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Uniform-sized water-in-oil vaccine formulations enhance immune response against Newcastle disease and avian influenza in chickens

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ABSTRACT

Water-in-oil (W/O) emulsion is one of the best "one-shot" delivery system for antigen to generate high and durable antibody response. Here a facile method using premixed Shirasu Porous Glass (SPG) membrane emulsification technique was employed to fabricate uniform-sized emulsion and mechanical stirring was employed to prepare non-uniform-sized emulsion as control. In release kinetics studies, uniform-sized emulsion showed low initial release, and during the followed 17 days, uniform-sized emulsions released antigen faster than emulsions of non-uniform size. In addition, vaccination studies against Newcastle disease virus (NDV) and avian Influenza virus (AIV) demonstrated that emulsion of uniform size induced higher HI antibodies and antigen-specific IgG titers. Furthermore, chickens vaccinated with emulsion of uniform size had a significantly greater ratio of $CD8^+$ T cells to $CD4^+$ T cells and a higher percentage of $CD8^+CD4^+$ T cells. Taken together, these results indicated that emulsion of uniform size induced a more potent immune response than emulsion prepared by mechanical stirring, and warranted the use of SPG membrane emulsification technique in generating new avian vaccines.
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1. Introduction

Animal infectious diseases, especially virus diseases, are worldwide concern as they usually cause great loss in domestic animal and poultry industry [1]. Although there are strict immune programs in farms, some infectious diseases, such as Newcastle disease and avian influenza, are still hard to be controlled [2–5]. Thus, the application of immune adjuvants has attracted more and more attention in improving the efficacy of vaccines [6–11].

Effective adjuvants should augment antigen presentation and promote activation of T and B cells [12,13]. Thus, antigen persistence in the lymph node or at the site of injection is likely to be an important factor impacting the durability of the resulting immune response. Oil-based adjuvants form a deposit of antigen and retain the antigen at the site of injection, thereby performing a depot function by preventing the antigen from rapid release. It was proved that the rate of antigen release from the injection site was a crucial factor in maintaining antigen-specific immune responses [14].

Like most drug delivery systems, the physicochemical properties of emulsions are likely to influence the kinetics of antigen release [15–17]. Previous studies demonstrated that the particle size played an integral role in the subsequent release of antigen [18,19]; however, the relationship among the size distribution of vaccine emulsions, antigen release and the resulting immune response has not been thoroughly investigated. In this study, we employed a facile method using Shirasu Porous Glass (SPG) membrane emulsification technique to fabricate uniform-sized emulsion and also prepared non-uniform-sized emulsion by mechanical stirring as control. And then we investigated the effect of emulsion droplet size distribution on the kinetics of antigen release and the followed immune response.

2. Materials and methods

2.1. Materials and reagents

Inactivated Newcastle disease virus (NDV), avian Influenza virus (AIV) and mineral oil were obtained from Beijing Sinder Weite Technology Co., Ltd. (China). Shirasu porous glass (SPG) membrane (2.8 μm pore size) was provided by SPG Technology Co. Ltd. (Japan). The National Engineering Research Center for Biotechnology (China) provided the premix membrane emulsification equipment (FMEM-500 M). Concanavalin A was purchased from Roche (Germany). Roswell Park Memorial Institute (RPMI) 1640, and fetal bovine serum (FBS) were

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purchased from Gibco (USA). All other reagents were of analytical grade.

2.2. Vaccine preparation

Uniform-sized emulsions were prepared by premix membrane emulsification technique using the SPG membrane [20]. Briefly, the water phase containing inactivated Newcastle disease virus (NDV) and avian Influenza virus (AIV) or Bovine Serum Albumin (BSA) was employed as the dispersed phase. Mineral oil contained Tween80 was used as the continuous phase. The coarse emulsions were first prepared by pouring the dispersed phase into the continuous phase under mechanical stirring, which was then transferred into the premix reservoir. Subsequently, uniform-sized microdroplets were achieved by repeatedly extruding the coarse emulsions through the membrane pores under a high pressure.

Non-uniform emulsions were prepared by mechanical stirring. The dispersed and contiguous phases were generated as described above. The dispersed phase was added to the contiguous phase and premixed on a homogenate machine (JJ-2B, Jintan Ronghua Instrument Manufacture Co., LTD, China) at 3500 rpm for 3 min. Then the shear forces were increased to 10,000 rpm for 5 min to generate non-uniform coarse emulsions.

2.3. Measurement of size distribution of emulsions

Size distribution measurements generated by a sub-micrometer particle size analyzer (ZetaPlus, Brookhaven Instruments Corporation, USA) were used to analyze the size distributions of emulsions.

2.4. Kinetics of antigen release

The inherent antigen-release characteristics of the emulsion preparations were determined by in vitro release kinetics studies. BSA (500 mg/ml) was used in the emulsifications instead of inactivated NDV and AIV. BSA-containing emulsions (1 mL) were injected into a Float-A-Lyzer (Spectrum Laboratories, Inc, USA) dialysis cassette and suspended in 140 ml isotonic 0.01 M phosphate-buffered solution (pH 7.2) at 37 °C. Samples were collected from the buffer at different time points over a period of 20 days. Micro-BCA was used to determine the concentration of BSA in each sample.

2.5. Animals

Specific pathogen-free female BALB/c mice were purchased from the Vital River Laboratories, and SPF female White Leghorn chickens were purchased from the Merial-Vital Laboratory Animal Technology Company. All animals were housed in a specific pathogen-free facility, and treated according to the regulations of Chinese law and the local Ethical Committee.

2.6. Cell recruitment studies

For cell recruitment studies, mice ($n = 6$) were immunized subcutaneously with 100 μl of uniform or non-uniform emulsions containing NDV and AIV. At the indicated time points, mice were sacrificed, and the injection site was excised, washed thoroughly and cut into small pieces. The tissue was then digested at 37 °C for 2 h in PBS containing 0.2% collagenase D (Roche Diagnostics, Germany) supplemented with 0.8 U/ml dispase (Gibco, Invitrogen, CA) and 2% fetal bovine serum. Single cell suspensions were prepared by passing the digested tissue through a 40 μm cell strainer and stained with combinations of the following antibodies: Ly6C-APC, CD11b-eFlour450, Ly6G-FITC, CD11c-APC-Cy7, F4/80-PE (all from eBioscience). Recruited immune cells in the tissue were then analyzed by flow cytometry with a CyAnTM ADP flow cytometer (Beckman Coulter, USA).

2.7. Expression of MHC molecules and co-stimulatory molecules on dendritic cells in secondary lymphoid organs

Balb/c mice ($n = 5$) were subcutaneously (S.C.) vaccinated with different vaccine formulations. At the indicated time points (24, or 80 h post-immunization), mice were euthanized. Axillary and sciatic lymph nodes were harvested and processed into single cell suspension. Cells were stained with fluorescence-labeled anti-mouse antibodies against CD11c, MHC II, and CD86 (eBioscience). CD11c, the common marker of DCs subsets in lymph nodes, was used to indentify DCs. The expression of MHC molecules and co-stimulatory molecules on dendritic cells was determined by CyAn™ADP flow cytometer (Beckman Coulter, California, USA) and analyzed using Summit software (version 4.3.02).

2.8. Immunization of animals

Seven-to-ten-day-old specific pathogen-free (SPF) female White Leghorn chickens (n = 10/group) were vaccinated subcutaneously with 0.25 ml of the various vaccines outlined above. Blood samples for serological tests were obtained on days 10, 15, 22, and 28 after vaccination. Virus hemagglutination inhibition (HI) tests and ELISA were performed to determine the levels of antiviral serum antibody titers.

2.9. Hemagglutination inhibition (HI) assay

Serum levels of AIV- and NDV-specific antibodies were determined by hemagglutination inhibition (HI) assay. Serial two-fold serum dilutions were prepared in microtiter plates and mixed with an equal volume of four hemagglutinating units/50 μl AIV or NDV antigen. Titers were expressed as the reciprocal of the highest dilution that gives complete inhibition of hemagglutination of red blood cells (1% (v/v)) in buffered saline.

2.10. Determination of antigen-specific antibody titres (IgG) by indirect ELISA

Blood from vaccinated chickens was allowed to clot at room temperature and then centrifuged. The serum supernatant was then harvested. ELISA plates (96-well) were coated with inactivated AIV or NDV in coating buffer (50 mM Na_2CO_3-$NaHCO_3$, pH 9.6) overnight at 4 °C. After washing three times with PBS–0.05% Tween 20 (v/v), the plates were blocked with 2% (w/v) BSA in PBS for 1 h at 37 °C. The concentration of antigen-specific IgG in sera was monitored using a 1:400 dilution. The plates were incubated at 37 °C for 1 h and washed six times with PBS-0.05% Tween 20. HRP-conjugated anti-chicken antibody (Bethyl, USA) was then added into each well at a 1:20000 dilution and incubated at 37 °C for 40 min. The plates were washed six times and developed with TMB solution in the dark for 20 min. The enzymatic reaction was stopped by adding 2 M H_2SO_4, and the OD450 values were read using a microplate reader (Tecan, Schweiz).

2.11. Flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) from vaccinated chickens were purified from heparinized peripheral blood by Ficoll density gradient centrifugation. The blood was then diluted 1:1 with phosphate-buffered saline (PBS) and layered on to an equal volume of 1.077 g/ml density Nycoprep™ (Nycomed Pharma, Norway) before centrifugation at 800 ×g for 20 min. The PBMC containing interface was subsequently collected, transferred to new tubes, and washed twice with PBS. The PBMC were stained with anti-chicken CD4-FITC (clone CT-4) or anti-chicken CD8-PE (clone CT-8) (SouthernBiotech, USA). The percentage of $CD4^+$ and $CD8^+$ cells was analyzed by flow cytometry with a CyAnTM ADP flow cytometer (Beckman Coulter, USA).

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