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PREPARATION AND EFFICACY OF ANTIHERPES TYPE 1 AND 2 SUBUNIT VACCINES

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Summary. — DNA free antiherpes subunit vaccines were prepared from diploid human embryonic lung cells infected either with type 1 or type 2 herpes simplex viruses (HSV). Virion and membrane-bound virus-specific glycoproteins were solubilized with Nonidet P-40 and separated by ultracentrifugation. The antigenic properties of the vaccine were tested in guinea pigs. Antibody response was followed by virus neutralization and complement fixation. The vaccine itself was low-immunogenic, however its immunogenicity has considerably increased by usage of suitable adjuvans. In virus neutralization test higher antibody titre was found against homologous virus. The antibody response was related to protein content and to the frequency of vaccination.

Key words: *herpes simplex virus*; *viral DNA*; *subunit vaccine*; *immune response*; *adjuvans*; *serologic reactions*

Introduction

Antiherpes vaccines are believed to be effective in reducing the frequency and severity of recurrencies (Katagava, 1973; Fanta *et al.*, 1974; Schmersahl and Rudiger, 1975; Maevskaya and Shubladze, 1975; Shubladze *et al.*, 1976; Nasemann, 1976; Wise *et al.*, 1977; Wassilew *et al.*, 1979; Barynsky *et al.*, 1983). In our country a formalin-inactivated antiherpes vaccine had been used for 8 years with good success (Andonov *et al.*, 1979; Dundarov *et al.*., 1982; 1983). Recently subunit vaccines have been tested in many countries ultilizing different procedures (Hilleman *et al.*, 1981; Skinner *et al.*, 1982; Cappel *et al.*, 1982; Bertland and Lampson, 1982). Their advantage is the removal of a great deal of viral DNA and higher degree of purification of virus-specific proteins. Perspectives of a wide usage of such vaccine need simple and inexpensive technologies and enhancement of their immunogenicity. In this work we present the results on preparation of subunit antiherpes vaccine from infected cells and testing of its antigenic properties in guinea pigs.

Materials and Methods

Cell cultures. Diploid human embryonic lung fibroblasts were used in passages 5—10. The cells were propagated in Eagle's medium with 10% calf serum.

Viruses. The HSV-1 strain DP and HSV-2 strain Kenru were used as prototype strains.

Solubilization of virus-specific glycoproteins from infected cells. When cytopathic effect developed in the infected cell monolayer, the cells were washed with phosphate-buffered saline (PBS) at 4 °C. Then they were scraped off and resuspended in PBS (10^7 cells per ml). After sonication for 2 min in disintegrator Branson (B 12/150 W, 20 Kc) the cells were treated with 2% Nonidet P-40 solution for 20 min at room temperature and once more sonicated. The 2 ml aliquots of sonicated suspension were layered on a 15—60% sucrose gradient in PBS or on a 20% sucrose cushon in PBS. Centrifugation followed for 5 hr at 100 000 g in MSE 6×14 rotor. The 0.5 ml vol fractions were collected. The detergent was removed from HSV antigen-containing fractions by dialysis or by precipitation with 10 vol of acetone. The degree of detergent removal was tested by erythrocyte haemolysis test according to a standard procedure using 1% cock erythrocytes. The cell suspension and the lysates at individual phases of their preparation were tested by complement fixation.

Isolation of DNA. DNA of purified virus labelled with ^{3}H-thymidine was extracted with 1% sodium laurylsarkosinate and 1% sodium dodecylsulphate according to Murray *et al.* (1974); the HSV DNA was separated by equilibrium density centrifugation in CsCl for 48 hr at 40 000 rev/min.

Separation of native and sonicated DNA by calcium phosphate chromatography. The separation of native and sonicated virus DNA was made according to Bernardi (1965). To fill up a column of d = 1.3 cm and h = 5 cm, fresh calcium phosphate was prepared according to Tiselius *et al.* (1956). On each column was applied 1 ml sample of ^{3}H-thymidine-labelled DNA in 5 mmol/l sodium phosphate starting buffer. For elution we used 0.24 mol/l and 0.48 mol/l phosphate buffers; the speed of elution was 15 ml/hr at room temperature, the vol of the collected fractions was 2.5 ml.

Immunization of experimental animals. Guinea pigs 300—350 g were inoculated by intramuscular route either with formaline inactivated whole virus vaccine or with the subunit vaccine standardized according to complement fixation activity and protein content. The animals were immunized with 5 doses at 7 days intervals. In some experiments two additional boosters were given in 30 days intervals. At first and last injections, as well as at each booster Freund's incomplete adjuvant or aluminium hydroxide were added in equal vol to the vaccine preparation containing 1.25 mg of protein per dose. Blood was drawn from the animals by cardial punction 20 days after last antigen dose. The antibody response of each animal was determined by neutralization test (in the absence of complement) and by complement fixation as described (Dundarov *et al.*, 1979; Dundarova, 1975).

Results

Isolation of HSV surface antigens from infected cells

The subunit vaccine was prepared from infected cell suspensions containing 10^6—10^7 PFU of virus per 0.1 ml. The virus-specific membrane-bound glycoproteins were extracted by NP-40 treatment. The non-stripped virions and the nucleocapsids containing the viral DNA were removed from the lysate by ultracentrifugation in sucrose gradient. HSV-specific antigens in the titre of 512 were found in four upper fractions ("subunit vaccine preparation" by CF reaction. In these fractions no infectious virus was detected in two subsequent passages in diploid P cells. The degree of virus DNA removal was determined by mixing of ^{3}H-thymidine labelled purified virus material with the infected cells before detergent treatment. After centrifugation in the sucrose gradient, the radioactivity has been found

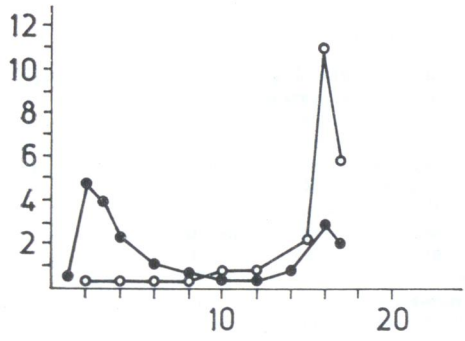

Fig. 1.
Distribution in sucrose density gradient of ^{14}C-labelled proteins and ^{3}H-labelled DNA purified from HSV-1 after NP-40 treatment
Abscissa: fraction number; ordinate: cpm $\times 10^{-3}$
Full circles: ^{14}C-radioactivity counts; empty circles: ^{3}H-radioactivity counts

on the bottom (Fig. 1). Similar distribution of radioactivity has been noted after centrifugation of the lysate on 20% sucrose cushon. After detergent treatment of ^{3}H-thymidine labelled infected cells, a given part of the label remained in upper fractions (Fig. 2). Under the same centrifugation conditions in sucrose gradient the intact purified virus was found in fractions 10—14 and the DNA isolated from it in fractions 2—6. One may assume that the lysate of infected cells contained exogenous DNA either cellular or perhaps viral, which did not sediment at given centrifugation forces.

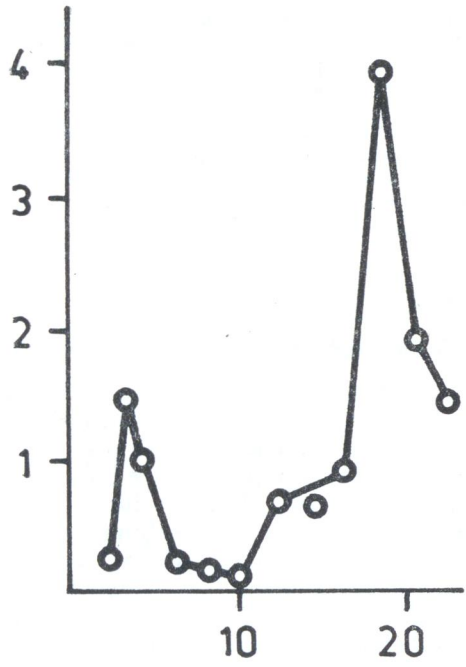

Fig. 2.
Distribution in sucrose density gradient of ^{3}H-thymidine labelled DNA from infected cells after NP-40 treatment
Abscissa: fraction number; ordinate: cpm $\times 10^{-3}$

To test the nature of DNA molecules, portions of pooled upper fractions were separated on calcium phosphate columns after dialysis against 0.0005 mol/l phosphate buffer. On a parallel column were applies native nonsonicated and twice sonicated virion DNA as control. As given in Table 1, nearly all radioactive label of the upper fraction pool was eluted with 0.24 mol/l

Table 1. Separation on calcium phosphate of native and sonicated HSV-1 DNA from the cell lysate

Fraction No.	Material applied to column		
	Pooled upper fractions*	Native HSV DNA	Sonicated HSV DNA
1 A	180**	70	60
2	8744	92	3070
3	4920	160	1803
4	810	130	195
5	190	100	120
6 B	144	100	100
7	270	4600	93
8	260	3700	62
9	74	280	60

Fractions 1—5 (A) — eluted with 0.24 mol/l phosphate buffer
Fractions 6—9 (B) — eluted with 0.48 mol/l phosphate buffer
* from sucrose gradient centrifugation of the lysate
** c.p.m.

phosphate buffer alike to sonicated virion DNA. In the 0.48 mol/l phosphate buffer about 3.5% radioactivity of the "upper fractions pool" was eluted, while the total native DNA was found in this fraction.

The results of erythrocyte haemolysis have shown that removal of the detergent NP-40 by prolonged dialysis was insufficient. After precipitation of extracted glycoproteins with chilled acetone according to Skinner *et al.* (1982) the detergent was fully removed; no NP-40 was found by the erythrocyte haemolysis test in the sediment resuspendend in PBS. The acetone precipitate contained about 0.75% of initial radioactivity.

Antigenic properties of the subunit vaccine

The effect of antigen dose on immune response was followed in 9 groups of guinea pigs 5 animals each, which were inoculated with whole virus, the subunit vaccine with or without Freund's adjuvant. High degree of correlation was found between protein content and CF antigen titre under standardized conditions of antigen preparation. The results given in Fig. 3 show the correlation between antibody titre in neutralization and complement fixation tests. Proportional relationship was found between antibody level and protein

concentration in the vaccine. This allowed us to consider the total protein content in the vaccine preparation as indicator of virus-specific antigens. The subunit vaccines seem 8 times less immunogenic than the whole virion vaccine at the same protein concentration. The addition of Freund's adjuvant enhances the immunogenicity of the vaccine four-fold.

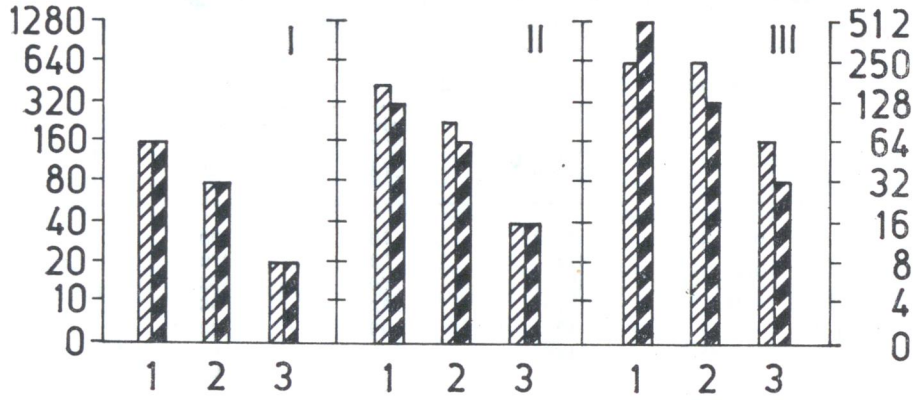

Fig. 3.
Immunogenicity of whole virus subunit vaccine prepared from HSV-1 infected P cells
Dashed columns (thin lines): neutralizing antibody titre (left ordinate); striated columns (thick lines): complement fixing antibody titre (right ordinate)
1 — immunization with the whole virion vaccine
2 — immunization with the subunit vaccine and Freund's adjuvant
3 — immunization with the subunit vaccine only
I — Protein content of 250 μg/ml
II — Protein content of 500 μg/ml
III — Protein content of 1500 μg/ml

In other experiments the antigenicity of subunit vaccines from HSV-1 and HSV-2 with the same protein content of 1 500 μg/ml and CF titre of 512 were compared. The vaccines were given to 5 guinea pigs in each group. Antigenicity was tested in comparison with the whole virion vaccine and "placebo". The results are shown in Fig. 4. They confirm that the whole virion vaccine and the subunit vaccine were more antigenic in combination with Freund's adjuvant. All subunit vaccines from HSV-1 and HSV-2 with similar parameters showed closely similar antigenicity. They induce in higher titres homologous virus-neutralizing antibodies. No such difference was found in CF reaction.

During the preparation of subunit vaccine it is difficult to keep the product sterile. Fresh subunit vaccine preparations were treated with formalin under conditions used for inactivation of whole virus vaccine preparations (Andonov *et al.*, 1979). The results have shown that CF titres after formalin

treatment were not changed substantially. No essential difference in immunogenicity was found between formalin-treated and untreated subunit vaccines. For further immunization experiments the formalin-treated vaccine was used, but $Al(OH)_3$) was used as adjuvant. The results of 3 experiments with type -1 and type-2 subunit vaccines are shown in Fig. 5. It can be seen that

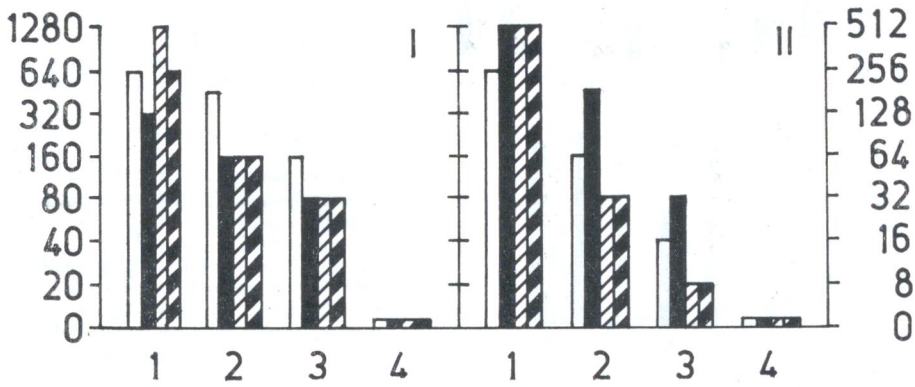

Fig. 4.
Immunogenicity of the subunit vaccine prepared from HSV-1 and HSV-2 infected P-cells
I — HSV-1; II — HSV-2
1 — immunization with the whole virion vaccine
2 — immunization with the subunit vaccine and Freund's adjuvant
3 — immunization with the subunit vaccine only
4 — immunization with non-infected NP-40 treated cells (placebo)
Empty columns: neutralizing antibody titres to HSV-1; black columns: neutralizing antibody titres to HSV-2 (left ordinate; serum dilution reciprocals);
Dashed columns (thin lines): complement fixing antibody titre to HSV-1; striated columns (thick lines): complement fixing antibody titre to HSV-2 (right ordinate; serum dilution reciprocals)

$Al(OH)_3$ stimulates a considerable immune response when compared with Freund's adjuvant. The following relationships were observed: between the intensity of immune response and protein (antigen) content, a closely similar immunogenicity of both types subunit vaccines and enhanced virus--neutralization titre to the homologous serotype.

All experiments showed a clear relationship between the degree of immune response, protein content and CF titre of the vaccine. The usage of concentrated vaccine preparations, however, was not desirable. Follow-up immune response dynamics allowed to select the optimum immunization schedule. In a further trial we used more diluted antigen (protein content 250 μg/ml) for single dose immunization or for two booster doses 30 days apart. The results are shown on Fig. 6. Neutralizing antibody titres by the end of immunization decreased reached the half of original level within 30 days. After booster dose the antibody titre increased confirming that the humoral antibody response was prolonged by the booster dose.

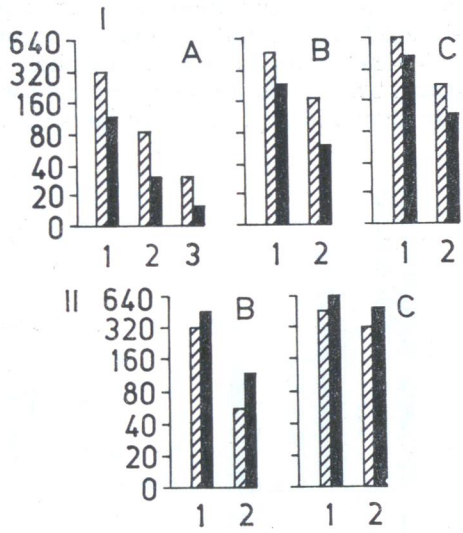

Fig. 5.
Comparative immunogenicity of formalin-treated whole virion vaccine versus subunit vaccines type 1 and 2
I — vaccine type 1; II — vaccine type 2
A — Protein content 250 μg/ml; B — protein content 500 μg/ml; C — protein content 1 mg/ml
1 — immunization with the whole virion vaccine
2 — immunization with the subunit vaccine and Freund's adjuvant
3 — immunization with the subunit vaccine only
Dashed columns: neutralizing antibody to HSV-1; black columns: neutralizing antibody titre to HSV-2
Ordinate: serum dilution reciprocals

Discussion

The majority of subunit vaccines was prepared from highly purified virus (Cappel, 1976; Cappel *et al.*, 1982; Hilleman *et al.*, 1981; Hilfenhaus *et al.*, 1981; Bertland and Lampson, 1982). This complicated the vaccine preparation and enhances its costs. It is well established that HSV glycoproteins are produced in excess in infected cells (Bocharov *et al.*, 1965; Kutinová et al., 1977). Therefore, cells are more economical source of vaccine preparation. Because the majority of virus-specific proteins is membrane-bound, nonionic detergents were used for their solubilization (Spear *et al.*, 1970; Vestergaard, 1973; Cappel, 1976; Kutinová *et al.*, 1977; Skinner *et al.*, 1978; Hilfenhaus *et al.*, 1981). Our experiments showed that NP-40 solubilized considerable amounts of antigens from HSV-1 and from HSV-2-infected P cells. Viral DNA has been removed by ultracentrifugation of the lysate according to Skinner *et al.* (1978) and Kutinová *et al.* (1977). In accordance with Skinner we found that the lysate from infected cells contains exogenous DNA molecules of cellular and possibly viral origin, which are fragmented due to repeated sonication of the cell suspension. The fragmented DNA is removed to a considerable degree by acetone precipitation (up to 99%). The residual DNA can be inactivated by formalin treatment.

The great amount of experiments on experimental animals has shown that the HSV type 1 and 2 subunit vaccines induce a considerable humoral immune response when given in suitable antigen concentration in combination with adjuvants. Our experimental results coincide with those of other authors (Cappel, 1976; Skinner *et al.*, 1978; Kutinová *et al.*, 1979). They demonstrate, at least in experimental animals, the following relationships: 1) when a vaccine preparation of lower protein content is used, repeated booster doses

should be administered; 2) subunit vaccine should be inoculated in combination with an adjuvant; 3) $Al(OH)_3$ seems to be a suitable adjuvant for humans as it enhances the immunogenicity similarly to Freund's adjuvant.

Further studies of subunit vaccines on volunteers may yield more complete

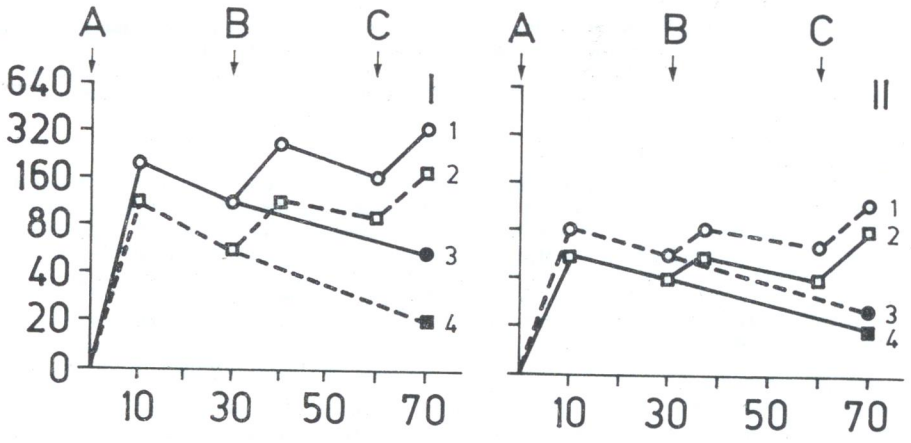

Fig. 6.
Antiherpes antibody formation after multiple inoculation of the subunit vaccine
Full line: neutralizing antibody titre to HSV-1; interrupted line: neutralizing antibody to HSV-2
(○) and (□) — three inoculation doses
(●) and (■) — single inoculation dose
I — subunit vaccine from HSV-1; II — subunit vaccine from HSV-2
Abscissa: days post-inculation; ordinate: neutralizing antibody titre

information on immunization schedule, intervals among the injections and the therapeutic effect. Preliminary results with two volunteers seemed encouraging for extending experimental vaccination of humans.

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