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Comparative Study of a Cationic Liposome and its Pegylated Cationic Liposome for Newcastle Disease Virus Vaccine

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ABSTRACT

This study compares the immune response produced by stearylamine liposome and PEGylated stearylamine liposome. Multilamellar vesicles comprising phosphatidylcholine, cholesterol, stearylamineand polyethylene glycol were formulated by lipid film hydration technique. The vesicle morphology was assessed by transmission electron microscopy (TEM). The immune response produced by stearylamine liposome was compared to the PEGylated stearylamine liposome. This was assessed by haemagglutination inhibition test. The TEM pictures showed spherical and tightly packed vesicles.Antibody titreselicited by PEGylatedstearylamine liposome was 24.4 % increase over the the primary immunization antibody titre while that of the stearylamine liposome was 11.7 % increase. The positive control produced a 9 % increase over the primary immunization antibody titre.The vaccine delivery of the stearylamine liposome was thus improved by the addition of PEG 1500. PEGylating stearylamine liposome offered a good potential for encapsulating newcastle disease antigen.

Keywords:stearylamine, polyethylene glycol, liposome, vaccine, newcastle disease vaccine, immunity.

INTRODUCTION

Newcastle disease (ND) is a highly contagious infection of poultry caused by avian paramyxovirus serotype 1 (Newcastle disease virus, NDV). The virus of Newcastle disease is classified within the genus *Paramyxovirus*of the family *Paramyxoviridae*. The genetic material of NDV is RNA[1-9].Chickens infected with NDV may die without showing signs of illness, therefore improving on the immunogenicity of the vaccine would be of benefit to the poultry farmers. Cationic liposomes are structures that are made of positively charged lipids and are increasingly being researched for use in vaccine delivery due to their favourable interactions with negatively charged cell membranes [10,11]. They are internalised by cells by a classical receptor-mediated endocytosis using cell surface receptors which contain specific binding sites for, and are able to internalise cationic molecules. Most eukaryotic cells are negatively charged and as a result positively charged liposomes will bind to antigen presenting cells and other immune cells [12-16]. This leads to better uptake and *in vivo* cytotoxic T-lymphocyte induction and humoral responses. Cationic liposomes are potential carriers for oral vaccine delivery due to their protective effects on encapsulated antigens and their ability to be taken up by Peyer's patches in the intestine. They provide enhanced antigen processing through their ability to target phagocytosis by professional antigen presenting cells [17]. Camouflaging the liposomes so as to fool phagocytes into ignoring them has also become a key objective of pharmaceutical chemists. The result of their efforts was a process called PEGylation, in which countless molecules of a synthetic, non toxic polymer, polyethylene glycol (PEG), are attached, at one end of the polymer chain, to the surface of the liposome. The long, slender, highly flexible PEG molecules slosh around the liposome like spaghetti boiling in a pot. Because of their chemical affinity for water molecules, they are heavily hydrated. To phagocytes, this molecular cloak of water of hydration makes the PEGylatedliposomes look like

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little watery blobs rather than something edible so they tend to leave them alone. The two main advantages of PEGylated liposomes for delivering drugs are increased bioavailability and the possibility in some cases of targeted delivery to the organs or tissues that most need them. This research, therefore, aims to evaluate and compare the immune response of birds to Newcastle disease virus vaccine encapsulated in a stearylamine and PEGylatedstearylamine cationic liposome.
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MATERIALS AND METHODS

Materials

Cholesterol (Sigma Grade, minimum 99 % Sigma Aldrich Chemie, GmbH, St. Louis, USA), PEG 1500 30 % solution (Fluka Sigma-Aldrich, Steinheim, Switzerland), stearylamine (Sigma-Aldrich, USA), phosphatidylcholine (Nattermannallee, Köln, Germany), methanol (extra pure phEur, NF,ScharlauChemie S.A.), chloroform (Sigma-Aldrich, Germany), La Sota (National Veterinary Research Institute, Jos)

Chicks

Eighty day old chicks were obtained from CHI Farms, Ogun State. The chicks were raised from day old until termination of the experiment. The chicks were reared under clean isolated conditions until they were free of maternally derived antibodies (MDA) to Newcastle disease virus (NDV), as assessed by haemagglutination inhibition test. Feed and water were available *ad libitum.*

METHODS

Preparation of dry films

Phospholipid, cholesterol, stearylamine and PEG 1500 were weighed as indicated in Table 1 below.Multilamellar vesicles (MLV) were prepared using a technique based on lipid hydration method [18]by dissolving 1:1 molar ratio of phospholipid: cholesterolin chloroform/methanol system (2:1) in a 100 ml round bottom flask. The solvent mixtures were evaporated to obtain thin dry films on the walls of the flasks. The film was hydrated with 5 ml of PBS, pH 7.4 containing 0.2ml/dose of the Newcastle disease virus vaccine at 8 ^{o}C with gentle shaking during which vesicles were formed. The vesicles were allowed to anneal for 30 min.

Table 1: Combining molar ratio of the cholesterol and phosphatidylcholine for the cationic liposome

Cholesterol(µm)	Phosphatidylcholine (µm)	Stearylamine(mg)	Polyethylene glycol (mg)
250	250	2	
250	250	2	35

Morphology of the vesicles: The prepared cationic liposomes suspension were processed by using copper grids to adsorb cationic liposome particles from the suspension, then stained in 2.5 % uranyl acetate for 30 seconds and dried [19]. The specimens were observed under JEM – 1010 Transmission Electron Microscope (JEOL, Japan) operated at 80 kV.

Immunization of the birds: Eighty (80) specific pathogen free (SPF) birds were divided into four groups of twenty birds each. The negative control was left unvaccinated. The positive control was given 0.2 ml/ bird of La Sota$^{(R)}$ vaccine. The third group was given 0.2 ml /bird of stearylamine liposome vaccine while the fourth group was given 0.2 ml/bird of PEGylated stearylamine liposome vaccine. The vaccinations were done at 3 weeks and 6 weeks of age.

Haemagglutination inhibition test: At 5 weeks and 8 weeks of age corresponding to primary and secondary post vaccination respectively, the birds in the various groups were bled through the jugular vein. Serum samples were collected and tested for the presence of Newcastle disease antibodies using hemagglutination inhibition (HI) test [20]. All animal handling and experiments were conducted following the guidelines stipulated by University of Nigeria Research Ethics Committee on animal handling and use. HI was read to be the highest dilution of the serum causing complete inhibition of the antigen. By comparing the result against the negative control serum which showed no haemagglutination (zero titre), the result was validated.

Statistical analysis

The results of the experiments were subjected to analysis of variance (ANOVA) and variant means were separated by the least significant difference (LSD) method using SPSS 16·0 software. Significant difference was accepted at the probability level, $p<0{\cdot}05$. Results of all the

determinations were presented as means with standard error for each of the groups.

RESULTS AND DISCUSSION

Transmission electron microscopy of the liposomes

Liposomes are spherical vesicles in which an aqueous volume is entirely enclosed by a membrane composed of phospholipids. When these lipids are exposed to an aqueous environment,interactions between them (hydrophilic interactions between polar headgroups and Van der Waals' interactions between hydrocarbon chains) and with water lead to spontaneous formation of closed bilayers [21]. They can be prepared so that they entrap materials both within their aqueous compartment (water-soluble materials) and within the membrane (oil-soluble materials). The net surface charge of liposomes has been modified by the incorporation of a positively charged lipid,stearylamine and can be made more hydrophilic by attaching polyethylene glycol to the surface of the liposomes.The technique used for the preparation of the liposomeswas lipid film hydration technique (hand shaking method) which formed films on the wall of the flasks and on hydration with phosphate buffer solution (pH 7·4) produced thick, gel-like, milky colloidal dispersion. The morphology of the vesicles were studied by means of transmission electron microscopy at x3600 magnification. From the negative staining electron micrographs, Figures 1 and 2 show the vesicles of the stearylamine liposome and PEGylated stearylamine liposome at a magnification of x3600. The vesicles were spherical, rigid and tightly packed. At a magnification of x3600, the vesicles of the PEGylated stearylamine liposomes gave larger and more compact vesicles This could be as a result of the increased hydration conferred by the polyethylene glycol. The tightly packed vesicles will prevent insertion of serum proteins that destabilize vesicles *in vivo*. The stability of the liposomal membrane, i.e., its mechanical strength as well as its function as a permeability barrier, depends on the packing of the hydrocarbon chains of the lipid molecules.[22] Size and hydrophilicity of the particles have been clearly described as important factors influencing intestinal absorption[23].

Fig. 1: TEM images of stearylamine cationic liposomes

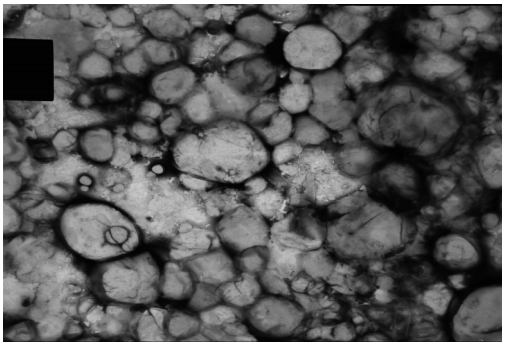
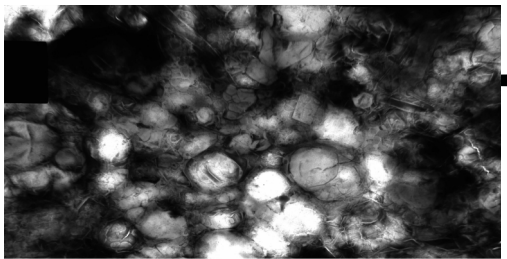


Fig. 2: TEM images of PEGylated stearylamine liposomes

Immune response of birdsproduced by stearylamine and PEGylated stearylamineliposomal vaccines.

The basis for encapsulation of drug in liposomes is to shield the drug from rapid enzyme degradation by proteases and to target the drug to the required tissue or organ. This can result in longer circulation and increased efficacy. Circulation time can also be increased by the inclusion of PEG 1500 surface coating. This polymer acts as a steric barrier and reduces the level of plasma protein binding and uptake by phagocytic cells [24,25]. Encapsulation of drug in liposomes can also reduce the volume of distribution and decrease toxic effects in healthy tissues [26]. From the Table below, there is evidence of a slow and sustained release of the antigen from the liposomal stearylamine. This will result in a pulsatile delivery of the antigen over a long period of time, leaking antigen gradually to the lymphoid cells of the gut associated lymphoid tissue. The immunotitres of the PEGylated stearylamine indicate an eventual higher and sustained immune response. Antibody titresproduced by PEGylatedstearylamine cationic liposomeswas a 24.4 % increase over the primary immunization titre while that of the stearylamine liposome was 11.7 % increase. The positive control



produced a 9 % increase over the primary immunization titre. There is a broad consensus that M cells associated with Peyer's patches are the main target for vaccination purposes [27].

Table 2. Antibody titres of the birds to the different vaccine formulations

Groups of birds	1st immunization ($\log_2$)	2nd immunization ($\log_2$)
Unvaccinated (negative)	0.00	0.00
LaSota (positive)	5.50± 0.67	6.00 ± 0.63
Stearylamine cationic liposome	5.10± 0.31	5.70± 0.15
PEGylatedstearylamine cationic liposome	4.90± 0.69	6.10 ± 0.28

CONCLUSION

The Newcastle disease virus vaccine formulated as stearylamine liposome and PEGylated stearylamine liposome gave spherical and stable vesicles whose sizes were in the nanometer range. The immune response of stearylamine showedslow release of the entrapped Newcastle disease antigen which gave higher response on attachment of polyethylene glycol to the surface of the stearylamineliposomes. Formulating a vaccine with PEGylated stearylamine liposome may hold a good potential in improving vaccine delivery for Newcastle disease vaccine.

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