hour and the column was washed at the same flow rate with 3 liters of TST. The beads were apportioned into three 250-ml Erlenmeyer flasks containing 100 ml of alkaline peptone broth and were incubated for 12 to 18 hours at 34°C. Growth from the alkaline peptone broth was streaked onto agar containing thiosulfate, citrate, bile salts, and sucrose (TCBS) (Difco) in petri plates, and colonies were allowed to develop at 34°C for 20 to 24 hours. Colonies characteristic of *V. cholerae* were selected and the bacteria identified biochemically and serologically by established procedures (*8*).

We ran numerous experiments with known mixtures of a non-sucrose-fermenting noncholera vibrio (non-O1 serovar) and O1 Inaba in saline which were passed through the column. We were able to isolate the Inaba strain adsorbed to the beads when the ratios of non-O1 serovars to O1 Inaba were as high as 300, with a concentration of 60 Inaba cells per liter. Also, we were successful in isolating Inaba from pondwater devoid of indigenous vibrio to which we added Inaba cells in low numbers.

Table 1 shows the results of our isolation method applied to 1 liter of water collected from a bayou in south central Louisiana. Characteristic colonies on TCBS were selected for identification. Five colonies were identified as O1 serovars. None of the sucrose-positive colonies were non-O1 vibrios. One isolate, W-19, was a Gram-positive coccus which agglutinated with antiserum containing antibodies against the A antigen, but did not agglutinate with B or C antiserum. Isolates W-1, W-11, W-12, W-15, and W-16 reacted serologically as O1 Inaba. Isolates W-1 and W-12 showed the biochemical reactions of the El Tor biotype. One isolate, W-1, possessed arginine dihydrolase and did not decarboxylate ornithine, both of which are atypical reactions. The status of the isolate has not been established. Isolate W-12 has characteristics of *V. cholerae* Hikojima. We have applied the method to four water samples. Three samples have yielded O1 serovars. The fourth sample was devoid of *Vibrio*.

The efficiency of the method is emphasized by two observations. All the isolates were serologically typical of O1 Inaba or possessed antigens present in O1 Inaba. In addition, sediment collected from column effluent and column rinse water by centrifugation was inoculated into alkaline peptone broth and subsequent growth was streaked onto TCBS. We did not isolate a sucrose-fermenting *Vibrio* from these inoculums. Thus, the column appears to sequester most or all the bacteria with O1 antigens or cross-reacting material.

The method we have developed may prove useful for the specific isolation of other microorganisms from water, including such pathogens as *Salmonella*, *Shigella*, and *Legionella*. It may also be possible to apply the principle of the method to ecological studies and to the isolation of specific organisms from any habitat including pathogens from pathological specimens.

K. W. HRANITZKY
A. D. LARSON
D. W. RAGSDALE
R. J. SIEBELING

Department of Microbiology, Louisiana State University, Baton Rouge 70803

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16 May 1980; revised 28 July 1980

Latency of Herpes Simplex Virus in Absence of Neutralizing Antibody: Model for Reactivation

Abstract. *Mice inoculated with herpes simplex virus (type 1) by the lip or corneal route and then passively immunized with rabbit antibody to herpes simplex virus developed a latent infection in the trigeminal ganglia within 96 hours. Neutralizing antibody to herpes simplex virus was cleared from the circulation and could not be detected in most of these mice after 2 months. Examination of ganglia from the antibody-negative mice revealed latent virus in over 90 percent of the animals, indicating that serum neutralizing antibody is not necessary to maintain the latent state. When the lips or corneas of these mice were traumatized, viral reactivation occurred in up to 90 percent of the mice, as demonstrated by the appearance of neutralizing antibody. This study provides a model for identifying factors that trigger viral reactivation.*

Herpes simplex virus (HSV) produces recurrent epithelial lesions in humans. In the mouse, viral inoculation on epithelial surfaces results in infection of local sensory ganglia (*1*). For approximately 2 weeks, infectious virus can be recovered from cell-free ganglionic homogenates (acute phase). After 2 weeks, infectious virus can no longer be recovered, but can be reactivated by explantation of ganglia (latent phase). The state of the viral genome during latency is not known. Some reports favor a low level of virus replication or intermittent spontaneous reactivation (*2*). Other reports support a block in the transcription of the viral genome during latency (*3*). Irrespective of the mechanism, the virus can be reactivated in vivo by several techniques including neurectomy, irritation of epithelial surfaces, and administration of immunosuppressive agents (*4*, *5*).

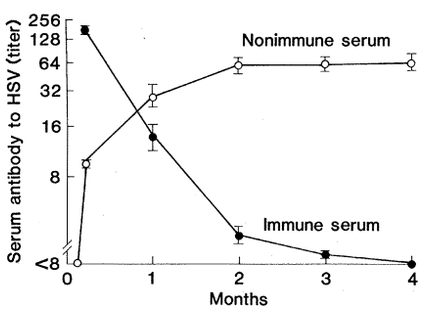

Fig. 1. Antibody titers in HSV-infected mice after administration of nonimmune or immune rabbit antiserum to HSV. The mice were infected with HSV by the lip route and given 0.2 ml of rabbit serum intraperitoneally 3, 48, 96, and 144 hours later. At the times indicated, the animals were bled and neutralizing antibody titers were determined. Each point represents the geometric mean ± standard deviation for 12 to 20 mice assayed individually.

Although there is considerable information on the various immunological mechanisms involved in eliminating the acute phase of the infection, relatively little is known about the importance of immunological factors in establishing or maintaining the latent phase. We now report that the latent state can be maintained in the absence of neutralizing antibody and that virus reactivation can be demonstrated by an increase in neutralizing antibody titers.

Female BALB/cJ mice, 6 to 8 weeks

0036-8075/80/1128-1026$00.50/0

old, were inoculated with the F strain of HSV (type 1) by the lip or corneal route (*6*) and passively immunized by intraperitoneal injection of hyperimmune rabbit antiserum to HSV (titer, 1000 per 0.05 ml) (*5*). Serum antibody titers were determined on rabbit kidney cells by microneutralization (*7*), and trigeminal ganglia were assayed for virus by explantation or homogenization (*6*).

In previous experiments (*6*), up to 100 percent of mice inoculated with HSV developed a productive ganglionic infection within the first week, but latency could not be detected until after the appearance, in 1 to 2 weeks, of neutralizing antibody. The present studies show that latency can be established in 48 to 96 hours if the animals are passively immunized with antiserum to HSV immediately after viral inoculation. As seen in Table 1, a productive infection was found in only 17 to 18 percent of the mice, while a latent infection was found in 59 to 61 percent. Passive immunization thus appears to eliminate or at least markedly reduce the productive phase of the infection, resulting in rapid development of latency. However, when passively immunized animals that had been inoculated with HSV by the corneal route were treated with cyclophosphamide, a productive infection ensued in 79 percent of them, compared to 17 percent of the controls. Similarly, when passively immunized mice that had been inoculated with HSV by the lip route were traumatized on the lips with dry ice, a productive infection developed in 38 percent of them, compared to 18 percent of the control mice. Thus, the same treatments that reactivated virus in mice with latent infections 2 months after viral inoculation (*5, 8*) also reactivated virus in the rapid latency phase induced by passive immunization.

Central to the question of latency is the role of antibody in maintaining the latent state. Our approach was to establish a latent infection while limiting as much as possible the host's immune response to the virus by passive immunization with antibody to HSV. In HSV-infected mice given serum from nonimmune rabbits, the neutralization titer rose to 64 (Fig. 1). In contrast, in HSV-infected mice given hyperimmune rabbit antiserum to HSV, the neutralization titer decreased to less than 8 (geometric mean) in most of the mice. The presence of low levels of neutralizing antibody in some animals 2 and 3 months after passive immunization probably represents a transient immune response by the mouse to the initial HSV infection rather than the effects of residual rabbit antibody.

Table 1. Rapid establishment of latent infection by passive immunization: reactivation by cyclophosphamide and lip trauma. Mice were inoculated with HSV by the corneal or lip routes and 0.2 ml of antiserum to HSV was administered by intraperitoneal injection 3 and 48 hours later. The mice were killed 48 or 96 hours after viral inoculation. One trigeminal ganglion was explanted and a second trigeminal ganglion was homogenized and assayed for infectious virus.

Treatment	Number of mice	Ganglionic infection* (%)	Productive infection† (%)	Latent infection‡ (%)
None	59	76	17	59
Cyclophosphamide§	58	96	79	17
None	39	79	18	61
Lip trauma‖	39	79	38	41

*Determined by explantation 48 or 96 hours after infection (measures both productive and latent infection). †Determined by homogenization 48 or 96 hours after infection (measures only productive infection). ‡Latent infection represents the difference between the percentage of mice with ganglionic infection and the percentage with productive infection. §Cyclophosphamide (200 mg/kg) was given intraperitoneally 1 day before and 1 day after corneal inoculation with HSV. In some experiments, an additional dose (15 mg/kg) was given on the third day. Results for ganglia assayed 48 or 96 hours after infection were similar, and the data from several experiments are combined. ‖At 24, 48, and 72 hours after the mice were inoculated with HSV by the lip route, dry ice was applied for 10 seconds to the areas of inoculation. Ganglia were assayed by explantation or homogenization 96 hours after inoculation. Control mice were lightly anesthetized with ether at the same time but were not treated with dry ice. The data represent the combined results from four experiments.

To determine whether the antibody-negative animals had latent infections, we killed 90 of them 1½ to 13 months after their passive immunization. Approximately 85 percent of the ganglionic explants yielded virus. Ganglionic homogenates, however, were negative for virus in all animals tested. These findings indicate that latency had been established and was being maintained in the apparent absence of neutralizing antibody (*9*).

Evidence that the antibody-negative mice with a latent infection (ANLI) were not immunologically tolerant was obtained by reinoculating the mice with HSV by the corneal route. In this experiment, the neutralizing antibody titer increased in all ten mice tested from less than 8 to 69 (geometric mean).

Table 2. Reactivation of HSV as measured by increase in titer of neutralizing antibody. All mice (treated and untreated) were inoculated with HSV by the lip route and given rabbit antiserum to HSV (0.2 ml, intraperitoneally) 3, 48, 96, and 144 hours after inoculation. At the end of 9 weeks the mice were bled and serum antibody titers were determined. Only animals with titers less than 8 were used in the subsequent experiments.

Group	Neutralization titer								
	Experiment 1* (weeks after inoculation)					Experiment 2* (weeks after inoculation)			
	9	12	15	18	22	9	16	20	25
Traumatized†									
1	< 8	8	32	64	64	< 8	< 8	< 8	16
2	< 8	8	16	16	32	< 8	< 8	32	64
3	< 8	< 8	32	32	16	< 8	8	< 8	< 8
4	< 8	8	32	256	256	< 8	16	16	16
5	< 8	32	64	64	64	< 8	< 8	< 8	8
6	< 8	8	8	128	64	< 8	32	32	64
7	< 8	< 8	8	16	256	< 8	8	128	128
8	< 8	16	32	32	32	< 8	< 8	16	64
9	< 8	8	16	16	16	< 8	< 8	8	16
10	< 8	< 8	8	64	32	< 8	8	8	64
11	< 8	< 8	16	64	64	< 8	< 8	8	32
12	< 8	< 8	< 8	16	64	< 8	16	16	32
13	< 8	< 8	16	16	16				
14	< 8	< 8	< 8	< 8	16				
15	< 8	16	64	32	32				
16	< 8	< 8	16	16	16				
17	< 8	< 8	< 8	< 8	< 8				
Untraumatized									
1	< 8	< 8	< 8	32	32	< 8	16	16	32
2	< 8	< 8	< 8	32	32	< 8	32	16	32
3 to 11	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
12 to 18	< 8	< 8	< 8	< 8	< 8				

*Each mouse was numbered, and serum neutralizing antibody to HSV was measured at the times indicated. †Dry ice was applied to the lips for about 10 seconds daily for three 10-day courses. In experiment 1 the mice were traumatized at 9, 12, and 15 weeks after inoculation; in experiment 2, at 9, 14, and 23 weeks.

The demonstration that ANLI mice could respond immunologically to HSV was exploited as a method for detecting viral reactivation. The lips of these mice were traumatized by application of dry ice. As shown in Table 2, antibody titer increased ($\geq$ 16) in 90 percent of the traumatized mice. In contrast, an increase in antibody titer was detected in only 4 of 29 control mice, suggesting that spontaneous reactivation had occurred in these mice. No neutralizing antibody was detected in uninfected control mice subjected to repeated dry ice trauma, despite the fact that some were caged with infected animals undergoing HSV reactivation.

In another experiment, ANLI mice were subjected to three 10-day courses of electric shock to the lips (36 V for 15 seconds). An increase in neutralizing antibody titer ($\geq$ 16) occurred in 11 of 14 shocked animals (79 percent) but in only 3 of 18 latently infected mice that were not shocked (17 percent). In still another experiment, an increase in antibody titer occurred in 8 of 11 mice treated with phorbol myristate acetate (73 percent), but in only 1 of 11 untreated mice (9 percent).

Support for the idea that epithelial irritants reactivate the virus by acting on nerve terminals corresponding to latently infected ganglion cells comes from experiments in which surfaces innervated by different branches of the trigeminal nerve were infected and traumatized. In ANLI mice that had been inoculated with HSV by the lip route, lip trauma induced reactivation, whereas corneal trauma did not. Conversely, in ANLI mice that had been inoculated with HSV by the corneal route, only corneal trauma induced reactivation (*10*).

What role the host's immune response plays in establishing latency is still not clear. There are at least three possibilities. First, ganglion cells may be primarily permissive for HSV replication. The host's immune response may modulate the productive infection and bring about a conversion to a nonproductive (latent) state. Second, there may be two populations of ganglion cells: one permissive and the other nonpermissive. The host's immune response may eliminate the productively infected cells (the acute phase of the infection), allowing detection of the underlying (latent) infection in the nonpermissive cells. Third, ganglion cells may be primarily nonpermissive for HSV replication. Various stimuli may act as a signal to bring about a switch from the nonpermissive to the permissive state. Replication of the virus at the epithelial surface and the ensuing inflammation may directly or indirectly provide such a signal, making ganglion cells permissive for HSV. During the course of the natural infection, the host's immune response shuts off this signal by decreasing replication of the virus at the epithelial surface. Ganglion cells then revert to the nonpermissive state and latency ensues. This could explain the rapid establishment of latency by passive administration of antibody (Table 1).

Earlier studies showed that systemic cyclophosphamide or lip trauma (*5, 8*) given to mice with a latent infection 2 months after viral inoculation resulted in reactivation of HSV. Our study shows that these stimuli also cause reactivation in the rapid latency model (Table 1). Presumably, the mechanisms of reactivation are the same in both models. Although the underlying molecular basis for latency and reactivation remains undefined, the idea that a variety of stimuli, including epithelial infection, may make ordinarily nonpermissive ganglion cells permissive for HSV replication has appeal. By analogy, lymphocytes are nonpermissive for HSV but become permissive after exposure to mitogens—and HSV itself can act as a B cell mitogen (*11*).

Regardless of the role of antibody in establishing the latent infection, the passive immunization experiments argue that serum neutralizing antibody is not needed to maintain the latent state. Moreover, the presence of latent virus in the absence of detectable amounts of neutralizing antibody suggests that viral antigens are not expressed during latency or that they are present in insufficient amounts to substantially boost the neutralizing antibody response. These experiments also support the idea that diminished immunosurveillance cannot be fully responsible for the reactivation induced by immunosuppressive agents (*5*). Of additional interest is the observation that about 9 percent of the untreated animals did show an increase in neutralizing antibody titer, suggesting that reactivation occurred in some animals either spontaneously or secondary to repeated trauma from eye bleeding.

In earlier studies we looked for reactivation by attempting to recover infectious virus from ganglia at various times after challenge with potential reactivating agents (*5*). This technique had limited usefulness because the animals had to be destroyed and only one point in time could be evaluated. In the present study, we found that lip trauma, which induced viral reactivation in only 8 percent of animals as measured by recovery of infectious virus (*8*), resulted in an increase in neutralizing antibody titer in 90 percent of the ANLI animals. The measurement of antibody thus makes it possible to determine whether reactivation occurred, even when separated by a long time period from the reactivating event, and the evaluation can be made without killing the animals. If the factors that trigger viral reactivation can be identified, it might be possible to prevent or greatly reduce the incidence of recurrent herpetic disease.

TSUYOSHI SEKIZAWA
HARRY OPENSHAW
CHARLES WOHLENBERG
ABNER LOUIS NOTKINS
Laboratory of Oral Medicine,
National Institute of Dental Research,
National Institutes of Health,
Bethesda, Maryland 20205

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12. We thank G. Lewis and F. Shaw for technical help and E. Rian for editorial assistance.

4 June 1980; revised 15 August 1980