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

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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.

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 depot 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
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 (J-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 0.8 U/ml dispase (Gibco, Invitrogen, CA) and 2% fetal bovine serum. 0.2% collagenase D (Roche Diagnostics, Germany) supplemented with pieces. The tissue was then digested at 37 °C for 2 h in PBS containing 0.8 U/ml dispase (Gibco, Invitrogen, CA) and 2% fetal bovine serum.

Subsequently, uniform-sized microdroplets were achieved by repeated extruding the coarse emulsions through the membrane pores under a high pressure.

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.

2.11. 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 CyAn™ADP flow cytometer (Beckman Coulter, USA).
2.12. Statistics

All statistical analyses were performed using GraphPad Prism® 5 software (San Diego, CA, USA). Results are expressed as mean ± SEM. Differences between two groups were compared using an unpaired, two-sided Student’s t-test. Differences among more than two groups were evaluated by one-way ANOVA with significance determined by Tukey-adjusted t-tests.

3. Results

3.1. Characterization of vaccine emulsions

In this study, a facile method, premix SPG membrane emulsification, was employed. The coarse emulsions with big size and broad size distribution were obtained by mild conventional emulsification and then extruded through SPG membrane with quite high pressure to form uniform-sized microdroplets. As shown in Table 1, the mean droplet size of both emulsion preparations was similar. However, emulsions prepared via premixed membrane emulsification method had a polydispersity index (PDI) that was significantly lower and closer to zero than the emulsions prepared using mechanical stirring method. The PDI values close to zero depict a monodisperse sample. Thus, a smaller PDI represents a narrower size distribution, and a larger PDI represents a broader size distribution. These data suggested that emulsions prepared by the membrane emulsification method had a size distribution that was significantly narrower than emulsions prepared by mechanical stirring.

3.2. Emulsions prepared by different methods displayed various antigen release kinetics in vitro

We first determined whether emulsions prepared by different methods had different kinetics of antigen release. Using BSA as model antigen, protein release curves were generated in a dialyzer by determining the time-dependent release of BSA from uniform-sized or non-uniform-sized emulsions into the surrounding medium. As shown in Fig. 1, during the first three days, the emulsion prepared by mechanical stirring showed an initial release, so-called burst effect, while the emulsion prepared by membrane emulsification showed low initial release. During the next 17 days, however, the rate of antigen release from emulsions prepared by membrane emulsification was much faster than that from non-uniform-sized emulsions.

3.3. Emulsions enhanced the influx of immune cells to the injection site

We next sought to determine the composition of immune cells recruited after W/O vaccination. Mice were immunized with emulsions containing NDV and AIV, and then the skin around the injection site was digested and analyzed by multi-color flow cytometry. Recruitment of immune cells into the skin occurred specifically at the injection site of emulsions, and we could not find elevated cell numbers in the non-treated sites at any time point (data not shown). Mice were immunized with emulsions prepared by different methods, and we found there was no obvious difference in recruitment of immune cells into the skin between these two groups. As shown in Fig. 2, a transient increase of neutrophils and monocytes occurred at early time-points (<2 days).

Table 1

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<th>Size/nm</th>
<th>PDI</th>
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<tr>
<td>Membrane emulsification</td>
<td>2346 ± 51.62</td>
<td>0.057 ± 0.028</td>
</tr>
<tr>
<td>Mechanical stirring</td>
<td>2256 ± 98.48</td>
<td>0.187 ± 0.202</td>
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This was followed by an increase in macrophages at day 2 and DCs at day 3. After 8 days, the cell numbers returned to base line levels.

Effective antigen uptake by DCs is a critical step in triggering an adaptive immune response. DCs circulate throughout peripheral tissues uptaking antigens, and then migrate to the lymph nodes where they present these antigens to various cells of the adaptive immune system [21,22]. We hypothesized, therefore, that the different antigen release rates of the emulsion preparations would influence the antigen uptake, which would influence maturation of antigen presenting cell and followed immune response differently.

3.4. Emulsion prepared by membrane emulsification enhanced expression of MHC II and the co-stimulatory molecule CD86 on DCs in draining lymph nodes

Maturation of antigen presenting cell is a prerequisite of antigen presentation and subsequent T cell activation. MHC molecules and co-stimulatory molecules (CD86) are important surface markers indicating the maturation level of APCs. Therefore, we next determined the expression of MHC II and CD86 on dendritic cells in secondary lymphoid organs by flow cytometry. As shown in Fig. 3, compared to emulsion prepared by mechanical stirring, emulsion prepared by membrane emulsification showed higher level of MHC II and CD86 significantly at 80 h post-immunization. While these two emulsions showed similar expression of MHC II and CD86 at 24 h post-immunization

3.5. Emulsion prepared by membrane emulsification induced a stronger immune response

To determine if the size distribution of emulsions influenced the followed immune response, inactivated AIV and NDV were simultaneously entrapped in uniform or non-uniform emulsions and used to vaccinate chickens. Serum was collected 10, 15, 22, and 28 days after vaccination to measure HI titers. As shown in Fig. 4a, vaccination with either emulsion preparation induced significantly higher HI titers than vaccination of antigen alone. These results indicated that W/O adjuvants significantly promoted the humoral immune response. Interestingly, emulsions of uniform size induced significantly higher AVI HI titers on days 10 and 22 after immunization (p < 0.05 and p < 0.01, respectively). These data suggested that vaccination with emulsions of uniform size may induce a greater humoral immune response. Similarly, the NDV HI titers induced by emulsion of uniform size were significantly higher (p < 0.001) on day 10 after immunization as compared to non-uniform emulsions or antigen alone (Fig. 4b). However, there was no
significant difference in NDV HI titers between the two emulsification preparations at days 15, 22, or 28.

We next evaluated the presence of anti-virus antibodies by ELISA. Anti-AIV and anti-NDV IgG antibodies were detected in all chickens except the free antigen control group due to low HI titer. The mean OD values of serum anti-AIV IgG antibodies from chickens immunized with emulsion of uniform size were significantly higher on days 10, 22, and 28 than chickens immunized with non-uniform emulsion (Fig. 5a). The same trend was observed for anti-NDV antibodies (Fig. 5b). These data correlate with the results of the HI tests.

Our data thus far indicated that the vaccine fabricated by membrane emulsification was better able to induce anti-AIV antibodies and anti-NDV antibodies; however, whether this vaccine could further improve the cellular immunity remained unclear. Therefore, we next evaluated the ratio of CD8+ to CD4+ T cells in chickens subcutaneously immunized with either emulsion preparation. PBMC were harvested at the end of each experiment and stained with anti-CD4 and anti-CD8 fluorescently labeled monoclonal antibodies. As shown in Fig. 6a, the ratios of CD8+/CD4+ in chickens vaccinated with either emulsion preparation were significantly higher than animals vaccinated with free antigen alone. Again, this indicated that adjuvants enhanced the immune response. Consistent with antibody titers, a significantly greater ratio of CD8+ to CD4+ T cells was observed in animals vaccinated with uniform-sized emulsions ($p < 0.05$).

CD4+ and CD8+ T cells are the main effector cells of the adaptive immune response and are required to clear viral infections [23]. Interestingly, a subset of CD4+CD8+ double positive (DP) T cells appeared in the PBMC of chickens 28 days after immunization. As shown in Fig. 6b, the percentage of CD8+CD4+ DP T cells in chickens vaccinated with uniform-sized emulsions was significantly greater ($p < 0.001$) than the percentage in chickens vaccinated with non-uniform-sized emulsions.

4. Discussion

Water-in-oil (W/O) emulsion has been used as adjuvant in vaccine formulation for many years. 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 SPG membrane emulsification technique to fabricate vaccine emulsion and also prepared emulsion with the same composition by mechanical stirring as control. The kinetics of antigen release of these two kinds of emulsions and followed immune response were investigated.

In measurement of size distribution of emulsions, the results revealed that size distribution of emulsion prepared by mechanical stirring, was broader than emulsion prepared by membrane emulsification. Previous studies showed that the solubility of the disperse phase in the...
continuous phase is dependent upon the radius of droplet. The solubility increased with decreasing radius [24]. To reach a more thermodynamically stable state, smaller droplets dissolving into the continuous phase and then diffusing to, and redepositing upon larger ones leading to an overall increase in average size of the emulsion, which is called Ostwald ripening [24]. This process in emulsions will lead the release of antigen. Because of the broad size distribution of emulsion fabricated by mechanical stirring, Ostwald ripening was much more significant, leading to the obviously initial release of antigen. However, the size distribution was narrow for emulsion prepared by membrane emulsification, leading to slower Ostwald ripening. With the occurrence of Ostwald ripening, there was an increase in the average radius of the emulsion droplets fabricated by mechanical stirring. Owing to this, the surface area-to-volume ratio of the droplets decreased, which led the release rate of antigen to be slow [25]. Our results indicated that uniform-sized emulsion showed low initial release, and during the followed 17 days, it showed a faster release rate of antigen.

For adjuvant of W/O emulsions, three major mechanisms are possible: (1) act as antigen-retaining depot at the injection site, releasing antigen over time; (2) directly attract antigen presenting cells (APC) to provide antigen; or (3) provide immune response-activating signals to local or recruited APCs [14]. Our results indicated that the emulsion size distribution directly affected the kinetics of antigen release. Thus, emulsions with different size distributions should induce different immune responses. Here, uniform-sized emulsion induced higher HI antibody and antigen-specific IgG than non-uniform-sized emulsion. Furthermore, the significantly greater ratio of CD8+ to CD4+ and the percentage of CD8+CD4+ cells were observed in animals vaccinated with uniform-sized emulsion than non-uniform-sized emulsion.

Mature CD4+ and CD8+ single positive T cells differentiate from immature, non-functional CD4+CD8+ double-positive T cells in the thymus [26]. Increased proportions of circulating DP T lymphocytes have been observed during acute viral infections [27]. In addition, there are multiple reports that DP T cells are functional effector/memory T cells specific for antigens from a variety of viral pathogens. For example, Nascimbeni et al. [28] broadened the perception of this population when they demonstrated that peripheral CD4+CD8+ DP T cells took part in the adaptive immune response against infectious pathogens. Desfrancois et al. [29] also reported that DP T cells displayed the phenotype and cytotoxic potential of effector/memory activated CD8+ T cells, but differed from these cells by the high production of IL-4, IL-5 and IL-13. Therefore, our results indicated that uniform-sized emulsions may induce a more powerful cellular immune response by increasing the percentage of DP T cells.

5. Conclusions

In this study, we employed a facile method using SPG membrane emulsification technique to fabricate vaccine emulsion with narrow size distribution. Release kinetic analysis revealed that uniform-sized emulsions released antigen faster overall than emulsions of non-
uniform size. Using Newcastle disease virus (NDV) and avian influenza virus (AIV) as antigens for vaccine studies, we found that emulsion prepared by membrane emulsification induced higher NDV and AIV HI antibodies and antigen-specific IgG titers than emulsions of non-uniform size. Furthermore, the ratios of CD8+ to CD4+ T cells and the percentage of CD8+ CD4+ double positive cells were significantly increased in chickens vaccinated with emulsions of uniform size as compared to chickens vaccinated with emulsions of non-uniform size. Taken together, these results indicated that W/O emulsion prepared by membrane emulsification should be considered to enhance the efficacy of new of avian vaccines.

Acknowledgements

This work was financially supported by the earmarked fund Special Fund for Agro-scientific Research in the Public Interest (grant no. 201303046), the 973 Program (grant no. 2013CB531500) and the 863 Program (grant no. 2012AA02A406).

References


