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RECONSTITUTED NEWCASTLE DISEASE VIRUS ENVELOPES AS A SPLIT VACCINE

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Summary. - Isolated envelope proteins of Newcastle disease virus (NDV) were inserted into the lipid bilayer of artificial vesicles to create a viral envelope-like structure. The structure-containing viral antigens at high density elicited a strong immune response, in contrast to purified viral proteins. The artificial envelopes or immunosomes possessed several advantageous properties when used as vaccines. They elicited a faster response and the immunity lasted longer in animals treated with these vesicles than in controls vaccinated with envelope proteins in combination with traditional adjuvants. A further useful feature of the artificial envelopes is that no inflammatory lesions develop at the site of their injection.

Key words: subunit vaccines; immunosomes; liposomes; surface antigens; immunogenicity; virosomes

Introduction

Most of vaccines used in medical and veterinarian practice are based on killed or live attenuated microorganisms. Several components of these organisms may be irrelevant to the generation of the immune response while others cause undesired immunological reactions. The goal to develop vaccines containing essential antigens only is not new. Recently more and more data support the concern of "genetical pollution" by the genomes of domesticated animals and human beings with RNA and DNA from attenuated or killed organisms used as vaccines. Unfortunately, vaccines based solely on isolated and purified antigens and/or on synthetic immunogenic peptides suffer from poor immunogenicity and the obligatory use of damaging quantities of adjuvants. It was previously shown that monomeric forms of enzymes are significantly less immunogenic than their multimer forms (Wahren *et al.*, 1987; Seligman, 1978; Martin *et al.*, 1986) and several viral proteins were reported to elicit very poor immune response if injected in the monomeric form (Gross *et al.*, 1977; Quinnan *et al.*, 1982; Morein *et al.*, 1984; McAleer *et al.*, 1984).

Reconstitution of viral surface-like structures, in general, vesicles with very high density of immunogenic peptides and proteins might solve the problem. Immunosomes or reconstituted viral envelopes were reported to allow restoration of the immunological properties of Semliki Forest Virus envelope proteins (Morein, 1978), of influenza haemagglutinin (Thibodeau *et al.*, 1981) and of rabies glycoprotein (Perrin *et al.*, 1985). Here we show that NDV envelope proteins rearranged on the surface of artificial liposomes are potent challengers of the immune system that can be used with or without adjuvants.

NDV is a relatively simple virus. Its membrane, as the membrane of other paramyxoviruses, is composed of a lipid bilayer and two glycoproteins that project from the viral surface (Scheid and Choppin, 1974). One of the proteins, haemagglutinin, HN, is responsible for the attachment of the virus to neuraminic acid (and sialic acid) containing receptor molecules on the cells outer surface. The protein also exhibits neuraminidase activity. The other, F (fusion) protein is responsible for several biological activities of the virus, all of which result from the fusion of the viral membrane with the cellular plasma membrane (Tom and Six, 1980). Posttranslational proteolytic cleavage of the F protein is required for its fusogenic activity.

Materials and Methods

Viruses, virus purification. Newcastle Disease Virus (NDV) was grown on embryonated chick eggs. Allantoic fluid was collected five days after inoculation, as described by Moore *et al.* (1974). Cellular debris was removed by low speed centrifugation (2500 g, 20 min). Virions were concentrated by pelleting (Hitachi PR 19, 48 000 g, 2 hr, at 4 ^{0}C). The virus pellet was suspended in NTE buffer (100 mmol/l NaCl, 19 mmol/l Tris-HCl, pH 7.2, 1 mmol/l EDTA) and pelleted through a 30% sucrose cushion (Hitachi SRP 28 SA, 131 000 g, 16 hr, at 4 ^{0}C) and suspended in NTE buffer.

Isolation of viral antigens. Viral envelope proteins were purified according to Helenius and Simons (1975). After solubilization of the viral membrane by 1% Triton X-100 (for 1 hr at room temperature) the aggregates were removed by low speed centrifugation and the supernatant was layered on top of 20% sucrose solution in TE buffer (10 mmol/l Tris-HCl, pH 7.2, 1 mmol/l EDTA). Nucleocapsids were pelleted by 160 000 g for 1 hr. Envelope proteins, recovered from the top of the sucrose cushion, were concentrated and freed from most of the detergent by ammonium sulphate precipitation and dialysis against TE buffer. Purity of the preparation was tested on SDS slab gel electrophoresis.

Immunization. SPF White Leghorn male birds 2-weeks-old were used (9 - 11 per group). The 1 ml aliquots of different vaccines (usually containing equal amounts of proteins) were injected subcutaneously. A second 1 ml booster was given after 2 weeks. Suspension of inactivated virions was used as control, with and without complete and incomplete Freund adjuvant (Freund, 1965). Purified viral protein micelles were used as a further control.

Antibody titres were measured by ELISA (Marquardt *et al.*, 1985; Snyder *et al.*, 1985) and haemagglutination inhibition at different times after immunization.

Purification of lipids and preparation of liposomes and immunosomes. Phosphatidyl lipids were extracted from ox brain by the fractional precipitation method of Folch (Folch, 1942), purified further by chromatography (Salem *et al.*, 1976); the composition of lipids was determined by two dimensional thin layer chromatography on silica gel (Salem *et al.*, 1976).

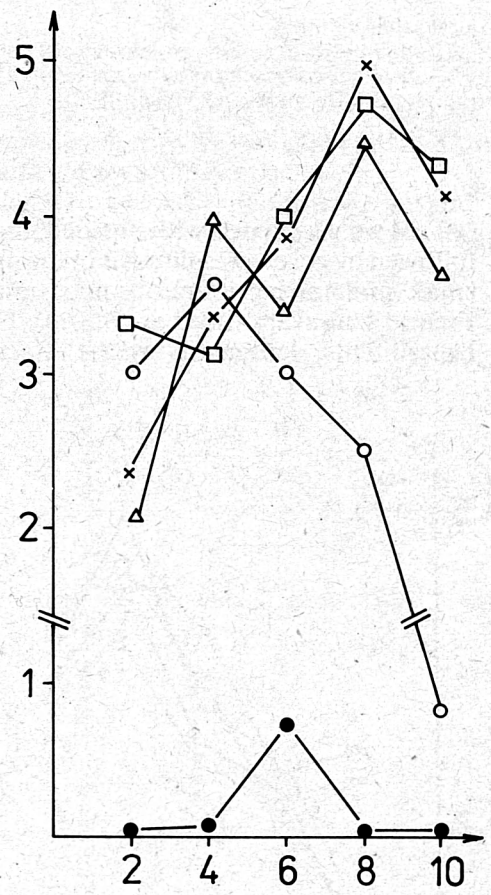


Fig. 1
Antibody titres (measured by ELISA) induced by different NDV vaccine preparations
1 ml of each vaccine as used per dose as described in Materials and Methods.
× inactivated virions, with adjuvant, containing 260 μg protein/ml
□ multilamellar liposomes, 33 μg protein/ml
△ small, unilamellar vesicles, 33 μg protein/ml
○ protein micelles, 33 μg protein/ml
● phys. salt sol. (non-immunized animals)
The inactivated virions contained all viral proteins, the liposome preparations and protein micelles only the envelope proteins.
Abscissa: time (in weeks); ordinate: (ELISA titre) dilution reciprocals.

Multilamellar vesicles (MLVs) and small unilamellar vesicles (SUVs) were produced according to standard procedures. Briefly, solutions of phospholipids and cholesterol (molar ratio was 1:1) in chloroform of chloroform-methanol (3:1) were dried onto the wall of a large glass beaker (Buchi rotational evaporator) in vacuum. The lipid film was wetted with the aqueous solution of viral envelope proteins and rotated for 15 minutes. Low energy sonication of the suspension was carried out in an ultrasonic cleaner bath for 2 - 5 min., or until microscopic (less than 2 μm) MLVs were formed (followed in a light microscope). SUVs were prepared from these vesicles by high energy sonication of the suspension (Bronson cell discruptor) until turbidity decreased significantly. Glutaraldehyde activated vesicles were prepared from phospholipid vesicles by 1% glutaraldehyde treatment, followed by a Sephadex G-25 chromatography to remove unreacted aldehyde molecules.

A further type of vesicles (DRVs, produced by detergent removal) was prepared from phospholipid-cholesterol mixtures solubilized in Triton X-100 (or octyl-glycoside). These mixtures were added to preparations of detergent solubilized envelope proteins and the detergents were removed by resins (Amberlite XAD-2 or BioRad SM-2) and subsequent dialysis against physiolo-

gical saline solutions.

Electron microscopy. The protein micelles and immunosome preparations were studied by electron microscopy using negative statining with 1% phosphotungstic acid, according to standard procedures (Schimmel and Volgel, 1970).

Results

First we vaccinated with purified protein micelles (protein composition was followed by gel electrophoresis in the presence of SDS), with multilamellar and small unilamellar immunosomes and with inactivated virus mixed with Freund's adjuvant (used as control). Fig. 1 summarizes the results of several experiments, demonstrating the effectivity of immunosomes in the vaccine.

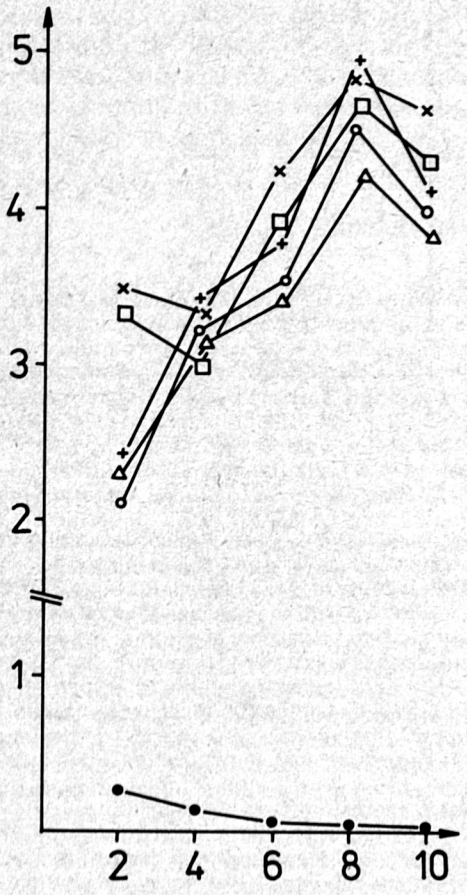


Fig. 2
Dose dependence of immunosome induced antibody titres (measured by ELISA) in immunized animals
+ inactivated virions, with adjuvant, containing 260 μg protein/ml
× multilamellar liposomes, 330 μg protein/ml
□ multilamellar liposomes, 33 μg protein/ml
△ multilamellar liposomes, 8.2 μg protein/ml
○ multilamellar liposomes, 4.7 μg protein/ml
● phys. salt sol. (non-immunized animals)
For further legend see Fig. 1

Antibody titres determined by ELISA technique were comparable in the cases of adjuvated preparations of inactivated virions and non-adjuvated immunosomes, even if the used antigen concentrations were much lower in the liposome preparations. Immunosome provoked immunity did not decrease significantly after 6 – 10 weeks unlike that elicited by protein micelles.

We followed the dependence of antibody titres on the amounts of viral antigens presentesd as immunosomess in the next series of experiments. Protein

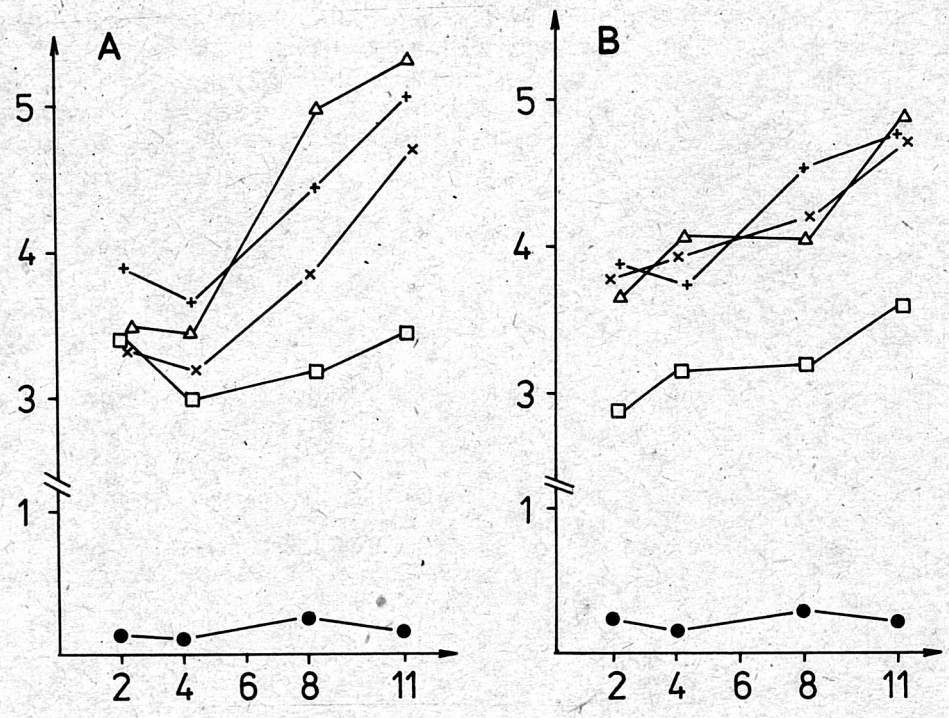
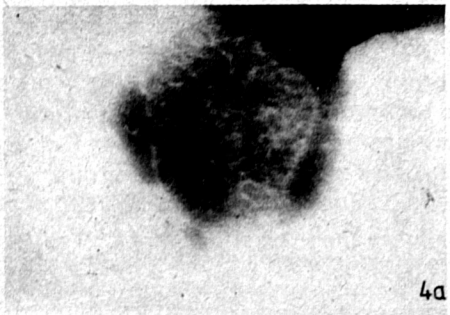
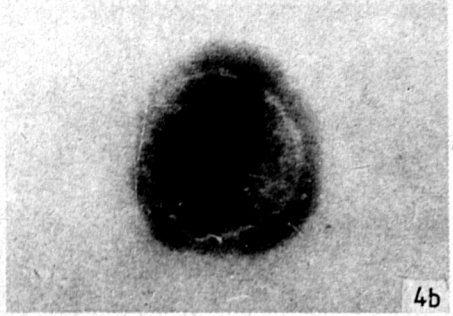
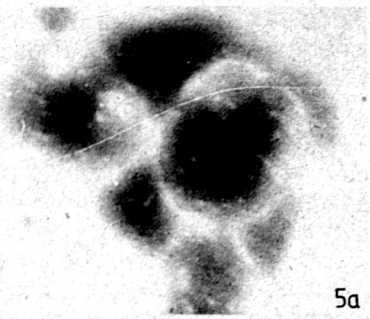


Fig. 3

Effect of adjuvants on antibody titres after vaccination with immunosome preparations
A: + inactivated virions, with adjuvant, containing 260 μg protein/ml
□ multilamellar liposomes, 33 μg protein/ml
△ multilamellar liposomes, with mineral oil adjuvant, 33 μg protein/ml
× multilamellar liposomes, with mineral oil and *B. pertussis* adjuvant, 33 μg protein/ml
● phys. salt sol. (non-immunized animals)
B: + inactivated virions, with adjuvant, containing 260 μg protein/ml
□ REV immunosomes (produced by detergent removal), 33 μg protein/ml
△ REV immunosomes, with mineral oil adjuvant, 33 μg protein/ml
× REV immunosomes, with mineral oil and *B. pertussis* adjuvant, 33 μg protein/ml
● phys. salt sol. (non-immunized animals)







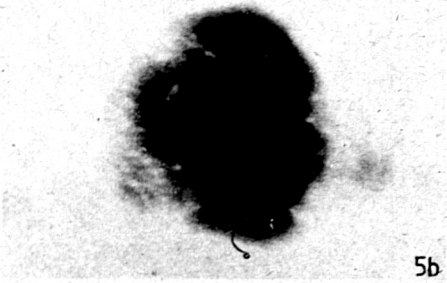


Fig. 4 - 5
Electron microscopic image of NDV immunosomes and protein micelles
Figs. 4a and *4b*: Immunosomes in which the protein: lipid ratio is similar to that one found in the viral envelope. Diameters of the vesicles are 300 and 190 nm, respectively.
Figs. 5a and *5b*: Immunosomes with 6-times lower protein: lipid ratio. Size of the shown vesicles are between 90 - 150 and 100 - 220 nm, respectively.

- lipid ratio was adjusted to achieve similar antigen density on the surface of the (outher layer of the multilamellar) vesicles as it was originally present on the surface of virions. ELISA and haemagglutination inhibition was used to measure antibody titres after immunization with multilamellar immunosomes and vesicles produced by detergent removal. Inactivated virus particles mixed with adjuvants were used as control. As shown on Fig. 2, 40- and 70-fold dilution of the original immunosome preparation resulted in somewhat reduced responses, but even after 10 weeks the antibody titre was reasonably high. Vaccination with 10-fold diluted immunosomes gave titres comparable to control values of inactivated virus preparations.

Comparison of antibody titres measured by ELISA after vaccination with protein micelles and immunosomes has proved the superiority of the proteins

incorporated into artificial lipid membranes as antigens, as they invoked higher and longer lasting antibody titres than the protein micelles. Figs. 4-6 show the electron microscopic appearance of immunosomes.

We did not use adjuvants to increase immunogenicity of the liposome preparations in any of the above-mentioned experiments. As can be seen in Fig. 3, immunogenicity of multilamellar immunosomes and vesicles prepared by detergent removal (DRVs) can be further increased by the use of mineral oil based adjuvants. While incomplete Freund adjuvant increased the potency of immunosomes significantly, the presence of B. pertussis did not improve the results achieved with immunosomes and incomplete adjuvant.

It is interesting to add that local inflammation and lesions were almost always encountered when mineral oil was used to improve immunogenity of protein micelles (or inactivated virions or liposomes). No such problem was apparent when immunosomes were used alone.

Discussion

There is now a trend towards developing vaccines that contain only purified

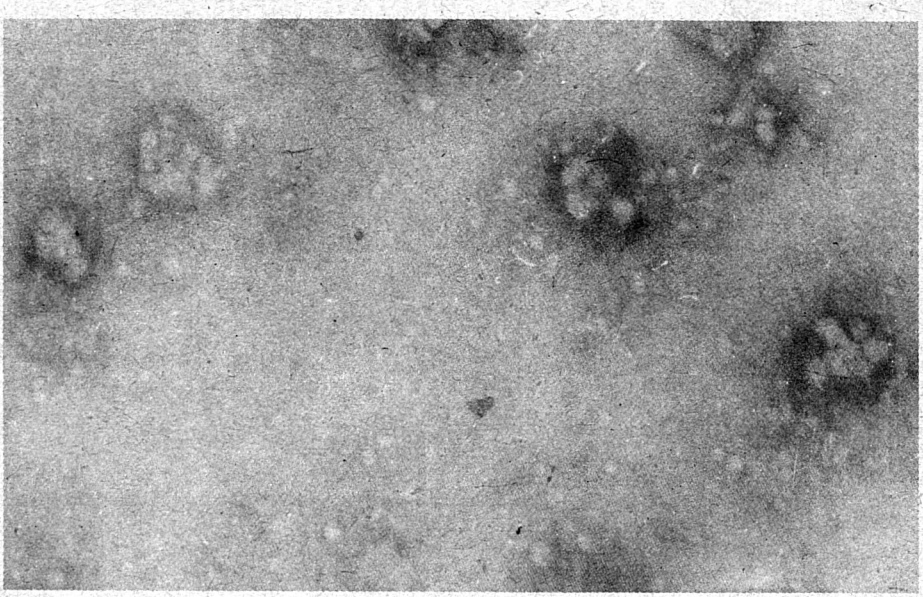


Fig. 6
Micelles of purified NDV envelope proteins (1 μm – 47 μm)

protein antigens of different viruses, bacteria, and parasites or synthetic peptides corresponding to epitopes of antigenic proteins. Large scale production of the proteins and peptides involved can be achieved by either recombinant DNA technology or chemical synthesis. However, in most cases the purified proteins and peptides are not immunologically competent or show considerably reduced immunogenicity.

We have constructed different types of immunosomes based on multilamellar and small unilamellar phospholipid vesicles, using partially purified NDV proteins. We found that several types of immunosomes elicited just good (or better) responses as did the killed viral particles. Similar results we found with surface glycoproteins of pseudorabies virus (Tóth and Duda, unpublished observations). Freund's adjuvant can be used to increase the effectiveness of immunogenic lipid vesicles. A characteristic feature of the vaccination via immunosomes is a complete lack of local lesions and necroses frequently encountered using conventional adjuvants. In each case immunosomes elicited faster and longer lasting immune responses than proteins, just like inactivated virions.

We think that immunosomes are promising means to develop effective vaccines for medical and veterinarian use with purified antigenic molecules and synthetic epitope peptides. Even those proteins that are not hydrophobic enough to be incorporated into the lipid bilayer of liposomes can be covalently bound to the surface of vesicles by conventional techniques to create high antigen density areas. The role of liposomes as immunological adjuvants is well documented (Tom and Six, 1980) and some authors write about antigens "packaged" in liposomes (Berezin *et al.*, 1988). Experiments (not shown) prove that entrapped proteins are not immunogenic and liposomes increase immunogenicity by non-specific adsorption of proteins on their surface.

The vesicle-bound protein/peptide vaccines pose none of the risks involved in the use of conventional vaccines, since no nucleic acids or other potentially harmful components are present.

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