Acute and Latent Infection of Sensory Ganglia with Herpes Simplex Virus: Immune Control and Virus Reactivation

By HARRY OPENSHAW, LUDMILLA V. SHAVRINA ASHER, CHARLES WOHLENBERG, TSUYOSHI SEKIZAWA AND ABNER LOUIS NOTKINS

Laboratory of Oral Medicine, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205, U.S.A.

(Accepted 5 January 1979)

SUMMARY

The role of antiviral antibody in controlling the acute and latent phases of herpes simplex virus (HSV) infection in sensory ganglia of mice was studied in vitro and in vivo. Organ cultures of ganglia inoculated in vitro with HSV produced infectious virus for at least 3 weeks. In the presence of antiviral antibody, the titre of virus was markedly reduced, but the infection was not eliminated. Similarly, passive administration of antibody to HSV-infected immunodeficient (nude) mice reduced the virus titre but did not eliminate the acute phase of the ganglionic infection. Suppression of the cell-mediated immune response in latently infected immunocompetent mice by treatment with cyclophosphamide and/or X-irradiation resulted in reactivation of HSV in up to 70% of the animals. Reactivation was demonstrated by recovering infectious virus in cell-free homogenates of ganglia and eye globes and by finding virus antigens in ganglia by immunofluorescent staining. Reactivation occurred both in vitro and in vivo in the presence of high concentrations of neutralizing antibody. It is concluded that antibody alone is not sufficient to eliminate the acute phase of the ganglionic infection and that cytotoxic agents known to suppress the host’s cellular immune response can reactivate virus in the presence of neutralizing antibody.

INTRODUCTION

Recent studies have shown that herpes simplex virus (HSV) can remain latent for months or years in sensory and autonomic ganglia of animals and man (Stevens & Cook 1971; Bastian et al. 1972; Baringer & Swoveland, 1973; Price et al. 1975a, b). These findings support the view that recurrent epithelial eruptions are due to reactivation of latent virus within ganglia and neural transport of HSV to the epithelial surface.

Much of the information on the pathogenesis of HSV has been obtained from animal models. Inoculation of mice with HSV by various epithelial routes results in infection of local ganglia (Stevens & Cook, 1971; Price et al. 1975b). During the first 2 weeks of the infection (acute phase) infectious virus can be recovered from ganglia by homogenization. After 2 weeks (latent phase), infectious virus can no longer be recovered from cell-free homogenates but can be recovered by explantation and co-cultivation of ganglia. Reactivation of HSV by a variety of methods including physical manipulation of the nerve (Carlton & Kilbourne, 1952; Walz et al. 1974; Nesburn et al. 1977; Price & Schmitz, 1978), stimulation of the epithelial surface (Blyth et al. 1976; Hurd & Robinson, 1977; Hill et al. 1978), systemic injection of prednisone in hairless mice (Underwood & Weed, 1974) and intra-
In some reports, reactivation was determined solely by the demonstration of skin lesions or the detection of virus at the periphery, whereas, in other reports, infectious virus was detected in ganglia without evidence of clinical disease at the epithelium. The role of immunity in controlling the acute phase and maintaining the latent phase of the ganglionic infection is poorly understood and, in general, attempts to reactivate HSV by immunosuppressive agents alone have been unsuccessful (Stevens & Cook, 1973; Blyth et al. 1976; Hurd & Robinson, 1977; Martin et al. 1977; Price & Schmitz, 1978).

The experiments reported here show that the host's immune response plays a critical role in controlling the acute phase of the ganglionic infection and that immunosuppressive agents can induce virus reactivation as determined by the recovery of infectious virus in ganglionic homogenates from latently infected animals.

**METHODS**

*Preparation of virus.* The F strain of HSV-I, obtained from the American Type Culture Collection, Rockville, Md., was grown in primary rabbit kidney (PRK) cells. A stock pool of $10^8$ plaque-forming units (p.f.u.) per ml, concentrated by ultracentrifugation, was used for animal and organ culture inoculation. Maintenance media for all cultures consisted of Eagle’s minimal essential medium (MEM) containing 2% heat-inactivated foetal bovine serum, 0.03% glutamine, 100 units penicillin/ml, 100 µg streptomycin/ml and 100 units mycostatin/ml.

*Animals and inoculation methods.* Except where indicated, 6 to 8 week-old female Balb/c mice were inoculated by placing a drop of stock virus containing $10^8$ p.f.u. on the skin of the right thigh or on both corneas and scarifying the epithelium with a 29 gauge needle (Price et al. 1975b). In some experiments, Balb/c nude (nu/nu) mice were used. Immune serum was obtained from New Zealand white rabbits after multiple intravenous inoculations of $10^8$ p.f.u. of HSV. One neutralizing unit (NU) of antibody represents the reciprocal of the highest twofold serum dilution in 0.2 ml which produces a 50% reduction of HSV plaques. Non-immune serum was obtained from uninoculated rabbits.

*Detection of virus in tissues.* To detect infectious virus, ganglia were homogenized as previously described (Walz et al. 1974), frozen and thawed three times and plated on PRK cells. Eye globes were removed and frozen at $-70$ °C until used. After thawing, paired eye globes from individual mice were homogenized in a 2 ml Ten Broeck glass tissue grinder (Bellco Glass, Vineland, N.J.), centrifuged at low speed and the supernatant fluid plated on PRK cells. After a 2 h adsorption of either the eye or ganglion homogenate, the inoculum was removed and MEM added. The plates were observed for virus cytopathic effect (c.p.e.) for 5 days.

The virus titre was determined in two ways. In some experiments, virus plaques were assayed using an overlay media containing MEM with 2% heat-inactivated pooled human serum as the source of anti-HSV antibody (Hampar et al. 1968). In other experiments, the virus titre was calculated (Reed & Muench, 1938) as the infectious dose that produced cytopathology in 50% (ID$_{50}$) of PRK microtitre wells, using four wells per virus dilution.

Since infectious virus cannot be found in cell-free homogenates during the latent phase of ganglionic infection, explantation procedures were used to detect virus. In some experiments, the explants were co-cultivated with PRK cells and virus was detected by the appearance of c.p.e. In other experiments, the explants were added to MEM without indicator cells and incubated at 37 °C. At various times thereafter, cell-free ganglionic homogenates were prepared and assayed for infectivity on PRK monolayers as already described.
Ganglionic organ culture: infection in vitro. A total of 40 to 60 pooled non-infected trigeminal ganglia were incubated at 37 °C for 6 h with \(10^5\) p.f.u./ml of HSV in a volume of 25 ml MEM. The ganglia were then washed and individually transferred to plates containing MEM with immune (500 or 10000 NU) or non-immune serum that had been heat-inactivated at 56 °C for 30 min. The culture media together with immune or non-immune serum were replenished every 3 days. At various times, ganglia were removed and washed. Ganglia that had been incubated with non-immune serum were treated for 1 h at 37 °C with immune sera (500 or 10000 NU) to neutralize extracellular virus. Residual immune serum was removed by washing three times in MEM. It was shown in preliminary experiments that all detectable neutralizing antibody was removed by these washings. All ganglia were then homogenized and the yield of infectious virus determined.

Immunosuppression of mice. Three regimens of immunosuppression were used. In the first, cyclophosphamide, obtained from Mead Johnson (Evansville, Ind.), was administered intraperitoneally (i.p.) in a dose of 200 mg/kg on days 1 and 3 of treatment and then 15 mg/kg daily beginning on day 5 until the animals were sacrificed. In the second regimen, X-irradiation was given in a single dose of 550 rads on day 1. The third regimen combined X-irradiation of 550 rads on day 1 and cyclophosphamide 15 mg/kg i.p. daily beginning on day 3 until the end of the experiment.

The degree of immunosuppression was determined by measuring spleen weights, counting peripheral blood leukocytes, titrating anti-HSV neutralizing antibody and measuring the responsiveness of spleen cells to phytohaemagglutinin (PHA) stimulation. The PHA assay was performed as described (Oppenheim & Rosenstreich, 1976). In brief, triplicate cultures of \(2\times10^6\) leukocytes in RPMI media were incubated with 6 μg PHA (Microbiological Associates, Bethesda, Md.). One μCi \(^3\)H-thymidine (New England Nuclear, Boston, Mass.) was added for the last 4 of the 72 h incubation. After harvesting the cells (Skatron Automatic Harvester, Flow Laboratories, Rockville, Md.), the incorporation of \(^3\)H-thymidine into DNA was determined and the data were expressed in terms of the stimulation index (ratio ct/min in cultures with PHA to ct/min without PHA). A stimulation index of less than 2.5 was considered negative.

Detection of virus antigens in infected ganglia. Six μm frozen sections of trigeminal ganglia from infected and uninfected mice were cut in a cryostat and dried at room temperature. After 20 min fixation with acetone, sections were stained for 45 min at 37 °C with fluorescein-conjugated rabbit anti-HSV serum purchased from Microbiological Associates, Bethesda, Md. Slides were examined with a Zeiss fluorescent microscope. Approximately 20 cross-sections were examined from each ganglion.

RESULTS

Effect of antibody on acute ganglionic infection in vitro

To study the effect of anti-HSV antibody on the acute phase of the infection, a ganglion organ culture system was used. Pooled trigeminal ganglia were inoculated with HSV. After an adsorption period of 6 h, individual ganglia were transferred to Petri dishes and cultured in the presence of immune or non-immune serum. On various days thereafter, ganglia were homogenized and assayed for infectious virus. The data in Fig. 1 show that in the absence of antibody, the virus titre increased from \(10^3\) ID\(_{50}\) per ganglion on day 2 to \(10^5\) ID\(_{50}\) on day 7, and slowly declined over the next 2 weeks. In contrast, in the presence of anti-HSV antibody (500 NU), the virus titre did not exceed \(10^3\) ID\(_{50}\) at any time during the course of the experiment. Thus, antibody reduced the amount of virus in the ganglion, but did not eliminate the infection.
Fig. 1. Effect of immune and non-immune serum on the acute ganglionic infection. Pooled trigeminal ganglia from uninfected mice were placed in culture and inoculated with HSV (see Methods). After a 6 h adsorption, individual ganglia were incubated with 500 NU of immune serum (●—●) or an equal concentration of non-immune serum (○—○). At various times thereafter, individual ganglia were homogenized and assayed for infectious virus. Each point represents the geometric mean ± standard error of four ganglia, homogenized and assayed individually.

Fig. 2. Effect of immune and non-immune serum on in vitro reactivation and production of infectious virus. Dorsal root ganglia from latently infected mice (4 to 8 weeks after inoculation) were placed in culture in the presence of 5000 NU of immune serum (●—●) or an equal concentration of non-immune serum (○—○). At various times thereafter, individual ganglia were homogenized and assayed for infectious virus. Each point represents the geometric mean ± standard error of approx. four ganglia assayed individually. In some of the cultures, antibody was removed on days 4, 7 and 11 and ganglia were incubated for an additional 48 h in non-immune serum before homogenization. The virus titre (△—△) represents the geometric mean of three to five ganglia assayed individually.

In vitro reactivation of latent infection: effect of antibody

Infectious virus cannot be detected in the homogenates of ganglia from latently infected mice (Stevens & Cook, 1971; Price et al. 1975a). However, if the ganglia are incubated in culture for 36 h or more prior to homogenization, infectious virus can be detected. To determine whether anti-HSV antibody would prevent virus reactivation, dorsal root ganglia from animals that had been infected 4 to 8 weeks earlier were placed in culture in the presence of immune or non-immune serum. At the end of 48 h, the ganglia were homogenized and assayed for infectious virus. The results in Table 1 show that neither 500 nor 5000 NU of antiviral antibody prevented in vitro reactivation. The data in Fig. 2 show that although virus was reactivated in the presence of antiviral antibody, the amount of virus produced was less than 0.1% of that found in cultures without antibody. However, when antibody was removed from the cultures, the virus titre in the ganglia increased 100- to 1000-fold within 48 h.

Effect of antibody on acute ganglionic infection in immunocompetent and immunodeficient mice

Although the in vitro studies showed that antibody did not eliminate the acute ganglionic infection, the organ culture system does not completely parallel the in vivo state since a major portion of each axon had been removed when the ganglia were placed in culture. Moreover, under in vivo conditions, antibody may have a greater range of action in the presence of complement and killer cells. For these reasons, the role of antibody in controlling the acute HSV ganglionic infection was studied in vivo.

HSV was inoculated bilaterally in the cornea of immunocompetent and immunodeficient
Table 1. Effect of immune and non-immune serum on in vitro reactivation of latent HSV

<table>
<thead>
<tr>
<th>Serum*</th>
<th>Virus reactivation†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reactivated/tested‡</td>
</tr>
<tr>
<td>Exp. 1  Immune (500 NU)</td>
<td>13/19</td>
</tr>
<tr>
<td>Non-immune</td>
<td>14/19</td>
</tr>
<tr>
<td>Exp. 2  Immune (10000 NU)</td>
<td>16/16</td>
</tr>
<tr>
<td>Non-immune</td>
<td>15/16</td>
</tr>
</tbody>
</table>

* The concentration of serum (immune or non-immune) was 4 % in experiment 1 and 78 % in experiment 2.
† Dorsal root ganglia from individual animals were placed in culture for 48 h, homogenized and assayed for infectivity.
‡ Number of ganglia reactivated/number of ganglia tested.

Fig. 3. Effect of passive administration of non-immune (a, b) and immune (c, d) serum on HSV titre in ganglia of immunocompetent and immunodeficient (nude) mice. Animals were infected on day 0. Non-immune or immune serum (5280 NU) was given i.p. 3 h after virus inoculation and on alternate days thereafter for 8 days. Each point represents the titre of HSV in the right trigeminal ganglion homogenates from individual mice. Bars represent the geometric mean ± standard error.

(nude) mice. Three hours after inoculation and every other day for 8 days the animals were given 0.2 ml of heat-inactivated immune (5280 NU) or non-immune serum i.p. At various times thereafter, the left trigeminal ganglion was homogenized and assayed for infectious virus and the right trigeminal ganglion was explanted on PRK cells and evaluated for the presence of infection. The data in Fig. 3(a) show that, at 4 days after inoculation, the mean titre in ganglia of immunocompetent mice was 10^3.5 p.f.u. Over the next 8 days, the titre of infectious virus declined to undetectable amounts. In contrast, the virus titre in ganglia
of immunodeficient mice (Fig. 3b) increased about 100-fold from day 4 to days 8 to 12. None of the animals in this group survived for more than 12 days. In the immunocompetent mice, the antibody titre was less than 10 NU on day 4, but decreased to 640 NU on day 8. In contrast, the antibody titre was always less than 10 NU in the immunodeficient mice. Thus, the quantity of infectious virus in ganglia appeared to show an inverse relationship to the amount of neutralizing antibody in sera.

To study further the role of antibody in controlling the acute phase of the ganglionic infection, other animals in the same experiment were inoculated with HSV and given anti-HSV antibody 3 h later and on alternate days for 8 days. At various times during the experiment, left trigeminal ganglia were explanted and right trigeminal ganglia were homogenized and assayed for infectious virus. The data in Fig. 3c show that only 1 out of 23 of the cell-free ganglionic homogenates from the immunocompetent, passively immunized mice contained detectable infectious virus. Proof that antibody did not prevent these animals from becoming latently infected came from explanting the left trigeminal ganglia. These experiments demonstrated that all the ganglia tested were positive for virus (data not shown). Thus, the antibody treatment prevented the development of the acute but not the latent phase of the infection. In contrast to the findings in immunocompetent mice, passively administered antibody did not completely prevent the development of the acute infection in the immunodeficient mice (Fig. 3d). Positive homogenates were seen in 2 of 12 mice (17%) on day 4, and 5 of 12 mice (46%) on days 8 to 12 despite the presence of high levels of neutralizing antibody (640 NU in both passively immunized nude and immunocompetent mice). After the administration of antibody was stopped on day 8, the median antibody level began to decline (60 NU) and positive homogenates were found in 95% of the mice examined after day 12. The data in Fig. 3(d) were selected to include only those mice that did not develop skin lesions. However, about 50% of the passively immunized nude mice developed typical herpetic skin lesions beginning on day 6 and all of these animals had positive ganglionic homogenates. The median serum antibody titres were the same in mice with or without skin lesions, but the ganglionic virus titre on day 8 was 100-fold greater in mice with skin lesions (data not shown). Thus, if mice with lesions were included in Fig. 3, the distinction between Fig. 3(c) and (d) would be even more marked. This failure of antibody to suppress the acute phase of infection in nude, but not immunocompetent mice, suggests that other components of the immune system are necessary to stop the acute ganglionic infection. In this connection, recent experiments showed that 10 out of 18 nude mice given immune spleen cells plus anti-HSV antibody cleared the acute phase of the ganglionic infection and developed a latent infection, whereas only one of 18 nude mice given antibody alone cleared the acute ganglionic infection (H. Openshaw, unpublished data).

**In vivo reactivation of latent infection**

As shown in Table 1 and Fig. 2, virus reactivation occurred within 48 h after explantation of ganglia from latently infected animals and could not be prevented by antiviral antibody. To determine whether HSV also could be reactivated in vivo, immunosuppressive agents were used. Latently infected mice (6 weeks after eye inoculation) were treated with cyclophosphamide or X-irradiation and the degree of immunosuppression was determined by measuring serum anti-HSV antibody titres, PHA stimulation of spleen cell culture, total leukocyte counts and spleen weights. During the first 2 weeks, neither treatment regimen decreased the median serum neutralizing antibody titre by more than one twofold dilution. In contrast, both cyclophosphamide and X-irradiation reduced the PHA stimulation index to less than 2-5. With cyclophosphamide, this effect was seen throughout 3 weeks of treatment; whereas with X-irradiation, the PHA stimulation index was consistently abolished only in the first week after treatment. Cyclophosphamide reduced the spleen weight by
Table 2. In vivo reactivation of latent HSV in trigeminal ganglia by cyclophosphamide

<table>
<thead>
<tr>
<th>Days*</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Expt. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reactivated/ tested†</td>
<td>%</td>
<td>Reactivated/ tested</td>
<td>%</td>
</tr>
<tr>
<td>2-5</td>
<td>0/10</td>
<td>0</td>
<td>1/12</td>
<td>8</td>
</tr>
<tr>
<td>7-9</td>
<td>—</td>
<td>6/12</td>
<td>50</td>
<td>—</td>
</tr>
<tr>
<td>12-15</td>
<td>7/10</td>
<td>70</td>
<td>0/24</td>
<td>0</td>
</tr>
<tr>
<td>19-25</td>
<td>3/14</td>
<td>18</td>
<td>0/13</td>
<td>0</td>
</tr>
<tr>
<td>34-41</td>
<td>1/14</td>
<td>7</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Days after initiation of cyclophosphamide treatment.
† Number of mice with HSV reactivation as determined by the presence of infectious virus in cell-free homogenates from paired trigeminal ganglia/number of mice tested.

Table 3. In vivo reactivation of latent HSV in trigeminal ganglia by total body X-irradiation and X-irradiation plus cyclophosphamide

<table>
<thead>
<tr>
<th>Days*</th>
<th>X-irradiation</th>
<th>X-irradiation plus cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reactivated/ tested†</td>
<td>%</td>
</tr>
<tr>
<td>5</td>
<td>1/10</td>
<td>10</td>
</tr>
<tr>
<td>7-9</td>
<td>2/10</td>
<td>20</td>
</tr>
<tr>
<td>13-16</td>
<td>2/15</td>
<td>13</td>
</tr>
</tbody>
</table>

* Days after initiation of treatment.
† Number of mice with HSV reactivation as determined by the presence of infectious virus in cell-free homogenates from paired trigeminal ganglia/number of mice tested.

47% and total leukocyte count by 83% on the fifth day of treatment, but both parameters tended to return to normal on the twelfth day of treatment. With X-irradiation, the reduction of both spleen weight (71 to 74%) and leukocyte count (70 to 88%) persisted for at least 2 weeks.

At different times after the initiation of cyclophosphamide or X-irradiation treatment, right and left trigeminal ganglia were removed and homogenates of the two pooled ganglia were tested for infectious virus. Isolates yielded only low amounts of virus (generally, <5 p.f.u./paired ganglia) and c.p.e. appeared in the cultures within 4 days. All isolates were successfully passed at least once in PRK cells and representative isolates were neutralized by anti-HSV serum.

The effect of cyclophosphamide on virus reactivation is shown in Table 2. Peak reactivation occurred during the second week when up to 70% of the mice had positive homogenates. Although reactivation occurred in four out of four experiments, the per cent reactivation was quite variable among experiments and at different times during the experiments. Similarly, X-irradiation or combined X-irradiation and cyclophosphamide (Table 3) resulted in variable degrees of virus reactivation. Maximum reactivation occurred at 7 to 9 days. Ganglionic homogenates from over 100 latently infected mice not treated with cyclophosphamide or X-irradiation failed to yield infectious virus.

To determine whether HSV reactivation in ganglia could result in neutral transport of HSV and ultimately the presence of infectious virus at epithelial surfaces, paired eye globes from latently infected mice treated with cyclophosphamide for 9 to 25 days were homogenized and assayed for infectious virus. As seen in Table 4, HSV was detected in eye homogenates from 10 to 22% of the mice in four separate experiments. Approximately the same percentage of reactivation occurred in the ganglia of these four experiments (data not shown), but a positive correlation between recovery of virus in the eyes and ganglia of the
Table 4. Detection of infectious virus in cell-free eye homogenates from
animals treated with cyclophosphamide*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Positive/tested†</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2/9</td>
<td>22</td>
</tr>
<tr>
<td>B</td>
<td>2/9</td>
<td>22</td>
</tr>
<tr>
<td>C</td>
<td>2/20</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>2/21</td>
<td>10</td>
</tr>
</tbody>
</table>

* Animals latently infected with HSV were treated with cyclophosphamide (see Methods). Eye globes were removed between 9 and 25 days after initiation of treatment and cell-free homogenates assayed for infectious virus.
† Number of paired eye homogenates containing infectious virus/number of mice tested.

Table 5. Amplification of virus reactivation by in vitro cultivation of ganglia from latently-infected cyclophosphamide-treated mice

<table>
<thead>
<tr>
<th>Days*</th>
<th>Ganglia cultured in vitro†</th>
<th>Ganglia not cultured in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline-treated</td>
<td>Cyclophosphamide-treated</td>
</tr>
<tr>
<td></td>
<td>Reactivated/tested</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>3/10</td>
<td>30</td>
</tr>
<tr>
<td>12</td>
<td>0/10</td>
<td>0</td>
</tr>
</tbody>
</table>

* Days after initiation of cyclophosphamide treatment.
† Paired trigeminal ganglia from individual mice were placed in culture for 24 h, homogenized and assayed for infectious virus.

same animal was found in only one case. Control eye homogenates from 40 latently infected, untreated mice were all negative for infectious virus.

Virus reactivation in trigeminal ganglia was also demonstrated by immunofluorescent techniques. Sections from trigeminal ganglia of latently infected mice were stained with FITC-labelled anti-HSV antibody. Virus antigens were not detected in any of the sections from untreated latently infected mice (20 ganglia from ten mice), while virus antigens were found in isolated cells in approx. 10% of mice treated for 9 to 10 days with cyclophosphamide. There was little or no evidence of virus spread to adjacent cells.

The possibility that reactivation had occurred in a higher percentage of animals, but that the assay techniques (i.e. recovery of infectious virus in ganglionic homogenates and detection of virus antigens by immunofluorescence) were not sensitive enough to detect the virus, prompted an attempt to amplify virus recovery. This technique was based on the assumption that if HSV had been reactivated *in vivo* after cyclophosphamide treatment, the ganglia, when cultured *in vitro*, would be able to produce infectious virus sooner than ganglia from untreated mice. To test this possibility, paired trigeminal ganglia from latently infected mice that had been treated for 9 days with cyclophosphamide or saline were incubated in culture for 24, 30 and 36 h. The ganglia were then homogenized and assayed for infectious virus. At 24 h, infectious virus was detected in seven of ten paired ganglia from cyclophosphamide treated mice as compared to two of ten paired ganglia from untreated mice. At 30 and 36 h, the difference between the two groups was no longer apparent and reactivation approached 100%. Using this method of amplification, ganglia were removed from latently infected mice 7, 9 and 12 days after cyclophosphamide or saline treatment. The ganglia were placed in culture for 24 h, homogenized and assayed for infectious virus. The data in Table 5 show that the per cent recovery was considerably higher in the ganglia from cyclophosphamide treated mice at each of the times examined.
In contrast, if ganglia were homogenized directly upon removal without in vitro cultivation for 24 h, less than 10% of the paired ganglia from cyclophosphamide-treated mice were positive in this experiment. Thus, cyclophosphamide may be producing a higher rate of reactivation than is apparent by the usual methods of detection.

DISCUSSION

Previous studies have shown that the host's immune response markedly decreases virus replication at the epithelial site of HSV inoculation (Walz et al., 1977). Little attention, however, has been given to the function of the immune response in (1) the control of the acute phase of ganglionic infection, (2) the establishment of latency and (3) the maintenance of latency. Both the in vitro and in vivo experiments reported here show that the immune response plays an important role in controlling the acute phase of the ganglionic infection. In organ cultures, antibody reduced the virus titre, but did not eliminate the infection. The simplest explanation for these findings is that by neutralizing extracellular virus, antibody allows only cell-to-contiguous-cell spread of HSV (Notkins, 1974). The rise in virus titre when antibody is removed from ganglionic cultures (Fig. 2) is best explained by infection of nearby or distant cells secondary to the extracellular spread of the virus.

Although the in vitro organ culture system provided useful information about immune control, it has some obvious deficiencies. In ganglionic cultures, the virus may enter the cut end of the axon and spread to other nerve bodies in a different way from that which occurs in vivo. Moreover, most components of the host's immune system are not present in organ culture. For these reasons, an in vivo system was used to evaluate the host's immune response to HSV ganglionic infection. The results (Fig. 3) were very similar to those obtained from organ culture; i.e. antibody reduced the virus titre, but did not eliminate the acute ganglionic infection. In addition, the demonstration that passively administered antibody eliminated the acute phase of the ganglionic infection in immunocompetent but not immunodeficient mice provided support for the argument that cell-mediated immunity is a necessary component of the host's defence against HSV ganglionic infections.

What role, if any, the host's immune response actually plays in establishing latency is not known. There are two possibilities. First, the host's immune response may modulate a productive infection in neurons and bring about a conversion to a non-productive (latent) infection, perhaps in a way similar to the proposed modulation of measles virus infection by antibody (Joseph & Oldstone, 1974, 1975). Second, the host's immune response may have nothing to do with establishing latency. Instead, there may be two populations of infected cells in the ganglia: one productively infected and the other latently infected. The host's immune response may eliminate the productively infected cells (i.e. the acute phase of the infection) thereby allowing detection of the latently infected cells by explantation of the ganglia. At the present time, we cannot distinguish between these two possibilities.

The part played by the host's immune response in maintaining the latent phase of the infection also is unknown. Our experiments with immunosuppressive agents showed that close monitoring of ganglionic homogenates was required to detect reactivation. Lack of such close monitoring in a sufficient number of animals may be one of the reasons why others have not found reactivated virus when immunosuppressive agents were given as the only treatment (Stevens & Cook, 1973; Blyth et al., 1976; Hurd & Robinson, 1977; Martin et al., 1977; Price & Schmitz, 1978).

Precisely how treatment with cyclophosphamide and X-irradiation produce reactivation is unclear. The observation of reactivation without immune manipulation by nerve trauma (Carlton & Kilbourne, 1952; Walz et al., 1974; Nesburn et al., 1977; Price & Schmitz, 1978) and epithelial stimulation (Blyth et al., 1976; Hurd & Robinson, 1977; Hill et al., 1978)
shows that diverse stimuli may induce reactivation. The underlying mechanism is particularly difficult to define since the state of the virus during latency is not known. If, in fact, a low level of virus replication is constantly or intermittently taking place in the latent stage (Nesburn et al. 1967; Baringer & Swoveland, 1974; Scriba, 1975; Hill & Blyth, 1976; Schwartz et al. 1978), suppression of the host's immune defence mechanisms may give the virus a better opportunity to replicate and spread. Alternatively, if infectious virus is not being made (Stevens & Cook, 1971; Walz et al. 1974; Puga et al. 1978), but a portion of the virus genome is expressed (Yamamoto et al. 1977), then immune lymphocytes in the vicinity of the infected cells might interact with virus products (e.g. cell surface antigens) and/or release mediators (e.g. interferon) that could modulate virus synthesis. In our experiments, the cell-mediated response was suppressed, but anti-HSV antibody titres remained elevated at the time of virus reactivation. Although high serum antibody levels did not prevent reactivation, the low amount of infectious virus recovered and the sparsity of immunofluorescent positive cells in reactivated ganglia may be explained, at least in part, by the prevention of extracellular virus spread by antibody. This effect of antibody on extracellular virus spread also might explain Stevens' observation that fewer infected cells were observed in latently infected ganglia transplanted to the peritoneal cavity of immune as compared to non-immune mice (Stevens & Cook, 1974).

Cyclophosphamide and X-irradiation also might reactivate HSV by means other than suppression of the cell-mediated response. Both of these agents can damage DNA (Ludlum, 1975) and might act directly on ganglia. Possibly, through the action of cell DNA excision and repair (Cleaver, 1974), virus DNA replication is in some way stimulated. The capacity of cyclophosphamide and X-irradiation to reactivate HSV in ganglia may thus serve as a useful model for obtaining new information on the factors that control latency.

The authors wish to thank Mr Granville Lewis for his excellent technical assistance.

REFERENCES


(Received 4 October 1978)