Photoinactivation of Latent Herpes Simplex Virus in Rabbit Kidney Cells

J. J. KELLEHER* AND J. VARANI

Microbiology Department, University of North Dakota School of Medicine, Grand Forks, North Dakota 58201

Received for publication 16 March 1976

The photoinactivation of actively and nonactively growing herpes simplex virus by neutral red and proflavine was studied in rabbit kidney cells. Active virus growth was inhibited by both dyes under conditions which did not destroy the cells. Neutral red caused a much greater inhibition than proflavine. Neutral red also caused a reduction in the reactivation rate of latent virus when the infected cells were treated during the latent period. In the treated cultures that did re-activate virus, the average length of the latent period increased over the control value. Proflavine treatment did not reduce the rate of reactivation of latent virus and did not increase the average latent period of the treated cultures.

Herpes simplex virus (HSV) is inactivated by photosensitizing dyes such as neutral red and proflavine after exposure to visible light (1, 11, 17). Experiments with other viruses have indicated that these dyes bind to the viral nucleic acid, resulting in the breakdown of guanine residues (3, 12-14, 18). A number of clinical studies and studies using laboratory animals concerning the therapeutic effects of neutral red and proflavine have been reported (2, 4, 5, 7-10, 16). These studies include cases of herpetic eye infections (7, 9, 16) and genital herpes lesions (4, 5) as well as primary and recurrent oral and skin lesions. The overall effectiveness of photosensitization therapy has yet to be proven, and potential hazards of the treatment have been brought up (11). In experimental studies virus has been inactivated by exposure to light both after extracellular virus was treated with dye (1, 17) and after virus was grown in cells treated with dye and then separated from the cellular material (11). In the present study we have treated cultures with neutral red and proflavine in an attempt to inactivate virus within the infected cells. We have treated cultures in which virus was actively growing and cultures in which virus was not actively growing and was considered to be latent (6). We have found that production of infectious virus could be reduced by neutral red treatment employing conditions that were tolerated by the cells and that reactivation of latent virus could also be inhibited under these conditions. Proflavine treatment was much less effective in reducing active virus growth and did not prevent the reactivation of latent virus.

MATERIALS AND METHODS

Cell cultures and virus. Primary rabbit kidney (RK) cells were used throughout this study. The preparation of cell cultures and the maintenance of the cells for long-term studies have been described in a previous report (6). The virus used in this study was the MS strain of HSV, a type 2 strain obtained from the American Type Culture Collection, Rockville, Md. The routine handling of virus including preparation of virus stocks, infection of RK cells, isolation, identification, and quantitation of the virus recovered from RK cells have all been previously described (6).

Latent infection. To establish latent infection RK cells were infected with a low multiplicity of virus (5,000 plaque-forming units [PFU] of virus per culture containing 10⁶ cells) and incubated at 41°C for 2 to 6 days. Virus growth did not occur at 41°C, and the infectious virus disappeared at a logarithmic rate. After incubation at 41°C, the cultures were transferred to 37°C. Virus growth resumed in some of the infected cultures immediately after transfer to 37°C. However, there were extensive delays in many of the cultures at 37°C, before active virus growth resumed. The period of time during which virus growth was not observed at 37°C was defined as the lag period. Lag periods of 4 or more days indicated some delay in virus replication after transfer to 37°C, because virus growth could always be seen within 3 days when infectious virus was inoculated directly onto cells at 37°C. Of the 200 cultures initially infected and incubated at 41°C, 82 had lag periods of 4 or more days and 48 had lag periods of 7 or more days after transfer to 37°C. The longest lag period was 45 days, and the average lag period of the last 48 cultures was 15.3 days. When active virus growth resumed, a cytopathic effect (CPE) could always be seen. Infectious virus was recovered from the cultures at this time.
The virus was passed once in RK cells and neutralized by commercial anti-HSV serum. The details of this model latent infection have been previously reported (6).

Photosensitizing dyes. A sterile solution of neutral red dye (1:300) was purchased from Grand Island Biological Co. (Grand Island, N.Y.). This stock solution was diluted 1:1,000 with Earle balanced salt solution. Cultures containing 1 ml of maintenance medium were treated with 0.2 ml of the diluted dye. The cultures were then incubated in the dark for 2 h. After 2 h the dye was removed, and the cultures were washed once with Earle balanced salt solution. They were then exposed to light from three 6-watt, cool-white fluorescent bulbs at a distance of approximately 10 cm for a period of 2 h. Proflavine dye was obtained from Pfaltz & Bauer, Inc. (Corona, N.Y.). A 0.1% solution of dye was prepared in Earle balanced salt solution and filter sterilized. This stock solution was diluted 1:1,000 with Earle balanced salt solution, and cultures containing 1 ml of the maintenance medium were treated with 0.2 ml of the diluted dye. The proflavine-treated cultures were then handled in the same manner as the neutral red-treated cultures. The concentrations of dyes used were chosen because they were the highest concentrations that were tolerated by the cells under the conditions of the experiments. At the concentrations used, a few of the cells detached from the monolayers after exposure to light. However, most of the cells appeared normal, and the monolayers remained intact.

RESULTS

Effect of neutral red treatment on virus growth at 37°C. Cultures of RK cells were infected with approximately 500 PFU of virus (multiplicity of infection, 0.0005) and incubated at 37°C for 4 h to allow virus to adsorb. The cultures were then treated with neutral red and photosensitized as described in the Material and Methods section. After photosensitization, the cultures were incubated in the dark at 37°C along with untreated control cultures. By 48 to 72 h after infection the control cultures showed widespread CPE. CPE could also be seen in all treated cultures, although it was only seen in a few focal areas in each culture. The cultures were freeze-thawed three times and assayed for virus in RK cells. The cultures were wrapped in aluminum foil during handling to minimize their exposure to visible light. The neutral red treatment greatly reduced the amount of virus produced. In the control cultures 5 × 10^4 to 1.5 × 10^5 PFU of virus per ml were recovered, whereas in the neutral red-treated cultures only 17 to 20 PFU of virus per ml were recovered. Some of the neutral red-treated cultures were allowed to continue incubating in the dark for an additional 3 days. Virus spread throughout these cultures and eventually did infect all of the cells. Some cultures were treated with neutral red but were not exposed to visible light. No reduction in CPE was observed in these cultures and as much infectious virus was produced in these cultures as in the control cultures.

Effect of neutral red treatment on latent virus. Cultures of RK cells were infected with 5,000 PFU of virus and incubated at 41°C for 2 to 3 days. After incubation at 41°C the cultures were transferred to 37°C and separated into two groups. One group was kept as a control, and the other group was treated with neutral red and photosensitized as described in the Materials and Methods section. All cultures were then incubated in the dark at 37°C and examined daily for the appearance of CPE. From previous work (6) we knew that once CPE appeared, active virus growth was occurring and infectious HSV could be recovered from the cultures at this time. The date on which CPE first appeared was noted. The cultures that did not show CPE were routinely incubated up to 45 days before being discarded. At the conclusion of the experiment the overall reactivation rates and the average lag periods of the control and treated groups were determined (Table 1). In the control group virus was recovered from 124 of 164 cultures for a reactivation rate of 76%. The average lag period of the cultures in this group was 5.0 days. In the neutral red-treated group virus was recovered from 82 of 153 cultures for a 54% recovery rate. The average lag period of the cultures in this group was 10.7 days. The differences in the overall reactivation rates and average lag periods between the control group and treated group were both highly significant statistically (P < 0.001).

In one experiment the neutral red-treated cultures were not exposed to visible light. Virus was recovered from these cultures and from

TABLE 1. Effects of neutral red treatment on the recovery of HSV from cells infected with latent virus

| Treatment* | No. of cultures treated | No. of cultures in which virus was recovered after incubation at 37°C (% | Avg lag period (days)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>164</td>
<td>124 (76%)</td>
<td>5.0</td>
</tr>
<tr>
<td>Neutral red</td>
<td>153</td>
<td>82 (54%)</td>
<td>10.7</td>
</tr>
</tbody>
</table>

* Cultures were infected with 5 × 10^4 PFU of virus and incubated at 41°C for 2 to 3 days. After incubation at 41°C, the cultures were transferred to 37°C. Half were kept as controls, and half were treated with neutral red and photosensitized as described in the Materials and Methods section. All cultures were incubated in the dark and examined daily for CPE. At the conclusion of the experiment, the recovery rates and average lag periods were determined.
matched control cultures at the same rate, and there was no significant difference in the average lag periods of these two groups. In another experiment cultures of RK cells were infected with virus and incubated at 41°C for 1 day. After 1 day the cultures were separated into two groups. One group was kept as a control, whereas the other was treated with neutral red and photosensitized. After photosensitization the cultures were immediately returned to 41°C incubation for an additional 2 days. The cultures were then transferred to 37°C, incubated in the dark, and examined daily for CPE. The photosensitization treatment followed by 41°C incubation affected the virus in the same manner as photosensitization followed by 37°C incubation. Virus was recovered from 17 out of 27 control cultures for a reactivation rate of 63% and from 5 out of 24 treated cultures for a reactivation rate of 21%. The average lag period of the treated cultures was increased from 3.8 to 5.6 days.

Effect of proflavine treatment on virus growth at 37°C. Cultures of RK cells were infected with approximately 500 PFU of virus (multiplicity of infection, 0.0005) and incubated at 37°C for 4 h. The cultures were then treated with proflavine and photosensitized. The treated cultures and untreated control cultures were then incubated in the dark at 37°C. By 48 h the control cultures showed widespread CPE. The amount of CPE observed in the proflavine-treated cultures was reduced, although the reduction was not as great as that seen with the neutral red treatment. The control and proflavine-treated cultures were freeze-thawed three times and assayed for infectious virus in RK cells. Again care was taken to keep the treated cultures from being exposed to visible light. The amount of virus produced in the treated cultures was reduced from the control value. In the control cultures 5.5 x 10^5 PFU of virus per ml were recovered, whereas in the proflavine-treated cultures only 5 x 10^4 PFU of virus per ml were recovered.

Effect of proflavine treatment on latent virus. Cultures of RK cells were infected with 5,000 PFU virus, incubated at 41°C for 2 to 3 days, and then transferred to 37°C. The cultures were separated into two groups; one group was kept as a control group and the other cultures were treated with proflavine and photosensitized as described in the Materials and Methods section. After photosensitization the cultures were incubated in the dark and observed daily for CPE. The date on which CPE was first observed was recorded and at the completion of the experiment the overall reactivation rates and the average lag periods of the control and treated groups were determined (Table 2). Unlike neutral red treatment, proflavine treatment did not reduce the overall reactivation rate. Virus was actually recovered from a higher percentage of the proflavine-treated cultures (70%) than from the control cultures (63%), although this difference was not statistically significant. In addition, the average lag period of the treated cultures was slightly reduced from the control value. This difference was statistically significant (P < 0.001).

**DISCUSSION**

The inactivation of viruses by the various photosensitizing dyes in the presence of visible light is thought to be the result of the dye binding to the viral nucleic acid and causing the breakdown of guanine residues in a photooxidation reaction (3, 12-14, 18). Extracellular HSV particles have been shown to be photosensitive either when extracellular virus is exposed to neutral red or proflavine (1,17) or when the virus is grown in cells in medium containing the dye (11). It was the aim of this study to look at the photosensitizing ability of these agents on virus within viable cells. In this type of study, the concentrations of dyes that can be used is limited by the inherent toxicity of these agents for the cells. In this study experiments were run to test the agents against actively growing virus and against virus in a nonproductive infection. Against actively growing virus both neutral red and proflavine were effective in reducing virus yields at dye concentrations tolerated by the cells. Proflavine treatment reduced production by about 90%. Neutral red was even more effective and caused a reduction of 4 to 5 logs.

![Table 2](http://aac.asm.org/)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cultures treated</th>
<th>No. of cultures in which virus was recovered after incubation at 37°C (%)</th>
<th>Avg lag period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>214</td>
<td>135 (63%)</td>
<td>6.9</td>
</tr>
<tr>
<td>Proflavine</td>
<td>214</td>
<td>150 (70%)</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* Cultures were infected with 5 x 10^9 PFU of virus and incubated at 41°C for 2 to 3 days. After incubation at 41°C, the cultures were transferred to 37°C. Half were kept as controls and half were treated with proflavine and photosensitized as described in the Materials and Methods section. All cultures were incubated in the dark and examined daily for CPE. At the conclusion of the experiment, the recovery rates and average lag periods were determined.
A very important aspect of this study was to look at the effects of these agents on virus that was not actively multiplying. In the model latent infection that we used all attempts to demonstrate the presence of infectious virus have been negative. We have never been able to isolate infectious virus; the infected cells do not appear to be altered morphologically by the virus, and agents such as bromodeoxyuridine and iododeoxyuridine do not eliminate the virus from the cells (6, 15). In this study treatment of latent virus-infected cells with neutral red significantly reduced the number of cultures from which virus was recovered and also increased the average lag period of the cultures that eventually did begin producing virus. If the increase in the average lag period were the only finding, this could be due simply to the elimination of actively growing virus. When the cultures are transferred to 37°C some of the cultures begin producing infectious virus immediately and CPE is seen within 3 days. If this virus that is actively growing were eliminated, it would reduce the number of cultures with short lag periods. However, this should not reduce the overall reactivation rate, since these cultures would still contain nonactively growing virus in addition to the virus that is actively multiplying. The fact that we did see a reduction in the reactivation rate leads us to suspect that the neutral red treatment affects the latent virus in addition to the actively growing virus.

Furthermore, in one experiment cultures were photosensitized and then returned to a 41°C incubation for 2 days. This treatment also resulted in a reduction of the overall reactivation rate. Although we do not refer to nongrowing virus at 41°C as being latent, it definitely is not actively growing. Finally, it has been shown that free viral nucleic acid can be inactivated by photosensitization (12) so there is no theoretical reason why the nongrowing virus in our infection should be resistant to the treatment.

The apparent elimination of latent virus by the neutral red photosensitization treatment indicates that latent HSV in our model system is not necessarily completely resistant to chemical attack. These results are in contrast to our previous results with bromodeoxyuridine on the same model infection (15). In the previous study we found that the latent infection could be modified by prior treatment of the cells with bromodeoxyuridine, but that once the latent infection was established the reactivation rate could not be reduced by treatment. Even though neutral red treatment did reduce the reactivation rate, there was still a large amount of latent virus that was not affected by the neutral red treatment. A total of 82 of the 153 neutral red-treated cultures did eventually reactivate virus and, of these, 45 had lag periods of 7 or more days. Perhaps we might have been able to eliminate all of the virus if we were able to use a higher concentration of dye and a longer exposure to visible light.

When proflavine was used in place of neutral red, the overall reactivation rate was not reduced. In fact, virus was recovered from a few more of the proflavine-treated cultures than control cultures. We also observed a slight shortening of the average lag period of the proflavine-treated cultures. This reduction was actually statistically significant (P < 0.001), although we hesitate at this point to say that virus reactivations were induced by the proflavine treatment.

It is not possible to explain the different results observed with the two dyes. The mechanisms of action of the two dyes may, in fact, be different, or it may be that neutral red is able to penetrate the cell more easily than proflavine. The concentration of dyes used was limited by their toxicity for the cell cultures. We were able to use a slightly higher concentration of neutral red in these experiments. Perhaps if a higher concentration of proflavine could have been used, we would have obtained results similar to those obtained with neutral red.

With regard to the therapeutic usefulness of photosensitization treatment for herpetic infections, the questions regarding effectiveness and safety remain to be answered. It appears from this study, however, that substantially different results may be obtained when different agents are used. In this study neutral red was more effective than proflavine in inhibiting active virus growth in RK cells. Furthermore, neutral red reduced the number of latent virus reactivations and prolonged the average lag period before reactivations occurred. Proflavine did not have these affects.

ACKNOWLEDGMENTS

This study was supported in part by Public Health Service research training grant 5T 01 GM 0 1097 from the National Institute of General Medical Sciences and by grant 5 S01 RR05407 from the Division of Research Resources.

LITERATURE CITED