaccine and based on an emulsion of water and vaseline oil [3]. Later Jules Freund developed paraffin oil-based emulsions in the absence or presence of heat-killed *Mycobacteria*, called Freund's incomplete and complete adjuvant, respectively [4].

These were all water-in-oil (W/O) type of emulsions, composed of antigen-containing water droplets (internal phase) trapped within an external, continuous oil phase (Fig. 1). Herbert showed that W/O emulsions retain the antigen at the injection site, allowing a sustained release of antigen [5]. This persistent release of non-replicating antigens likely explains prolonged humoral immunity, caused by provision of antigen to B cells. However, due to high viscosity W/O emulsions are not easy to inject. Moreover, these types of emulsion may induce necro-ulcerative lesions at the injection site [6].

By contrast, oil-in-water (O/W)-type emulsions exhibit low viscosity and are generally well-tolerated, but induce only short-term immune responses [7]. The antigen is located in a water phase that also contains oil droplets. The oil droplets of O/W emulsions may not or minimally associate with antigen [8,9]. Administration of O/W emulsion as a particulate immunostimulator has been shown to result in recruitment of and interaction with antigen-presenting cells at the site of injection. Upon movement towards the draining lymph nodes, they increase the efficiency of antigen presentation to T cells [9]. In general, the composition of O/W emulsions differs from those of W/O-type emulsions. The oil content of O/W emulsions is generally lower and O/W production often requires, besides special equipment, like high-pressure homogenizers, the use of emulsifiers with high hydrophilic lipophilic balance (HLB) value.

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doi:10.1016/j.vaccine.2006.03.074

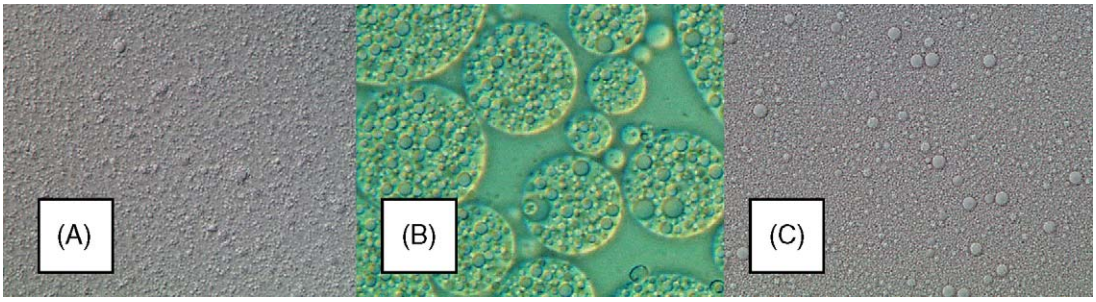


Fig. 1. Microscopic view (magnification 1000×) of prototypic emulsion structures: (A) W/O; (B) W/O/W; and (C) O/W.

An intermediate type of emulsion exists as a multiple emulsion or the so-called double emulsion. Double (W/O/W) emulsions contain water droplets trapped within larger oil droplets, which are dispersed in an external water phase. Antigen may be trapped either within the internal water droplets or the external water phase. W/O/Ws may show acceptable adjuvant activity and duration with local reactions that are possibly more acceptable when compared to those of W/O emulsions [10]. However, physical stability is limited and can only be maintained by the use of special polymeric type of emulsifiers [11].

Each emulsion type requires a special formulation procedure and suitable emulsifiers. It is therefore difficult to relate the adjuvant properties of the emulsion type to a composition or structural feature. In the present study we were able to compare vaccine formulations based on different emulsion structures using an identical composition of ingredients. W/O, O/W and W/O/W emulsions were composed of the same type of oil, a similar oil-to-water ratio and an identical amount of emulsifiers. For all variants either non-metabolizable mineral oil or Miglyol 840, a metabolizable medium-chained triglyceride, was used. The formulations contained the inactivated New Castle Disease Virus (NDV) as antigen, and were administered as experimental vaccines to 3–4-week-old chickens. Goal of this study was to determine the significance of emulsion structure for the efficacy of the vaccine formulation. We conclude that all mineral oil-based formulations showed better efficacy compared to Miglyol-based formulations. W/O was superior to W/O/W or O/W adjuvants. However, O/W emulsions showed best safety profiles.

## 2. Materials and methods

### 2.1. Materials

The oil phases tested were mineral oil (Marcol 52 from Exxon, USA) and Miglyol 840 (medium-chained triglyceride from Huls, Germany) and Tween 80 and a polymeric emulsifier, Arlacel (both Uniqema, UK), were used as surfactants in all the vaccines.

Formalin-inactivated New Castle Disease Virus, strain Clone 30, Infectious Bronchitis virus (IBV), strain Massachusetts 41, both produced in eggs, were the viral avian antigen examined. The total concentration of virus suspension used was 8% (w/v) of the final vaccine. Because of its high stability Infectious Bursal Disease virus (IBDV), strain D78, produced in vero cells, was used in similar emulsion variants in order to perform in vitro release studies. The aqueous phase containing the virus suspension was diluted with 0.01 M phosphate buffer, pH 7.2.

### 2.2. Preparation of the vaccines

Stable and reproducible emulsions were prepared with 0.2% (w/v) Tween 80 and 0.5% (w/w) Arlacel P135. Tween 80 was added to the aqueous phase and Arlacel was mixed into the oil phase at 60 °C. All the inactivated antigen material was part of the aqueous phase. The water-to-oil ratio of all emulsions tested was 60% (w/w) aqueous phase and 40% (w/w) oil phase. By using identical emulsion compositions, but different preparation techniques, we prepared three different types of emulsions, including a water-in-oil (W/O), a water-in-oil-in-water (W/O/W) and an oil-in-water (O/W) emulsion.

The W/O emulsions were prepared by adding the aqueous phase into the oil phase, while mixing was performed with high shear forces at 20,000 rpm using the Ultra Turrax Type T25 (IKA, Germany). In case of a W/O/W emulsion the water-to-oil-to-water ratio was 30 to 40 to 30% (w/w). All the inactivated antigen material was added to the inner aqueous phase. The preparation was performed in two steps. First, the primary W/O emulsion was prepared as described above. The obtained W/O emulsion was then mixed into the secondary aqueous phase at 1000 rpm using the Eurostar mixer (IKA, Germany) provided with a propeller blade.

The production of the O/W emulsion required the use of a high-pressure homogenizer (Microfluidizer type M-120 E; Microfluidics, USA). A premix was prepared by adding a part of the oil phase to 0.01 M phosphate buffer, pH 7.2. This premix was subsequently microfluidized at a pressure of 800–850 bar. All the inactivated antigen material was added. Finally, the remainder of the oil phase was added using the Ultra Turrax Type T25 at 11,000 rpm.

The droplet size of the emulsions was determined by interference microscopy at 1000× magnification (Olympus,