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Original Research Article

Evaluation of Immunomodulatory Effect of Immunace on Adjuvanticity of 1,2-Dioleoyl-3-trimethylammoniumpropane-based Liposomes

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Abstract

Purpose: To investigate the antibody effect of immunace on the adjuvanticity of 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP)- based liposomes.

Method: The vesicles of the liposome-based ND vaccine were prepared by lipid film hydration method and physically characterized for shape, particle size and zeta potential. Forty experimental birds were divided into an unvaccinated group, a liposomal Newcastle disease (ND) vaccine group, combined liposomal ND vaccine and immunace group, and a live La Sota® vaccine group. The liposomal ND vaccine, liposomal ND vaccine and immunace and a live La Sota® vaccine groups were vaccinated orally at 3 and 6 weeks of age. Haemagglutination inhibition test was carried out after primary and booster doses.

Results: The log₂ mean antibody titre induced by the liposomal ND vaccine after secondary immunization of the birds was 9.60 ± 0.95 while that of the combined liposomal ND vaccine and immunace group was 7.00 ± 1.71, and that of the live La Sota vaccine® was 6.00 ± 0.63. There was no detectable antibody in the unvaccinated group throughout the experiment. At *p* < 0.05, the liposomal ND vaccine group, after secondary immunization, produced antibodies which were significantly higher than those of the combined immunace-liposomal ND vaccine group.

Conclusion: There was a boost in the immune response of the birds immunized with liposomal ND vaccine and immunace after primary immunization only.

Keywords: Antibody, Newcastle disease vaccine, La Sota, DOTAP, Immunace, Immunity, Adjuvant

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INTRODUCTION

Newcastle disease (ND) is a contagious and fatal viral disease affecting both wild and domestic avian species [1,2]. The impact of ND is most notable in controlled poultry due to its high susceptibility [2]. ND has been reported to represent a stronger drain on the world economy than any other animal viral disease. It is probably the most serious global disease of poultry. Vaccination programmes are now designed to

hyperimmunize chickens effectively to protect them from field/virulent strains of the ND virus, and for the vaccines to be effective it is important that they are delivered properly and effectively.

Lately, the advances in dosage forms have resulted in the development and improvement of drug delivery systems. From earliest times, man has devised different approaches and mechanisms to introduce therapeutic agents into the body. Currently, the approaches have

evolved into cutting-edge nanotechnologies that have improved drug release profile, absorption, targeting and overall bioavailability. Many medications such as peptides and proteins, antibody, vaccines, gene based drugs which are subject to enzymic degradation by proteinases, peptidases, nucleases etc have been protected by encapsulating in carriers like liposomes, niosomes, virosomes, dendrons, nanoemulsions, nanospheres and polymer based nanoparticles [3,4]. They are effective and they target the drug to specific sites for effective and safe delivery.

Vaccines are immunological products that are capable of inducing the production of antibodies. Vaccines based on recombinantly made subunit and synthetic- peptide antigens are usually non-immunogenic, and the need for adjuvanticity is essential. They are however not ideal because they induce only humoral immunity but not cell-mediated and cannot be lyophilized. Recently, there has appeared a vaccine adjuvant candidate, liposome, with a liposome-based vaccine (against hepatitis A) being licensed for use in humans [4,5]. Vaccines based on novosomes (non-phospholipid liposomes formed from single-chain amphiphiles, with or without other lipids) have been licensed for the immunization of fowl against Newcastle disease virus and avian reovirus [5,6]. Other liposomal or novosome-based vaccines against bacterial and viral infections are under development. Liposomes are biodegradable and non-toxic and can elicit both humoral and cell-mediated immunity and are easy to prepare [6,7]. They are highly versatile in their structural characteristics which can be modified/manipulated to improve their adjuvanticity [6,7,9], e.g. surface charge, lamellarity, fluidity, pH sensitivity, vesicle size, antigen association, etc. There are detailed information available on the fate of liposomes after uptake by cells and the subsequent induction of cytotoxic T-lymphocyte responses. Liposomes are immunological adjuvants that are capable of augmenting immune responses to entrapped and surface-adsorbed antigens [8]. The basis for this immune-potentiating response lies in the natural targeting of liposomes to antigen-presenting cells (APC), most notably the macrophages [9,15]. Cationic liposomes are lipid-bilayer vesicle with a positive surface charge that has re-emerged as a promising new adjuvant technology [10,17]. Although there are some evidence that cationic liposome itself can improve the immune response against co-administered vaccine antigens, their main functions are to protect the antigens from clearance in the body and deliver the antigens to

professional antigen presenting cells (APC) [11]. An immune booster immunace (Vitabiotics, UK) contains a comprehensive spectrum of antioxidants, vitamins and essential nutrients designed to maintain immune resistance and long-term cell protection (from free radicals and peroxides). Generally, immunace and other immune boosters increase the immunity of humans and animals by supplying the essential nutrients [12,24]. Immune boosters such as immunace are given for revitalization and improvement of the immune response, especially in those with low immunity. Immunace increases the amount of antibodies produced, by improving and maintaining the immune resistance [12]. In this study, the researchers combined two immune boosters, DOTAP-based liposome and immunace to know the effect of immunace on the established adjuvanticity of DOTAP.

EXPERIMENTAL

Materials

Cholesterol (Sigma grade, minimum purity 99 %; Sigma Aldrich Chemie, St Louis, Missouri, USA), DOTAP (Sigma-Aldrich, donation from Prof. Godwin Nchinda, Rockefeller University, New York, USA), phospholipon 90H (donation from Phospholipid GmbH Nattermannallee, Cologne, Riedel-de Haen, Seelze Germany), methanol(extra pure; ScharlauChemie S.A.), chloroform (Sigma-Aldrich GmbH, Germany), live La Sota[®] vaccine (National Veterinary Research Institute, Jos, Nigeria), Immunace .

Preparation of the liposome suspension

Lyophilized La Sota[®] vaccine containing 200 doses/vial was reconstituted with phosphate-buffered saline by dissolving a vial in 40 ml phosphate buffer solution. Then 196 mg phosphatidylcholine, 96.7 mg cholesterol and 50 mg DOTAP were dissolved in 3 mL chloroform/methanol system (2:1) in a round-bottom flask. The solvent mixture was evaporated at room temperature and the flask rotated until a smooth, thin, dry film was obtained on the wall of the flask [13,26]. Reconstituted vaccine (5 ml) was used to hydrate the dry film and agitated gently until a milky colloidal dispersion was formed.

Transmission electron microscopy

The prepared liposome-encapsulated ND vaccines were processed using copper grids to adsorb the particles from the suspension, staining in 2.5 % uranyl acetate for 30 sec and

drying. The specimens were observed under a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV at x10,500 magnifications [14,27].

Vesicle size and zeta potential

The z-average vesicle diameter and zeta potential of the ND vaccine liposomes in phosphate buffer solution (pH7.4) were determined by photon correlation spectroscopy using a nanosizer 3000 HS (Malvern Instruments, Malvern UK). The zeta potential was calculated from the mean of three runs. Each sample was diluted with bi-distilled water and the electrophoretic mobility determined at 25 °C and a dispersant dielectric constant of 78.5 and pH 7. The obtained electrophoretic mobility values were used to calculate zeta potentials using DTS software version 4.1 (Malvern Instruments) and applying Henry's equation [15,28].

$$UE = \{2\varepsilon Zf(K)\}/3\eta \dots\dots\dots (1)$$

where Z is the zeta potential, UE the electrophoretic mobility, ε the dielectric constant, η the viscosity of the medium and f(K) is Henry's function.

Vaccination schedule of the birds

The chicks were raised from 1 day old until termination of the experiment. All bird handling and experiments were conducted following the guidelines stipulated by University of Nigeria Research Ethics Committee on animal handling and use. Eighty birds were divided into four groups of 20 birds each; the unvaccinated group, the liposomal ND vaccine group, the liposomal ND vaccine + immunace group and the La Sota[®] vaccine group. The different groups were kept in separate rooms before vaccination. The liposomal ND + immunace group was given 25 mg/mL of immunace from day old to two weeks. At 2 weeks of age, all of the birds were tested for the absence of residual haemagglutination inhibiting (HI) antibody to Newcastle disease.

At 3 weeks of age, the liposomal ND vaccine group and liposomal ND vaccine + immunace group were given 0.2 ml/bird (a dose) each of the liposomal ND vaccine orally while the La Sota[®] vaccine group were given 0.2 ml/bird (a dose) of the reconstituted La Sota[®] vaccine orally. The unvaccinated group served as the control. Two weeks after primary vaccination, 10 ml of blood was withdrawn from the jugular vein and serum samples assessed for antibody to ND virus by

haemagglutination inhibition (HI) technique [16,29]. Booster vaccination was done at 6 weeks of age. Two weeks after secondary vaccination, all birds were bled and serum samples assessed for antibody to ND virus. The results are presented as the mean with standard deviation for each of the parameters.

Data analysis

The results are presented as log₂ mean \pm standard deviation (SD). To determine statistically significant difference among the means, one-way analysis of variance was applied at $p < 0.05$ using SPSS (version 16.0, SPSS Inc, USA).

RESULTS

Transmission electron micrographs

The technique used for the preparation of the liposomes was the lipid film hydration technique, which formed films on the walls of the flasks and on hydration produced a thick, gel-like, milky colloidal dispersion. The images of the vesicles, were closely packed and had near spherical shapes.

Particle size

The vesical size and zeta potential were evaluated using a zeta sizer. The mean size distribution and zeta potential were <100 d.nm and 24 mV respectively.

Immune response of the birds

All the birds screened prior to administration of vaccine were negative for HI antibody. All control (unvaccinated) birds had no antibody throughout the experiment. The immune response of the birds is shown in Table 1. After primary vaccination, the liposomal ND vaccine + immunace group had the highest mean antibody titre (log₂) and standard deviation of 6.60 \pm 0.0, the liposomal ND vaccine group had an antibody titre of 5.3 \pm 0.56 while the La Sota[®] vaccine had a mean antibody titre of 5.50 \pm 0.67 after primary immunization. After secondary vaccination the chickens further sero-converted, the liposomal ND vaccine had the highest mean antibody titre (log₂) of 9.60 \pm 0.95 which was significantly higher than the liposomal ND + immunace group with a mean antibody titre of 7.00 \pm 1.71 and the LaSota[®] vaccine group with (log₂) 6.00 \pm 1.30.

Table 1: Immune response of birds after primary and secondary immunization

Treatment group	No. of chickens	Primary immunization (log ₂) ± SD	Secondary immunization (log ₂) ± SD
Unvaccinated control	10	0.0 ^A	0.0
Liposomal ND vaccine	10	5.30+0.56 ^B	9.60+0.95 ^C
LiposomalND+immunace vaccine	10	6.60+0.0 ^B	7.00+1.71 ^B
La Sota@vaccine	10	5.50+0.67 ^B	6.00+1.30 ^B

* Concentration of the serum antibodies is expressed as HI titre to log base 2. Results presented as the mean with standard deviation. B is significantly higher than A and C is significantly higher than A and B

DISCUSSION

Liposomes are spherical vesicles composed of an aqueous core which is entirely enclosed by a membrane composed of phospholipids and cholesterol. When these lipids are exposed to an aqueous environment, interactions between themselves and with water lead to spontaneous formation of closed bilayers [17,30]. The fluidity or rigidity of the liposomal bilayer, when it is made from a single lipid depends on the lipid phase transition temperature (T_c) [18,31]. Increasing the temperature changes the membrane fluidity from a solid-gel phase, where the lipid hydrocarbon chains are in an ordered state, to a fluid liquid-crystalline phase, a disordered state, where molecules have more freedom of movement.

Membrane permeability is highest at the phase transition temperature, and is lower in the gel phase than in the fluid phase. A look at the liposomes showed vesicles that were mostly spherical in appearance, entrapping the vaccine within their core. The vesicles appeared tightly packed under the transmission electron microscope, indicating that the phosphatidylcholine, cholesterol and DOTAP, which are amphipathic lipids, thermodynamically organized themselves to form stable self-closed vesicles. The tight packing of the vesicles may also have reduced the binding/insertion of proteins, which normally destabilize membranes and mark liposomes for removal by phagocytic cells. Generally, the more ordered and hence tightly packed the membrane of a liposome, the less permeable it is [19,32].

Particle size is also one of the determining factors for macrophage clearance. Therefore, large liposomes are rapidly removed from circulation. Photon correlation spectroscopy using a zetasizer is a technique that measures time-dependent fluctuations in the intensity of scattered light and was employed to measure size distribution of the particles. If the particle size of the liposomes is below 200 d.nm, it would escape phagocytosis and the circulation time will

be prolonged [20,33]. If the circulation time is prolonged, there will be more contact time of the liposomes with the immune cells, resulting in higher immunity. Encapsulation of the ND vaccine in the liposomes may also have reduced the volume of distribution of the vaccine and increased targeting to the immune cells [20,33].

Knowledge of the zeta potential of the liposomal ND vaccine would help to predict its fate *in vivo*. Positively charged liposomes will normally fuse with the negatively charged cells. Once internalized, the particles are offered to the continuous lymphoid tissue so as to protect the antigen against degradation on mucosal surfaces, and enhance its uptake in mucosa-associated lymphoid tissue. The vaccines used for ND control must be able to protect the susceptible poultry against velogenic strains of the virus. Antibody titres of 2³ are protective, but current poultry practices are now designed to hyperimmunize chickens to protect them more effectively from field/virulent strains of the ND virus [21,34]. This could be achieved by administering live plus inactivated vaccination to boost the antibody titres to as high as 2⁷.

In the study, the objective was to investigate the effect of immunace on the established immunoadjuvanticity of DOTAP. The primary immunization of immunace and DOTAP-based ND vaccine produced an antibody titre which was higher than DOTAP-based ND vaccine alone. After the secondary immunization, the antibody titre of DOTAP-based ND vaccine was significantly higher than DOTAP-based ND vaccine showing that immunace has a short-acting effect on the immune cells while DOTAP has a longer lasting effect on the immune cells. Immunoadjuvants are useful in low-biosecurity areas with high ND prevalence [22,35]. Liposomal ND vaccine, after secondary immunization, had a significant effect on the immune cells, inducing antibody titres as high as 2⁹. Association of antigen with liposomes has been known to allow the antigen to gain access to both the major histocompatibility (MHC) class I and class II pathways in antigen presenting cells

(APCs). As a result, liposomal antigen can stimulate antibody production as well as cellular immune responses [23,36].

CONCLUSION

DOTAP-based cationic liposome, based on the findings of this study, has demonstrated its ability to function as immune adjuvant. Immunace, on the other hand, demonstrated a short-lived co-immunoadjuvanting activity. There was no significant immunological relevance for combining DOTAP-based ND vaccine and Immunace since DOTAP-based cationic liposome still gave a significantly higher immune response after secondary immunization.

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CONFLICT OF INTEREST

No conflict of interest associated with this work.

CONTRIBUTION OF AUTHORS

EO, VO and AA designed this work, VO and AA supervised it while EO and AN collected and analysed the data and prepared the manuscript which was then approved by all authors.

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