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A SIMPLE NOVEL PROCEDURE FOR PREPARATION OF HERPES SIMPLEX VIRUS SUBUNIT VACCINE

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Summary. — A simple procedure for preparation of herpes simplex virus type 1 (HSV-1) subunit vaccine is described. The method is based on treatment of virus-infected cells with a nonionic detergent, removal of cell nuclei by low speed centrifugation, separation of viral nucleocapsids by high speed centrifugation through a sucrose cushion and, finally, precipitation of viral and cellular glycoproteins and proteins with ammonium sulphate (AS). Unlike to acetone precipitation, affinity chromatography on lectins or hydroxylapatite chromatography, AS precipitation repeatedly yielded the best and most reliable results as judged by relative protein content, antigenicity and immunogenicity of the final vaccine product.

Key words: herpes simplex virus, subunit vaccine, ammonium sulphate precipitation, vaccine preparation

Introduction

Several approaches have been adopted for the preparation of subunit herpes simplex virus (HSV) vaccines. Such vaccines should not contain a measurable amount of high molecular viral DNA in contrast to inactivated vaccines prepared from infected cell suspensions. First vaccines of this type were inactivated with formalin (Rodovský *et al.*, 1971), ultraviolet radiation (Cappel, 1976), high temperature (Naseman, 1976) or combination of formalin with high temperature (Benda and Dbalý, 1971). Some inactivated vaccines have been made as polyvalent vaccines containing both type 1 and type 2 HSV strains (Weitgasser, 1977; Barinsky *et al.*, 1977; Dundarov *et al.*, 1982). DNA free subunit vaccines can be prepared either from purified virions or by treatment of infected cells with nonionic detergents. The first approach implies purification of virions by ultracentrifugation in sucrose gradient followed by stripping off of the envelope with a nonionic detergent and separation of envelope glycoproteins (Hilfenhaus and Moser, 1981; Klein *et al.*, 1981; Cappel *et al.*, 1982; Yoshino *et al.*, 1982; Thomson *et al.*, 1983). Although this technique allows to prepare relatively pure viral

glycoproteins, it is rather time consuming and probably more expensive for large scale vaccine production.

Several investigators described, therefore, the preparation of subunit HSV vaccine by detergent treatment of infected cells avoiding virion purification (Kitces *et al.*, 1977; Kutinová *et al.*, 1977; Skinner, 1978; Kutinová *et al.*, 1980; Thornton *et al.*, 1982; Mertz *et al.*, 1984). This has the advantage that the virus-coded glycoproteins synthesized in excess in infected cells including those not incorporated into virions may be extracted from infected cells. In order to halt the nucleocapsid envelopment, Skinner *et al.* (1980) added lithium chloride to infected cells. The detergent as well as the residual high molecular viral DNA must be removed from the mixture of cellular and viral glycoproteins. This was done by acetone precipitation (Skinner *et al.*, 1978), by affinity chromatography to Concanavaline A-Sepharose (Kutinová, 1985) or to lentil lectine (Mertz *et al.*, 1984). We describe a simple procedure based on the precipitation of proteins and glycoproteins with ammonium sulphate (AS). The latter procedure is highly efficient as judged by low protein loss and the high antigenicity of the final vaccine product. In addition, we present our experience with other procedures for preparing an immunogenic antigen extract from HSV-1 infected cells. The results of experimental vaccination will be described elsewhere.

Materials and Methods

Cells. Quail embryo cells (QEC) were prepared from Japanese quail embryonated eggs (inbred line CI_2 aguti) by trypsinization with 0.1 % chypsin solution (quail embryos were provided by the breed VUCHŠH, Ivanka pri Dunaji near Bratislava). The cells were seeded into 1200 ml Roux flasks (5×10^7 cells per flask) and grown to confluency in medium TM Sevac II (modified M-199) supplemented with 10 % inactivated bovine serum (IBS) within 48—72 hrs.

Virus. HSV type 1 strain HSZP (Szántó, 1960) was adapted to chick embryo cells during at least 16 passages in these cells. The average titre of the chick cell adapted HSZP strain propagated in QEC ranged from 5×10^7 to 1×10^8 PFU/ml as tested in Vero cells.

Preparation of infected cell extract. QEC monolayers were inoculated with 0.2 PFU/cell of the HSZP strain and incubated for 20 hrs. Infected cells were scraped off into 3 ml of hypotonic, reticulocyte buffer (10 mmol/l Tris-HCl, 10 mmol/l KCl, 15 mmol/l $MgCl_2$ pH 7.5) per flask, pooled and then equal amount of the same buffer containing 1 % Nonidet P-40 and 2 mmol/ dithiotreitol has been added. After 10 min incubation with the detergent, the nuclei were removed by centrifugation ($800 \times$ g). Then the supernatant was further incubated for 20 min at room temperature and thereafter centrifuged at $100.000 \times$ g through 30 % sucrose cushion for 3 hr (Skinner *et al.*, 1978). Formalin was added to a final concentration of 0.1 % and allowed to stand at least for 4 hr at room temperature. The product referred to as infected cell extract (CE) was stored at −70 °C.

Labelling of the infected CE. QEC were labelled from 4—10 hr post-infection (p.i.) using BEM/10 (Basal Eagle Medium with ten times reduced amino acids except of arginine) supplemented with 1 % IBS containing 74 kBq/ml of ^{14}C-amino acid hydrolysate. At 20 hr p.i., the radioactive medium was removed, the cells were rinsed with cold phosphate buffered saline (PBS) and processed as described above.

To label the viral DNA, ^{3}H-thymidine (370 kBq/ml) was added to the medium from 4—20 hr p.i. In certain experiments, the cells were pulsed from 4—6, 6—8 and 8—10 hr p.i. respectively, chased until 20 hr p.i. and harvested as described.

Acetone precipitation. Stock solution of NaCl (3 mol/l) was added to the infected CE to achieve a final concentration of 0.3 mol/l. The infected CE containing 0.3 mol/l NaCl was then added dropwise to 10 times larger volume of acetone at −20 °C. After 60 min at −20 °C the precipitate was sedimented at 3000 rev/min for 20 min. The sediment was dried in a vaccum chamber at

4 °C for 2 days and dissolved in sterile distilled water to the half of the original volume of the CE.

Precipitation of the infected CE with AS. Saturated AS solution was added dropwise to the continuously mixed infected CE. The precipitate was allowed to stand for 1 hr in the ice bath, then it was sedimented at 2000 × g for 20 min at 4 °C, dissolved in distilled water to obtain the half of original volume and dialyzed under sterile conditions against 4 changes of PBS for several days at 4 °C. The vaccine was stored lyophilized at 4 °C or frozen at −70 °C (at protein concentration of 1 mg/ml/ampoule).

Hydroxylapatite chromatography. Hydroxylapatite (Calbiochem) was resuspended in the starting buffer (0.005 mol/l phosphate buffer, pH 7.2). The fine debris were decanted and the column (bed vol 20 ml) was filled up and washed with the starting buffer. About 50 mg protein of the infected CE was dialyzed against the starting buffer and applied to the column. To remove the nonadsorbed material, the column was washed with 2 bed vol of the starting buffer. One bed vol of 0.2 mol/l phosphate buffer was used for elution.

Affinity chromatography on lectins. Spheron-Concanavaline A (30 ml) was saturated with 0.1 % bovine serum albumin (BSA) in the presence of 0.2 mol/l glucose for 12 hr using a magnetic stirrer at 4 °C. After filtration phosphate buffered saline was added (pH 7.2) and the column was packed with the gel. After profound washing, the infected CE was applied to the column in 0.01 mol/l PBS pH 7.2. The glycoproteins were eluted with 0.2 mol/l glucose in the same buffer.

Affinity chromatography on lentil lectine. Lentil lectine-Sepharose was washed with acetate buffer pH 4.5 and then with 0.15 mol/l NaCl. The infected CE was applied to the lectin column after dialysis against this buffer in an amount of 50 mg protein per 1 g lectine. The glycoproteins were eluted with 0.2 mol/l mannoside or glucose in 0.01 mol/l phosphate buffer.

Both procedures, i.e. chromatography on lentil lectine-Sepharose as well as on Con-A Spheron were repeated on minicolumns using ^{14}C-amino acids labelled infected CE.

Protein concentration measurements were made according to Lowry (1951).

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were analysed on 8 % PAG (cross-linked with methylenbisacrylamide) as previously described (Matis and Rajčáni, 1980). The gels were fixed and treated with 1 mol/l sodium salicylate to provide fluorographic enhancement (Medix-Rapid X-ray film, Hradec Králové).

The following sera were used for immunoprecipitation procedures:

- swine immune serum (IgG fraction) against semipurified strain Kupka virus
- rabbit immune serum prepared against semipurified strain KOS virions by single high speed centrifugation through Ficoll gradient (Matis *et al.*, 1975),
- a mixture of 4 monoclonal antibodies reacting with gB of HSV-1 (kindly provided by dr. G. Russ, Institute of Virology, Bratislava),
- human convalescent serum showing high neutralizing antibody titre to HSV-1,
- control (nonimmune) rabbit serum.

The following labelled antigens were used for immunoprecipitation before SDS-PAGE:

- ^{14}C-amino acid labelled infected cell extract
- ^{14}C-amino acid labelled acetone-precipitated vaccine
- ^{14}C-amino acid labelled AS-precipitated vaccine
- QEC labelled with ^{14}C-amino acids from 4—10 hr after infection with the HSZP strain
- Vero cells labelled with ^{14}C-amino acids from 4—10 hr after infection with the KOS strain.

Radioimmunoprecipitation procedures with either serum protein-A from the *Staphylococcus* A Cowan were described earlier (Raučina *et al.*, 1984).

Rocket immunoelectrophoresis. Glas plates 6 × 9 cm were covered with a thin layer (1 mm) of 1 % agarose in veronal buffer pH 8.6 containing 200 μl of rabbit imune serum raised against the KOS strain of HSV. At the edge of the plate 8 wells were made for application of antigen samples; into each well was given 10 μl of antigen containing approximately 1 mg/ml protein. The same antigens (including positive and negative controls) were tested in parallel against nonimmune rabbit serum. The electrophoresis was run for 4 hr at 15 mA (3 V/cm^2), then the gels were thoroughly washed in acid alcohol, air dried and stained with Coomassie brilliant blue (for details see Fuchsberger and Borecký, 1979).

Enzyme immunoassay (*ELISA*). Immunoplates KOH-I-NOOR (Dalečín, Czechoslovakia) were coated with the swine immune serum (IgG fraction) to HSV strain Kupka. The IgG was diluted in 0.01 mol/l phosphate buffer pH 7.2 in a concentration of 5 μg/ml. The antibody was allowed to absorb overnight at 4 °C. After washing with PBS-Tween 20, the wells were saturated

Table 1. Preparation of the subunit HSV vaccine

1. Choice of the cell system and virus strain
2. Selection of a suitable interval for the harvest
3. Extraction of glycoproteins from cytoplasmic membranes of infected cells.
4. Removal of cell nuclei and viral nucleocapsids
5. Inactivation of the residual viral DNA with formalin
6. Precipitation of glycoproteins with ammonium sulphate
7. Dissolving of precipitated glycoproteins in phosphate buffered saline, dialysis of the ammonium sulphate
8. Measurement and adjustment of the protein content; immunogenity and toxicity tests

with 2—5 % bovine serum albumin (BSA) solution in PBS, the the antigen (vaccine) was added at dilutions from 1 : 10 (100 μg/ml) to dilution 1 : 320 (3 μg/ml). After incubation overnight the wells were washed with PBS-Tween, then rabbit immune serum to HSV strain KOS was added in the dilution of 1 : 5000. After incubation for 2 hr at 37 °C, the wells were washed, then reacted with the peroxidase-labelled conjugate Sw AR/Px (Sevac, Prague) diluted 1 : 400. After the last washing the substrate-ortophenylendiamine mixture were added for 30 min, then the reaction was stopped with 2N H_2SO_4 and the absorbancy was measured at OD_{490}. The reaction was scored positive if the OD_{490} measured in the well containing the vaccine antigen was 2.1 higher than the OD_{490} value with the same dilution of mock-infected cell extract. The antigenic activity of the vaccine was expressed in antigen units per 1 μg protein, the minimum activity being 0.5—1 units per 1 μg.

Immunogenicity of the vaccine was tested in guinea pigs and mice. The former received 2 or 3 subsequent intraperitoneal and/or intramuscular injections of the vaccine (300 μg) mixed with Al-Span-Oil (Sevac, Prague) adjuvant in a total volume of about 1 ml on the days 1, 28 and 35. Blood was drawn 10 days after the last injection. Mice were given 75—100 units of the antigen mixed with the Al-Span-Oil adjuvant in the total volume of 0.3 ml per intraperitoneal route according to a similar administration schedule.

Results

Replication of the HSZP strain in quail embryo cells

The replication of HSZP strain in QEC was excellent. When infected at a multiplicity of 0.2 PFU/cell, the harvests were in the range of 6—80 PFU/cell. In thirty batches, each prepared from 20—25 bottles (about 5×10^8 cells), the total yield ranged between 3 — 40 $\times$ 10^9 PFU. As compared to virus input, this was a 60—400 fold increase. At 20—22 hr p.i. the extracellular virus ranged from 6—50% of total infectivity depending on actual density of the QEC monolayer. The data are presented in Tables 2—4.

Alternative final procedures for vaccine preparation

The infected QEC extract was prepared as shown in Table 1. The standard steps included NP-40 treatment, the low and high speed centrifugations. Variations in the final procedure were compared to prepare a standard mixture of virus-coded and cellular proteins devoid of the detergent and of the majority of viral DNA.

When acetone was used for precipitation of the QEC extract, no standardzed results were obtained (Table 2). It was difficult to dissolve the precipi-

Table 2. Results of acetone precipitation of the NP-40 extract from HSV-1 infected cells

Vaccine lot no.		Infectivity of cell suspension	Protein content in the crude extract	Protein content in the vaccine	ELISA units per 1 µg/protein	Immunogenicity for mice	Immunogenicity IELPHO
14	Ac	4×10^9 (80 %)*	130 mg/145 ml	60 mg/60 ml (46 %)	0.4	n. d.	n. d.
16	Ac	14×10^9 (93 %)	118 mg/110 ml	44 mg/56 ml (37 %)	0.2**	n. d.	slightly positive
19	Ac	12×10^9 (98 %)	176 mg/160 ml	43 mg/40 ml (24 %)	1.5	32/256	posit
22	Ac	25×10^9 (60 %)	134 mg/110 ml	54 mg/40 ml (40 %)	4	64/512[a]	posit
37	Ac	35×10^9 (88 %)	43 mg/27 ml	3 mg/16 ml (8 %)	2±	32/128	posit
37	AS		16 mg/10 ml	10 mg/7 ml (60 %)	> 5	32/128	posit
37	AS		16 mg/10 ml	9 mg/5 ml (53 %)	> 5	32/128	posit

* per 500×10^6 cells (percentage of cell-associated infectivity in brackets)
** similar results with lots no. 17, 28, 20, 27, 29, 36 ($\leq 0.2-0.5/\mu g$)
[a] mean neutralizing antibody titre in the absence and presence of complement in 6—10 mice

Table 3. Results of ammonium sulphate precipitation of the NP-40 extract from HSV-1 infected cells

Vaccine lot no.		Infectivity of the cell suspension	Protein content in the crude cell extract	Protein content in the vaccine	ELISA units per 1 μg protein	Immunogenicity for guinea pigs	Immunogenicity IELPHO
38	AS fr. 1	10×10^9 PFU (71 %)	39 mg/86 ml	9 mg/25 ml (23 %)	8	n. d.	n. d.
	AS fr. 2			7 mg/30 ml (17 %)	> 1	n. d.	n. d.
39	AS fr. 1	8×10^9 PFU (63 %)	92 mg/95 ml	16 mg/23 ml (17 %)	5	128/256	posit
	AS fr. 2			7 mg/20 ml (8 %)	0.5	32/32	posit
40	AS	9×10^9 PFU (75 %)	224 mg/140 ml	128 mg/90 ml (57 %)	1	64/256	posit
41	AS	2×10^9 PFU (90 %)	58 mg/78 ml	29 mg/44 ml (50 %)	1	64/256	posit
42	AS	10×10^9 PFU (66 %)	99 mg/94 ml	37 mg/75 ml (38 %)	2	64/256	posit
43	AS*	9×10^9 PFU (77 %)	70 mg/116 ml	26 mg/46 ml (37 %)	> 5	n. d.	posit
44	AS	4×10^9 PFU (57 %)	86 mg/139 ml	54 mg/85 ml (64 %)	5	n. d.	posit
31/33	AS	20×10^6 PFU (90 %)	140 mg/140 ml	77 mg/77 ml (55 %)	2	n. d.	posit

* continuous coolong of the precipitation mixture
For further explanations see legend to Table 1

Table 4. Results of affinity chromatography of the extract prepared from HSV-1 infected QEC

Vaccine lot No.	Chromatography procedure	Protein content		ELISA units	Immunogenicity	
		cell extract	vaccine	μg	mice	IELPHO
24	Amberlit	60 mg/50 ml	29 mg/45 ml (48 %)	1.3	4/32*	n. d.
26	Sferon-Con A	48 mg/40 ml	28 mg/60 ml (60 %)	1	n. d.	n. d.
32	Hydroxylapatite	69 mg/60 ml	32 mg/20 ml (46 %)	1	16/16	posit
35 Lect	Lentil lectine	15 mg/20 ml	5 mg/ 7 ml (33 %)	1		posit
35 Ac	none	100 mg/40 ml	65 mg/20 ml (65 %)	< 0.1		negat

* before chromatography antigen induced higher neutralizing antibody production in mice (16/128)

Table 5. Immunization of mice with different batches of the subunit HSV vaccine

Mouse number	Antigen	Neutralizing antibody c+ / c−
10	vaccine lot no. 22	128—1024 / 16—64*
10	cell extract lot. no. 24	32— 128 / 8—16
10	vaccine lot. no. 24	16— 64 / 4
10	vaccine lot no. 20	8 / 4**
	booster with the vaccine lot no. 22	512 / 16

* range of neutralizing antibody titre in the presence (c+) and absence (c−) of complement
** average titre in the serum mixture

tate and the protein recovery was often unsatisfactory. Out of 12 batches only 3 showed a good antigenicity (lot Nos 19, 22 and 37). The highest amount of the recovered protein did not exceed 40 %. In contrast, AS precipitation enhanced protein recovery as well as improved the antigenicity of the final vaccine product allowing to recover at least 50 % of protein with the antigenic activity of 1—2 ELISA units per 1 μg protein (Table 3).

Several trials with affinity chromatography are summarized in Table 4. Relatively good results were achieved with hydroxylapatite. The use of lectins seemed tedious and less effective due to the problems with the sterility and the need to concentrate the eluted vaccine. Amberlit has been used to remove the detergent, but because of the lack of a reliable method for detergent determination and the low immunogenicity of the eluted vaccine we refrained of the further use of this resin.

Immunogenicity tests

In vitro tests were made by rocket immunoelectrophoresis (Fig. 1) and ELISA as described in Mat. and Methods. If the antigenic activity of the vaccine was in the limits of at least 0.5—1.0 ELISA units per 1 μg protein, the rocket immunoelectrophoresis results were positive (Tables 2—4). On the other hand, when the vaccine reacted weakly in ELISA (less than 0.5 units per 1 μg protein) no clear-cut precipitation lines were detected at protein concentration of 1 mg/ml.

In vivo tests were made in mice and guinea pigs. When mice were given by i.p. route about 75 μg protein with antigenic activity of 0.5—1 units per 1 μg in 3 doses with Al-Span-Oil adjuvant they developed neutralizing antibodies in titres up to 1024. After immunization with low-immunogenic batches, which antigenic components had remained undissolved in the precipitate, a single booster dose with the highly antigenic batch increased the antibody titre considerably (Table 5).

Table 6. Incorporation of ^{3}H-thymidine into nuclear and cytoplasmic fractions of HSV-1 infected cells

Origin	cpm	Per cent
1. Total cell suspension	5×10	100%
2. Cytoplasmic fraction	1.67×10	34%
3. Nuclear fraction	3.35×10^{7}	66%
4. Supernatant after high speed centrifugation*	2.2×10^{6}	25.8%
5. Sucrose cushion	1.5×10^{6}	20.2%
6. Nucleocapsids** (sediment)	4×10^{6}	54%

Cells harvested by 22 hr p.i. from 2×1200 Roux flasks; labelled with 370 kBq/ml from 5 to 22 hr p.i.

* The QEC extract was further precipitated either with AS or acetone; the residual cpm was in the range of 1.7—2.5%

** Used for preparation of HSV DNA

Virus-coded glycoproteins in the subunit vaccine

The presence of virus-coded glycoproteins and proteins in the vaccine was controlled by preparing ^{14}C-amino acids-labelled QEC extracts. The infected QEC contained a prominent band corresponding to the major capsid polypeptide (VP5, 155 kD). This band was missing after centrifugation through the sucrose cushion (Fig. 2).

The polypeptides from solubilized infected QEC were compared in the NP-40 treated extract, in the extracts subjected to low and high speed centrifugations and acetone precipitation (Fig. 3). VP5 was seen in lane 1 (low speed sediment), lane 2 (low speed centrifugation supernatant), lane 3 (sediment after high speed centrifugation) and lane 10 (infected QEC extract). VP5 was not seen in the supernatant after high speed centrifugation (lane 4) and in its acetone precipitates (lanes 6, 8 and 9). Although acetone precipitated the main envelope glycoproteins, PBS alone was not sufficient to dissolve it (lanes 5 and 7). When SDS was used to solubilize the acetone precipitate, the same main glycoproteins were seen (lanes 6, 8 and 9). In contrast to acetone, the AS precipitate could be well dissolved in PBS (Fig. 2, lane 3). No virus-coded polypeptides remained in the supernatant after AS precipitation.

The ^{14}C-labelled vaccine prepared by AS precipitation was immunoprecipitated with various antisera: rabbit immune serum to strain KOS, a mixture of monoclonal antibodies to gB and swine immune serum to strain Kupka (Fig. 4). We assume that the second major band immunoprecipitated with polyclonal sera (lanes 3—6) is the truncated gC of the HSZP strain which electrophoretic mobility was faster than that of gB. Additional less prominent bands of gE and gD were immunoprecipitated with the polyclonal sera, the latter being precipitated with the swine serum rather than with the rabbit serum.

DNA tracing with ^{3}H-thymidine labelling

Incorporation of ^{3}H-thymidine into nuclear and cytoplasmic fractions of HSZP strain-infected QEC is shown in Table 6. The nucleocapsids sedimented through the sucrose cushion were further used for viral DNA extraction by the phenol-chloroform procedure (details not shown). After precipitation of fraction 4 with either acetone or AS, the vaccine contained about 1.7—2.5 % of the original label, i.e. at least 10 times less radioactivity than fraction 4.

Discussion

Unlike to other subunit vaccines, the presented one has been prepared in QEC. The advantage of QEC is the lack of endogeneous retroviruses, which may contaminate chick embryo cells. The HSZP strain used has been adapted to chick embryo cells. After infection of QEC at the multiplicity of 0.1 PFU/ /cell, the majority of virus has remained cell-associated at 20 hr p.i. The adapted strain HSZP is a syn strain, which cytopathic effect is easy to control. The synthesis of HSZP glycoproteins and their transport to the plasma membrane was analysed in Vero and BHK cells (Raučina *et al.*, 1985). Although there was some delay in the processing of the syn strain glycoproteins, their accumulation was similar to that of the strain KOS glycoproteins. The gC 1 of the syn strain HSZP showed lower affinity to *Helix pomatia* lectin (Raučina *et al.*, 1984) and was found underglycosylated because of the lack of O-linked oligosacharides. The mobility of such truncated gC 1 may be faster than that of gB revealing an apparent m.w. as low as 92—110 kD (Cohen *et al.*, 1980; Eberle and Courtney, 1980; Wenske and Courtney, 1983).

Procedures used for preparation of the subunit vaccine were essentially the same as described by Skinner *et al.* (1978; 1980) and Kutinová *et al.* (1977; 1982). Our approach differed in using QEC as substrate and ammonium sulphate for precipitation of viral and cellular proteins. The latter seems useful for obtaining the most optimal ratio between protein content and antigenic activity. For example, the vaccine revealing an antigenic activity of 1 ELISA unit per μg protein would be less effective than the batch with a potency of 5 units per μg. The variations among batches confirm the difficulties in comparing the immunogenicity of various subunit vaccines (Meignier, 1986). In our hands, AS precipitation seemed cheap, efficient and promising procedure for preparing representative amounts of protein with sufficient antigenic potency. Expressing the antigenicity of the vaccine in ELISA units per μg protein seemed useful to assess differences between individual batches. Nevertheless, further standardization of reagents and conditions of ELISA are desirable.

The basic requirement of subunit vaccine is the removal of viral DNA from the final product (reviewed by Hall and Katrack, 1986; Miegnier, 1986). High molecular HSV DNA was recovered from the nucleocapsid pellet which represented more than 50 % of the label incorporated into the TCA precipitated material. After phenol-chloroform extraction and CsCl density

gradient centrifugation its purity was confirmed by electrophoresis in agarose gel (data not shown). The rest of the label was present in the supernatant fluid subjected to high speed contrifugation as well as in the sucrose cushion itself indicating that it corresponded to the fragmented low molecular DNA as described by others (Kavaklova *et al.*, 1986). About 90 % of the latter label has remained in the supernatant after AS or acetone precipitations. The residual fragmented DNA — representing not more than 2.5 % of the original cytoplasmic radioactivity — was inactivated with formalin.

The described procedure is simple and inexpensive. The vaccine product obtained contains all 3 glycoproteins (gC, gB and gD) which seem essential for immunogenicity of virions (Kümel *et al.*, 1985) and possibly present the most important tools of their virulance.

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Legend to Figures (Plates XLII—XLIV):

Fig. 1. Rocket immunoelectrophoresis of the subunit vaccine. Agarose was soaked with 0.1 ml (200 μg) of immune IgG from anti-HSV-1 serum
I — Antigens in the wells: 1 — control QEC extract; 2 — vaccine lot No. 22 (posit); 3 — vaccine lot No. 23 (posit); 4 — vaccine lot No. 20 (negat); 5 — vaccine lot No. 18 (slight positivity); 6 — vaccine lot No. 17 (negat); 7 — vaccine lot. No. 16 (slight positivity).
II — Antigens in the wells: 8 — infected QEC extract batch 34; 9 — vaccine lot No. 22 (posit); 10 — uninfected QEC; 11 — vaccine lot No. 32 (slightly posit); 12 — vaccine lot No. 35 Amberlit (posit); 13 — vaccine lot No. 35 acetone (negat); 14 — vaccine lot No. 23 (posit).

Fig. 2. PAGE of HSZP strain-infected QEC labelled with ^{14}C amino acids from 4—20 hr p.i. Lanes: 1— QEC harvested at 20 hr p.i.; 2 — NP-40 treated QEC extract centrifuged through the sucrose cushion; 3 — as under 2 after precipitation with AS (dissolved to 1/3 of original volume (155 kD major capsid polypeptide indicated by arrow); 4 — the rest of material 2 not precipitated with AS.

Fig. 3. SDS-PAGE of acetone-precipitated proteins in the ^{14}C-labelled HSV-infected QEC extract and vaccine product (for details see Mat. and Methods).
The NP-40 treated QEC extract after low speed centrifugation: lane 1 — sediment; lane 2 — supernatant.
The supernatant after low speed centrifugation subjected to high speed centrifugation through sucrose cushion: lane 3 — sediment; lane 4 — supernatant.
The supernatant after high speed centrifugation precipitated with acetone, dissolved in PBS

and centrifuged: supernatant (lanes 5 and 7 — soluble protein) sediment (lanes 6 and 8 — insoluble proteins).
The acetone precipitate is in lane 9, the HSZP-infected QEC before detergent treatment are in lane 10.
The position of some virus-coded proteins is indicated in the right.

Fig. 4. SDS-PAGE and immunoprecipitation of AS-precipitated subunit vaccine.
Lane 1 — AS precipitated supernatant after high speed centrifugaton through sucrose cushion dissolved in PBS; lanes 2 and 9: immunoprecipitation of the material from lane 1 with monoclonal antibody to gB (KOS); lanes 3 and 4: immunoprecipitation of the same material with swine immune IgG; lanes 5 and 6: immunoprecipitation of the same material with rabbit immune serum; lane 7 — control swine serum; lane 8 — control rabbit serum.
The position of viral glycoproteins is indicated in the right.