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Colonization of Murine Ganglia by a Superinfecting Strain of Herpes Simplex Virus

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We report on the colonization of murine trigeminal ganglia after sequential infection of mice by herpes simplex viruses (HSVs). In preliminary studies, we have established that whereas the HSV-1(F) strain efficiently colonizes ganglia when inoculated by either the ear or eye routes, the HSV-1 × HSV-2 recombinant C7D colonizes ganglia when inoculated by the eye route only. The experimental design consisted of inoculating the right eye with C7D on day 1 and with HSV-1(F) in both left and right eyes on day 26. Both right and left trigeminal ganglia were removed and analyzed independently for latent virus on day 52. Our studies indicate that HSV-1(F) viruses were recovered from all left trigeminal ganglia but from only a small number of right trigeminal ganglia. Some right trigeminal ganglia yielded no viruses, whereas others yielded both C7D and HSV-1(F) viruses identified on the basis of plaque morphology and restriction enzyme cleavage patterns of viral DNA. The results indicate that more than one virus may colonize the same ganglion and that trigeminal ganglia may be protected from colonization by a superinfecting virus by determinants acting at a local level in the absence of demonstrable virus.

The ability of herpes simplex virus 1 and 2 (HSV-1 and HSV-2) to remain latent and cause recurrent lesions underlies in large part the physical and emotional trauma associated with HSV infections in humans. The viral and host factors which determine the establishment of latency are a major focus of research on these viruses. We report three observations: (i) the capacity of HSV to establish latency in the mouse may be dependent on the route of inoculation, (ii) more than one HSV strain may reside in latent form in the same ganglion, and (iii) HSV may induce a state of resistance to colonization to the sensory ganglion innervating the site of inoculation and independent of demonstrable levels of latent virus.

The circumstances which led to our studies are as follows. Previous studies in experimental animals have shown that prior immunization with one strain of HSV precluded or reduced the probability of establishment of latent virus by a second infecting virus of at least the same serotype (2, 12, 17). In apparent accord with these conclusions, analyses of viruses recovered from human ganglia postmortem by restriction endonuclease indicated that only one virus can be recovered from a colonized sensory ganglion (10). Further support was obtained from studies showing that only the first infecting virus established latency in rabbits inoculated sequentially with marked HSV-1 strains (2). Nevertheless, these observations are not consistent with the observation that humans may be infected with more than one strain of the same serotype (1, 25). The results presented in this report were obtained from studies designed initially to determine whether it is possible to colonize mouse sensory ganglia with more than one strain of HSV.

MATERIALS AND METHODS

Virus and cells. The virus strains used in these studies were HSV-1(F) (3) and HSV-1 × HSV-2 recombinants C7D and D1E1 described in detail elsewhere (14, 15). These recombinants were selected because of diminished capacity to cause lethal infections and latency in the mouse (19). Virus stocks were made either in HEp-2 [HSV-1(F)] or Vero cells (C7D and D1E1) overlayed with mixture 199 supplemented with 1% inactivated calf serum. The cells were propagated in Dulbecco modified minimal essential medium of Eagle, supplemented with 10% fetal calf serum.

Mice and methods of infection. Six- to eight-week-old BALb/c AnN mice (Goodwin Institute for Cancer Research, Plantation, Fla.) were used in all experiments. For inoculation, the animals were anesthetized with sodium pentobarbital by intraperitoneal injection.

Eye inoculation was done by scarifying the cornea with an hypodermic needle and bathing the eye with about 10 μl of viral suspension. Virus was allowed to

adsorb for 10 to 30 min while the mice remained anesthetized.

Ear inoculation was done by injecting 10 μl of virus suspension into the right pinna.

Assay of virus from ganglia. Mice were anesthetized with sodium pentobarbitol and exsanguinated. Appropriate ganglia were immediately removed and processed. Virus assays were based on the general consensus that during acute phase of the infection (less than 14 days), infectious virus could be recovered from ganglia both by homogenization and explantation, whereas during the latent phase of infection (21 days postinfection and later) only latent virus could be recovered by explantation of the ganglia (26). To assay infectious (nonlatent) virus, ganglia were homogenized in 0.5 ml of maintenance medium in a Belco tissue grinder; the suspension was subjected to three cycles of freezing and thawing and transferred on Vero cells (25-cm^2 plastic flasks). After a 1-h adsorption at 37°C on a rotator shaker, the inoculum was removed, maintenance medium was added, and the flasks were incubated at 37°C. Cultures were examined daily for 7 days for cytopathic effects characteristic of HSV-infected cells.

Assay of latent virus. Ganglia were first incubated 4 or 5 days in 0.5 ml of maintenance medium at 37°C equilibrated with 4% CO_2. They were then homogenized as above and the suspension transferred on Vero cells. Adsorption, medium addition, incubation, and examination were done as above (26).

When necessary for better detection, and characterization of the cytopathic effects, Vero cells were fixed with methanol, stained with Giemsa stain, and air dried.

Plaque purification. Clones were isolated by two rounds of subcultures from plaques developed under liquid overlay consisting of mixture 199 supplemented with 1% calf serum and 0.1% of standard immune serum globulin (3).

Analyses of the restriction enzyme patterns of HSV DNAs. DNA was extracted from roller bottle cultures of HEp-2 cell infected at a multiplicity of 0.01 PFU-/cell, purified by gradient centrifugation, cleaved with restriction endonucleases, and subjected to electrophoresis in agarose gels as described elsewhere (1, 14, 25).

Immune precipitation of [^{35}S]methionine-labeled soluble HSV-1 proteins. Vero cells propagated as monolayers in 150-cm^2 plastic flasks were infected with HSV-1(F) at a multiplicity of infection of 5 PFU/cell. After 1 h of adsorption virus was aspirated and the cells were overlayed with 10 ml of minimal essential medium with 1% fetal calf serum. At 6 h postinfection, the medium was removed and 10 ml of methionine-free labeling medium with 1% FCS and supplemented with 20 μCi of [^{35}S]methionine (New England Nuclear Corp. Boston, Mass.; specific activity, <400 Ci/mmol, Dreieich, Germany) per ml was added. After 2 h of labeling, the cells were washed in PBS and scraped off the flask. The cells were pelleted and resuspended in 0.8 ml of 0.18 M Tris–0.076 M barbital buffer, pH 8.6, with 1% Triton X-100 and 10^{-5} M of TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone) and TLCK (*N*-α-*p*-tosyl-L-lysine chloromethyl ketone-hydrochloride), respectively. The soluble proteins were prepared by sonication of the cells and clarification of the suspension by centrifugation at 100,000 × *g* for 1 h at 4°C in a SW60 rotor (16). A 25-μl volume of the soluble antigens were absorbed for 30 min at 4°C with a pellet of formalin-fixed staphylococcus (strain Cowan I) obtained from 25 μl of a 10% (vol/vol) suspension of the bacteria. The fixation of the staphylococcus was done according to the procedure of Kessler (6). After removal of the bacteria the supernatant was incubated with 50 μl of pooled serum from exsanguinated mice for 1 h at 4°C. The immune complexes were pelleted after binding to fixed staphylococcus coated with rabbit-anti mouse IgG (DAKO, Copenhagen) as previously described (19).

The pellet was washed extensively and the precipitated HSV proteins were solubilized in 50 μl of disruption mixture (15). The proteins were electrophoretically separated in 9.25% (wt/vol) sodium dodecyl sulfatepolyacrylamide gels which were stained and processed for autoradiography as described by Morse et al. (15). Exposure time was 10 days on a XPR-1 Kodak film.

RESULTS

Selection and characteristics of the virus strains. The virus strains used in these studies were selected for their low virulence, ability to establish latent infections, differential plaque morphology, and restriction enzyme patterns of their DNA. Specifically, HSV-1(F), a wild-type HSV-1 strain has a PFU/50% lethal dose (LD_{50}) ratio of 30 by intracerebral inoculation (19). In cell cultures, it causes infected cells to round up and clump (3). The virus is relatively nonneurovirulent by peripheral routes as illustrated by the observation (Table 1) that all mice given 10^7 PFU by the ear or eye routes survived infection. Inoculation of the virus into the pinnae of ears caused intense erythema and edema in 1 day, small vesicles within 3 to 4 days, and yellow crusts on day 6. Healing began about 20 days after inoculation. Infection of eyes with HSV-1(F) resulted in severe conjunctivitis in 2 to 3 days; the infection was characterized by photophobia, edema, and progressively expanding crusts on the lids. Extension of lesions to the nose was not uncommon. Healing took place during the week 3 after inoculation.

TABLE 1. Role of the route of inoculation in the establishment of latent virus in the mouse trigeminal ganglia

Virus	PFU/ mouse	Eye-inoculated		Ear-inoculated
		Mice yielding infectious virus/total tested	Mice yielding latent virus/total tested	Mice yielding latent virus/total tested
HSV-1(F)	1×10^7	0/5	5/5	10/10
C7D	2×10^7	0/10	12/20	0/9
D1E1	2×10^6	ND[a]	0/10	0/9

[a] ND, Not determined.

The PFU/LD_{50} ratio by the intracerebral route of the HSV-1 × HSV-2 recombinant C7D was shown to be 6 × 10^5 (19). The virus causes the infected cells to fuse into polycaryocytes and the *syn*$^-$ phenotype is readily differentiated from the *syn*$^+$ phenotype of HSV-1(F) plaques. The virus was also relatively free of neurovirulence by the peripheral route. Inoculation into ears resulted in some erythema of the pinna between days 1 and 20 in all animals, but no crusts ensued. In eyes, C7D produced only mild conjunctivitis in half of the inoculated animals, although all mice showed some signs of photophobia between 3 and 10 days postinoculation. The PFU/LD_{50} ratio by the intracerebral route of the HSV-1 × HSV-2 recombinant D1E1 was 1.3 × 10^6 (19).

Another characteristic which we considered important in the selection of strains for these studies was based on the preliminary studies which showed that the immune response of mice inoculated wtih C7D was lower than that of mice inoculated with wild-type strains and other HSV-1 × HSV-2 recombinants. This observation is discussed in greater detail below.

Establishment of latent infection depends in part on the route of inoculation. Preliminary experiments have shown that in a number of relatively avirulent HSV-1 × HSV-2 recombinants, the establishment of latency in the trigeminal ganglion of the mouse was determined in part by the route of inoculation (Table 1). In this experiment, mice were inoculated into the right ear or eye. At days 26 to 33, the mice were sacrificed, and ganglia were removed and assayed for latent virus as described in above. In the case of mice infected by the ear route, the second, third, and fourth right cervical ganglia were pooled and examined for latent virus. Virus was readily recovered from mice inoculated with HSV-1(F) but not from those inoculated with the recombinants C7D or D1E1. To ensure that the ganglia were negative, the Vero cell cultures inoculated with suspended ganglionic cells were harvested after 7 days, frozen, thawed, and subcultured on fresh Vero cell culture. A further 7-day period of observation failed to detect virus.

Trigeminal ganglia from mice infected by the eye route were assayed for both infectious virus and latent virus. As shown in Table 1, latent, but not infectious, virus was detected. Furthermore, latent virus was recovered from ganglia of mice infected with HSV-1(F) and C7D but not with recombinant D1E1.

It should be noted that HSV-1(F) established a latent infection in 100% of the mice tested, whereas C7D established latent infection in approximately 50 to 70% of mice. It is also of interest that Vero cell monolayer cultures inoculated with suspensions of ganglionic cells carrying latent HSV-1(F) developed approximately 100 to 1,000 plaques. In contrast, cultures inoculated with suspensions of ganglionic cells carrying latent C7D developed approximately 10 plaques each.

Latent infection with recombinant strain C7D does not preclude establishment of latent infection with HSV-(F). In the first experiment in this series, 20 mice were infected in the right eye with 2 × 10^7 PFU of recombinant C7D per mouse. At 26 days, 10 of these mice (group A) were inoculated in both eyes with 10^7 PFU of HSV-1(F) per mouse. The remaining 10 mice (group B) were kept as a control. At the same time, 7 previously uninoculated mice were given 10^7 PFU of HSV-1(F) per mouse in both eyes (group C). All animals were sacrificed 26 days later. The left and right trigeminal ganglia were removed and individually assayed for the presence of latent virus.

These studies showed that all 7 mice inoculated with HSV-1(F) (group C) retained the virus in latent form. Among the 10 mice (group B) inoculated with C7D, only 1 mouse carried latent virus in the right (ipsolateral) ganglion 52 days after inoculation. Among the 10 mice inoculated with both C7D and HSV-1(F) (group A), 5 mice carried in latent state both HSV-1(F) and C7D as judged by plaque morphology (Table 2). From one of these, C7D was isolated from both left and right trigeminal ganglia. Of particular interest is the observation that 4 of 10 right trigeminal ganglia were negative for both HSV-1(F) and C7D, even though all left ganglia yielded HSV-1(F).

It is of interest also that when the suspension of ganglionic cells yielded both *syn*$^+$ and *syn*$^-$ plaques, the wild-type HSV-1(F) *syn*$^+$ plaques outnumbered the *syn*$^-$ plaques of C7D by a factor ranging from 10 to 100. To confirm the morphologic identification of HSV-1(F) and

TABLE 2. Recovery of virus from trigeminal ganglia of mice 5 days after eye inoculation with recombinant C7D and 26 days after inoculation of HSV-1(F)

Virus isolated	No. of positive ganglia	
	left	right
HSV-1(F) only	9/10	0/10
C7D only	0/10	1/10
C7D and HSV-1(F)	1/10	5/10
Negative	0/10	4/10
HAV-1(F) only	15/10	5/10
C7D only	0/10	0/10
C7D and HSV-1(F)	0/10	3/10
Negative	0/10	7/10

C7D, the syn^- and syn^+ plaques were harvested, and the virus was plaque purified. The electrophoretic patterns of the viral DNA (Fig. 1) extracted from infected Vero cell cultures and digested with restriction endonucleases confirmed the identity of syn^+ and syn^- plaque-forming viruses as HSV-1(F) and C7D, respectively.

In the second experiment in this series, 50 mice were inoculated into the right eye with 2×10^7 PFU of C7D per mouse. At 26 days, 30 mice (group A) were inoculated in both eyes with 10^7 PFU of HSV-1(F) per mouse. These mice were sacrificed 26 days later; the left and right ganglia were removed and individually tested for infectious virus (15 mice, group A) and latent virus (15 mice, group B). Of the remaining 20 mice inoculated with C7D, 10 mice each were sacrificed 26 days (group C) and 52 days (group D) after inoculation; the left and right trigeminal ganglia were removed and tested for the presence of latent C7D virus.

The results of this experiment were as follows. (i) No virus was isolated from any of the ganglia of 15 mice tested for the presence of infectious virus (group A). (ii) Latent C7D virus was isolated from right (ipsolateral) ganglia of 3 mice at 26 days after eye inoculation (group C) and from the right ganglion of one mouse 52 days after eye inoculation (group D). (iii) Among the mice inoculated with both C7D and HSV-1(F) (group B), all 15 mice yielded HSV-1(F) from the left ganglion, but only 3 mice yielded both virus from the right ganglion and furthermore, no virus was isolated from 7 of the 15 right trigeminal ganglia.

It is of interest that the severity of the lesion after challenging with HSV-1(F) varied depending on inoculation history. Thus, the lesions in the right eye which had been previously inoculated with C7D were milder than those in the left eye.

Immune response of mice inoculated with HSV-1(F), C7D, and D1E1. Mice inoculated with HSV-1(F) by either the eye or the ear route made precipitating antibodies to nearly all of the antigens available for immune precipitation (Fig. 2, lanes 2 and 3). Among the strong immunogens were the glycoproteins gA, gB, gC and gD, and the infected cell polypeptides (ICP) 5, 35, 36, 43, and 44.

Recombinants C7D and D1E1 were less immunogenic than HSV-1(F). Furthermore the induction of antibody was less effective by the eye route than by the ear route (Fig. 2, lanes 5 through 8). The glycoproteins gC and gD as well as the polypeptides ICP 43 and 44 were not detected in the precipitates obtained with sera from mice infected by the eye route with either C7D or D1E1 (Fig. 2, lanes 5, 7).

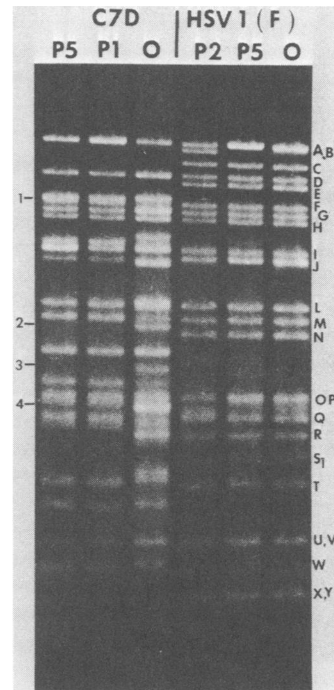

FIG. 1. Photograph of C7D and HSV-1(F) DNAs digested with *BamHi* restriction endonuclease and electrophoretically separated in agarose gels. The procedures for purification of HSV DNA, digestion with restriction endonucleases, electrophoresis in agarose gels, and staining with ethidium bromide were as cited in the text. The lanes marked C7D-0 and HSV-1(F)-0 contain digests of DNAs extracted from cells infected with parental viruses. All other lanes contain digests of viral DNAs derived from viruses contained in a latently infected right trigeminal ganglion. Briefly, 5 syn^- and 5 syn^+ plaques were harvested from one Vero monolayer culture seeded with virus derived from a latently infected trigeminal ganglion; this ganglion was removed from a mouse inoculated in the right eye 52 days earlier with C7D and 26 days earlier in both eyes with HSV-1(F). The virus contained in each plaque was plaque purified in Vero cells twice more before virus stocks were made. The electrophoretic patterns of the DNAs extracted from HSV-1(F) plaques no. 2 (P2) and no. 5 (P5) were similar to that of the parent HSV-1(F) except for the electrophoretic mobility of the *BamHi* B fragment. As noted elsewhere, the *BamHi* B DNA fragment varies in electrophoretic mobility (18, 19). Virus obtained from C7D plaques no. 1 and 5 (P1 and P5) resemble the parent virus with respect to the syn phenotype but differ with respect to the mobility of several DNA bands. The bands numbered 1, 2, and 3 correspond to terminal, junction, and subterminal fragments which tend to vary with respect to electrophoretic mobility (8, 11, 18, 22, 25; B. Roizman and M. Tognon, Curr. Top. Microbiol. Immunol., in press). Consistent with the conclusion of others (11), one variant of C7D and HSV-1(F) was the predominant isolate from the latently infected ganglia.

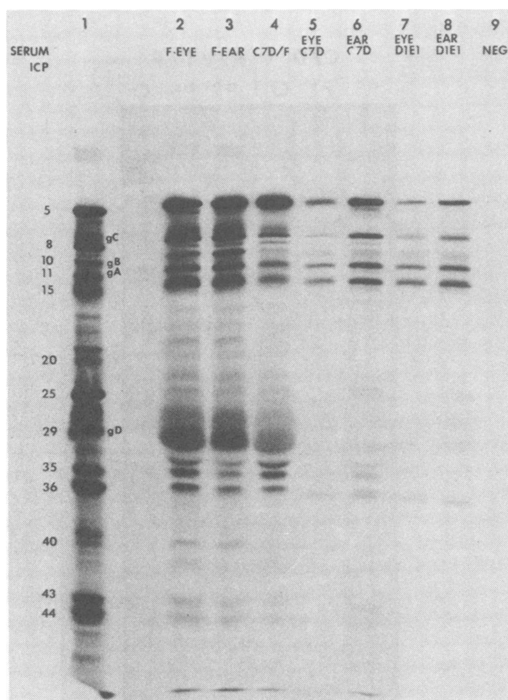

FIG. 2. Autoradiographic images of HSV-1 polypeptides electrophoretically separated in polyacrylamide gels. Lysates of Vero cells infected with HSV-1 (F) labeled with [^{35}S]methionine from 6 to 8 h postinfection were clarified by high-speed centrifugation and then reacted with pooled murine sera as described in the text. Lane 1, high speed supernatant fluid containing the labeled HSV-1(F) polypeptides available for precipitation. Electrophoretic profile of HSV-1(F) polypeptides precipitated by immune sera are shown in lanes 2 to 8; no visible precipitation was obtained with serum from uninfected mice (lane 9).

The pattern of viral polypeptides immune precipitated by sera from mice infected sequentially with C7D and HSV-1(F) was similar to that obtained with serum from mice infected with only HSV-1(F), except that the amounts of gC and ICP 10 and 40 were reduced.

DISCUSSION

The salient features of the results presented in this report are as follows: (i) both viral genotype and the route of inoculation determine the establishment of latency in the mouse, (ii) more than one HSV strain can establish latency in the same ganglion, and (iii) prior inoculation of virus may prevent subsequent colonization of the ganglion which innervates the sites of inoculation even though no latent virus can be detected after the first inoculation. We consider the significance of each of these points separately.

Determinants of the capacity to establish latency by HSVs. That viral genetic determinants play a significant role in the establishment of latency has been previously well established through the use of *ts* (9, 13, 21) and of thymidine kinase-deficient mutants (4, 5, 23, 24) which may have reduced capacity to establish latency. Whereas the failure of at least some *ts* mutants to establish latency has been attributed to a mutation in a gene required for that purpose (9, 13), the reduced capacity of thymidine kinase-deficient mutants to establish latency has been attributed to their inability to multiply to a sufficiently high level at the site of inoculation or in the ganglion (9). We show that viral genotypes and routes of inoculation are interactive determinants for the establishment of latency. Two hypotheses which could explain the establishment of latency after inoculation of C7D by the eye and not by the ear routes are a higher level of multiplication in the eye or a shorter route to the ganglion. The use of viruses differing from each other in multiple characteristics and the experimental design of these studies precluded a differentiation among these alternatives. It should be noted, however, that if the immune response reflects the amount of antigen produced during virus multiplication, then the extent of multiplication of C7D was similar to that of D1E1. It would also follow that the differences between C7D and D1E1 with respect to their abilities to establish latency are not related to the extent of viral multiplication.

The role of prior virus infection in the establishment of latency by superinfecting virus. Previous studies have established that only one HSV strain defined by a single restriction endonuclease pattern can be recovered from human ganglia. These studies proposed the hypothesis that the first virus to colonize a ganglion prevents another virus from entering it (10). Consistent with this hypothesis, Centifanto-Fitzgerald et al. (2) observed that only one infecting virus can be recovered from latently infected ganglia of experimental animals sequentially inoculated with two marked strains. The objectives of the current studies were to investigate some of the parameters of the apparent exclusion of a second virus by a first infecting virus. To accomplish this aim, we chose for the first infecting virus an HSV-1 × HSV-2 recombinant with a reduced capacity to establish latency or cause lethal infection. For the second virus we chose a wild type with reduced peripheral pathogenicity and which under the conditions tested established latency in 100% of the animals. The significant conclusions of our studies are as follows.

(i) The first infecting virus can induce a state of resistance to colonization of the ganglion by a second infecting virus. The state of resistance was confined specifically to the ganglion which

innervated the site of inoculation with the first virus and not to the one on the opposite, previously uninoculated site. Thus, in mice previously infected with C7D in the right eye, HSV-1(F) colonized all left trigeminal ganglia in both experiments but only approximately half of the right trigeminal ganglia. Whereas the nature of the resistance is not known, the data argue that it is not disseminated throughout the body but acts at the site of inoculation or along the route to the ganglion of the first infecting virus to prevent establishment of latency. In this respect the state of resistance described in this report differs from the immunity to establishment of latency by immunization at anatomically unrelated sites (2, 12, 17). It is of interest also that the severity of the lesion caused by HSV-1(F) varied depending on the eye; the lesion was more severe in the peviously uninoculated left eye than in the right eye which had been previously inoculated with C7D. One possible explanation for the reduced frequency of HSV-1(F) isolation from the right trigeminal ganglion and the less severe lesions induced by HSV-1(F) in this eye is the alteration of the clinical course of infection by immunoglobulin A induced by the prior C7D infection.

(ii) There is no absolute or categorical condition which precludes two viruses from residing in the same ganglion simultaneously even though they enter by the body sequentially. We interpret our observations to indicate that the state of resistance induced by the first infecting virus was inadequate or insufficient to preclude the colonization of the ganglion by the second virus.

We cannot at this time define the mechanism or describe the requirements for the establishment of the resistant state. However, the experimental design employed in this study allows the operational definition of the resistant state and should enable its elucidation. It should be noted, however, that irrespective of its mechanism of action, the resistance of a ganglion to colonization from a previously inoculated site bears directly on the "round trip" hypothesis proposed by Klein (7) to explain maintenance of virus in ganglia notwithstanding repeated activation of the virus. The hypothesis envisions that upon activation in the ganglion the virus is transported to the portal of entry (mucocutaneous junction of the lip, cornea, genitals, etc.) where the virus multiplies. The progeny virus then colonizes de novo the ganglion. The accumulated evidence on the state of resistance to specific colonization of ganglia previously exposed to the virus suggest that either the hypothesis is trivial or patients with frequent recurrent infections are incapable of establishing a resistant state.

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