INCORPORATION OF BOVINE HERPESVIRUS 1 PROTEIN SUBUNITS INTO LARGE UNILAMELLAR AND MULTILAMELLAR LIPOSOMES

J. HAMPL, J. ŠTĚPÁNEK, J. FRANZ and I. SVOBODA

Veterinary Research Institute, 621 32 Brno

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Abstract

Hampl J., J. Štěpánek, J. Franz, I. Svoboda: *Incorporation of Bovine Herpesvirus 1 Protein Subunits into Large Unilamellar and Multilamellar Liposomes.* Acta vet. Brno, 61, 1992: 29–36.

Virus proteins, obtained by the treatment of purified bovine herpesvirus 1 with detergents, were incorporated into large unilamellar (LUV) and multilamellar (MLV) liposomes. Their antigenicity was compared with that of virus proteins adsorbed onto LUV surface in mice. Both antibody levels and dynamics of antibody responses, examined by ELISA and RIA, confirmed a marked immunostimulatory effect of the liposomes. The most pronounced antibody responses were observed in animals injected with virus proteins incorporated into LUV, prepared from egg phospholipid, or adsorbed onto their surface.

Bovine herpesvirus 1, virus proteins, antibody response, large unilamellar liposomes, multilamellar liposomes

One of the current innovation trends in the production of vaccines against virus diseases of farm animals is the development of subunit vaccines. The principle consists in the selection from the whole complex of virus structural proteins of those, which elicit an immune response providing the protection of vaccinates (protective antigens). The question whether the proteins are obtained by degradation of complete virus particles, propagated by conventional methods, or are prepared in vitro by techniques of molecular biology, is irrelevant.

A common drawback, limiting the immediate use of such proteins, is their rather weak immunogenicity, which is much lower than that of the same proteins as a part of complete virion structures. All attempts to compensate for this drawback by the use of current non-specific immunostimulants or adjuvants have failed (Dougan 1985; Rowland 1986; Zanetti et al. 1987; Liu and Cepica 1990).

A certain effect was evident, however, when the protein subunits were incorporated back into multimolecular, corpuscular structures, such as ISCOMs (immunity stimulating complex), or various types of liposomes. In both cases, the size and shape of the particles resemble those of virions.

Enhancement of antigenicity of proteins, incorporated into ISCOMs or liposomes, was reported by Boudreault and Thibodeau (1985), Bakouche et al. (1987), Trudel et al. (1987), Elguink (1989), Tan et al. (1989), Brynestad et al. (1990), Gregoriadis (1990) and Lövgren and Morein (1991).

Infectious bovine rhinotracheitis (IBR) is an economically important disease, caused by bovine herpesvirus (BHV-1). Specific prophylaxis of this worldwide occurring disease has been limited to the use of live and inactivated vaccines, prepared from the virus propagated in cell cultures. Development of subunit vaccines, as a way of improving their efficacy and quality, has become a topical task (Trudel et al. 1987, 1988; Merza et al. 1988).

Therefore our aim was to prepare BHV-1 protein su units, to acquire skills in methods for incorporating them into liposomes and to examine the immunogenicity of such complexes.

Materials and Methods

Virus (its propagation and purification). The strain Los Angeles of BHV-1, propagated in primary calf kidney cell cultures, was used. The virus was harvested after 2—3 days of incubation of an infected cell culture by repeated freezing and thawing. After the removal of cell debris, the infectious tissue fluid was concentrated by ultrafiltration and the concentrated virus was purified by ultracentrifugation (Beckman L8-88M) in a potassium tartrate density gradient (Trudel et al. 1987). Virus-containing fractions were pooled and centrifugated and the sediment was resuspended in TEN buffer. The virus content was checked by titration in cell cultures and by electron microscopy.

Virus protein subunits. A suspension of the purified virus was incubated with non-ionogenic detergents. Either Triton X-100, or 1-0-n-octyl-glucopyranoside (OG) were used. Viral nucleocapsids were separated by ultracentrifugation in a saccharose density gradient. The protein content in the supernatant was determined by the method of Lowry (1951). The detergent and saccharose were removed by dialysis before the incorporation of the proteins into liposomes.

Liposomes. Large unilamellar liposomes (LUV) were prepared by the method of Zumbühl and Weder (1981). The molar ratio phospholipid: sodium deoxycholate was 0.62. Continuous dialysis was performed on the LIPOPREP apparatus (Dianorm A. G.). No virus proteins were added to the mixture of the phospholipid and detergent when "empty" liposomes were prepared. The liposome suspension was then incubated with the virus proteins at 37 °C for 2 hours. Proteins which did not adsorb to the surface of liposomes were washed off by ultracentrifugation.

Egg phosphatidylcholine (PC), or the soya phospholipid asolectin (PL) were used for the preparation of LUV, the molar ratio in the first being as follows: phosphatidylcholine (PC): phosphatidylethanolamine (PE): cholesterol (C): sphingomyeline (SM) = 12 : 4 : 4 : 1.

Multilamellar liposomes were prepared from PC by the method described by Bangham et al. (1972). The MLV suspension was then sonicated (ARTEC SONIC Model 300) at 120 W, 19.8 kHz for 2 minutes. The incorporation and adsorption rates were checked using a homologous protein, labelled with ^{125}I.

Electrophoresis in polyacrylamide gel (SDS-PAGE). The method of Laemli (1970) with 10 % polyacrylamide under reducing conditions and in the presence of SDS (Bio-Rad Laboratories) was used. 5 to 10 µl samples of the virus proteins and liposomes were left to separate at 120 V for 1.5 hour. The gel was stained with silver after the separation (Heukeshoven and Dernick 1985).

Antibody assay by ELISA. Antibody titres were determined by indirect ELISA using complete BHV-1 as the antigen. Porcine, anti-mouse antibodies, purified by affinite chromatography and labelled with horse radish peroxidase, served as the conjugate and hydrogen peroxide and 5-aminosalicylic acid as the substrate.

Antibody assay by RIA. Procedures and components of the commercial set RIA-test-IBR (Rodák et al. 1983; Hampl et al. 1987) were used. Porcine anti-mouse antibody, purified by affinite chromatography and labelled with ^{125}I served as the conjugate.

Immunization experiments. The immunogenicity of free virus protein subunits and subunits incorporated into large unilamellar and multilamellar liposomes was examined in homogenous groups of Balb/c mice. The experiments were evaluated in terms of dynamics of specific antibody titres in blood sera of experimental animals (determined by serological methods). Two immunization experiments were conducted on the lines of the following designs:

Experiment No 1

27 mice were divided into 5 groups and treated with the following antigen forms:

Group 1 (n = 6) — Suspension of LUV, prepared from PC, with incorporated protein;
Group 2 (n = 6) — Suspension of preformed LUV, prepared from PC, with adsorbed protein;
Group 3 (n = 4) — Suspension of MLV, prepared from PC, with incorporated protein;
Group 4 (n = 5) — Suspension of LUV, prepared from PL, with incorporated protein;
Group 5 (n = 6) — Free virus protein.

All suspensions were adjusted to the concentration of 0.03 µg protein per 1 dose, i. e. 0.2 ml. The mice were injected intraperitoneally 3 times at 4-week intervals. No serological examinations were done during the immunization period. The mice were sacrificed 14 days after the last treatment and blood sera were obtained for examination.

Experiment No 2

52 mice were divided into 4 equal groups of 13 and treated with the following antigen forms:
Group 1 — Suspension of LUV, prepared from PC, with incorporated protein;
Group 2 — Suspension of preformed LUV, prepared from PC, with adsorbed protein;

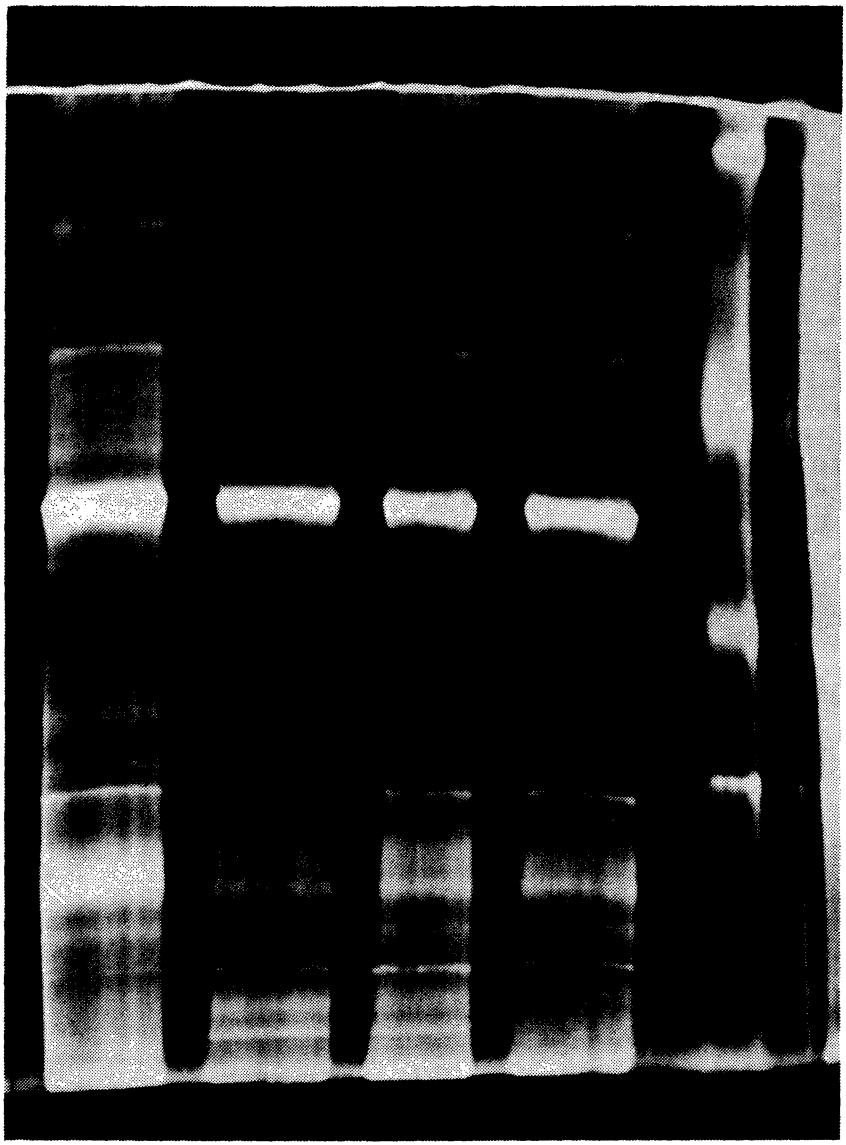

Fig. 1 — Electrophoresis SDS-PAGE
— From left to right Molecular weight standards, 91 000, 67 000, 43 000, 30 000 desc.
— MLV with incorporated virus protein
— LUV with incorporated virus protein
— LUV with adsorbed virus protein
— free BHV-1 proteins

Group 3 — Suspension of MLV, prepared from PC, with incorporated protein;
Group 4 — Free virus protein.

All suspensions were adjusted to the concentration of 0.015 μg protein per 1 dose, i.e. 0.2 ml. The mice were injected twice i. p. at an interval of 3 weeks. In each group, 4 mice were sacrificed 3, 4 and 9 weeks after the first treatment and blood sera were obtained for examination.

Results

The incorporation of virus proteins into LUV and MLV was demonstrated by two methods.

Fig. 1 shows the distribution of virus protein subunits after SDS-PAGE. The subunits separated into more than 25 fractions with molecular weights between 150 and 30 kD. The spectra of proteins, incorporated into any of the three liposome types, were identical and corresponded to the electrophoretical pattern of free virus subunits.

The incorporation of virus proteins into LUV and MLV was further demonstrated by the radioisotope tracer technique (Table 1). The incorporation rate ranged between 2.3 and 2.8 %. The level of the binding capacity of liposomes approached the estimated incorporation rate.

The immunization experiments confirmed the premise that immunogenicity of virus proteins is markedly enhanced by incorporation into liposomes (Table 1). The best effects were obtained with LUV, prepared from PC, no matter whether the proteins were incorporated into the structure, or adsorbed onto the surface of preformed "empty" liposomes. This has been demonstrated by serological examination of mice, treated 3 times at 4-week intervals. The average antibody titre (n = 12) was $3.6 \cdot 10^4$ and in 8 animals the titre reached or exceeded $1.0 \cdot 10^5$. Antibody responses in mice, treated with virus proteins incorporated into MLV, were much weaker and, in practical terms, antibody titres in this group did not differ from those in animals treated with free virus proteins.

The immunostimulating effect of LUV, prepared from asolectin, were negligible.

These results were confirmed in Experiment 2 (Table 2). Responses to the treatment with free virus proteins were very weak and only a mild immunizing effect was observed in mice treated with proteins incorporated into MLV. On the other hand, a pronounced effect was evident in mice treated with proteins incorporated into, or adsorbed onto the surface of LUV.

Table 1

Highest antibody titres obtained

Group	No of mice*	Incorporation rate (%)	Treatment	Number of mice with RIA antibody titres					
				450	1 850	4 050	12 150	36 950	109 450
1	6	2.7	LUV–PC with incorporated protein	0	0	0	0	2	4
2	6	2.3	LUV–PC with adsorbed protein	0	0	0	0	2	4
3	4	2.8	MLV–PC with incorporated protein	0	0	0	3	1	0
4	5	2.7	LUV–PL with incorporated protein	2	3	0	0	0	0
5	6		free virus protein	0	0	3	3	0	0

* All animals were treated 3 times at 4-week intervals.
One dose contained 0.03 μg virus proteins.

Table 2

Dynamics of antibody titres in mice treated with virus proteins incorporated into liposomes, prepared from PC

Group/number*	Treatment	Average antibody titres ELISA/RIA		
		Days after the first treatment		
		21	42	63
1/13	LUV with incorporated protein	150/450	14 985/46 050	4 212/11 340
2/13	LUV with adsorbed protein	50/150	7 875/24 525	3 744/7 020
3/13	MLV with incorporated protein	25/75	1 785/4 950	1 500/4 140
4/13	free virus protein	50/125	600/900	400/1 200

* All animals were treated twice with a 3-week interval.
One dose contained 0.015 μg virus proteins.

The generally weaker antibody responses in Experiment 2 were obviously due to the fact that, compared with Experiment 1, a half-dose of the antigen, i.e. 0.15 μg protein, was used.

Discussion

One of the drawbacks of conventional vaccines against virus diseases is the presence of a large amount of proteins coming from culture media and disintegrated cells used for obtaining the virus suspension. The antigenic structure of such vaccines is not adequate to the purpose of vaccination and represents a considerable stress to vaccinates.

An alternative way how to obtain improved biologicals is the production of subunit vaccines, containing protective antigens predominantly. However, hopeful are only those procedures, in which the isolated subunit proteins are integrated into corpuscular, multimolecular structures, such as liposomes or ISCOMs (Naylor et al. 1982; Perrin et al. 1984; Bakouche et al. 1987; Tan et al. 1989; Morein et al. 1987; Trudel et al. 1987). Properties of liposomes and the ensuing biological activities depend on many factors. Both the properties and the amount of the incorporated virus protein are determined by the method of preparation and its variations. In our experiments, only a rather low degree of incorporation, not exceeding 2.8 %, was achieved, which is not sufficient for the production of commercial vaccines.

Incorporation of more than 25 BHV-1 protein fractions into both LUV and MLV was demonstrated by SDS-PAGE using a highly sensitive staining method. In a previous study, four specific antigenic zones were identified among them (Štěpánek et al. 1990). This finding corresponds to those of Misra et al. (1981) and Babiuk et al. (1987), who identified four immunologically active glycoproteins, gp-1—gp-4 of BHV-1.

The amounts of proteins, incorporated into LUV or MLV and administered in the experiments, were 30 or 15 ng per dose. This difference was reflected in antibody responses in individual groups of mice. Gregoriadis et al. (1987) stated that the intensity of the antibody response depended not only on the amount of the protein incorporated in liposomes, but also on the phospholid: protein ratio. For the tetanus toxoid, administered to mice, they considered the range 30:1 to 2 049 : 1 as the optimum and recorded only very weak immune

responses if the ratio reached or exceeded 90 361 : 1. In our experiments, the phospholipid: protein ratio was $3.5-3.7 \cdot 10^5 : 1$ and the intensity of the antibody response was proportional to the amount of proteins administered.

Differences of immunogenicity between antigen incorporated into liposomes or adsorbed onto their surface have been a frequent topic of discussions (Shek and Sabiston 1982; Gregoriadis et al. 1987).

Ostro (1987) concluded that the number of antigenic determinants available on the surface of liposomes and capable of binding with B lymphocytes or macrophages, with the ensuing binding to T and B lymphocytes, is the decisive factor for the intensity of the immune response.

This view contradicts that of Shek and Sabiston (1982), who reported a higher immunogenicity of antigens incorporated into liposomes. Van Rooijen and Van Nieuwmegen (1983) and Van Rooijen et al. (1983) concluded that liposomes with antigens adsorbed onto their surface stimulated the formation of IgG, but above all IgM, during the primary immune response.

In our Experiment 1 (3 doses of 30 ng protein), no differences were observed between groups that received liposomes with incorporated or adsorbed proteins. On the other hand, in Experiment 2 (2 doses of 15 ng protein), the immune response was weaker in mice treated with liposomes with adsorbed proteins.

Weak antibody responses were observed in mice treated with MLV. The lower immunogenicity was probably associated with the incorporation of the virus protein into their phospholipid bilayers and interlamellar spaces. The same degree of incorporation as in LUV was demonstrated by the radioisotope tracer technique. MLV obviously lack a sufficient number of antigenic determinants, necessary for their binding with B lymphocytes, and are taken up and subsequently digested by the cells of RES. No immunostimulating activity, similar to that of LUV, was demonstrable in MLV.

Inkorporace proteinových subjednotek viru BHV-1 do velkých jednolamelárních a multilamelárních liposomů

Virové proteiny získané degradací purifikovaného a koncentrovaného viru BHV-1 pomocí detergentů byly inkorporovány do velkých unilamelárních (LUV) a multilamelárních (MLV) liposomů. V imunizačních pokusech na myších byla jejich antigenní účinnost srovnána s antigenní účinností virových proteinů adherovaných na povrch LUV. Úroveň i dynamika protilátkové odpovědi u myší, hodnocená ELISA a RIA testy, potvrdila výrazný imunostimulační efekt liposomů. Nejvýraznější protilátková odpověď byla zaznamenána u zvířat imunizovaných virovými proteiny inkorporovanými do LUV připravených z vaječného fosfolipidu nebo adherovaných na jejich povrch.

Проникновение протеиновых подъединиц вируса BHV-1 в крупные однопластинчатые и многопластинчатые липозомы

Вирусные протеины, полученные деградацией очищенного и концентрированного вируса ELISA с помощью детергентов вводили в крупные однопластинчатые (LUV) и многопластинчатые (MLV) липозомы. В иммунизационных экспериментах у мышей сопоставляли их

антигенное действие с антигенной эффективностью вирусных протеинов, адгезированных на поверхность LUV. Уровень и динамика ответа антител у мышей, оцениваемые BHV-1 и тесты RIA, подтвердили выразительный иммуностимулирующий эффект липозомов. Самый выразительный ответ антител наблюдали у животных, иммунизированных вирусными протеинами, вводимыми в UV и подготовленными из ячного фосфолипида или адгезированные на их поверхность.

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