

Reactivation of Latent Herpes Simplex Virus After Pneumococcal Pneumonia in Mice

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Received for publication 13 November 1974

In attempts to reactivate latent herpes simplex virus, we instilled *Diplococcus pneumoniae* intratracheally into mice harboring latent infections in sacrosiatic spinal ganglia. All mice developed a severe pneumonia within 24 h and were given penicillin therapy. Representative mice that survived the pneumonia were sacrificed at daily intervals, and appropriate tissues were examined for evidence of viral reactivation. Herpes simplex virus was reactivated in the ganglia and appeared to travel both proximally and distally in associated nerve trunks. Clinically apparent disease due to the virus was not detected in any mice.

As we have discussed in considerable detail elsewhere (J. G. Stevens, *Curr. Top. Microbiol. Immunol.*, in press), an attractive, general hypothesis explaining recurrent herpetic disease involves initial infection and replication of herpes simplex virus (HSV) in the skin, mucous membrane, or eye, with subsequent centripetal passage in sensory nerves to corresponding ganglia where a latent infection is established. After "reactivation" by one of many diverse stimuli, the virus would then pass centrifugally in the sensory nerve, ultimately reaching the surface where lesions would again be produced. To establish the validity of this hypothesis, three phenomena must be shown to occur. First, and most importantly, after infection of tissues at the body surface, virus must be shown to reach and then to be selectively harbored in sensory ganglia. Second, the infection must be demonstrated to travel to and from the ganglia in corresponding nerve trunks. Finally, a defined manipulation of the experimental animal must be followed by reactivation of virus and subsequent reappearance of lesions on the external surface.

In this laboratory, mice have served as most useful animals for study of this hypothesis. In this system, we have been able to show that, after cutaneous infection, virus travels in nerves and induces latent infections in corresponding sensory ganglia (5, 8). In addition, it seems quite likely that the neuronal route taken is axonal and that the virus is harbored in neurons (4, 5). Present evidence also suggests that the latent infection is maintained by conservation of nonreplicating virus (J. G. Stevens, in press) and that the latent infection is somehow modu-

lated by antiviral immunoglobulin G (J. G. Stevens and M. L. Cook, *J. Immunol.*, in press). The findings concerning latency in sensory ganglia have now been generalized and extended to another experimental animal (2, 9) and, most importantly, to natural infections in man (1, 3, 7).

In this communication, we deal with the unresolved phenomenon of reactivation and show that after severe pneumococcal pneumonia, infectious virus is reactivated from murine sacro-sciatic spinal ganglia and subsequently travels both centripetally and centrifugally in the associated nerves. As yet, however, clinically apparent cutaneous (or neurologic) disease has not been demonstrated.

MATERIALS AND METHODS

Virus and bacteria. The prototype HSV type I strain (MacIntyre) used throughout this investigation has been described previously (5). In the initial experiments, virus previously passed 17 to 23 times in mouse brains was used as the inoculum. Later, after it was found that equivalent results could be obtained with virus stocks prepared by standard methods in RK₁₃ cell monolayer cultures, all inocula were prepared by this more simple method.

The *Diplococcus pneumoniae* employed was a type III strain kindly supplied by M. J. Pickett, Dept. of Bacteriology, University of California, Los Angeles. Upon receipt, it was inoculated intraperitoneally, reisolated from the blood of mice four successive times, and then maintained for less than four passages on blood agar. Inocula for mice consisted of 24 h cultures of the organism in brain-heart infusion broth containing 10% newborn calf serum.

Mice and viral inoculation. Four-week-old outbred Swiss Webster mice obtained from local suppliers were used throughout. Latent HSV infections

were established by application of about 5×10^3 RK₁₃ cell plaque-forming units of virus to scarified, inflamed rear footpads. As has been detailed elsewhere (5, 8), all mice surviving the central nervous system disease that follows this procedure harbor latent virus in sacrosclatiac spinal ganglia.

Bacterial inoculation and sequelae. Mice with latent herpetic infections were anesthetized with pentobarbital, and the trachea was exposed surgically and then instilled with approximately 5×10^7 bacteria in 0.025 ml of brain-heart infusion broth. As will be noted, in early experiments the inoculum also contained 3% gastric mucin. The surgical wound was then closed with wound clips (9-mm autoclips, Clay Adams, Parsippany, N.J.) and mice were allowed to recover from the anesthesia. About 24 h after this procedure was completed, all mice developed an extensive lobar pneumonia with exudative pleuritis and pericarditis from which they rapidly succumbed unless penicillin therapy was instituted. Therefore, at this time and twice daily thereafter until termination of the experiment, 5,000 U of aqueous penicillin was given intraperitoneally to all animals. In the usual experiment, about 15% of mice died during or soon after bacterial inoculation, and about 20% of those remaining died as a result of the bacterial infection.

Direct virus isolation from ganglia. Spinal ganglia from the infected side of each mouse were ground together in 0.75 ml of Eagle minimal essential medium with 5% fetal calf serum in a Ten Broeck homogenizer and inoculated directly on RK₁₃ cell monolayer cultures in 2-oz (ca. 60 ml) French square bottles. After a 1-h adsorption period, 5 ml of the same medium was added, and the cultures were incubated at 37 C, scored daily for viral-induced cytopathic effects, and finally discarded after 4 days. This method of employing smaller volumes and inoculation of the entire homogenate was done to increase the sensitivity of our previous assays for infectious virus (8). Possible artifacts due to reactivation of virus from neurons that may not have been destroyed by homogenization were obviated by termination of the assay at day 4. This was justified since, in our previous assays for viral induction in ganglia from hundreds of latently infected mice, we never observed a positive culture before 6 days of incubation. Therefore, any virus detected before 4 days could be considered to be the progeny of infectious virus present in high concentration in the ganglia at the time of explant. When viral cytopathic effects were suspected, a portion of the supernatant fluid was frozen at -70 C for further study.

Co-cultivation of tissues. Tissues to be co-cultivated were chopped into small pieces with iris scissors and co-cultivated with monolayer cultures of RK₁₃ cells. These cultures were maintained for 25 days and fed intermittently with minimal essential medium supplemented with 5% fetal calf serum. As with the ganglionic cultures, cultures suspected of having viral-induced cytopathic effects were frozen for further study.

In situ hybridization techniques. For in situ nucleic acid hybridization experiments, HSV deoxyribonucleic acid (DNA) was purified and used to

prepare radioactive complementary ribonucleic acid, which was subsequently applied to sections of ganglia. Our method of preparing this ribonucleic acid, employing it to detect viral DNA in sections, and establishment of its specificity have been detailed elsewhere (4).

Serological identification of viral isolates. Each of eight viral isolates taken at random in these experiments was identified as HSV by the immunological neutralization methods described earlier (4).

RESULTS

Reactivation of latent infection. In the initial experiments, mice harboring a latent infection in sacrosclatiac spinal ganglia were instilled intratracheally with *D. pneumoniae* suspended in 3% mucin. At daily intervals, representative animals were sacrificed and appropriate tissues were removed and processed. Here, the sacrosclatiac spinal ganglia were either subjected to in situ hybridization methods specific for viral DNA or ground and assayed for infectious virus. The appropriate proximal and distal nerve roots, the sciatic nerve trunk, and the skin from the sole of the foot were chopped and co-cultivated separately with RK₁₃ cell monolayers. Pooled data from five experiments (Table 1) show that the procedure results in an increase in the numbers of neurons in which viral DNA can be detected and an induction of infectious virus in the ganglia early after pneumonia developed. Of equal significance is the later marked increase in the number of animals from which infectious virus can be recovered by co-cultivation of proximal and distal nerve roots or the sciatic nerve, with the greatest percentage (39%) reached on day 4. In this table, results from the three segments of the peripheral sacrosclatiac nerve are pooled, so that if virus was recovered from any one of them, the animal was scored as positive. When the individual segments from this group and those reported in the next section were scored separately and these totals were compared, 53% of the positive tissues consisted of distal roots and 17% were proximal roots. Sciatic nerves accounted for 19%, and various combinations of the three segments made up the remaining 11%. Infectious virus was never recovered from skin of the soles of the feet by these techniques of co-cultivation.

From these experiments, it can be concluded that pneumonia induced by pneumococci suspended in mucin results in synthesis of nascent viral DNA and infectious virus in ganglia latently infected with HSV. When the procedure is followed by co-cultivation techniques involving the corresponding nerves, a significant number of nerves then replicate infectious virus. The

TABLE 1. *Reactivation of latent HSV in sacrosciatic spinal ganglia of mice ill with pneumococcal pneumonia^a*

Day	Viral DNA detected by in situ nucleic acid hybridization ^b		Virus recovered by direct assay of ganglion ^c		Virus recovered by in vitro cultivation of sciatic nerve and roots ^d	
	Mice positive/mice tested	% Positive	Mice positive/mice tested	% Positive	Mice positive/mice tested	% Positive
0	1/10	10	0/22	0	1/22	5
1	6/9	67	1/15	7	4/15	27
2	0/8	0	2/14	14	4/14	29
3			1/15	7	4/15	27
4			0/18	0	7/18	39
5	1/6	17	0/13	0	1/13	8

^a Representative isolates were identified as HSV by neutralization with specific antisera.

^b Ganglia were removed from latently infected mice at the intervals noted and subjected to in situ nucleic acid hybridization procedures to detect HSV DNA. After processing, individual sections from four to seven ganglia could be detected in each group sectioned. As reported previously (5), when taken from latently infected mice, these ganglia contain ≤ 0.05 neurons in which viral DNA can be detected. In the experiments reported here, an arbitrary value of ≥ 2 neurons positive for viral DNA/ganglionic section (40 times the normal background) was taken as the minimum number to qualify the animal as being positive.

^c Ganglia were removed from latently infected mice at the intervals noted and prepared for direct assay of virus as described in the Methods section.

^d In these assays, the results from proximal or distal nerve roots and sciatic nerves were pooled. Thus, if infectious virus was recovered from any one of the tissues, the animal was scored as positive. A breakdown into the frequency of virus recovery from each tissue is summarized in the text. It is important to note that the percentage of positive mice at 4 days varied between 20% (one of five) and 75% (three of four) in individual experiments.

obvious interpretation that virus is induced in the ganglion and passes to the nerve will be discussed later.

Distinct role for the pneumococcus in the phenomenon. This reactivation of HSV was instituted by intratracheal instillation (by surgical operation during general anesthesia) of bacteria suspended in 3% gastric mucin. Therefore, before concluding that pneumococcal

pneumonia precipitated the reactivation, it was necessary to determine the role of mucin and the experimental procedures employed.

The effect of mucin was studied by intratracheal instillation of either a complete mixture or the mucin without bacteria. After 3 and 4 days, representative mice were sacrificed and the nerve roots and sciatic nerves were co-cultivated by the usual methods. The data (Table 2) indicate that the mucin treatment alone reactivates virus in a significant number of animals, but when bacteria are also present the effect appears to be more pronounced (60% versus 30% on day 4). However, the number of animals studied on each of the 2 days was too small for statistical analysis. When the results of the experiments for both day 3 and 4 were combined, the total number of virus reactivations was not significantly different between the two groups of mice ($P > 0.05$ by chi-square test with Yates correction for continuity). From these experiments, a role for the bacteria was not clearly demonstrated.

More instructive were similar experiments in which mucin was deleted completely and bacteria alone were compared to instillation of media. Here (Table 3) it is clear that the bacteria were effective in inciting the reactivation, since those animals receiving bacteria gave the usual level of reactivation but those in which media alone was used possessed a reactivation rate

TABLE 2. *Recovery of reactivated HSV in sacrosciatic nerve roots and nerves from mice instilled intratracheally with D. pneumoniae suspended in mucin or with mucin alone^a*

Day after intratracheal instillation	Virus recovered from mice instilled with mucin + bacteria		Virus recovered from mice instilled with mucin alone	
	Mice positive/mice tested	% Positive	Mice positive/mice tested	% Positive
3	3/10	30	3/11	29
4	6/10	60	3/10	30
Totals	9/20	45	6/21	29

^a Mice harboring a latent herpetic infection in the sacrosciatic spinal ganglia were instilled intratracheally with bacteria suspended in 3% gastric mucin or with the mucin alone. Animals in both groups were given penicillin therapy on the time and dosage schedule outlined in the text. At the time intervals noted, representative animals were sacrificed, and the sacrosciatic nerve roots and sciatic nerve were co-cultivated with RK₁₃ cells, which were scored for cytopathic effects induced by HSV. Representative isolates were identified as HSV by neutralization with specific antisera.

TABLE 3. Recovery of reactivated HSV in sacrosiatic nerve roots or nerves from mice instilled intratracheally with *D. pneumoniae* or with the suspending medium^a

Day after intratracheal instillation	Virus recovered from mice instilled with media + <i>D. pneumoniae</i>		Virus recovered from mice instilled with media alone	
	Mice positive/mice tested	% Positive	Mice positive/mice tested	% Positive
3	5/8	63	2/10	20
4	5/11	45	1/10	10
Totals	10/19	53	3/20	15

^a Mice and appropriate tissues were manipulated as in Table 2, except that mucin was not used in inocula.

only slightly higher than the "background" level seen in previous experiments (Table 1). Again, the number of experimental animals used was too small for statistical analysis on each day of the experiment. However, when the results of the studies for both days 3 and 4 were combined, the number of virus reactivation among mice infected with pneumococci was significantly greater than among mice given media alone ($\chi^2 = 8.02$, $P < 0.05$).

DISCUSSION

From the experiments reported here, it is clear that infectious HSV is reactivated from sacrosiatic spinal ganglia after severe pneumococcal disease in mice. In this regard, it has been recognized for many years that herpetic lesions are not an uncommon companion of pneumococcal disease in man (2). In addition, we tentatively conclude that the infection travels centrifugally and centripetally in nerves. Although it might be argued that virus in the nerves is being reactivated from cells resident in those tissues, the following findings rule against this interpretation. First, viral DNA and significant amounts of infectious virus appear in the ganglia before virus is found in the greatest number of nerves. Second, as noted earlier, a greater percentage of distal nerve roots demonstrate virus than do the corresponding sciatic nerve trunks (which, incidentally, are of greater length). Since the virus went through the sciatic nerve trunk to get to the ganglia and the trunk is essentially a "conglomerate" of the distal roots, if virus were latent in the nerve, the trunks would be expected to be as least as efficient as distal roots in harboring a latent infection. Finally, all of our previous evidence is

consistent with latent virus being selectively associated with neuronal somas (4). Nerves contain only supporting cells.

It should be pointed out here that Walz et al. (10), by surgical section of distal nerve roots, recently accomplished the first experimental reactivation of latent virus in this system. A few days after this sectioning procedure had been accomplished, infectious virus could be recovered directly from the ganglia in about 1/3 of the mice studied. This result is obviously of great significance, but the surgical procedure used limits the usefulness of that model for subsequent studies of pathogenesis since the proposed "conduit" to the skin is severed. The system presented here and others now being studied in our laboratory present no such restrictions, since they have been selected so that the peripheral nervous system remains intact.

Finally, it should be emphasized again that the experimental proof for the general hypothesis concerning pathogenesis of herpetic disease is not yet complete, since we have not yet been able to detect cutaneous disease after the reactivation and then prevent this disease by surgical interruption of appropriate nerves. If this can be accomplished by the additional manipulations now being attempted, the significant, general features of the hypothesis concerning pathogenesis of recurrent herpetic disease will all have been experimentally satisfied. Beyond this, a complete definition of the biochemical basis for latency and reactivation is a challenge that will not be so easily accomplished.

ACKNOWLEDGMENTS

The technical assistance provided by E. Scott and V. Bastone is gratefully acknowledged.

This research was supported by Public Health Service grants AI-06246 and NS-08711 from the National Institute of Allergy and Infectious Diseases and the National Institute of Neurological Diseases and Stroke, respectively.

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