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THE COMPLEX FORMATION OF INFLUENZA VIRUS ENVELOPE GLYCOPROTEINS WITH OUTER MEMBRANE PROTEINS OF *NEISSERIA MENINGITIDIS* OR *BORRELIA BURGDORFERI*

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Summary. – The isolation of influenza virus envelope glycoproteins was achieved by one-step procedure consisting of treatment of purified virus with zwitterionic detergent and separation of viral constituents by sucrose density gradient centrifugation. Viral glycoproteins and proteins of outer membrane of *N. meningitidis* or *B. burgdorferi* formed complexes after removal of the detergent by dialysis. Complexing of viral glycoproteins and bacterial proteins was monitored by gel chromatography on Sepharose 6B, polyacrylamide gel electrophoresis and electron microscopy. It was demonstrated by immunoblot analysis, that virus-spirochete complexes elicited formation of antibodies in mice directed against osp A and osp B of spirochete, as well as against viral glycoproteins, respectively.

Key words: influenza virus glycoproteins; spirochete outer membrane proteins; complex formation; antibodies

Introduction

The lipopolysaccharide (LPS)-devoid outer membrane proteosomes of gram-negative bacterium *N. meningitidis* were reported to be a potent carrier for complexing with synthetic peptides equipped with hydrophobic terminus (Lowell *et al.*, 1988).

As glycoproteins of enveloped viruses span envelope structures with hydrophobic terminal tails of their molecules, we analyzed the possibility to complex them with meningococcal proteosomes. We reported earlier that it was possible to complex glycoprotein E of tick-borne encephalitis virus (TBEV) with such bacterial proteosomes (Slávik *et al.*, 1991).

Our present intention was to complex envelope glycoproteins of influenza virus, where inside the envelope structure the glycoproteins primarily interact with matrix (M) polypeptide. In contrary to TBEV, influenza virus contains helical, but not icosahedral capsid.

B. burgdorferi is the causative agent of Lyme disease (Burgdorfer *et al.*, 1982), which is a chronic disorder with dermatologic, rheumatologic, cardiac and neurologic manifestations. Its immunologically relevant outer surface proteins, i. e. osp A and osp B are lipoproteins (Brandt *et al.*, 1990). When present in organism prior to infection, antibodies against osp A are protective (Edelman, 1991). Controversial data were reported about the presence of LPS in outer membrane *of B. burgdorferi* (Beck *et al.*, 1985; Takayama *et al.*, 1987).

In the present work we have attempted to complex influenza virus glycoproteins with proteins of outer membrane of *N. meningitidis or B. burgdorferi.*

Materials and Methods

Virus. Influenza virus A/Singapore/1/57 (H2N2) was propagated in 10–11 day-old chick embryos at 35 °C for 48 hrs. The haemagglutination titer of the harvested allantoic fluid was about 512–1024 HAU/ml, and the infectious titer ranged between 10^7 and 10^8 $TCID_{50}$/ml.

Virus purification. The allantoic fluid was clarified by low speed centrifugation and gauze-filtered, and the virus was pelleted at 30 000 x g for 90 mins. Pellets were resuspended in 0.05 mol/l Tris pH 7.2, clarified, and sedimented in 25–50 % discontinuous succrose density gradient at 93 000 x g for 120 mins. Visible virus band was taken and diluted about 20-times with Tris pH 7.2 and again pelleted. Pellets were resuspended in Tris pH 7.2, clarified and pooled.

About 0.5–1.0 ml of partially purified virus (up to 1 mg protein) was layered onto 25 ml of 20–40 % sucrose density gradient made in PBS pH 7.6 and electrophoresed for 24 hrs at 100 V, 10 mA, and 4 °C (Rosenbergová *et al.*, 1981). Virus-containing fractions from several runs were pooled, diluted 20–30-times with Tris pH 7.2, and the virus was pelleted at 186 000 x g for 80 mins. Pellets were resuspended in Tris pH 7.6 (electrophoretically purified virus).

Isolation of viral glycoproteins. Electrophoretically pure virus (up to 500 μg) was heated at 37 °C for 1 hr in the presence of 1 % Empigen BB (Calbiochem), layered over 4 ml of 3–15 % sucrose density gradient containing 0.5 % of the same detergent and centrifuged in rotor SW 55 Ti (L-8 Beckman ultracentrifuge) at 45 000 rpm for 4.5 hrs. After fractionation, aliquots of fractions were analyzed by polyacrylamide gel electrophoresis (PAGE).

Preparation of bacterial proteosomes. Meningococcal proteosomes were prepared as described earlier (Slávik *et al.*, 1991).

Strain B 31 of *B. burgdorferi* was grown in modified Barbour-Stoener-Kelly (BSK II) medium (Barbour, 1984) at 32 °C for 3–4 days. Spirochetes were pelleted at 10 000 x g, washed with PBS, lyophilized and stored at –18 °C. Reconstitution and subsequent washings were performed once with PBS and twice with TNE buffer (0.05 mol/l Tris pH 7.4, 0.15 mol/l NaCl, 0.01 mol/l EDTA). Spirochetes were then treated in 1 % Empigen BB containing buffer at 37 °C for 1 hr, precipited with ammonium sulphate (500 mg/ml) for 1 hr at 4 °C and pelleted in microcentrifuge for 3 mins. Pellets were resuspended in 1 % Empigen BB containing buffer; the precipitation of spirochete proteins and the pelleting of precipitate were repeated two times.

The resulting pellet was resuspended in Empigen BB in TNE, diluted to about 1.5 mg of protein/ml and layered in 1 ml aliquots onto 4 ml of 5–20 % sucrose density gradient made in 0.5 % Empigen BB in TNE. Tubes were spun in rotor SW-50.1 for 3.5 hrs at 36 000 rpm and 4 °C. After fractionation aliquots of fractions were analyzed by PAGE. Fractions containing spirochete outer surface proteins occupied upper 40 % of centrifuge tube content. They were pooled and protein was recovered by ammonium sulphate precipitation.

Complexing of viral and bacterial proteins. Meningococcal proteosomes were mixed with influenza virus glycoproteins and dialyzed as previously described (Slávik *et al.*, 1991). Outer membrane

proteins isolated by detergent treatment and subsequent sucrose density gradient centrifugation of *B. burgdorferi* were mixed with viral glycoproteins in a ratio of 1.5:1.0 (w/w of proteins). They were dialyzed against TNE pH 8.0 and then against PBS for 1 week at 4 °C.

PAGE of proteins was performed on 10 % slab gels 140x140xl mm (Laemmli, 1970). Samples were boiled in loading buffer (2 % SDS and 5 % 2-mercaptoethanol) for 3 mins, loaded and run for 3 hrs at 150 V and 20 mA. Gels were fixed and stained with 0.25 % Coomassie brilliant blue R-250, and destained in methanol-acetic acid-water solutions.

Gel chromatography of complexes. Dialyzed preparations were 3-times washed on membrane XM-300 (Amicon) with PBS, clarified in microcentrifuge, and finally purified on Sepharose 6B column run with PBS pH 7.2. About 2 mg of complexed proteins in 1 ml were applied on 0.9x41 cm column, chromatographed and fractionated under monitoring the absorbance at 254 nm. Aliquots of fractions were analyzed by PAGE for presence of virus and spirochete polypeptides.

Immunoblot analysis. Purified virus or lyophilized spirochete were dissolved in boiling buffer and run separately in polyacrylamide gels. Separated polypeptides were electroblotted onto nitrocellulose membranes overnight at 60 V (Towbin *et al.*, 1979). Membranes were incubated with sera of mice immunized twice in 14 day-interval and collected 14 days after the second dose. Sera were diluted in 5 % defatted milk-containing buffer (0.05 mol/l Tris pH 8.0, 0.08 mol/l NaCl, 0.002 mol/l $CaCl_2$, 0.2 % Triton X-100) and applied onto membranes overnight at 4 °C (Sláviková *et al.*, 1987). Nitrocellulose membranes were then twice washed with the latter buffer, twice with TNE buffer containing 0.05 % Tween-20, and finally incubated for 2 hrs at 4 °C with ^{125}I-labelled protein A ($5x10^6$ cpm/ml). Strips were washed 8-times with TNE-Tween-20 buffer, dried and autoradiographed.

Electron microscopy. Negative staining of samples and their electron microscopical examination were described previously (Slávik *et al.*, 1991).

Proteins were assayed with the Folin phenol reagent in a standard way.

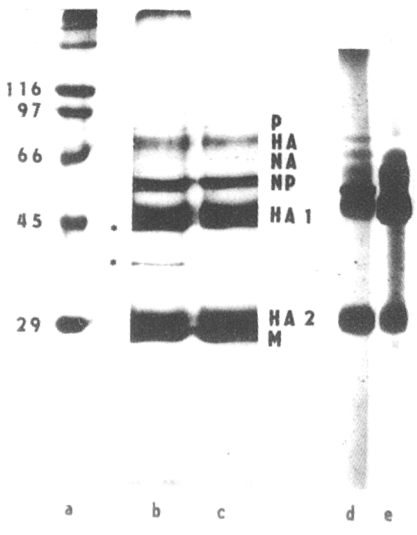

Fig. 1
PAGE of influenza virus proteins 10 % slab gel (lanes a–c) and 7 % tube gels (d, e). Staining with Coomassie brilliant blue R-250 (a–d) or Schiff's basic fuchsin (e). Molecular weight marker proteins (a). Partially purified virus (b). Electrophoretically purified virus (c–e). P – polymerases, HA – haemagglutinin, NA – neuraminidase, NP – nucleoprotein polypeptide, M – matrix polypeptide.

Results

Isolation of viral glycoproteins

In preparations of influenza virus purified by differential centrifugation and sucrose density gradient centrifugation, non-virion polypeptides were detected by PAGE; most prominent of them was a polypeptide with M_r corresponding to actin, and a 36 K polypeptide (Fig. 1b). In electrophoretically pure virus preparations, contaminating polypeptides were not detected by PAGE (Fig. 1c).

As molecules of neuraminidase (NA) were not visualized by protein staining in slab gels (broader bands than in tube gels), electrophoretically pure influenza virus was electrophoresed in tube gels in phosphate buffer. Broad band of NA was detected by staining for proteins and for glycides as well (Fig. 1d, e).

After treatment of electrophoretically pure influenza virus with Empigen BB, virus constituents were separated in sucrose density gradient. Under conditions used, the polypeptide M sedimented slowly and remained in the upper quarter of the tube. Virus glycoproteins sedimented to the border of upper and lower half of the tube. Nucleoprotein polypeptide was not detected in any fraction of

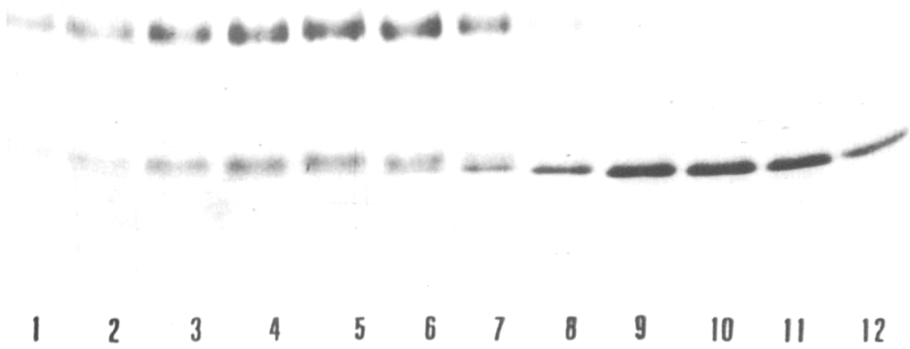

Fig. 2

Separation of influenza virus structural proteins by sucrose density gradient centrifugation Electrophoretically purified virus after detergent treatment was used. Fractions from upper half of centrifuge tube were analyzed by PAGE in slab gel. Staining with Coomassie brilliant blue R-250.

sucrose density gradient by PAGE, indicating that virus nucleocapsid sedimented to the pellet under conditions used (Fig. 2).

After separation of glycoproteins from polypeptide M, pooling appropriate fractions and measurement of protein content in respective pools, it was found that glycoproteins and polypeptide M represented about 30 % and 50 % of the total protein of influenza A/Singapore/1/57. These values were used, when equal amount of glycoproteins was applied in form of virus-spirochete complex or formaldehyde-inactivated virus in immunization experiments.

Complexes of viral glycoproteins with meningococcal proteosomes

Virus glycoproteins were complexed with proteosomes of N. meningitidis in equal weight proportions. During gel chromatography on Sepharose 6B column, complexed molecules eluted behind the void volume with slight accumulation of eluted material in fractions No. 8 and 11 (Fig. 3, insert). Small portion of detergent present in complexed material eluted as single peak free of proteins,

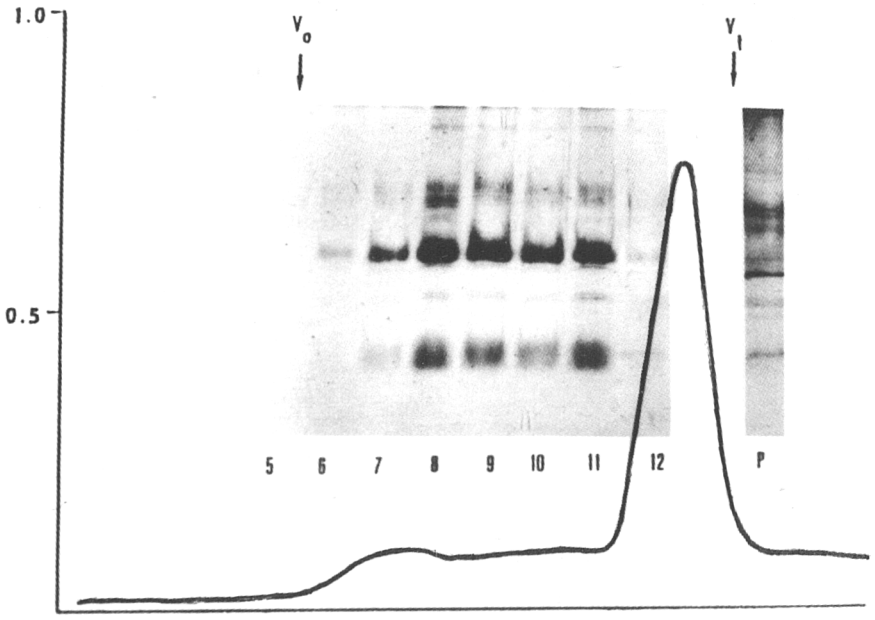

Fig. 3

Gel chromatography of complexes of influenza virus glycoproteins with meningococcal proteosomes Aliquots of eluted fractions were electrophoresed in slab gel. Insert: staining with Coomassie brilliant blue R-250. Lane P: non-complexed meningococcal proteosomes. Abscissa: fraction No. Ordinate: A_{254}.

just prior to total volume of column. For comparison, the polypeptide profile of non-complexed meningococcal proteosomes is shown in Fig. 3 (insert, lane P).

The smallest spherical virion-like particles visualized by negative staining in preparations of virus-meningococcal proteosomes complexes exhibited diameter of about 40–60 nm (Fig. 4, arrow). They were covered on their surface with "spiky" projections. There were also present large stripped parts of bacterial outer membranes of leaf-like appearance, covered by viral glycoproteins-and side-clustered to a different extent (Fig. 4).

Complexes of viral glycoproteins with proteins of B. burgdorferi

When gel chromatography of virus-spirochete complexes was performed, a peak of material appeared, which was eluted with void volume of the column. Slight elevation of absorbance baseline was observed in fractions eluting between V_o and V_t of the column; symmetrical peak of detergent eluted

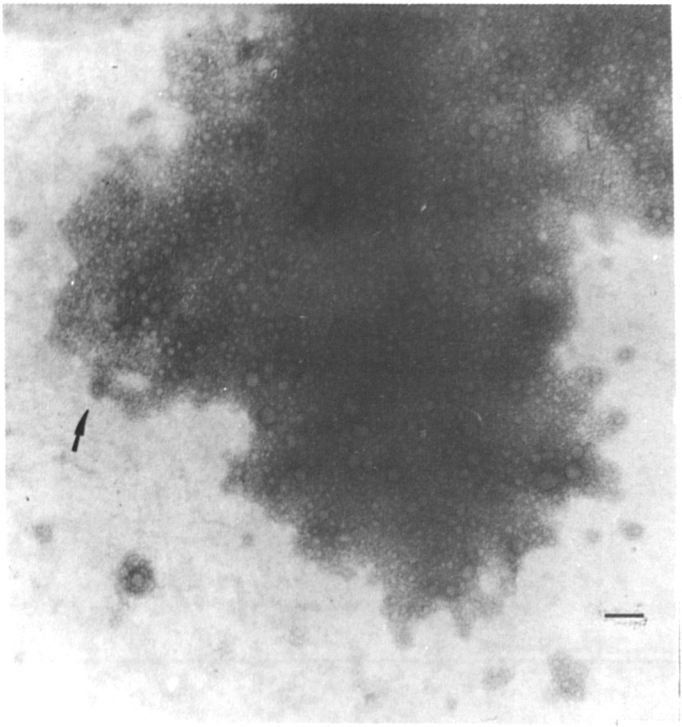

Fig. 4

Electron microscopy of negatively stained complexed particles consisting of influenza virus glycoproteins and meningococcal proteosomes

Arrow indicates one of virus-like particles. Bar = 100 nm.

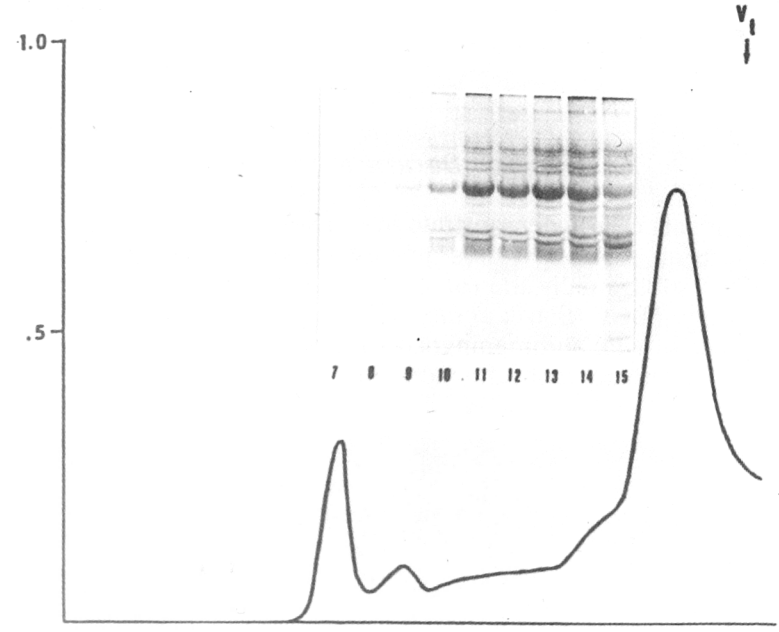

Fig. 5
Gel chromatography of complexes of influenza virus glycoproteins with outer membrane proteins of *B. burgdorferi*
PAGE of aliquots of respective fractions in slab gel. Insert: staining with Coomassie brilliant blue R-250. Abscissa: fraction No. Ordinate: A_{254}.

similarily as above (Fig. 5). Virus glycoproteins together with spirochete outer membrane proteins were detected in polyacrylamide gel mainly in fractions No. 11–15, while flagellar 41 K polypeptide accumulated mostly in fractions No. 14 and 15 (Fig. 5, insert).

In fractions eluted in void volume of the column, negative staining revealed disrupted particles originating from protoplasmic cylinders of spirochete. In fractions No. 10–15, there appeared material sparsely covered with viral glycoproteins. Virus molecules exhibited uniform clustering and no virus-like particles, similar to those found in meningococcal proteosomes complexes were detected in spirochete complexes by electron microscopy (Fig. 6).

Purity of virus-spirochete complexes

For immunization of mice, the combined fractions No. 10–13 (Fig. 6) were used. In sera with antibodies elicited by complexed preparations, only bands corresponding to glycoproteins of influenza virus were detected by immunoblot

analysis (Fig. A, lanes a–d). The same sera used subsequently for the immunoblot reaction with proteins of whole *B. burgdorferi* revealed only bands corresponding to osp A(32)K and osp B(34 K) of outer membrane of spirochete (Fig. 7B, lanes a–d).

Discussion

The present data clearly demonstrate that envelope glycoproteins could easily be isolated from a RNA viruses possessing helical capsid. The isolation procedure was substantially the same as that previously used for polypeptide E isolation from TBEV (Slávik *et al.*, 1991).

The presence of viral and meningococcal (spirochete) polypeptides eluted in the same gel chromatography fractions indicated that complexing of protein

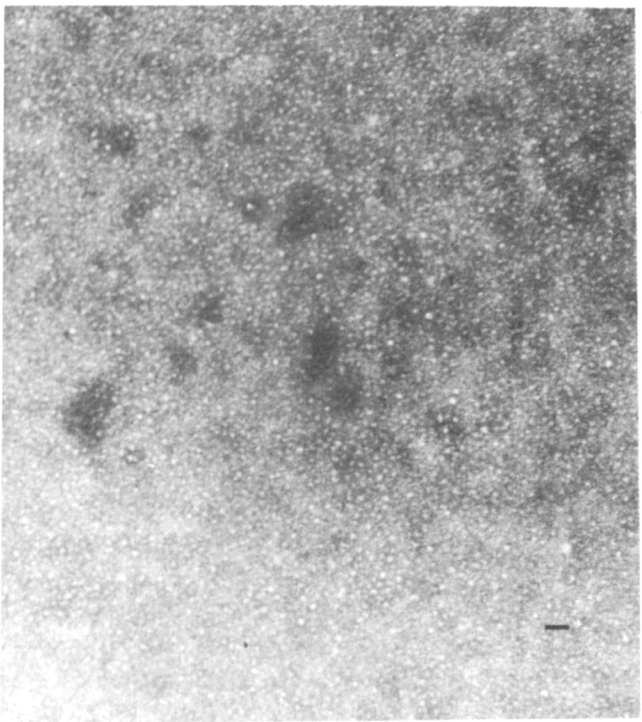

Fig. 6
Electron microscopy of negatively stained complexed particles consisting of influenza virus glycoproteins and outer membrane proteins of *B. burgdorferi*
Bar = 100 nm.

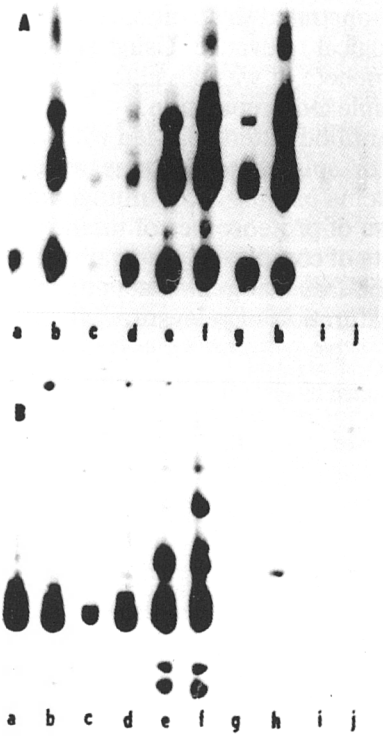

Fig. 7
Immunoblot analysis of proteins of influenza virus and of whole *B. burgdorferi*
A: influenza virus proteins. B: *B. burgdorferi* proteins. Proteins detected with sera pooled from 15 mice immunized with following preparations: Lanes: A/a, B/a – 2 μg of complexed prepartion consisting of virus glycoproteins (HANA) plus outer membrane proteins of *B. burgdorferi* (PBB); A/b, B/b – 2 μg of (HANA + PBB) applied together with 2 mg of alum (Al); A/c, B/c – 200 ng of (HANA + PBB); A/d, B/d – 200 ng of (HANA + PBB) + Al; A/e, B/g – 3 μg of formaldehyde-inactivated virus (FV); A/f, B/h – 3 μg of FV + Al ; A/g, B/i – 300 ng of FV; A/h, B/j – 300 ng of FV + Al; A/i, B/e – 1 μg of PBB + Al; A/j, B/f – 100 ng of PBB + Al.

molecules occured even in the presence of traces of detergent in preparations. Moreover, a low absorbancy of complexed material and the elution of symmetrical peak of detergent suggested that gel chromatographic procedure was an efficient step for separation of detergent from complexed molecules. The position of peak of detergent, corresponding to material with M_r of about 70 K indicated that Empigen BB was present in micellar form in the solution.

Glycoproteins of influenza virus were more prominent after visualization by negative staining, than the polypeptide E of TBEV (Slávik *et al.*, 1991). Therefore, when there were found virion-like particles covered on their surface with "spiky" projections of viral glycoproteins, it probably meant that hydrophobically binding sites were present on the surface of meningococcal proteosomes in high density. As compared to meningococcal proteosomes, outer membrane proteins of *B. burgdorferi* exhibited lower density of such binding sites. It might represent an advantage in presentation of spirochete epitopes to immunocompetent cells of organism.

Analysis of purity of virus-spirochete complexes by immunoblot analysis demonstrated that complexed particles were prepared from molecules of immunological relevance. Using simple purification procedures structurally complex spirochete, it was possible to reach high degree of purity of its proteins in final complexed preparation.

Antibody titers reached with viral glycoproteins complexed with meningococcal or spirochete proteins were comparable. It might mean that spirochete proteins exhibit B-cell mitogenicity and T-helper cells activities comparable to those of proteosomes of meningococcus (Lowell *et al.*, 1988). High immunogenicity of complexed preparations and quality of antibodies could be ascribed also to the fact that gentle isolation and purification procedures used might prevent denaturation of relevant viral and/or bacterial polypeptides.

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References

Barbour, A. G. (1984): Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med.* **57**, 521–525.

Back, G., Habicht, G. S., Benach, J. L., and Coleman, J. L. (1985): Chemical and biological characterization of a lipopolysaccharide extracted from the Lyme disease spirochete *(Borrelia burgdorferi). J. inf. Dis.* **152**, 108–117.

Brandt, M. E., Riley, B. S., Radolf, J. D., and Norgard, M. V. (1990): Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. *Infect. Immun.* **58**, 983–991.

Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E., and Davis, J. P. (1982): Lyme disease, a tick-borne spirochetosis? *Science* **216**, 1317–1319.

Edelman, R. (1991): Perspective on the development of vaccines against Lyme disease. *Vaccine* **9**, 531–532.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lowell, G. H., Smith, L. F., Seid, R. C., and Zollinger, W. D. (1988): Peptides bound to proteosomes via hydrophobic feet become highly immunogenic without adjuvants. *J. exp. Med.* **167**, 658–663.

Rosenbergová, M., Slávik, I., and Hána, L. (1981): Zonal density gradient electrophoresis of influenza A virus. *Acta virol.* **25**, 78–86.

Slávik, I., Kuzemenská, P., Kožuch, O., Maťoška, J., Pokorný, J., and Sekeyová, M. (1991): The complex of flavivirus envelope polypeptide with meningococcal proteosomes elicits formation of virus-neutralizing antibodies. *Acta virol.* **35**, 313–321.

Sláviková, K., Kettmann, R., Reinerová, M., Zajac, V., Portetelle, D., and Burny, A. (1987): Provirus integration of bovine leukaemia virus into DNA of infected human myeloma cells. *Neoplasma* 34, 653–657.

Takayama, K., Rothenberg, R. J., and Barbour, A. G. (1987): Absence of lipopolysaccharide in the Lyme disease spirochete *Borrelia burgdorferi. Infect. Immun.* **55**, 2311–2313.

Towbin, H., Staehelin, T., and Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedure and some applications. *Proc. natn. Acad. Sci. U. S. A.* **76**, 4350–4354.