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RESTRICTION OF LATENT HERPES VIRUS INFECTION IN RABBITS IMMUNIZED WITH SUBVIRAL HERPES SIMPLEX VIRUS VACCINE

J. RAJČÁNI, *L. KUTINOVÁ, *V. VONKA

Institute of Virology, Slovak Academy of Sciences, 809 39 Bratislava; and
*Institute of Sera and Vaccines, 101 03 Prague, Czechoslovakia

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Summary. — The ability of an experimental herpes simplex virus type 1 (HSV) vaccine to influence the establishment of latent infection was examined. The virion-free vaccine was prepared from HSV 1-infected LEP cells by extraction with Nonidet P 40. Albino rabbits were immunized with three doses of the subviral vaccine (strain KOS) and challenged with 10^6 PFU of HSV 1 (Kupka strain) into the right scarified cornea. Non-immune controls and rabbits immunized with formalin-inactivated virion vaccine (strain KOS) were infected in a similar way. At intervals from 53 to 164 days p. i., the animals were killed and fragments of both trigeminal ganglia were kept separately in culture for 10 days. In 18 out of 22 immunized animals (81.8%), latency was established in the homolateral Gasserian ganglion. The proportion of HSV-yielding fragments, was considerably higher in ganglia from non-immune animals (42.6%) as compared to those from ganglia of rabbits immunized with the subviral vaccine (5.4%) or with the whole virion vaccine (14.6%). The immunity resulting from previous vaccination restricted about 5 times the number of ganglion cells, which become virus carriers.

Key words: herpes simplex virus type 1; subviral vaccine; immunization; latent infection

Introduction

Several reports described the effect of immunization on the establishment of latent infection with HSV 1. McKendall (1977) reported a markedly decreased incidence of latency in immunized versus non-immune mice. Kitces *et al.* (1977) found a decreased incidence of both acute and latent infections in the regional sensory ganglia of mice immunized with an HSV 1 vaccine prepared from detergent-treated virions. A lower frequency of the virus carrier state was also established in guinea pigs passively immunized with hyperimmune serum and challenged by corneal inoculation (Tenset

and Hsiung, 1977). On the other hand, Price *et al.* (1975) reported that intraperitoneal immunization with a non-lethal dose of living virus, which had been shown not to cause latency in the dorsal root ganglia, did not prevent mice from latent infection if challenged at a distant site, namely into the hind footpad. The number of neurons, which harboured the infectious HSV between days 2—10 post infection (p. i.), was approximately ten times lower in the dorsal root ganglia of immunized mice, than in the ganglia of non-immunized controls (Walz *et al.*, 1976).

The aim of the present work was to examine the capability of our experimental subviral vaccine (Kutinová *et al.*, 1977) to influence the establishment of latent HSV infection in rabbits. The experiments were also performed in effort to determine the immunogenicity of the vaccine in laboratory animals. The subviral vaccine induces neutralizing antibodies (NA) in different animal species and confers to them protection against intraperitonral (Kutinová *et al.*, in preparation) or intradermal (Šlichtová *et al.*, in preparation) inoculation of the virus.

Materials and Methods

Cells. VERO cells used for virus titration were grown in Eagle's basal medium (BEM) supplemented with 10% inactivated calf serum (ICS). Human lung embryo cells (LEP) (Řezáčová and Barešová, 1969) were used for preparation of the experimental vaccines.

Viruses. The KOS strain of HSV 1 was obtained through the courtesy of Dr. J. L. Melnick (Baylor College of Medicine, Houston, U.S.A.). The strain Kupka of HSV 1 was isolated and kindly supplied by Dr. R. Benda (Military Institute of Hygiene, Epidemiology and Microbiology, Prague).

Experimental vaccines. The subviral vaccine, denoted antigen mixture (AM), was prepared by extraction with Nonidet P-40 of HSV 1 (strain KOS)-infected LEP cells as described (Kutinová *et al.*, 1977). There was no surviving virus present in the AM. The HSV 1-specific antigenic activity of the AM was determined by the ^{51}Cr release inhibition test (CRIT) as described by Kutinová and Vonka (1978). The yield from each Roux bottle corresponded to 2 ml of AM suspension and had an activity of 2560 CRIT units per ml. The inactivated whole virion vaccine was prepared from the KOS strain grown in LEP cells. The cells were infected with 0.5 PFU per cell and incubated for 72 hr at 37 °C. The cultures were frozen and thawed twice, then clarified by low speed centrifugation. The virus was sedimented by centrifugation at $75000 \times g$ for 60 min in a Janetzki VAC 601 centrifuge. The pellet was resuspended in isotonic reticulocyte standard buffer (RSB), pH 7.4 to obtain 2 ml of virus suspension from each 1200-ml Roux bottle; the suspension was sonicated in an MSE sonic oscillator for 60 sec at grade 3. The titre of the preparation was 10^8 PFU/ml, which corresponded to 1.6×10^{10} physical particles as determined by electron microscopy. The virus was inactivated by 0.015% formalin for 48 hr at 37 °C. Formalin was removed by dialysis against RSB for 48 hr at 4 °C. No living virus was present in this preparation.

Animals, immunization procedure and virus challenge. Albino rabbits from the Dobrá Voda breed (2500—3000 g) were immunized either with three doses of the AM or with three doses of the whole virion vaccine. The dose of each antigen represented the harvest from one Roux bottle. The first two doses, mixed with an equal volume of incomplete Freund's adjuvant were administered intramuscularly on the 1st and 29th days. The third dose without adjuvant was applied into the ear vein 7 days later. Altogether 9 rabbits received the whole virion vaccine (group II) and 13 animals the AM (group III). Nine controls remained unvaccinated (group I). Seven days after the last antigen injection all animals were infected into the right scarified cornea with 10^6 PFU of the Kupka strain in 0.05 ml volumes. Swabs were taken from both conjunctival sacs on days 2, 3, 4, 6, 8 and 10 p.i. They were eluted into 1 ml of BEM for 1 hr at 4 °C. For titration, VERO cells were grown in plastic Petri dishes (50 mm diameter), 0.5 ml of individual dilutions were adsorbed on to the monolayers and overlaid with 0.8% methyl-cellulose in BEM

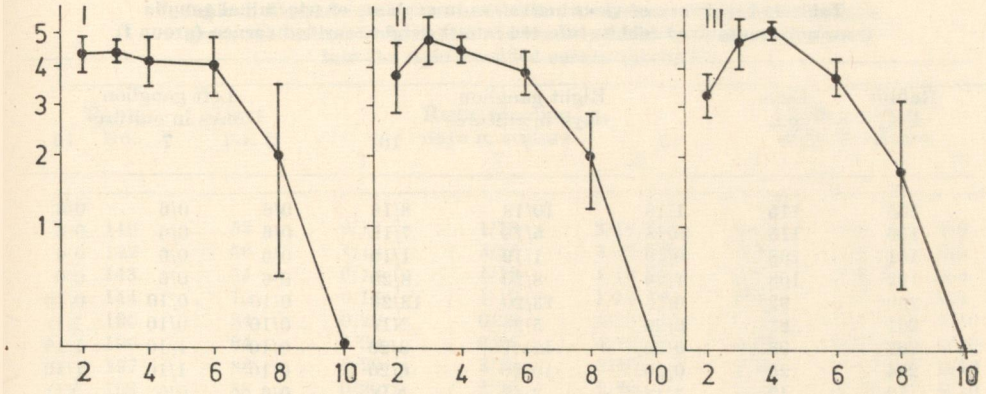

Fig. 1.

Replication of HSV 1 strain Kupka in the cornea of rabbits not immunized (I), immunized with subviral AM vaccine (II) and immunized with inactivated whole virion vaccine (III)

Abscissa: days p.i.; ordinate $\log_{10}$ PFU/ml (mean values and standard deviations)

containing 10% ICS. Plaques were counted after 4 days of incubation in a 3% CO_2 atmosphere at 37 °C. The mean PFU log values and their standard deviations were calculated from individual plaque counts for the three experimental groups at each time interval. The results were compared by the t test. Blood samples were drawn from each animal before immunization, before virus inoculation and before autopsy.

Explantation procedure. Both trigeminal nerves and Gasserian ganglia were removed from each rabbit at intervals from 53 to 164 days p.i. They were immediately immersed into phosphate buffered saline containing 3% foetal calf serum, minced into fragments and cultured as described (Benda *et al.*, 1973; Rajčáni *et al.*, 1975). The right ganglion pieces were distributed into 15 to 20 Leighton tubes, the left ones into 8—10 tubes. All were fed with medium CMRL-1415 supplemented with 10% foetal calf serum and antibiotics. The medium was replenished on the 3rd and 7th day of culture. The last medium sample was taken on the 10th day. The medium samples were assayed for virus in microplates (Dynatech AG) seeded with VERO cells (2×10^4 cells in 0.1 ml per well). Each sample was inoculated into 3 wells. Results were read after 3 days of incubation in a 3% CO_2 atmosphere at 37 °C.

Neutralizing antibody (NA) determination. Titres of complement non-requiring antibody were determined as described (Kutinová *et al.*, 1977). The KOS strain was used for the NA assay.

Examination of explants by immunofluorescence. Fragments from one ganglion were pooled, quickly frozen in liquid propan-butan, cut in a cryostat, fixed in acetone and stained by the indirect fluorescent antibody technique (Rajčáni *et al.*, 1977). The immune serum was prepared in rabbits immunized with HSV 1 virions purified by centrifugation in a Ficoll gradient (Matis *et al.*, 1975). Serum diluted 1 : 10 and adsorbed to uninfected rabbit brain suspension was used for immunofluorescence. The swine anti-rabbit conjugate (SwAR; SEVAC, Prague) was fractionated on DEAE-cellulose and the fraction revealing a molar fluorescein isothiocyanate to protein ratio of 1.5 was used for staining.

Results

Replication of HSV in the cornea of immunized and non-immune rabbits

The replication of the Kupka strain in the conjunctival sacs of immunized and control animals was followed by plaque assay. The mean titres were

Table 1. Frequency of virus activation in explants of trigeminal ganglia from non-immunized rabbits infected into the right scarified cornea (group I)

Rabbit No.	Days p.i.	Right ganglion days in culture 3	7	10	Left ganglion days in culture 3	7	10
155	115	1/18	10/18	8/18	0/6	0/6	0/6
156	115	0/18	5/18	7/18	0/6	0/6	0/6
161	108	0/19	1/19	1/19	0/6	0/6	0/6
162	108	1/20	8/20	8/20	0/6	0/6	0/6
200	92	0/20	13/20	13/20	0/10	0/10	0/10
201	92	0/20	5/20	ND	0/10	0/10	ND
202	95	0/20	15/20	3/20	0/10	1/10	1/10
204	95	0/20	10/20	6/20	0/10	1/10	1/10
72	47	3/18	3/18	ND	0/6	0/6	ND
Total		2/173 (1.2%)	70/173 (40.5%)	46/135 (34.1%)	0/70	2/70	2/54

ND = not done.
Numerator: number of positive fragments; denominator: total number of fragments tested.

calculated for each time interval in each animal group (Fig. 1). There were no marked differences between the titres found in the control rabbits (group I), those immunized with the whole virion vaccine (group II) and those immunized with the AM vaccine (group III). A comparison of the mean values by the t test showed that the values did not differ at a degree of significance $\alpha = 10\%$ ($P > 0.05$). Thus, the preceding systemic immunization did not influence virus replication in the cornea. No virus was isolated from the contralateral (uninfected) eyes.

Detection of latent virus infection in trigeminal ganglia of immunized and non-immune rabbits

Fragments from each right trigeminal ganglion were kept separately in 15—20 Leighton tubes, the left ganglia in 6—10 tubes. Medium samples removed on the 3rd, 7th and 10th day of culture were assayed for virus in VERO cells. Table 1 shows the incidence of virus-yielding fragments from ganglia of non-immune rabbits in which latency had been established. The majority of explants released the virus after 3 days in culture; only 2 out of a total of 70 virus-producing fragments shed HSV into the medium before the 3rd day. The proportion of virus-producing fragments decreased by day 10 in culture, possibly due to destruction of the ganglion tissue by the virus. The proportion of positive explants was the highest on the 10th day only in a single case (rabbit No. 156). The percentage of positive explants shown in Table 4 was calculated from those intervals, which revealed the maximal positivity rate.

Table 2. Frequency of virus activation in explants of trigeminal ganglia from rabbits immunized with the inactivated KOS strain and infected into the right scarified cornea (group II)

Rabbit No.	Days p.i.	Right ganglion days in culture			Left ganglion days in culture		
		3	7	10	3	7	10
140	53	0/18	1/18	3/18 FA	0/8	0/8	0/8
142	56	1/18	3/18	3/18 FA	0/8	0/8	0/8
143	54	0/18	1/18	1/18 FA	0/8	0/8	0/8
144	70	0/17	1/17	1/17 FA	0/8	0/8	0/8
195	84	0/20	0/20	4/20	0/10	0/10	0/10
196	84	0/20	2/20	4/20	0/10	0/10	0/10
197	88	0/20	3/20	3/20	0/10	0/10	0/10
198	88	0/20	2/20	2/20	0/10	0/10	0/10
199	158	0/20	2/20	4/20	0/10	0/10	0/10
Total		1/171 (0.6%)	15/171 (8.8%)	25/171 (14.6%)	0/82	0/82	0/82

FA = fluorescent antibody staining positive.
For other explanations see Table 1.

Table 2 shows the activation of latent virus in the explanted fragments from ganglia of animals immunized with the whole virion vaccine (group II). In contrast to the previous group, the proportion of virus-producing fragments was significantly lower. The number of positive medium samples on the 10th day was the highest or equal to that found on the 7th day in culture. No virus was shed from the explants of the contralateral ganglia. The average rate of 14.6% given in Table 4 was again calculated from intervals which revealed the highest proportion of positive samples.

The activation of HSV in explants of trigeminal ganglia from rabbits challenged after immunization with the AM vaccine is shown in Table 3. The reduction in the number of positive fragments was even more marked than in group II. Only 5.4% of cultured explants produced the virus. In 4 out of 13 animals no virus was isolated from the homolateral ganglia, although two of them revealed latency in the contralateral ganglion (Nos 147 and 172). In all but three animals (Nos 148, 173 and 194), the virus was shed by only a single fragment per ganglion. Such a degree of restriction of the latent infection was only exceptionally found in non-immunized rabbits (No. 161). The proportional incidence of latent infection in the three animal groups is shown in Table 4.

On day 10 after explantation, fragments from the same selected ganglia were pooled and examined for HSV 1 antigens by indirect immunofluorescence. As indicated in Tables 2 and 3, the fragments of all virus-producing ganglia were also positive for virus antigens, and vice versa. The HSV-specific antigens were seen in neurons and satellite cells. The occurrence of

Table 3. Frequency of virus activation in explants of trigeminal ganglia from rabbits immunized with AM and infected into the right scarified cornea (group III)

Rabbit No.	Days p.i.	Right ganglion days in culture 3	7	10	Left ganglion days in culture 3	7	10
145	74	0/18	1/18	1/18 FA	0/8	0/8	0/8
146	74	0/18	0/18	0/18 (FA)	0/8	0/8	0/8
147	80	0/18	0/18	0/18 (FA)	0/8	0/8	2/8
148	80	0/18	0/18	2/18 FA	0/8	0/8	0/8
171	164	0/18	1/19	1/18	0/8	0/8	0/8
172	164	0/15	0/15	0/15	0/8	2/8	4/8
173	68	0/20	2/20	ND	0/8	0/8	0/8
174	68	0/15	1/15	1/15	0/6	0/6	0/6
190	102	0/20	0/20	1/20	0/10	0/10	0/10
191	102	0/20	0/20	0/20	0/10	0/10	0/10
192	102	0/20	0/20	1/20	0/10	0/10	0/10
192	102	0/20	0/20	1/20	0/10	0/10	0/10
194	154	0/20	3/20	ND	0/10	0/10	0/10
Total		0/240	8/240 (3.3%)	8/200 (4%)	0/112	2/112	6/112

(FA) = fluorescent antibody staining negative, FA = fluorescent antibody staining positive
For other explanations see Table 1.

Table 4. Incidence of latent HSV infection as established in ganglia of immunized and control rabbits by inoculation into the right scarified cornea

Experimental group	Positive/total No. of rabbits RG	LG	Positive/total No. of ganglion fragments RG	LG
I — Control rabbits	9/9	2/9	72/173 (42.6%)	2/70 (2.8%)
II — Rabbits immunized with inactivated KOS strain	9/9	0/9	25/171	0/90
III — Rabbits immunized with the AM	9/13	2/13	13/240 (5.4%)	6/112
Total immunized	18/22 (81.8%)	2/22	38/411 (9.2%)	6/202 (2.9%)

RG and LG — right and left ganglion, respectively.

Table 5. Neutralizing antibodies in non-immunized and immunized rabbits before and after challenge with live HSV 1 and extent of latent infection in the right ganglion

Group	Rabbit No.	Neutralizing antibodies[1]) at challenge	Neutralizing antibodies[1]) at ganglion explantation	Extent of latent infection[2])
I	200	–	160	++++
	201	–	320	+++
	202	–	190	++++
	204	–	40	++++
			GMT 140	
II	140	226	113	++
	142	320	135	++
	144	381	113	+
	195	2168	226	++
	196	1812	226	++
	197	762	160	++
	198	1812	226	++
	199	762	67	++
		GMT 756	GMT 145	
III	145	762	56	+
	147	906	56	–
	148	1080	80	++
	171	906	270	+
	172	540	320	–
	173	270	160	+
	174	453	226	+
	190	1280	381	+
	191	1812	381	–
	192	1080	453	+
	193	1280	160	+
	194	906	ND	ND
		GMT 850	GMT 185	

[1]) Reciprocal of the highest serum dilution still inhibiting viral cytopathic effect in 50% of cultures; GMT = geometric mean titre.

[2]) –, +, ++, +++, ++++: no, <10%, 10–20%, 21–50%, >50% of explants positive, respectively.

antigen-positive cells in sections was rather focal and confined to single sections. This was in good agreement with the sporadic virus release from single ganglion fragments.

Results of neutralizing antibody determination

NA were found in the majority of animals (Table 5). At the time of challenge, the NA titres were comparable in both immunized animal groups. During the period of latency, the titres dropped markedly. Again, there was significant difference between groups II and III. In addition, the geometric

mean titres, as determined in these rabbits, were comparable to those found in non-immune rabbits after virus inoculation (group I). There was no apparent association between the extent of established latency and the actual level of NA prior challenge or at the end of the experiment.

Discussion

The molecular basis for latency and the role of the immune response in development of the recurrent herpetic lesions are still not fully understood. It has been established beyond doubt that HSV persists in the regional sensory ganglia (reviewed by Stevens, 1975; Baringer, 1975; Klein, 1976). In the course of primary infection, the virus enters the nerve endings and quickly migrates along the nerve to the body of the neuron (Cook and Stevens, 1973). In the present model, HSV could be recovered as soon as 16—20 hr p. i. from the homolateral ganglion explants (Rajčáni and Čiampor, 1978). Single cells, mainly neurons but also some satellites, positive for virus antigens between 2—6 days p. i. could be found in serial sections of the uncultured ganglia. Later on, the probability of finding such neurons decreases, although occasional neurons containing virus particles can be found by electron microscopy in sections of the ganglia of some rabbits with established latent infection (Baringer and Swoveland, 1974). Attempts to isolate the virus directly from the ganglion usually fail; only if highly sensitive neuron cultures are used as indicator cells, infectious virus can be directly recovered from some ganglion homogenates (Schwartz *et al.*, 1978). Even in this system, the probability of virus recovery from the homogenate decreases with time. As shown by Yamamoto *et al.* (1977), the virus-specific thymidine kinase activity decreases to undetectable levels in the ganglion suspension within a month p. i. The failure to demonstrate viral mRNA in the latently infected ganglion tissue also strongly indicates that the nonproductive persistence of the genome rather than continuous virus replication in single hidden cells is the basis of latency (Puga *et al.*, 1978).

The culturing of the ganglion explants seems to be the only way how to detect the virus carrier state in the ganglion tissue. We took the advantage of a semiquantitative modification of this technique. It has turned out that more than 40% of all ganglion fragments became virus producers between days 3—10 in culture. This confirms the notion that cells habouring the "silent" genome are evenly distributed in the ganglion tissue. Although the replication of HSV in the cornea was not influenced by previous immunization, the development of the virus carrier state was markedly suppressed by this procedure. The capability of the AM vaccine to restrict the extent of latency was higher than that of the whole virion vaccine. The conditions of the experiment, however, did not permit a full comparison of the efficiency of both vaccines.

As previously reported, some rabbits develop latency also in the contralateral trigeminal ganglion (Rajčáni *et al.*, 1977). This occurs probably due to the presence of contralateral nerve connections in the peripheral trigeminal

system. The two animals which developed latency in the contralateral but not in the homolateral ganglion, deserve special attention, but we cannot offer any reasonable explanation of this observation. In both these rabbits, medium levels of NA were detected at the time of infection, while at the time of explantation one revealed a NA level higher than the other. Thus, if there was a special course of infection which resulted in an atypical virus distribution, this was not reflected by the circulating NA titres. This is in agreement with the finding that the extent of the carrier state restriction apparently does not depend only on the actual serum NA titre. Such an interpretation of our findings is not contradictory to the well documented protecting effect of antibodies on the spread of HSV 2 from the inoculation site (Oakes and Rosembund-Hornbeak, 1978). Immune IgG prevents activation of latent infection in the ganglion both in vivo (Stevens and Cook, 1974; Lehner *et al.*, 1975) and in vitro (Rajčáni *et al.*, 1977). It seems that local accumulation of antibody molecules rather than circulating antibody prevents virus particles from entering nerve endings and establishing latency. The role of cell-mediated immunity in the control of HSV infection was repeatedly demonstrated (Mori *et al.*, 1967; Oakes, 1975; Roger-Zisman and Allison, 1976; Nagafuchi *et al.*, 1979). The accumulation of immunocompetent cells in the regional lymph nodes and the local traffic of T lymphocytes specifically responding to the antigenic stimulus are also related to the virus inoculation site (Jacobs *et al.*, 1976).

The influence of local virus multiplication at the site of primary infection on the penetration of HSV into the nerve endings in the course of a secondary challenge was demonstrated in a series of experiments (Klein *et al.*, 1978). HSV-infected mice with evidence of latency in spinal ganglia are protected against reinfection into both the hind footpad and the oro-facial area. Mice topically treated with phosphonoacetic acid, however, become resistant only to reinfection performed at the same inoculation site. If the virus is reinoculated at a distinct site, these mice develop latent infection in the correspondung regional sensory ganglion.

Our semiquantitative approach to the testing of the relative extent of the "latent" infection established in the regional sensory ganglion seems to be a suitable tool for scoring of the degree of protection, conferred to the experimental animal by previous immunization.

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