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Comparison of the adjuvant activity of emulsions with different physicochemical properties on the antibody response towards the venom of West African carpet viper (*Echis ocellatus*)

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ABSTRACT

Adjuvant emulsions are widely used to enhance the antibody response of the animals used as immunoglobulin source for producing antivenoms. Usually, the adjuvant activity of emulsions is attributed both to their ability to trigger "danger" signals from cells in which they induce death, and to form depots from which immunogens are slowly released. However, there is contradictory evidence suggesting that adjuvant activity of emulsions is independent of the dispersion type and the rate of immunogen release. In order to test how physical properties of emulsions, composed of mineral oil and water, affect their ability to enhance the antibody response towards snake venoms, we compared water-in-oil (W/O) emulsions prepared at volume ratios of 70/30, 50/50 or 30/70, a 50/50 oil-in-water (O/W) emulsion, and a water-in-oil-in-water (W/O/W) multiple emulsion. Comparison included their droplet-size, viscosity, rate of immunogen release and ability to enhance the antibody response of mice immunized with the venom of the African viperid snake *Echis ocellatus*. It was found that all emulsions released a low amount of venom, and that the 50/50 (W/O) and the multiple emulsion (W/O/W) were those that induced the higher anti-venom antibody response. Our results suggest that the ability of emulsions to enhance the anti-venom response is not associated to their ability to form depots from which the venom is slowly released.

1. Introduction

Emulsions are heterogeneous systems conformed by two or more immiscible liquids, one of which constitutes a discontinuous phase dispersed in the other, which in turn constitutes a continuous (or dispersing) phase. In order to stabilize the liquid/liquid interfaces, interfacial tension is reduced by the addition of surfactants (Brito et al., 2013). Simple emulsions are obtained by dispersing an aqueous solution in an oily phase (W/O emulsions) or by dispersing oil in an aqueous phase (O/W emulsions). On the other hand, multiple emulsions are obtained by the dispersion of a simple emulsion in a phase similar to that used as discontinuous phase. For example, a W/O/W emulsion is obtained by dispersing a W/O emulsion in a second aqueous phase (Florence and Whitehill, 1982; Muguet et al., 2001; Brito et al., 2013).

Similarly to many substances (e.g. mineral salts, surface-active agents, bacterial products, cytokines, hormones, polyanions and polyacrylics) and other vehicles (e.g. liposomes and biodegradable microspheres), emulsions have immunological adjuvant activity. This means that when used in combination with a specific immunogen, emulsions increase the immune response towards that immunogen, compared to the response induced by the immunogen alone (Hunter, 2002).

Because of their adjuvant activity, emulsions are extensively used for immunizing animals to produce hyperimmune sera. For example, Freund or Montanide ISA adjuvants are W/O emulsions used to induce high titers of neutralizing antibodies in the animals used as immunoglobulin source for the production of snake

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antivenoms (Waghmare et al., 2008). However, as these adjuvants induce severe local tissue damage, its use is regulated by international guidelines (Fukanoki et al., 2001; Jansen et al., 2007) and normally is limited to the first stages of the immunization schemes (León et al., 2011). In order to reduce the severity of local damage caused by adjuvant emulsions, the use of low dose, low volume, multi-site immunization protocols has been recommended (Pratanaphon et al., 1997).

The mechanism by which emulsions exert their adjuvant activity is complex and not completely understood. Usually, the adjuvant activity of emulsions is attributed to their ability to: a) trigger "danger" signals from cells in which they induce apoptosis or necrosis (Shen and Ya-Wun, 2012), and b) form depots from where immunogens are slowly released (Aucouturier et al., 2001), generating a sustained stimulus that enhance the antibody production (León et al., 2011).

However, when comparing the antibody response of mice immunized with emulsions prepared with ovalbumin dissolved in an aqueous buffer, squalene and emulsifiers with various hydrophilic-hydrophobic balance (HLB) values, Yang et al. (2005) obtained results suggesting that adjuvant activity of emulsions is independent of the dispersion type and the rate of immunogen release. Thus, the explanation of how adjuvant emulsions operate has become controversial. With the aim of ensuring that the surfactant was the only factor affecting the type of emulsion, Yang et al. (2005) minimized the effect of the phase ratio by preparing all the emulsions at a volume ratio of 50:50 (W/O or O/W).

In this work, we tested whether there are differences in the antibody response to a viperid snake venom when using emulsions with different physical properties obtained by using differential phase volume ratio and dispersion types (e.g. W/O, O/W or W/O/W).

2. Materials and methods

All procedures used in this study involving animals were approved by the Institutional Committee for the Care and Use of Laboratory Animals of Universidad de Costa Rica (approval number CICUA 82-06) and meet the ARRIVE guidelines (Kilkenny et al., 2011).

2.1. Snake venom

Venom of *E. ocellatus* was provided by Prof. Robert A. Harrison. Venom was collected from specimens captured in Nigeria and maintained in captivity at the herpetarium of the Liverpool School of Tropical Medicine. Venoms were stabilized by lyophilization and stored at 4 °C in dark bottles. Solutions of venoms were prepared immediately before use. The batch of venom used in this study had an intravenous median lethal dose (LD_{50}) (and 95% confidence interval) of 6.7 (5.6–8.5) μg/18–20 g mouse (Sánchez et al., 2016), and an intraperitoneal LD_{50} (and 95% confidence interval) of 32.5 (22.1–47.7) μg/16–18 g mouse (Vargas et al., 2015).

2.2. Preparation of emulsions

Emulsions were prepared by using two-syringes connected by a three-way valve. In the case of W/O emulsions, preparation of the mixture was started by thrusting a solution of snake venom, dissolved in 0.12 M NaCl, 0.04 M phosphate buffer, pH 7.2 (PBS), into a solution of 85% mineral oil and 15% of mannide monooleate (Arlacel A), in such a way that 8 μg of venom were contained in 200 μL of mixture. Then, the mixture was passed from one syringe to another until the water-drop test showed that the emulsion was formed. In order to obtain W/O emulsions of different physical properties, different proportions of water and oil were used (i.e. 70/30, 50/50 and 30/70). The O/W emulsion (50/50) was prepared by first thrusting the mineral oil into the venom solution, which in addition contained 0.8% of Span 80 and 1.2% of Tween 80. The W/O/W emulsion was generated by dispersing a W/O emulsion (50/50) in PBS containing with 1.6% Tween 80 and 0.4% Span 80. All emulsions were prepared immediately before use. The correct preparation of emulsions was confirmed by light microscopy.

2.3. Determination of the droplets size

The size of the droplets in W/O emulsions was determined by diluting the samples in hexane, followed by a dynamic light scattering analysis (Nanotrac u2250).

2.4. Determination of the apparent viscosity

Apparent viscosity of emulsions was determined using a rotational rheometer (Kinexus Pro Rheometer, Malvern Instruments, UK), equipped with a stainless steel cone-plate sensor. Measurements were carried out at different temperatures in the range of 20–35 °C and variable viscosity shear rate in the range of 1–100 s^{-1}. Steady state and dynamic oscillatory tests were performed.

2.5. Determination of the rate of venom release

One milliliter of emulsified or non-emulsified solution (control), containing 0.5 mg venom, was placed in a 100 mL-erlenmeyer flask, containing 25 mL of 2% bovine serum albumin (BSA; Sigma-Aldrich) and 0.25% phenol in PBS. The flask was incubated during seven days in a shaking incubator adjusted at 37 °C and 60 r.p.m. Assays were performed in triplicate for each emulsion or control evaluated. Samples of 0.5 mL were collected at 7 days and analyzed by enzyme-linked immunosorbent assay (ELISA) in order to determine their venom concentration. ELISAs were performed at room temperature (20–22 °C). Polystyrene plates (Costar 9017, Corning) were coated overnight with 100 μL of a solution of equine antibodies against *E. ocellatus* (anti-EoAbs) venom at 3 μg/100 μL, dissolved in PBS. Then, the plates were washed 10 times with distilled water and 100 μL of non-diluted samples, or of standards prepared by diluting the control samples in a solution of 2% BSA in PBS (PBS-2%BSA), were added and incubated for 1 h. The plates were washed 10 times with distilled water and 100 μL of anti-EoAbs conjugated with biotin and diluted 1:1000 with PBS-2%BSA were added to each well and incubated for 1 h. Next, the plates were washed 10 times with distilled water and 100 μL of avidin-peroxidase conjugate (A7419; Sigma-Aldrich), diluted 1/2000 with PBS-2%BSA, were added to each well and incubated for 1 h. The plates were afterwards washed 10 times with distilled water, and color was developed by the addition of H_2O_2 and *o*-phenylenediamine (P9029; Sigma-Aldrich). Color development was stopped by the addition of 2.0 M hydrochloric acid and absorbances were recorded at 492 nm using a microplate photometer (Multiskan FC; Thermo Scientific). Finally, the concentration of venom was calculated by interpolating the absorbance of samples in a calibration curve constructed by plotting the absorbance of the standards as a function of their venom concentration.

2.6. Immunization of mice

At intervals of two weeks, and during a period of eight weeks, groups of 10 CD-1 mice (male, 6–8 week old) were subcutaneously injected with 200 μL of PBS, or the same volume of different emulsions, containing 8 μg of the *E. ocellatus* venom. During immunization, all mice were housed in a facility maintained at room temperature and 12hr-light/dark cycle. All mice had *ad libitum*