Original article

Use of a dialyzable short-chain phospholipid for efficient preparation of virosome vaccines against Newcastle disease

Atthachai Homhuan[1]* and Sompol Prakongpan

Faculty of Pharmacy, Mahidol University, 447 Sri-Ayhuthaya, Rajathevi, Bangkok 10400, Thailand.
[1]Present address: Vaccine Research and Development Center,
National Health Research Institutes No. 35, Zhunan, Miaoli County, Taiwan
**Corresponding author: E-mail address: g4138458@yahoo.com*

Abstract:

Virosomes can be regarded as liposomes carrying the spike proteins of enveloped virus on their surface. To further advance the use of virosomes, we developed a solubilization/ reconstitution procedure for construction of Newcastle disease (ND)-virosomes, which would be easily applicable to industrial production. This procedure included the use of 1,2-dihexanoylphosphatidylcholine (DHPC) as a viral membrane solubilizer. DHPC is a short-chain phospholipid with detergent-like property and with a relatively high critical micelle concentration (CMC, 14 mM). Virosomes were prepared by solubilization of virus with DHPC followed by the removal of nucleocapsids by ultracentrifugation. The solubilized membrane components were then easily reconstituted by the dialysis removal of DHPC. Biochemical analysis revealed that ND-virosomes contained both hemagglutinin-neuraminidase (HN) and fusion (F) proteins, with preserved biological activity. The immunogenicity of ND-virosomes was determined in chickens after subcutaneous immunization. The relatively high hemagglutination-inhibition (HI) antibody titers and high clinical protection upon viral challenge were found in animals that received ND-virosomes. Using DHPC for the production of virosomes was uncomplicated and would allow further exploitation of virosomes as subunit-vaccines not only in an academic field, but also in an industrial setting.

Keywords: Fusogenic activity; Hemagglutinin-neuraminidase; Newcastle disease-virosomes; Short-chain phospholipid; Vaccines

Introduction

Reconstituted viral envelopes were first prepared from Sendai virus [1] and the term 'virosomes' was later introduced by Almeida *et al.,* who in 1975 reconstituted influenza virus envelopes [2]. Virosomes closely mimic the intact virus except that they do not contain virus replication machineries and therefore they represent a very useful system for presentation of antigens (Ag) in order to induce immunity against the incorporated Ag [3-5]. Reconstitution of viral membranes is usually relied on solubilization of the viral envelope with a detergent, removal of the viral nucleocapsid complexes by ultracentrifugation, and subsequent removal of the detergent from the solubilized viral membrane [6]. The schematic representation of virosome preparation is shown in Figure 1.

Solubilization and reconstitution are two physical processes which may cause inactivation of membrane proteins [7]. The extent of these processes is dependent mainly on the applied detergent. Unfortunately, a universal rule to determine the ideal detergent for the reconstitution of membrane proteins does not exist. A particular detergent can be very successful in the solubilization of certain membrane proteins, while it fails for others. Until now, the most frequently used detergents in the reconstitution of virus membranes have been non-ionic detergents, such as octaethyleneglycol mono (n-dodecyl) ether ($C_{12}E_8$), Triton X-100 and octylglucoside (OG) [6, 8-10]. Triton X-100 and $C_{12}E_8$, detergents with a low critical micelle concentration (CMC, 0.24 mM), have been applied successfully in the reconstitution of viral envelopes derived from various viruses [6, 10]. These virosomes exhibited relevant biological activity. However, the use of low-CMC detergents has a disadvantage in that they can not be readily removed from virosomes by dialysis, so the residual detergent in virosomes prepared is to be expected. For this reason, an attempt to prepare virosomes using the high CMC detergent namely, OG (CMC, 23 mM), which can be readily removed by dialysis has been performed. But the preparation of virosomes using OG frequently resulted in the formation of virosomes without protein functionality [8, 10]. Stegmann *et al.* have shown that influenza virosomes prepared from OG failed to preserve fusogenic activity of hemagglutinin (HA) proteins of influenza virus [8]. We also have previously reported that Newcastle disease (ND) virosome vaccines prepared from OG offer low clinical protection in animals upon challenge with virulent virus [11].

Recently, short-chain lecithins-phospholipids with a chain length of 6-8 carbon atoms-have gained much

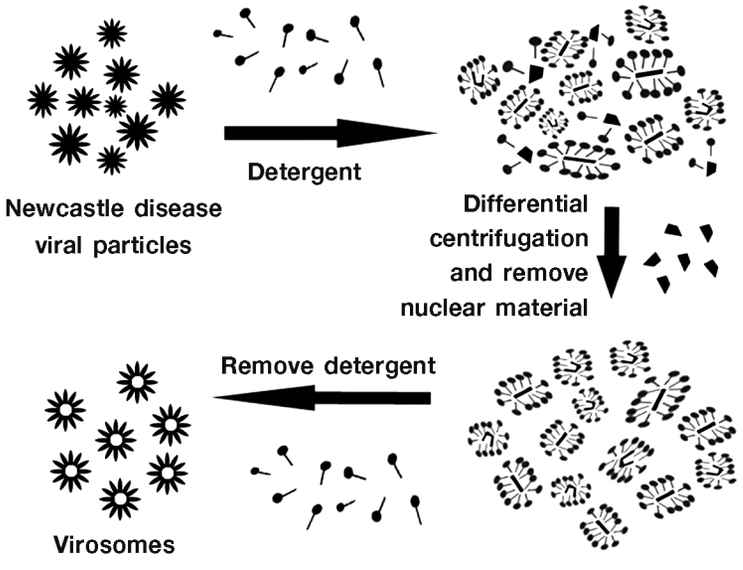

Figure 1 Schematic picture of virosomes formation. The incubation between intact NDV and detergent, e.g. short-chain lecithin, results in solubilization of the viral envelope while nucleocapsids can not be dissolved in detergent. After excluding the nuclear materials by differntial centrifugation, subsequent removal of detergent leads to spontaneous virosome formation.

interest over the past decade for use as solubilizing agents in the reconstitution of membrane proteins [12]. Several studies have shown that the short-chain lecithin, dihexanoylphosphatidylcholine (DHPC), is superior to various commonly used detergents in preserving the biological activity of membrane proteins during solubilization [12, 13]. Kessi *et al.* have postulated that short-chain phosphatidylcholine interacts primarily with the lipid bilayer of a membrane and very little with the membrane protein [13]. Accordingly, the membrane protein remains associated with its preferred intrinsic membrane lipids and therefore retains its native structure and function. Moreover, a relatively high CMC of short-chain lecithins (14 mM) alllows efficient removal of detergent molecules from the membranes by an easy dialysis method [12].

Newcastle disease (ND) is one of the most serious endemic diseases in a wide variety of birds. It always leads to considerable chicken death and economic losses in poultry production. Respiratory distress, diarrhea, and neurological lesions are the main characteristics of infected birds [14, 15]. The causative agent, Newcastle disease virus (NDV), is an enveloped RNA virus, belonging to the family of *Paramyxoviridae,* genus *Rubulavirus*. The viral envelope is composed of a lipid bilayer and two surface glycoproteins, namely hemagglutinin-neuraminidase (HN) and fusion (F). HN is responsible for the attachment of the virus to sialic-acid containing receptors of the host cells. F protein plays a major role in fusion of the viral membrane with the cellular plasma membrane [16]. These two membrane glycoproteins are the antigenic components that can induce a protective immune response [17]. Many different types of vaccines have been produced for protection against NDV outbreaks, including virosome vaccines [11, 18].

In this study, we describe and characterize the production of ND-virosome vaccines using a dialyzable short-chain phosphatidylcholine as a membrane solubilizer. We also examined the glycoproteins functionality of ND-virosomes prepared. The immune response was assessed by measuring serum antibody levels and clinical protection after NDV challenge. It was demonstrated that short-chain lecithin can be used successfully for ND-virosome preparation, with preserve proteins functionality. In addition, the virosomes were immunogenic and provided good protection against virus challenge.

Materials and Methods

Materials

Dihexanoyl-sn-glycero-3-phosphocholine (DHPC) was obtained from Sigma (St.Louis, USA). 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (pyrPC) was purchased from Molecular Probes (Eugene, USA). Murine monoclonal antibodies, IDNDV134.1 and IDNDV133.1, directed against hemagglutinin (HN) and fusion (F) glycoproteins, respectively were kindly provided by Dr. Maas R.A. (Department of State Quality Control and Standardization, CDIC, Lelystad, The Netherlands). All other chemicals were of analytical grade.

Chickens

Male BABCOCK chickens were obtained on the day of hatching from Kerd-Charoen Hatcheries (Bangkok, Thailand) and maintained in conventional cages, with food and water ad libitum. Guidelines and legislative regulations on the use of animals for scientific purposes of Mahidol University, Thailand were followed. The chickens were found to be free from maternal immunity to NDV.

Virus

Lentogenic Clone-30 strain of NDV (Boxtel, The Netherlands) was grown in the allantoic cavity of chick embryos. The virus-infected allantoic fluid was concentrated by cross-flow ultracentrifugation with a poly-ethersulfone filter with a pore size of 300 kDa. The virus was further purified by layering 8 vol. of concentrated virus on top of 4 vol. of 20% sucrose (w/w) in HBS (5.0 mM Hepes, 0.15 M NaCl, pH 7.4) and pelleting by ultracentrifugation (80,000 x g, 1 h, 4°C, Beckman SW41).

Preparation of ND-virosomes

An amount of purified virus corresponding to 200 nmol phospholipid was suspended in 1 ml of HBS. The resuspended virus was transferred to a glass-tube containing a dry film of 44 μmol DHPC and incubated for 1 h at ambient temperature with gentle shaking. The suspension was then layered on a cushion of 20% sucrose (w/v) in HBS and ultracentrifuged for 90 min at 100,000 x g to remove insoluble nucleocapsid. The optically clear supernatant above the sucrose solution was collected. To remove DHPC, the supernatant was dialyzed overnight using a Slide-A-Lyzer dialysis cassette with a molecular weight cut-off (MWCO) of 10,000 (Pierce, Rockford, IL, USA) at 4°C against two changes of HBS in 1000-fold volume excess.

Characterization of ND-virosomes

Mean hydrodynamic diameter of ND-virosomes was determined by dynamic light scattering (DLS) at 25 °C with a Malvern 4700 system equipped with a 75 mW Argon ion laser (488 nm, Uniphase, San Jose, CA, USA), a remote interface controller and PCS software, version 1.35 (Malvern Ltd., Malvern, UK). The particle size distribution was reflected by a polydispersity index (PI) ranging from 0.0 for an entirely monodisperse particles, up to 1.0 for heterodisperse particles. The protein content of ND-virosomes was determined according to Bio-Rad DC protein assay with bovine serum albumin (Pierce, Rockford, IL) as a relative standard. The phospholipid content was determined according to Rouser *et al.* with sodium phosphate (Merck, Damstadt, Germany) as a standard [19].

SDS-PAGE and glycoprotein staining

Protein composition was assayed by SDS-PAGE. A 12.5% acrylamide gel was used and the protein was visualized by Coomassie Brilliant Blue staining. To dye glycosylated proteins, the carbohydrate-specific periodic acid Schiff (PAS) staining method was performed according to the method described elsewhere [20].

Hemagglutination activity (HA)

A five-fold serial dilution of ND-virosomes in phosphate buffered saline (PBS; 10 mM Na_2HPO_4, 0.15 M NaCl, pH 7.4) was set up in a U-shaped 96-wells microtiter plate. Chicken erythrocytes were isolated from whole blood and 1% suspension of freshly isolated erythrocytes in PBS was prepared. An equal volume of 1% red blood cells was added to each well. The plate was gently shaken to mix the contents of each well. After incubation the plate for 60 min at 4°C, sedimentation of the cells was visually assessed in each well. The HA was expressed as hemagglutinating units (HAU)/μg protein; where HAU was defined as the lowest sample dilution able to inhibit the sedimentation of red blood cells.

Membrane fusion assay

Membrane fusion experiments were carried out essentially as described before [21]. To incorporate fluorescent probes -pyrPC- into the virosome membrane, solubilized viral components were added to pyrPC after removal the nucleoprotein complex by ultracentrifugation at 100,000 x g for 90 min. The amount of pyrPC relative to the total amount of phospholipid was 10%. After removal DHPC by dialysis as described above, the formed virosomes with pyrPC incorporated were collected. Virosomes at a concentration of 5 μM phospholipid were mixed with chicken erythrocyte ghosts (100 μM phospholipid). The virosome mixture was placed into a thermostatted and stirred cuvette that contained 1.4 ml of HBS. After a 2-min equilibration period, the pH of the medium in the cuvette was lowered to 5.5 and 4.0 by adding 70 μl of fusion buffer (0.1 M morpholinoethane sulfonic acid - 0.2 M acetic acid, pretitrated with NaOH and HCl, respectively). Fusion was continuously monitored at 37°C by measuring the decrease of pyrPC excimer fluorescence with an LS50B fluorescence spectrophotometer (Perkin- Elmer, Beaconsfield, UK) set at an excitation wavelength of 345 nm and an emission wavelength of 480 nm while the cuvette

contents were continuously stirred. The decrease of fluorescence was expressed relative to the difference in the initial fluorescence and the excimer fluorescence at 'infinite' dilution, which was obtained by adding 70 µl of 10% (w/v) Triton X-100 in HBS. Fusion extents (*f*) were calculated as: % fusion (f) = $[(E_0-E_t)/(E_0-E\infty)] \times 100$, where E_t represents the excimer fluorescence intensity at time (t), and E_0 and $E\infty$ correspond to the excimer fluorescence intensities at time zero and after addition of Triton X-100, respectively.

In-vitro antigenicity of ND-virosomes

Antigenicity of ND-virosomes was quantified by ELISA as previously described [22]. Briefly, virosomes were serially diluted in 96-well flat bottom plates, which were coated with 1 µg/ml anti-HN mAb (IDNDV134.1) or 1 µg/ml anti-F mAb (IDNDV133.1, CDIC, Lelystad, The Netherlands). After incubation for 2 h at room temperature, plates were washed and incubated for 1 h at room temperature with horseradish peroxidase conjugated-IDNDV134.1 or IDNDV133.1. Plates were washed and the color reaction was performed. The reference ND antigen, formalin-inactivated NDV strain Ulster (CDIC, Lelystad, The Netherlands), was also included in each plate. The results were shown as the ratio of relative ELISA titer to the protein concentration determined by Bio-Rad DC protein assay.

Immunization and virus challenge

At four weeks of age, the chickens were divided into three groups of 15 each. Chickens in group 1 received 0.5 ml commercial oil emulsion vaccine (INACTIVAC® CHICK-ND, Maine Biological Laboratory, USA) by subcutaneous (SC) injection. Chickens in group 2 were administered with ND-virosomes containing 30 µg viral protein via SC injection. A third group served as the control, receiving no vaccine. Four weeks after vaccination, all birds were challenged by oral administration of a virulent strain of NDV with an approximate dose of 10^4 EID_{50} per bird. After challenge, all birds were observed daily for clinical signs of ND. The protective efficacy was calculated by dividing the number of chickens that survived without showing any clinical evidence of ND during 14 days by the total number of challenged chickens.

Blood samples were collected for serological analysis at 1, 2, 3, and 4 weeks after vaccination. After heat treatment at 56°C for 30 min, sera samples were stored at -20°C and subjected to the hemagglutination inhibition (HI) test for assessment of antibody levels against ND. The antibody titers of each group were expressed as geometric mean titer ± standard error of mean (SEM). Analysis of variance was used for statistical evaluation of the data. The significance of the differences between geometric mean antibody titers was determined by the Fisher's least-significant test at a confidence level of 95%.

Hemagglutination inhibition (HI) assay

The HI assay was performed according to the method of Alexander [23]. In brief, dilution series of sera were incubated with four hemagglutinating units (HAU) of NDV La Sota strain at room temperature for 30 min. The HAU was titrated before each assay. Thereafter, chicken erythrocytes were added and agglutination was monitored after incubation at room temperature for 45 min. The HI titer was expressed as the reciprocal of the highest serum dilution completely inhibiting agglutination.

Results

Preparation and characterization of ND-virosomes

Reconstituted Newcastle disease viral envelope or 'virosomes' were prepared according to the detergent removal technique. In this study, dihexanoylphosphatidylcholine (DHPC), a short-chain lecithin, was used as a membrane solubilizer. Table 1 summarises the physicochemical properties of ND-virosomes prepared. The particle size of virosomes as measured by DLS was ~ca. 125 nm, which resembled the size of intact virions. The polydispersity index (PI) of virosomes was around 0.2, indicating a rather narrow particle size distribution. The high protein/phospholipid ratio (2.3) was also found in virosomes prepared. To determine the identity of membrane glycosylated proteins, HN

and F, the intact virus and virosomes were separated in several lanes on SDS-PAGE gels. The gel was stained with Coomassie blue to visualize the proteins, and another gel was stained with PAS to visualize the glycoproteins. These results are shown in Figure 2. The components of ND-virosomes were separated in lanes 2 and 3. Lane 2 was stained with Coomassie blue and lane 3 was stained with PAS. Unlike parent NDV (lane 1), ND-virosomes contained two prominent glycoproteins corresponding to HN and F of NDV.

Table 1 Physicochemical characteristics and hemagglutination activity of virosomes prepared

Particle size (nm), PI[a]	Protein/phospholipid ratio (w/w)[b]	Relative content/μg protein HN[c]	F[c]	Hemagglutination activity (HAU/μg)[d]
125, 0.17	2.3 ± 0.3	4.1 ± 1.2	2.0 ± 0.1	3,930

[a] Polydispersity index.

[b] An approximate molecular weight of phospholipid of 750 was used in the calculation. Data represent mean ± S.D. of three different samples for each preparation.

[c] Relative content (relative to that of reference antigen, inactivated NDV Ulster strain) as determined with IDNDV 134.1 and 133.1 monoclonal antibody in an ELISA divided by protein content. The results are shown as mean value (± upper/ lower value of duplicate means).

[d] Hemagglutination activity was defined as the highest dilution of samples able to inhibit the sedimentation of red blood cells; the hemagglutination titer was expressed as HAU/μg protein.

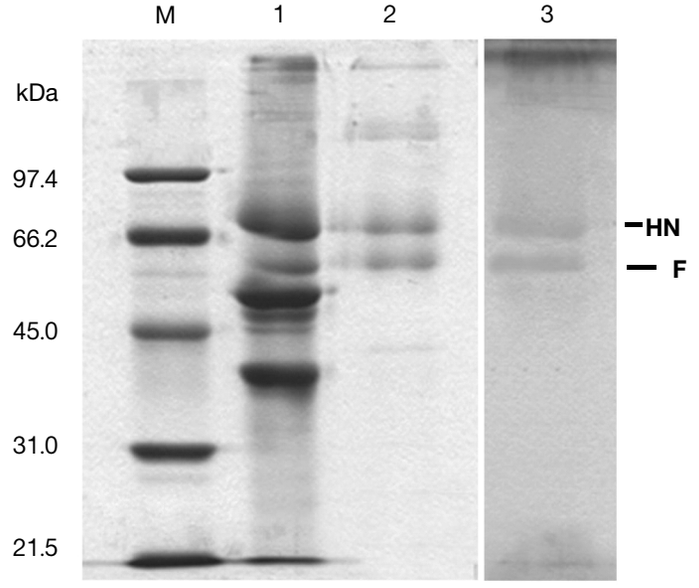

Figure 2 SDS-PAGE of NDV (lane 1) and ND-virosomes (lanes 2 and 3). On the left panel (lane 1, 2), the gel was stained with Coomassie Blue. On the right panel (lane 3), the gel was stained with PAS. The molecular weight (kDa) of reference proteins is indicated at the left (lane M).

To ensure the presence of HN and F protein in the ND-virosome vaccines, the relative quantification of HN and F antigens was determined by ELISA. The relative ELISA titer divided by the protein concentration was used as an arbitrary unit for the relative antigen contents of the formulations. ND-virosomes consisted of high HN content (4.1), whereas the F protein content was relatively low (2.0). The functional feature of HN incorporated in virosomes was also determined by an HA assay. ND-virosomes exhibited a high agglutination activity (3,930 HAU/μg). These results confirmed that the glycoprotein HN was truly incorporated in virosomes, and the HN incorporated still retained its biological activity.

Fusogenic activity of ND-virosomes

The fusogenic activity of ND-virosomes was determined by a lipid mixing assay. The fluorescence reporter molecules, pyrPC, were co-reconstituted in the virosomal membrane during the reconstitution of viral envelopes. It is well known that erythrocyte membranes are a rich source of sialic acid containing receptor, which can be recognized by the HN glycoproteins. Therefore, chicken erythrocyte ghosts were used as a model of biological target membrane in this study. Lipid mixing between fluorescent-labeled virosomes and an excess of erythrocyte ghosts was monitored continuously at different pH, i.e. 7.4, 5.5 and 4.0. The results show that ND-virosomes induced a low fusion activity at pH 7.4. Decreasing the pH of medium resulted in an increase of the fusogenic activity of ND-virosomes (Figure 3).

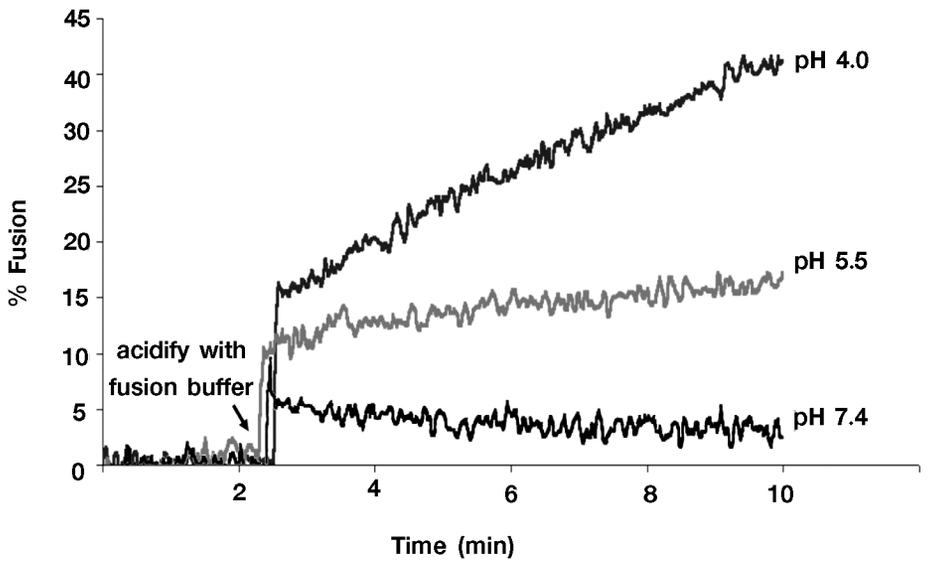

Figure 3 Activation of ND-virosomes fusion by low pH. Fusion activity of virosomes were determined by a pyrene fluorescence excimer quenching assay as described under "Materials and Methods". Data are representative of three repeated assay.

Immunological responses

Protection and immune responses were studied in chickens after single vaccination. Sera obtained from chickens at 1, 2, 3 and 4 weeks post-vaccination were analysed for the presence of HI-antibody. The kinetics of antibody induction are summarised in Table 2. One week after immunization with ND-virosomes and commercial vaccine, low antibody levels had developed in chicken's sera. The HI antibody level in the group receiving ND-virosomes was the highest at the third week post-vaccination (4.4 $\log_2$) and declined to 3.3 $\log_2$ at the fourth week after vaccination. The commercial W/O emulsion vaccine induced higher HI titers than those induced by ND-virosomes. All unvaccinated chickens were negative for NDV antibody (HI titer < 1) throughout the experiment. In the challenge experiment, the protection level in chickens vaccinated with W/O emulsion vaccine was 100%, whereas ND-virosomes gave 93% protection. Most of the unvaccinated chickens did not survive the challenge and exhibited 80% mortality (Table 2).

Discussion

The use of virosomes, in which the antigens are presented in multimeric form, has been reported as a promising approach for improving the immunogenicity of purified viral membrane proteins [4]. In this study, a systematic approach for development of ND-virosome vaccines was described. We have demonstrated for the first time that a short-chain lecithin, dihexanoylphosphatidylcholine (DHPC), could be used in the preparation of ND-virosomes successfully. The key step in preparation of virosomes is solubilization and reconstitution of membrane protein antigens with an appropriate detergent [10]. Some harsh detergents, such as sodium dodecyl sulfate (SDS) and cetyl trimethyl ammonium bromide (CTAB), are good solubilizing agents but they usually have a damaging effect on the protein functionality, in which the antigenicity of virosome vaccines might be reduced [7]. Preparation of virosomes is therefore relied on the use of mild and non-ionic detergent [6]. The use of a dialyzable DHPC as the viral membrane solublizer has several advantages over the

Table 2 Serum antibody responses and protection after challenge in chickens vaccinated subcutaneously with ND vaccine formulations[a]

Formulation	Antibody response at weeks after vaccination[b] (Number of responders/total)				Challenge[c] percentage protected
	1	2	3	4	
W/O emulsion vaccine	1.25 ± 0.25 (4/15)	3.40 ± 0.41^A (15/15)	5.33 ± 0.25^A (15/15)	4.93 ± 0.30^A (15/15)	100
Virosomes	1.60 ± 0.16 (5/15)	2.50 ± 0.13^B (15/15)	4.40 ± 0.13^B (15/15)	3.30 ± 0.32^B (15/15)	93
Negative control	<1 (0/15)	<1 (0/15)	<1 (0/15)	<1 (0/15)	20

[a] 30 μg protein/ dose for virosomal vaccine; 0.5 ml dose for W/O emulsion vaccine (commercial vaccine).

[b] Antibody response measured by HI assay represented as geometric mean titer, $\log_2$ ± standard error of mean (SEM). Groups, which are not statistically different at the level of α = 0.05, are indicated by the capital letter for each separate column.

[c] Challenge was done by oral inoculation of virulent NDV with an approximate dose of 10^4 EID_{50}.

traditional method of virosomes preparation that involves viral envelope solubilization with either Triton X-100 or $C_{12}E_8$ and subsequent removal of detergent by adsorption to hydrophobic, polystyrene beads [8]. For example, the use of DHPC as a mild detergent to solubilize virus spike proteins allows more efficient removal of detergent molecules from the virosomal membranes by dialysis, which favors membrane stability and provides the possibility to entrap vaccine adjuvants or other antigens inside these virosomes with remote loading procedure [12, 13, 24].

Sterilization, a process capable of destroying all forms of microbial life, is a major requirement for inactivated and subunit vaccines [14, 18]. The small size of virosomes (125 nm) and their highly homogeneous dispersion (PI < 0.2) allow the sterilization by filtration, indicating the potential use of virosomes for practical application. SDS-PAGE analysis demonstrated that NDV contained protein bands ranging from 40 to 70 kDa. Two major protein bands corresponding to the molecular weight of HN and F proteins were detected in the virosomes. Within the sensitivity limit of Coomassie blue staining, no contamination by other proteins was detected in virosomes. These results were in agreement with recent studies, showing that HN and F proteins could be incorporated into ND-virosomes prepared with Triton X-100 [11, 18, 25].

To ascertain the presence of HN and F proteins in virosomes, the *in vitro* antigen quantification was performed by ELISA. Relatively high HN and F contents were found (Table 1). In addition these proteins retained their biological activity. We found that ND-virosomes prepared with DHPC could preserve the capability to agglutinate chicken red blood cells. This finding was similar to the previous studies, in which virosomes prepared with Triton X-100 still retained hemagglutination activity (HA) [11, 18, 26]. Retaining of HN functionality implied that the orientation of HN proteins in virosomal membrane would be similar to that of intact NDV.

To determine the fusogenic activity of ND-virosomes, fluorescent probe (pyrPC) was co-reconstituted into virosomal membrane. Resealed erythrocyte ghosts prepared from fresh chicken's blood were used as non-labeled target membranes as they are a rich source of sialic acid containing receptor, which is necessary for *Paramyxovirus* to fuse with the host cells [27]. It was interesting to note that the degree of membrane fusion between labeled-virosomes and chicken erythrocyte ghosts was pH dependent: the more acidic the environment, the more fusion of virosomes was observed (Figure 3). This finding was similar to the results from previous investigation by Trybala, who observed that the degree of fusion between NDV la Sota and chicken erythrocytes occurring at acidic pH was higher than that occurring at neutral pH [27]. Román et al. also reported that the fusion of NDV Clone-30 strain with adherent monkey cell line COS-7 was enhanced at the acidic pH [28].

Geometric mean HI titers ranging from 2 $\log_2$ to 5 $\log_2$ have been proposed as a good predictor for clinical protection of chickens immunized with inactivated ND vaccines [29, 30]. Four weeks after vaccination, the mean serum HI antibody titers of groups receiving ND-virosomes (3.30 $\log_2$) correlated with a high level of protection (93%) against virulent NDV challenge (Table 2). However, the commercial W/O emulsion vaccine induced higher serum HI antibody titers throughout the vaccination study. Furthermore, a 100% clinical protection in chickens vaccinated with W/O emulsion vaccines was observed. According to our study, the ND-virosomes prepared are not better than the available inactivated ND vaccines in terms of immunity induction and clinical protection.

An obvious advantage of ND-virosomes is that it permits the differentiation of ND infected from vaccinated animals (DIVA). ND-virosome vaccines consist of HN and F glycoproteins and devoid other viral proteins, e.g. nucleocapsid complexes. Thus, the vaccines could only induce Abs against HN and F, since other Abs directed against nucleocapsid proteins could not be detected from the serum of the vaccinated animals. Consequently,

the virosome vaccines fulfill the criteria of a marker vaccine for NDV, which allows the differentitation of field virus infected birds from vaccinated individuals (DIVA strategy) [31, 32]. We suggest that the immunogenicity of virosome vaccines could be improved by the co-incorporation of lipopolysaccharide (LPS) or other Toll-like receptor ligand adjuvants in virosome vaccines, which could be the subject of the future study.

Conclusion

We have demonstrated the potential of a short-chain lecithin, DHPC, for successful preparation of ND-virosomes. The biophysical properties of ND-virosomes have been characterized and shown to be immunogenic in chickens. Vaccination with ND-virosomes provided good clinical protection from lethal NDV challenge. The results obtained from this study would be useful for further development of other enveloped veterinary and human virus vaccines.

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