HERPES SIMPLEX VIRUS TYPE 1 ENVELOPE SUBUNIT VACCINE NOT ONLY PROTECTS AGAINST LETHAL VIRUS CHALLENGE, BUT ALSO MAY RESTRICT LATENCY AND VIRUS REACTIVATION

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Summary. – A subunit vaccine containing the main antigenic components of herpes simplex virus 1 (HSV-1) was tested in Balb/c mice and albino rabbits. The mice were completely protected against challenge with 10 LD_{50} of the highly pathogenic SC16 strain given by intraperitoneal (ip) route when immunized with 1000 antigen units (ELISA) corresponding to 110 μg of protein. The animals were protected against lethal disease when immunized with 1 – 33 μg of protein per dose. Immunization of rabbits with 3000 antigen units prior to inoculation of strain Kupka into right scarified cornea limited the establishment of latency in the trigeminal ganglia. Both the number of animals in which latency had been established as well as the number of homolateral sensory ganglion cells which had become virus carriers were reduced. However, the effect of immunization was less striking at preventing HSV reactivation in rabbits vaccinated after infection. When shedding of reactivated HSV was elicited by repeated epinephrine iontophoresis to cornea, there was no quantitative difference between the immunized and mock-immunized groups, only the period between stimulation and the onset of virus shedding was prolonged in immunized rabbits (from 3.6 to 5.6 days, $p < 0.05$). But if the corneas were stimulated by a single iontophoresis procedure, the duration of virus shedding was significantly reduced from 5.6 days in the mock-immunized rabbits to 1.7 days in the immunized ones ($p < 0.025$). In the latter experiment, the total number of positive swabs during 14 days of the post-stimulation period was higher in the mock-immunized animals (31 of 171, 18.1%) than in the immunized ones (12 of 162, 7.4%; $p < 0.025$).

Key words: herpes simplex virus; subunit vaccine; mouse; rabbit; latency; virus reactivation

Introduction

Different antigen preparations of HSV (subunit or virion envelope vaccines, recombinant glycoproteins or their mixtures) as well as genetically engineered attenuated live vaccines have been tested in animals (reviewed by Meignier, 1985; Hall and Katrak, 1986; Burke, 1991; Meignier, 1991). Both the subunit envelope vaccines and the various recombinant glycoproteins have been repeatedly shown to protect mice, rabbits and guinea pigs against lethal challenge by intracerebral (ic) or peripheral routes even if HSV strains of heterologous type had been administered (reviewed in Table 1). When evaluating the effect of vaccination on the establishment of latent infection, the interpretations of different investigators ranged from significant reduction of the number of animals that developed latency (Hilfenhaus and Moser, 1981; Thomson *et al.*, 1983; Metcalf and Whitney, 1987; Cremer *et al.*, 1985; Rooney *et al.*, 1988; Willey *et al.*, 1988; Al-Ghamdi *et al.*, 1989; Manservigi *et al.*, 1990; Blacklaws *et al.*, 1990; Mishkin *et al.*, 1991) through a partial effect manifested by reduced frequency of virus-carrier ganglion cells (Rajčáni *et al.*, 1980) to insignificant results (Schneweis *et al.*, 1981; Klein *et al.*, 1981; Scriba, 1982; Sander and Sander, 1992). Šlichtová *et al.* (1982) found that immunization with a subunit vaccine reduced the extent of latent infection in mice when given before virus administration, but did not influence recurrencies when performed after infection.

The effect of vaccination was most frequently assessed in the mouse ear model (Hill *et al.*, 1975), in the guinea pig

Table 1. Efficacy of HSV subunit vaccine and recombinant HSV glycoprotein preparations in animal models

Effect	Reference
Baculovirus-expressed gE provided protection against lethal ip and ocular challenge in mice	Ghiasi *et al.*,1992
HSV-1 subunit vaccine did not prevent the establishment of latency in mice but decreased the intensity of inflammation during recurrencies as well as their frequency	Sander and Sander, 1991
Native purified gD from HSV-1 protected against lethal HSV-2 challenge and establishment of latency in mice	Mishkin *et al.*, 1991
HSV-1 ISCOM vaccine protected guinea pigs against intravaginal challenge with HSV-2	Ertürk *et al.*,1991
HSV-1 gD fusion protein (but not gC) expressed in E. coli protected mice against lethal challenge with HSV-1 as well as HSV-2 given id to hairless mice	Bröker *et al.*,1990
HSV-infected vaccinees deloped limited recurrent disease, the magnitude of initial virus shedding was low in guinea pigs that did not react by detectable antibodies to non-viral polypeptides	Bernstein *et al.*, 1990
From vaccinia virus recombinants expressing gB,gD, gE,gH and gI, those producing gB and gD induced NA and conferred highest protection against lethal challenge and establishment of latency; gE exerted partial protection only	Blacklaws *et al.*, 1990
Mice immunized with mouse L cells expressing gB and gD showed increased rate of virus clearance from ear pinna upon challenge into the ear	Blacklaws and Nash, 1990
Recombinant gB-1 expressed in human cells protected mice against lethal challenge and establishment of latency	Manservigi *et al.*, 1990
gD-1 vaccinia virus recombinants were protective for mice against HSV-2 challenge	Wachsman *et al.*, 1989
HSV-1-infected cell extract mixed with ISCOM had protective effect against ip challenge with HSV-1 as well as HSV-2 in Balb/c mice	Ertürk *et al.*, 1989
Zwitterionic detergent-extracted HSV-1 antigen preparation protected against establishment of latent infection and shortened the duration of ear erythema	Al-Ghamdi *et al.*, 1989
Recombinant gC protected mice against ic challenge with HSV	Weir *et al.*, 1989
Recombinant vaccinia virus expressing gB HSV protected Balb/c mice from acute and latent infection after corneal challenge	Willy *et al.*, 1988
Long term protection after immunization with recombinant gD reduced frequency of latent infection by 30%	Rooney *et al.*, 1988
Treatment with HSV gB/gD reduced spontaneous cervico-vaginal virus shedding in guinea pigs	Myers *et al.*, 1988
Reconstituted mixtures of HSV gB,gD,gE and gC protected mice and guinea pigs against ic or intravaginal challenge	Megnier *et al.*, 1987
HSV gD enhanced ear clearance in challenged mice	Martin and Rouse, 1987
Recombinant HSV gB protected mice against ip challenge with HSV	Cantin *et al.*, 1987
Immunization of outbred mice with F strain of HSV induced immune response and protection against herpetic ocular disease and reduced the incidence of latent trigeminal ganglion infection	Metcalf and Whitley, 1987
Immunization of mice with HSV-1 gB was protective against subsequent challenge with HSV-1 and HSV-2	Kino *et al.*, 1986
Mice immunized with vaccinia virus expressing HSV gD protected against lethal and latent infections with HSV-1	Cremer *et al.*, 1985
Purified HSV gB/gC protected Balb/c mice against ic lethal challenge	Roberts *et al.*, 1985
HSV gB protected mice against footpad challenge	Chan *et al.*,1985
HSV gD protected against lethal challenge with HSV-1 and HSV-2	Long *et al.*, 1984
Subunit virion envelope vaccine of HSV-1 protected labially infected mice from primary lesion, encephalitis and reduced latent trigeminal ganglion infection	Thomson *et al.*, 1983
HSV-1 gC protected mice against ip challenge	Schrier *et al.*, 1983
Guinea-pigs latently infected with HSV-2 were immunized with HSV-2 vaccine derived from extracts of infected cells. The vaccination had no effect onthe course of recurrent herpes in these animals	Scriba, 1982
In immunized mice the incidence of latent infection after ocular infection was limited in ophthalmic neurons	Tullo *et al.*, 1982

Table 1 (continued)

Subviral HSV-1 vaccine reduced latency and lowered the frequency of recurrent disease when administered before infection; when given post infection, the vaccine did not reduce recurrencies	Šlichtová *et al.*, 1982
HSV-2 subunit vaccine was antigenic in guinea pigs and protective against intravaginal challenge with HSV-2	Thornton *et al.*, 1982
HSV-1 killed vaccine preparations protected from lethal challenge but had only moderate effect on establishment of latency	Schneweis *et al.*, 1981
Immunization with HSV-1 envelope antigen did not prevent latent infection in nude mice but reduced reactivated virus titers in cultured ganglia	Klein *et al.*, 1981
Mice were protected by HSV-1 subunit vaccine against acute lethal infection and establishment of latency	Hilfenhaus and Moser, 1981
DNA-free HSV subunit vaccine did not prevent establishment of HSV latency in mice	Cappel *et al.*, 1980
Immunization with a DNA-free HSV subunit vaccine reduced the number of virus carrier ganglion cells but did not completly prevent the establishment of latency in rabbits after corneal challenge	Rajčáni *et al.*, 1980

genital model (reviewed by Stanberry, 1991), in the mouse or guinea pig footpad models, and/or by a simple ip or intradermal (id) virus challenge in mice. For demonstration of the protective vaccination effect we used HSV-1 strain SC16, which regularly causes hind leg paralysis. The effect of immunization on the establishment of latency was tested in the rabbit ocular model (Stevens *et al.*, 1972; Rajčáni *et al.*, 1975). Finally, to follow the possible effect of vaccination on virus reactivation, epinephrine iontophoresis to cornea was performed in rabbits (Kwon *et al.*, 1981).

Materials and Methods

Animals. Albino rabbits weighing 2500 – 3500 g were obtained either from the breed Velaz (Prague, Czech Republic) or Charles River (Sulzfeldt, Germany). The rabbits were infected at day 0 into the right scarified cornea with 2 x 10^6 PFU of HSV-1 strain Kupka in a volume of 0.05 ml. The animals were fed on standard synthetic diet and water *ad libitum*.

Balb/c mice were purchased from the breed Velaz and held on a standard diet in a separated room avoiding contacts with other virus-infected animals. For lethality prevention tests, the mice were infected with HSV-1 strain SC16 in a volume of 0.1 ml containing different virus doses ranging up to 100 LD_{50} (1 LD_{50} = 5 x 10^3 PFU).

HSV-1 strains. To establish latency in rabbits the non-encephalitogenic strain Kupka (passaged in rabbit lung embryo cells and Vero cells, stock titer 2 x 10^8 PFU/ml) kindly provided by Dr. R. Benda, Prague, was used. For testing the HSV-1 lethality for Balb/c mice the strain SCl6 highly virulent for mice (stock titer 6 x 10^8 PFU/ml, propagated in Vero cells) kindly provided by Dr. T. Hill, Bristol, England, was employed. For vaccine preparation the strain HSZP (adapted to chick embryo and quail embryo cells during more than 40 passages, stock titer 1 x 10^8 PFU/ml) was used.

For virus neutralization tests the strain KOS (propagated in Vero cells, stock titer 1 x 10^9 PFU/ml), kindly provided by Dr. V. Vonka, Prague, was employed.

Virus titrations. The spinal cord and adrenal glands removed from mice at days 2, 4, and 6 or 7 p.i. and/or the cultured trigeminal ganglion fragments were homogenized (10% organ suspensions in Basal Eagle's Medium (BEM)). The swabs from rabbit conjunctival sacs were eluted for 1 hr in BEM supplemented with 5% foetal calf serum (FCS) and antibiotics, and stored at -70 °C when not immediately inoculated. Testing of virus infectivity was made in 24-well microplates (Nunc) on which Vero cells were grown in monolayers for 24 – 48 hrs.

The infected cells were incubated for 3 – 4 days p.i. in 5% CO_2 atmosphere at 37 °C and CPE was read on days 2, 3 and 4 p.i. More exact plaque counts were made on 3 mm plastic Petri dishes (Nunc) under 0.9% methylcellulose overlay in BEM with of 10% newborn calf serum, glutamin and antibiotics.

Blood samples for serologic examinations were drawn from mice and/or rabbits before immunization and, when not otherwise indicated, 7 or 10 days after the second antigen injection. From immunized and infected mice blood samples were drawn on days 2, 4 and 6 after challenge. Blood samples from immunized and infected rabbits or conversely, from infected and immunized ones, were taken according to special schedules described in Results. Serum samples were stored at -20 °C.

Virus neutralization test was made in 24-well microplates (Nunc). About 100 PFU of strain KOS was mixed with equal amount of inactivated serum diluted from 1:8 to 1:8,192 and divided into two parts. One part of the mixture (0.2 ml) was incubated with 0.1 ml of fresh guinea pig serum (diluted 1:16) as the source of complement. After 1 hr incubation, the virus-serum and the virus-serum-complement mixtures were inoculated onto the Vero cell monolayers. Each test was run with negative and positive serum controls; virus titer controls were prepared in the presence and absence of complement. After incubation in 5% CO_2 atmosphere at 37 °C, the endpoints were determined by days 3 and 4 p.i. and scored according to prevention of CPE formation (control wells were inoculated with 100 PFU of virus).

Subunit vaccine was prepared as described previously (Rajčáni *et al.*, 1988). Briefly, quail embryo cells were infected with strain HSZP (0.2 PFU/cell). When showing complete giant cell formation, the cells were scraped off into buffer containing 10 mmol/l Tris-HCl pH 8.0 and 15 mmol/l $MgCl_2$, treated with the same buffer containing 1% Nonidet NP-40 and 1 mmol/l dithiothreitol, centrifuged at 1000 r.p.m. for 30 mins and then centrifuged at

100,000 x g for 3 hrs trough a 30% sucrose cushion. The supernatant was inactivated with formalin and precipitated with ammonium sulphate at 60% concentration. The sediment was dissolved in PBS and thoroughly dialyzed. The protein content and antigenic activity of the vaccine were determined, and the antigen potency was expressed in ELISA units per μg of protein.

ELISA of the antigenic potency of the vaccine was made in microplates coated with anti-KOS swine IgG. Serial dilutions of antigen were incubated with the bound antibody for 2 hrs at 37 °C, washed, incubated with swine antiserum diluted 1:1000, washed, incubated with rabbit anti-swine peroxidase-labelled antibody and washed again. Finally, the antigen binding was vizualized with DAB solution in the presence of H_2O_2.

Serum antibody titration was made in microplates coated with HSV-1 antigen prepared in human embryo lung (HEL) cells infected with strain KOS. The antigen was formalin-inactivated and semipurified by adsorption to activated CNBr-Sepharose bound anti-HEL IgG (prepared from a swine immunized with the uninfected HEL cell extract). The antigen was then dialyzed through a Diaflo ultrafiltration membrane (YM10, Amicon) and used for coating microplate wells (at concentration of 8 μg/ml or 4 antigen units per 0.1 ml). The antigen-coated microplates were incubated with corresponding sera in different dilutions, washed, incubated with anti-human IgG, labelled peroxidase with (Sevac, Prague) and the antibody was vizualized with DAB solution as described above (for details see Hsiung, 1982).

Immunization protocols. Mice were given subcutaneously (sc) two doses of the subunit vaccine containing 10 (group A), 100 (groups B1 and B2), 300 (group C), and 1000 (groups D1 and D2) ELISA units, respectively. In groups A, B1, C and D1 the vaccine doses were mixed with aluminium hydroxide (1.5%) and 10% volume of mineral oil (Al-Span-oil adjuvant from SEVAC, Prague), while in groups B2 and D2 the vaccine doses were mixed with aluminium hydroxide (1.5%) and sodium deoxycholate (0.25%). The rabbits were immunized intramuscularly (im)with 3000 ELISA units of the vaccine mixed with aluminium hydroxide. The mixture of antigen and adjuvant was allowed to stand for at least 1 hr at 4 °C at occasional mixing.

Virus reactivation procedure was performed as described by Kwon *et al.* (1981). In Rompun/Ketalar anaesthezia the cathode was attached to the wet right ear and the anode was applied to corneal limbus. Epinephrine (0.1%) was repeatedly dropped onto gently scarified right cornea and the iontophoresis was allowed to proceed for 8 mins under 2 mA current. In the first trial the iontophoresis was repeated daily within the next 3 days. In addition, the animals received daily 5 im injections of 0.4 ml of epinephrine (diluted 1:20). In the second trial a single iontophoresis procedure was combined with a single epinephrine injection. Conjuctival swabs were taken before starting the procedure (day 0), then on day 2 (before the third iontophoresis) 3, 4, 5, 7, 8, 9, 10, 11 and 14 since the beginning of the stimulation procedure.

Explantation of regional sensory ganglia was done as described previously (Rajčáni *et al.*, 1975). Briefly, both trigeminal ganglia were minced into small fragments and cultured in RPMI-1640 medium containing 10% FCS and antibiotics. For quantification purposes the minced ganglion fragments were kept separately in the wells of the 24-well microtitration plate. The medium from each well was tested for virus presence on days 3, 7 and 10 in culture. By the day 10 in culture, the minced fragments coming from 4 wells in a vertical line were collected to obtain 6 homogenized suspensions from each ganglion and tested for infectious virus content.

Statistical analysis of results was done by use of the Student t-test, the Scheffé test, the Mann-Whitney U-test, the chi-square test and the median test (Sachs, 1984).

Results

Protection test in Balb/c mice

The lethality of SC16 virus for Balb/c mice was determined in 2 experiments using 4 different dilutions of stock virus; it was found that 1 LD_{50} in unprotected mice corresponds to about 5 x 10^3 PFU. Mice infected ip died with signs of hind leg paralysis since day 6 p.i. At immunization according to each of the schedules shown in Table 2, the mice received different doses of antigen ranging from 10 ELISA units (group A) to 1000 ELISA units (group D).

Table 2. The experimental schedule for protection test in Balb/c mice

Day 0 : immunization with first vaccine dose, first serum sample
Day 28: immunization with second vaccine dose, second serum sample
Day 38: postimmunization serum sample
Day 50: challenge with 10 LD_{50} of SC16 virus by i.p. route
Animal groups: A - immunization with 10 ELISA units of the vaccine
B - immunization with 100 ELISA units of the vaccine
C - immunization with 300 ELISA units of the vaccine
D - immunization with 1000 ELISA units of the vaccine
E - immunization with 300 ELISA units of the crude antigen extract from strain HSZP-infected cells
F - "immunization" with the adjuvant only
G - no immunization

Table 3. Serum antibody levels in mice immunized with different doses of the subunit vaccine

Animal group	Antigen dose	Serum antibody titer ELISA	NT C^+	NT C^-
A[a]	10 U	640	64	16
B1	100 U	10 240	64	8
C1	300 U	20 480	128	16
D1	1000 U	40 960	256	32
E1	300 U	2 560	64	8
F1	none	< 160	<16	<4

Sera from 5 animals in each group were pooled. NT C^+, NT C^-: virus neutralization tests in the presence or absence of complement, respectively.
[a]No oil used as adjuvant. For the legend see Table 2.

Table 4. Serum antibody titers in immunized Balb/c mice after challenge with SC16 virus

Animal group	Days post immunization 2			4			6		
	ELISA	NTC$^+$	NTC$^-$	ELISA	NTC$^+$	NTC$^-$	ELISA	NTC$^+$	NTC$^-$
A (10 U)	640	16	4	2 560	4	4	20 480	64	16
B1(100 U)	2 560	32	8	20 480	64	16	40 960	2 048	64
B2(100 U)	640	16	4	640	16	4	10 240	64	16
C (300 U)	2 560	32	8	20 480	64	16	40 960	2 048	64
D1(1000 U)	40 960	128	16	81 920	256	64	160 000	2 048	128
D2(1000 U)	2 560	64	4	2 560	64	4	10 240	1 024	16
E2 (300 U)	640	16	8	640	64	16	2 560	1 024	64
F2	< 640	<4	<4	<640	<4	<4	<640	<4	<4
G	< 640	<4	<4	<640	<4	<4	<640	<4	<4

For the legend see Table 2.

The vaccine batch used had the activity of 9 ELISA units per 1 μg of protein. Positive controls were immunized with a crude formalin-inactivated virus (HSZP strain, cell extract), the mock-immunized mice were given adjuvant only. Another control group (G) consisted of non-immunized mice. All animals were challenged with 10 LD_{50} of SC16 strain by ip route.

A satisfactory antibody response, which was comparable with the titers found in mice immunized with the formalin vaccine developed in mice immunized with 100 ELISA units of the subunit vaccine (Table 3). All the immunized animals showed a booster response after challenge; the rapid antibody increase between days 4 – 6 post challenge was clearly different from the primary post-infection antibody response (group G, Table 4). As expected, the booster response in challenged mice was most intensive in animals, which had been immunized with the highest antigen dose (1000 ELISA units). The lethality of SC16 infection, which was 100% in mock-immunized and in non-immunized animals, decreased to 50% in mice protected with the low and medium vaccine doses and to nil in mice protected with the high vaccine dose (Table 5). In randomly selected animals examined between days 2 – 6 p.i., the unprotected mice regularly had high virus titres in both lumbal cord and adrenal glands. In partially protected mice (immunized with the low and medium antigen doses) infectious virus was occasionally found in the adrenal glands but not in the spinal cord (with the exception of one mouse in group A). Thus, HSV antigen doses ranging from 10 to 300 ELISA units (1 – 33 μg of protein) provided partial protection against neural spread and paralytic disease (Table 6).

The high antigen dose was fully protective enabling a complete clearence of the virus from target tissues. A statistical analysis of the lethality data (chi-square test) confirmed the significant difference between mock-immunized to immunized mice (groups A, D2, E2). The lethality rate seen after challenge of the high antigen dose-vaccinated mice differed from that of the mock-vaccinated as well as of the low dose-vaccinated animals (Table 7).

Table 5. Mortality of immunized Balb/c mice after challenge with 10 LD_{50} of SC16 virus

Animal group	Days p. i. 3	5	6	7	8	9	10	11	12	Total
A (10 U)						2	1	2		5/13
B1(100 U)				1			4			5/10
B2(100 U)								1	1	2/ 4
C (300 U)				1			3			4/ 8
D1(1000 U)							1			1/10
D2(1000 U)										0/ 8
E1 (300 U)				1						1/10
E2 (300 U)							2	2		4/19
F1			1	2	5	2				10/10
F2				3	3	4				10/10
G			3	4	2	1				10/10

For the legend see Table 2.

Antibody response in immunized rabbits

The mean neutralizing antibody (NA) titer from infected rabbits (group A) was compared with that in rabbits that had been first immunized and later infected (group B, latency protection test) and with that in rabbits that had been first infected and later immunized (group C). If challenged after immunization (group B), the mean NA titer increased by at least twofold ($p < 0.05$, Fig. 1). The mean NA titers from rabbits that had been infected first and then immunized (3000 ELISA units, group C) showed a higher increase (in average threefold, $p < 0.05$). The two booster effects also differed ($p < 0.05$) in the impact on the NA titer increase.

Table 6. Virus titration (PFU/ml) in adrenal glands and spinal cord of protected mice challenged with 10 LD_{50} of SC16 strain

Animal group	Days p. i. 2		4		6	
	AdG	SpC	AdG	SpC	AdG	SpC
A	$6x10^4$	n	n	n	n	n
	n	n	n	n	n	n
	n	n	$6x10^4$	n	n	n
	n	n	$6x10^4$	$6x10^0$	n	n
B1	$1x10^4$	n	n	n	n	n
	n	n	n	n	n	n
	n	n	n	n	n	n
B2	$5x10^4$	n	n	n	$2x10^2$	n
	n	n	n	n	n	n
	$2x10^3$	n	n	n	n	n
	n	n	n	n	n	n
C	$1x10^3$	n	n	n	n	n
	$1x10^3$	n	n	n	n	n
D1	n	n	n	n	n	n
	$1x10^4$	n	n	n	n	n
	n	n	n	n	n	n
	n	n	n	n	n	n
	n	n	n	n	n	n
	n	n	n	n	n	n
	n	n	n	n	n	n
E1	n	n	$1x10^4$	n	n	n
	n	n	n	n	n	n
	n	n	n	n	n	n
	n	n	n	n	n	n
F1	$3x10^4$	n	$5x10^5$	n	n	$1x10^1$
	$5x10^4$	n	$5x10^4$	$2x10^4$	$2x10^3$	$5x10^3$
F2	$6x10^3$	n	$> 10^5$	n	$6x10^2$	n
	$2x10^4$	n	$> 10^5$	n	$2x10^2$	$6x10^2$
	$2x10^3$	$1x10^2$	$> 10^5$	n	$3x10^1$	$6x10^2$
	$6x10^1$	n	n	n	$3x10^2$	n
G	$1x10^4$	n	$1x10^5$	n	$1x10^3$	$3x10^4$
	$1x10^4$	n	$1x10^5$	n	$1x10^3$	$3x10^2$
	$5x10^5$	n	$1x10^5$	n	$3x10^2$	$3x10^2$

n = negative; AdG = adrenal gland; SpC = spinal cord.

Table 7. Significance of differences between the mortality of mice in experimental groups A-F from Table 5

Experimental groups	Mortality of mice in groups A 5/13 38%	B2 2/4 50%	D2 0/8	E2 4/13 31%	F2 10/10 100%
A (10 U)	.	NS	p<0.05	NS	p<0.005
B2(100 U)	NS	.	NS	NS	NS
D2(1000 U)	p<0.05	NS	.	NS	p<0.001
E2	NS	NS	NS	.	p<0.001
F2	p<0.005	NS	p<0.001	p<0.001	.

For the legend see Table 2. NS = non-significant (p >0.05).

Summing up, in immunized rabbits the mean serum NA titers were similar even if they seemed slightly higher than in infected ones (p >0.05). In preimmunized rabbits the booster effect of infection caused a significant increase of the the mean NA level similarly as did the booster effect of vaccination in previously infected rabbits; however, in the latter case, this increase was more marked than in the former one (compared by the Scheffé method, difference significant at p <0.05).

Latency protection test

To demonstrate the effect of immunization on establishment of latency, the animals were divided into 2 groups. In one group, latency was established with the Kupka strain inoculated into the right scarified cornea. In another group, rabbits were first immunized with two vaccine doses (days

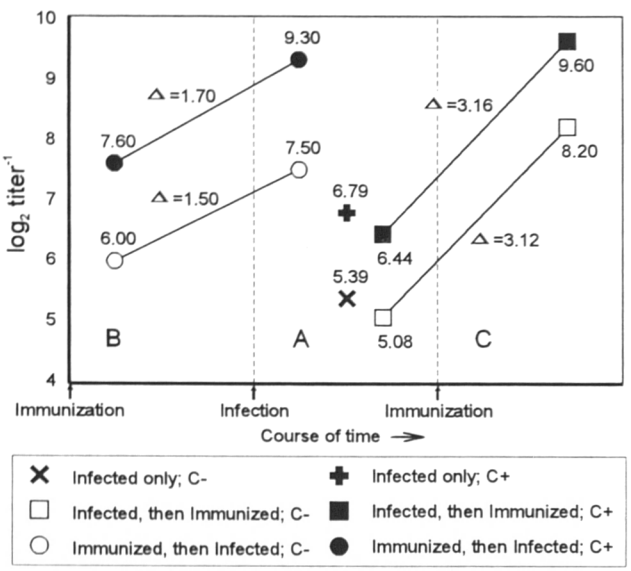

Fig. 1
The neutralizing antibody (NA) response in infected and immunized rabbits

The mean NA titers as measured in the presence and absence of complement were calculated for 12 sera of infected rabbits (group A), for 25 sera of immunized rabbits which were later infected (group B), and for 10 sera of rabbits which had been first infected and later immunized (group C). The mean NA titers in these groups fulfilled the homogeneity of variance test at p <0.05. Analysis of variance showed that the mean titer values for individual groups differ significantly p <0.01. Contrasts were evaluated for significance according to the Scheffé method, comparing the C^+ and C^- mean values in each group. The mean NA titer was slightly higher in 25 immunized rabbits (if compared to the infected ones, the difference was not significant, p >0.05); it increased by at least twofold (p <0.05) when the animals were infected during the latency protection test (group B). In the other 10 infected rabbits (group C), the mean NA titer resembled that found in group A (p >0.05) and increased by at least three dilution steps after immunization (significant at p <0.05).

0 and 28, 3000 ELISA units each) and then (22 days later, i.e. on day 50 of the experiment) the virus was inoculated into the right scarified cornea. Swabs were taken from both conjunctival sacs in 3 – 4 day intervals for 14 days; virus replication was detected in the cornea of each previously immunized rabbit for at least 7 – 10 days. In these animals blood was drawn on days 0, 38 and on the autopsy day; autopsies were performed 60 – 80 days later, i.e. 105 – 125 days since the beginning of the experiment.

Table 8. Establishment of latency in control and immunized rabbits

Rabbits	Positivity rate of rabbits	Tissue	Positivity rate of fragments	Positivity rate of medium samples
Control	11/12[a]	RTG	52/66 (79%) 52/72 (72%)*[b]	75/264 (28.4%) 75/288 (26%)*
	5/12	LTG	N.D.	11/60
Immunized	3/10[a]	RTG	6/18 (33.3%) 6/60 (10%)*[b]	8/72 (11.1%) 8/240 (3.3%)*
	0/10	LTG	N.D.	0/50

*Related to all the rabbits examined including negative ones. RTG = right trigeminal ganglion; LTG = left trigeminal ganglion. [a]difference significant at $p < 0.005$; [b]difference significant at $p < 0.001$. ND = not done.

In non-immunized controls blood was drawn on the day of corneal infection (day 0) and 50 – 70 days later on the day of autopsy. Table 8 shows that 11 of 12 non-immunized rabbits developed latent infection in the homolateral trigeminal ganglion, and 5 of them also in the contralateral ganglion. Quantitation of the ganglion fragments yielding virus in culture showed that 75 of 288 minced right ganglion fragments (28.4%) originating from control rabbits has released the reactivated virus from explanted ganglion fragments into the medium. Out of a total of 72 pooled and homogenized ganglion fragments coming from these rabbits 52 (79%) became infectious in the course of cultivation. Different results were obtained in immunized rabbits. Latency was established in 3 of 10 rabbits only; this difference was significant at $p < 0.005$. Furthermore, out of 240 minced ganglion fragments coming from the immunized rabbits only 8 (3.3%) released virus. No latency was found in the contralateral ganglia. Of the total of 60 suspensions prepared from the pooled right ganglion fragments by day 10 in culture, only 6 (10%) had reactivated virus ($p < 0.001$).

The extent at which immunization might have prevented the establishment of latency was calculated by comparing the proportion of virus yielding fragments to the total in each of two animal groups. Results were significant even if the differences were calculated for the number of fragments coming from rabbits which had become virus carriers (Table 8). Thus, a part of immunized rabbits was completely protected, while in the rest of immunized animals the number of ganglion cells, which harboured the latent genome was reduced.

Reactivation prevention test

In the first trial of this series, alltogether 53 rabbits were infected to the right scarified cornea with the Kupka strain (day 0 of the experiment). Then the animals were immunized either with the HSV vaccine in doses of 3000 ELISA units each on days 30 and 58 (22 rabbits), or with a mock-antigen (noninfected quail embryo cell extract) at the same intervals. The animals were subjected to the epinephrine iontophoresis from day 70. The corneas were stimulated for 3 consecutive days and the conjunctival sacs were swabbed daily for a period of 14 days in order to detect shedding of reactivated virus. Under given conditions, the number of rabbits with established latent infection who shed HSV after reactivation did not differ in both groups. Also mean period of virus shedding was the same ($p > 0.1$). Significant was only the prolonged lag period between starting corneal stimulation and the onset of virus shedding in vaccinated rabbits (Table 9).

To test the possibility that less potent stimulation of the cornea in rabbits subjected to a single iontophoresis procedure would demonstrate the desired vaccination effect, we examined the other 37 rabbits. The results of this experiment are shown in Table 10. The reactivation occurred in 8 of 19 mock-immunized and in 7 of 18 vaccinated animals. However, the mean period of virus shedding was reduced in immunized animals ($p < 0.025$). Also the rate of virus shedding as related to the total number of conjunctival swabs decreased after vaccination ($p < 0.025$).

Table 9. Comparison of ocular shedding after repeated epinephrine iontophoresis in control and vaccinated rabbits

	Control	Vaccinated
Number of rabbits	31	22
Number of shedders	17 (55%)[a]	11 (50%)[a]
Positive swabs of total	54/279 (19%)[b]	30/198 (15.2%)[b]
Duration of shedding (days)	4.0 ± 1.5[c]	3.2 ± 2.35[c]
Onset of shedding (the day)	3.6[d]	5.6[d]

Epinephrine iontoforesis performed daily for 3 consecutive days. [a,b,c]Non-significant differences ($p > 0.1$). [d]Significant difference ($p < 0.05$, the median test).

Table 10. Reactivation of latent HSV-1 after a single epinephrine iontophoresis procedure

	Animal group Vaccinated (A)	Controls (B)	Significance of difference between A and B
Number of rabbits which reactivated virus out of total tested	7/18 (38.8%)	8/19(42%)	NS
Number of positive swabs out of total tested[a]	12/162 (7.4%)	31/171(18.1%)	<0.025[c]
Number of positive swabsout of total (corrected)[b]	12/63 (19%)	31/72 (43%)	<0.05[c]
Duration of virus shedding (days)	1.7 ± 1.2	4.9 ± 1.6	<0.025[d]

[a]Swabs taken on days 2,3,4,5,6,8,9,10, and 11 since the iontophoresis procedure. [b]Calculated for the total number of swabs coming from rabbits which shed virus. [c]Chi-square test. [d]Mann-Whitney U-test. NS = non-significant.

Latency in non-shedders

The presence of non-productive (latent) infection among non-shedders, which did not release infectious virus upon epinephrine iontophoresis was tested in 12 randomly selected animals. Each of these reactivated latent virus in the right (homolateral) trigeminal ganglion fragments in the course of explantation.

Discussion

The immune response to HSV has been mostly analyzed in the mouse model (Mester and Roouse, 1991), though mice may show differences as compared to humans such as less potent antibody dependent cellular cytotoxicity and a less evident MHC class-II restricted cytotoxic cell-mediated response. Passive immunization with a hyperimmune polyclonal serum or with monoclonal antibodies against gB, gC, gD and gE reduces lethality after peripheral virus challenge (Table 11). NA would limit the extent of neural spread to the regional sensory ganglion in the course of primary infection (Walz *et al.*, 1976) and may reduce the extent of latency (McKendall *et al.*, 1979; Rajčáni *et al.*, 1980; Table 1); however, the latter effect can differ according to the conditions of the experiment (Klein, 1980; Schneweis *et al.*, 1981; Scriba, 1982; Sander and Sander, 1991). NAs suppress the reactivation of latent virus in culture (Rajčáni *et al.*, 1977; Oakes and Lausch, 1984) or in the ganglion tissue transplanted to peritoneal cavity of passively immunized recipients (Cook and Stevens, 1974). Passive immunization prevents acute ganglionic infection in normal outbred mice but not in nude mice (Openshaw *et al.*, 1979), suggesting the involvement of a cell-associated defense mechanism. In addition, Oakes and Rosemond-Hornbeak (1978) demonstrated the participation of a radiosensitive component in protection from neural spread of HSV by passive immunization; Rager-Zisman and Bloom (1974) showed that passive serum transfer was not sufficient to prevent virus spread in cyclophosphamide-treated mice. The cellular component which determines the natural resistance are mononuclear killer cells. The ability of low, subneutralizing doses of IgG to interact with killer cells and mononuclear phagocytes was demonstrated in antibody-dependent cytotoxicity test, which is more effective in the presence of whole IgG than in the presence of $F(ab)_2$ fragments (McKendall *et al.*, 1985).

Table 11. Overview of passive MoAb protection and lymphocyte transfer protection against HSV challenge in mice

Model	Reference
MoAbs to gB2, gC2, gD2, gE2 protect mice against footpad challenge with HSV-2	Balachandran *et al.*, 1982
Passive immunization with gB1, gC1, gD1 in mice protected against i.c. challenge	Kümel *et al.*, 1985
MoAbs against gC1 and gD1 protected mice against footpad challenge	Dix *et al.*, 1981
MoAbs to gB1, gC1, gD1 and gE1 protected mice against ocular challenge	Rector *et al.*, 1982
MoAbs to gB1 and gD1 protected nude mice from zosteriform spread of HSV-1	Simmons and Nash, 1985
Protection against herpetic ocular disease with MoAbs to gB1, gC1, gD1 and gE1	Metcalf *et al.*, 1988
Protection against zosteriform spread with MoAbs to gB1, gC1 and gD1	Mester *et al.*, 1990
CD4 (Lyt-1) lymphocytes identified as the effector cells involved in delayed hypersensitivity and ear tissue clearance from HSV-1	Nash and Gell, 1993
Recovery from lethal HSV-1 infection was mediated by cytotoxic T lymphocytes	Larsen *et al.*, 1983

The protective role of T lymphocytes in HSV clearance was demonstrated by adoptive transfer of splenocytes to syngeneic recipients, which became resistant to ic virus challenge (Ennis, 1973). The active cells were suggested to

be helper T cells (Lyt-1$^+$) but also cytotoxic Lyt-2$^+$ cells (Nash *et al.*, 1981; Howes *et al.*, 1979). In the ear model and in nude mice with zosteriform herpes the local virus clearance and prevention from neural spread was mediated predominantly by Lyt-1$^+$ cells (Nagafuchi *et al.*, 1982; Nash and Gell, 1983). This protective effect may be enhanced by simultaneous transfer of cytotoxic Lyt-2$^+$ cells (Wildy and Gell, 1988). Thus, while Lyt-1$^+$ cells are more important for virus clearance at the inoculation site, Lyt-2$^+$ cells act more efficiently at destroying virus antigen-producing cells in the regional sensory ganglion (Nash *et al.*, 1987). Target antigens for T cell-mediated cytotoxicity are HSV glycoproteins such as gB, gD and probably also gC (McLaughlin-Taylor *et al.*, 1988; Johnson *et al.*, 1990; Martin *et al.*, 1989), as well as nonstructural proteins ICP4 and ICP27 (Martin *et al.*, 1989). The latter phenomenon may be of great interest in preventing the replication of reactivated virus. Consequently, recent data provide evidence that MoAbs are protective by different mechanisms which depend on their epitope specificity (Eis-Hübinger *et al.*, 1991). MoAb to gB would act more effectively at preventing local replication of HSV after intracutane challenge or at virus inoculation to mucous membranes, while non-neutralizing anti-gC MoAb would prevent ganglionic infection.

How immune mechanisms could influence the establishment of latency is more unclear. Because local virus replication in the cornea of immunized rabbits is not reduced (Rajčáni *et al.*,1980), antibodies may possibly prevent – or rather reduce – the neuritic uptake (Walz *et al.*, 1976; Klein, 1980; Openshaw *et al.*, 1979). When reducing the extent of neural spread antibodies interact with the cell-mediated immune response at the portal of entry (Nash, 1985). Sekizawa *et al.* (1980) found that antibodies are not needed for the maintenace of the latent state, but other observations (Rajčáni *et al.*, 1977; Oakes *et al.*, 1984) showed that polyclyonal as well as some MoAbs may influence the reactivation rate of latent HSV in ganglion explants. The exact mechanism of this effect is unknown.

The presented results showed that (1) the immunization was effective in protecting mice from lethal outcome of virus challenge during acute infection (high vaccine dose led to an enhanced clearance resulting undetectable virus levels in target organs),(2) the immunization reduced the extent of latency established in the trigeminal ganglia of rabbits inoculated into scarified cornea, and (3) the immunization of infected rabbits influenced the replication of reactivated virus by prolonging the lag phase between stimulation and virus shedding and, under favourable conditions, the therapeutic vaccination of previously infected rabbits limited the duration and extent of reactivated virus shedding.

To understand the significance of latter observation, it is useful to consider briefly the molecular mechanisms of latent infection in neurons. During latency the HSV DNA is present as a circularized episomal non-integrated molecule harboured in the nuclei of neurons (Rock and Fraser,1985; Efstathiou *et al.*, 1986). The dispute on the extent of the transcription of non-structural virus-specific mRNAs during HSV latency was settled by the discovery of latency-associated transcripts (LAT) (Deatly *et al.*, 1987; Rock *et al.*, 1987; Puga and Notkins, 1987; Croen *et al.*, 1987). The protein specified by LAT has been expressed as a recombinant fusion protein and the antibody prepared against it recognized an antigen in latently infected neurons (Doerig *et al.*, 1991). LAT is not needed for the establishment of latency but is related to HSV reactivation and to the control of latency within neurons (Hill *et al.*, 1990; Nsiah and Rapp, 1991; Steiner *et al.*, 1989; Tenser *et al.*, 1993). The reactivation of latency is a multistep process. It precludes the overcoming of cellular regulatory mechanisms preventing transcription and translation of HSV immediate early polypeptides which transactivate the virus reproduction cycle (Mackem and Roizman, 1982; O'Hare and Hayward, 1985; Estridge *et al.*, 1990). The reactivated virus may spread to skin or mucous membranes by retrograde axonal transport and can replicate there (Klein, 1982, 1985). Immune mechanisms should restrict the growth of reactivated virus in the neuron as well as at the site of recurrent lesion. The effect of prolonged lag phase between stimulation of the cornea and virus shedding in immunized rabbits seems to confirm the two-step reactivation event initiated after lift-off of the blockade which had maintained the carrier state. This is followed by retrograde transport and replication of reactivated virus at the site of recurrent lesion. Shimomura *et al.* (1985) found that 24 hrs after stimulation of the cornea in rabbits with established latency the infectious virus was present in 100% of homolateral trigeminal ganglion homogenates but not in the homogenates of cornea.

The finding that previous immunization reduced the duration of recurrent shedding only if the reactivation stimulus was not too intensive, is not necessarily discouraging. Possibly, repeated adrenergic stimulation combined with repeated systemic epinephrine administration elicited such an extensive reactivation of latent HSV that subsequent virus shedding could not be prevented. The benefitial effect of vaccination was observed only after a mild epinephrine iontophoresis procedure. A similar therapeutic effect was observed with the same vaccine in the mouse ear model (Sanders and Sanders, 1991).

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