Effect of Treatment with 5-Ethyl-2'-Deoxyuridine on Herpes Simplex Virus Encephalitis in Normal and Immunosuppressed Mice

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5-Ethyl-2'-deoxyuridine (5-ethyl-dUrd), an analog of thymidine, was evaluated for its capacity to inhibit herpes simplex virus (HSV) replication in vitro and in vivo. The 50% effective dose concentration of 5-ethyl-dUrd for HSV types 1 and 2 (HSV-1 and -2) cultured in Vero cells was 6 and 9 μg/ml, respectively. Levels of 5-ethyl-dUrd 14-fold in excess of the 50% effective dose for HSV-1 did not inhibit the formation of confluent monolayers by Vero cells, suggesting that the compound was not cytotoxic or inhibitory for mammalian cells. In vivo studies showed that 5-ethyl-dUrd was effective in significantly reducing mortality when administered to young adult mice after subcutaneous infection with HSV-2. Intraperitoneal and intravenous inoculation of drug (250 mg/kg per day) resulted in a 50% survivor rate at 15 days. Comparative studies with adenine arabinoside at 250 mg/kg per day gave a 40% survivor rate. Intramuscular injection of 5-ethyl-dUrd at a concentration as high as 2,000 mg/kg per day for 10 days was well tolerated by uninfected animals, and HSV-2-infected mice treated at this dosage had a 100% survival rate. Treatment with 5-ethyl-dUrd at a concentration of 500 mg/kg per day significantly increased the mean survival times of HSV-1- and HSV-2-infected mice immunosuppressed by irradiation; however, the fatal course of the infection was not altered. Assay for virus in tissues showed that 5-ethyl-dUrd treatment delayed progression of the infection into the central nervous system, indicating suppression of virus replication in the tissues.

The treatment of herpes simplex virus (HSV) encephalitis is the subject of much active research. Still, only a small number of effective antiviral drugs have emerged (21, 24). Among these, the most promising currently undergoing clinical trials is adenine arabinoside (ara-A) (28). However, this antiviral agent is moderately cytotoxic in vitro for Vero cells at concentrations inhibitory to HSV (2) and teratogenic in rabbits (11) and has been reported to cause chromosome breakage in human lymphoid cells in vitro (17) and in vivo (29). An additional major disadvantage is its low solubility in water, a property which necessitates infusion of very large volumes over lengthy periods of time when the drug is used in antiviral therapy clinically (3). Although not considered sufficiently toxic to limit its use in life-threatening or severely debilitating situations involving HSV infections, ara-A, when given in doses of 20 mg/kg per day, has also been reported to cause anorexia, nausea, vomiting, weight loss, megaloblastosis in the erythroid series in bone marrow, and thrombophlebitis when given by the intravenous (i.v.) route (22).

In the search for an antiviral agent which might have less serious toxicity problems, we have investigated the efficacy of 5-ethyl-2'-deoxyuridine (5-ethyl-dUrd) for treatment of HSV encephalitis. 5-Ethyl-dUrd is a highly soluble thymidine analog which has been shown to have a striking degree of in vitro antiviral activity toward DNA viruses (1, 4). Other studies with 5-ethyl-dUrd have shown that it is therapeutically effective against superficial and deep HSV keratitis both experimentally (5, 6, 14) and in humans (6) when applied topically or injected subconjunctivally. Thus far, there is no evidence that it is mutagenic (7, 20, 25, 27), immunosuppressive (9), or inhibitory to corneal tissue regeneration (8). It has also been shown by Gauri et al. (10) that 5-ethyl-dUrd does not induce activation of oncogenic viruses in tissue culture.

We investigated the antiviral activity of 5-ethyl-dUrd against HSV type 1 (HSV-1) and HSV-2 in a murine encephalitis model. In vivo
tests were done with immunosuppressed as well as normal mice because disseminated herpesvirus infections have been recognized to be more severe in patients who are immunologically compromised (16).

MATERIALS AND METHODS

Cells and virus. Vero cells, obtained from Flow Laboratories, were maintained in 150-cm plastic tissue culture flasks (Corning Glass Works) in Eagle minimal essential medium supplemented with antibiotics, 10% fetal calf serum, and NaHCO₃.

HSV-1 (KOS strain) and HSV-2 (333 strain) were prepared from plaque-purified virus on Vero cells infected at a multiplicity of 0.1 plaque-forming units (PFU) per well. Virus was harvested from cells at 20 h after infection by freezing and thawing. Supernatants were clarified and titrated for virus on Vero cells as previously described (19).

Compounds. ara-A was purchased from the Sigma Chemical Co. and administered as a sterile aqueous solution at a concentration of 19.37 mg/ml. The low solubility of ara-A necessitated acidification with HCl followed by immediate back titration to pH 5.0 with sodium hydroxide. All solutions of ara-A were prepared immediately before each use. In vitro assay of the antiviral activity of ara-A against HSV-2 showed that preparation in this manner did not alter its activity as determined by comparison with ara-A prepared in 0.85% saline within the solubility limits (0.5 mg/ml) of the compound. The 50% effective dose concentration of ara-A preparations for HSV-2 in Vero cell cultures was 30 μg/ml.

5-Ethyl-dUrd was obtained from Summers Laboratories, Inc. and prepared as a sterile solution in 0.85% physiological saline at a concentration of 19.37 mg/ml. Preparations of 5-ethyl-dUrd were stable, remaining biologically active in solution at room temperature for at least 1 month.

In vitro determination of antiviral activity and toxicity. Confluent monolayer cultures of Vero cells were infected with 300 PFU of virus per culture, and the cultures were incubated at 37°C for a 1-h period of adsorption. The unadsorbed virus was removed by washing with minimal essential medium. 5-Ethyl-dUrd, dissolved in minimal essential medium, was added to each flask at appropriate concentrations and allowed to incubate for 1 h. Cultures were then overlaid with 0.5% methyl cellulose and incubated at 37°C for 72 h. Culture fluid was removed, and the monolayers were washed with saline, fixed in methanol, and stained with 1% crystal violet for plaque counts.

The minimum toxic dose, as defined by the concentration of 5-ethyl-dUrd required to reduce the total cell number of growing cell cultures by 50%, was determined by incubating freshly seeded cultures of Vero cells (9.6 x 10⁵ cells) in the presence of various levels of the drug for 72 h at 37°C.

Animals. Four-week-old SJL white mice (Jackson Laboratories, Bar Harbor, Maine) were used in all experiments.

Irradiation of mice. Mice were placed in groups of 18-inch (ca. 45.72-cm)-square Lucite boxes, located 80 cm from the target of an AECL model-8 irradiator containing 60Co emitting 6,268 Ci. The animals were exposed to 170 R/min for 2.2 min. Treatment in this manner has been shown to be immunosuppressive for mice (20). This dose of radiation was sublethal, and no visible signs of irradiation damage to the animals were observed. Animals were infected with virus 24 h after irradiation.

In vivo drug toxicity. To determine maximal levels of 5-ethyl-dUrd tolerated, various doses of the compound were administered daily for 6 days to young adult mice using intraperitoneal (i.p.), i.v., and intramuscular (i.m.) routes of injection. Concentrations of 5-ethyl-dUrd as high as 3,000 mg/kg per day for 6 days did not cause any visible signs of illness in animals (5/5) when administered by an i.p. or i.m. route. Administration of 5-ethyl-dUrd at 500 mg/kg per day produced no observable effects when administered by an i.v. route. Examination of the mice later confirmed the absence of grossly detectable pathology in 5-ethyl-dUrd-treated mice. Based on these considerations and drug availability, experimental protocols were designed to test 5-ethyl-dUrd efficacy at concentrations of 250, 500, and 2,000 mg/kg per day.

Toxicity levels were not determined for ara-A; however, the acute i.p. 50% lethal dose (LD₅₀) for ara-A in rodents has been reported to be in the range of 2,300 mg/kg (12).

Survival studies. Survival studies on immunosuppressed mice were conducted by infecting irradiated mice with 100 LD₅₀ of HSV-1 (3 x 10⁾ PFU) or HSV-2 (3 x 10⁶ PFU) subcutaneously in the right rear footpad. Antiviral compounds were administered at 3 h postinfection by an i.v. route twice daily for 5 days.

Studies with normal animals were carried out by subcutaneous inoculation of 100 LD₅₀ of HSV-2 only. Drug treatment was started at 3 h postinfection and was administered daily by routes and concentrations as indicated. Each daily dose was given in two separate injections for 10 days.

Assay of virus-infected tissues. Tissues to be assayed for virus were homogenized in a Ten Broek tissue grinder (Belo Glass, Inc., Vineland, N.J.) containing Hanks basal salts solution to give a 10% (wt/vol) suspension. The mixtures were frozen and thawed three times and centrifuged at 1,000 x g for 10 min at 22°C, and the supernatants were used for viral titration assays on monolayers of Vero cells.

Statistical analysis. An analysis of variance was used to calculate mean survival times and significant differences. The negative-exponential transformation method as described by Liddell (15) was used to compensate for censoring of mean survival times. An observation time (T) of 15 days and a theta value of 0.10 was used for computing transformation functions.

RESULTS

In vitro antiviral activity of 5-ethyl-dUrd. The antitherapic activity of 5-ethyl-dUrd was determined in Vero cell cultures. The data given in Fig. 1 show that 5-ethyl-dUrd was highly effective in reducing plaque formation by both HSV-1 and HSV-2. The concentrations of 5-ethyl-dUrd which gave 50% inhibition of plaque
formation were in the range of 6 and 9 \( \mu \text{g/ml} \) for HSV-1 and HSV-2, respectively. These results are in accord with previously reported data in HeLa cells (1) and within similar ranges obtained for the in vitro antiviral activity of ara-A (23). Our preparations of ara-A consistently showed a 50% effective dose concentration of 30 \( \mu \text{g/ml} \) in the Vero cell cultures.

Addition of 5-ethyl-dUrd to freshly seeded Vero cells resulted in a 50% reduction in total cell number of growing cultures at a concentration of 200 \( \mu \text{g/ml} \). These data indicate that the antiviral index of 5-ethyl-dUrd, as defined by the ratio of the concentration required to reduce total cell number by 50% to the concentration required to inhibit plaque formation by 50%, was 33 versus 22 for HSV-1 and HSV-2, respectively.

In vivo antiviral activity of 5-ethyl-dUrd in non-immunosuppressed mice. In vivo studies on the antiviral activity of 5-ethyl-dUrd were conducted by a subcutaneous route of infection (16). Studies on survival times with HSV-2-infected non-immunosuppressed animals showed a significant increase in survival rate of all animals in the treated groups as compared with controls. As shown in Table 1, mice treated with 5-ethyl-dUrd at doses of 250 \( \text{mg/kg} \) per day by either an i.p. or i.v. route of administration had a 50% survival rate at 15 days. A 40% survivor rate was observed with ara-A-treated animals. Animals surviving at 15 days were used for determining whether virus was present in various tissues. All tissues examined, which included the footpad, sciatic nerve, spinal cord, and brain, were devoid of virus.

Animals given 5-ethyl-dUrd at a concentration of 250 \( \text{mg/kg} \) per day by an i.p. route and surviving at 15 days were also immune to rechallenge with 100 \( \text{LD}_{50} \) of HSV-2 when given 30 days later and observed for a period of 60 days. A group of 10 mice of the same age, used previously only as 5-ethyl-dUrd drug controls, were found not to be immune to challenge with 100 \( \text{LD}_{50} \) of HSV-2, with all animals dying inside of a 15-day period.

By using an i.m. route of administration and higher levels of 5-ethyl-dUrd, an improved ther-

![Graph](image)

**Fig. 1.** In vitro antiviral activity of 5-ethyl-dUrd for HSV-1 and HSV-2 in Vero cell culture. Each assay contained virus at a level of 300 PFU per flask. Symbols: \( \bullet \), HSV-1; \( \triangle \), HSV-2.

**Table 1.** Effects of 5-ethyl-dUrd and ara-A treatment in preventing mortality in non-immunosuppressed mice after subcutaneous infection with HSV-2

<table>
<thead>
<tr>
<th>Drug dose per treatment (mg/kg per day)</th>
<th>Route of administration</th>
<th>5-ethyl-dUrd</th>
<th>ara-A</th>
<th>Survivors per total</th>
<th>5-ethyl-dUrd</th>
<th>ara-A</th>
<th>5-ethyl-dUrd</th>
<th>ara-A</th>
<th>Mean survival time (days)</th>
<th>ara-A</th>
<th>Mean survival time (days)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>9 days</td>
<td>15 days</td>
<td>Mean survival time (days)</td>
<td>9 days</td>
<td>15 days</td>
<td>Mean survival time (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>i.v.</td>
<td>7/10</td>
<td>5/10</td>
<td>11.4</td>
<td>10/10</td>
<td>4/10</td>
<td>12.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>i.v.</td>
<td>10/10</td>
<td>5/10</td>
<td>11.7</td>
<td>10/10</td>
<td>4/10</td>
<td>12.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>i.v.</td>
<td>10/10</td>
<td>6/10</td>
<td>12.9</td>
<td>10/10</td>
<td>4/10</td>
<td>12.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,000</td>
<td>i.v.</td>
<td>5/5</td>
<td>5/5</td>
<td>15.0</td>
<td>0/20</td>
<td>6.4</td>
<td>6.4</td>
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</tbody>
</table>

* Routes of infection and drug administration were as described in the text.

* All differences between drug-treated groups and controls were significant (\( P < 0.01 \)) as calculated by an analysis of variance. Animals surviving at 15 days were observed for an additional 30 days with no change in morbidity occurring.
apeutic effect was observed. Doses of 5-ethyl-dUrd at 500 mg/kg per day gave a 60% survival rate at 15 days, whereas the group receiving a maximal concentration of 2,000 mg/kg per day had a 100% survivor rate with no apparent signs of morbidity (Table 1).

In vivo antiviral activity of 5-ethyl-dUrd in immunosuppressed mice. Although immature mice, less than 12 days old, are susceptible to subcutaneous infection with both HSV-1 and HSV-2, these animals develop resistance to HSV-1 by this route of infection at about 4 to 5 weeks of age; however, immunosuppression of these mice results in spread of HSV-1 from subcutaneous sites of infection to the central nervous system (18). Hence, due to the apparent lower neurovirulence of HSV-1 in the animal model used in these studies, immunosuppression was required to establish a fatal infection.

Because the immune response may be an important adjunct to antiviral chemotherapy, the antitherpetic activity of 5-ethyl-dUrd in immunosuppressed animals was tested on both HSV-1 and HSV-2 subcutaneous infection. Figure 2 shows the results obtained when immunosuppressed animals infected with either HSV-1 (Fig. 2A) or HSV-2 (Fig. 2B) were treated with 500 mg/kg per day of 5-ethyl-dUrd by an i.m. route. Similar to non-immunosuppressed mice infected with HSV-2, all HSV-1 and HSV-2 control animals in these experiments died by day 5. As shown, treatment with 5-ethyl-dUrd did not alter the fatal course of the infection but produced a significant increase ($P < 0.01$) in mean survival time for both HSV-1- (3.6 days) and HSV-2- (2.6 days) infected animals.

Levels of virus in treated and control HSV-1-infected animals. On day 4 after HSV-1 infection, the levels of virus in tissues of 5-ethyl-dUrd-treated animals were markedly reduced. Results given in Table 2 show that on day 4 of infection apparently normal 5-ethyl-dUrd-treated animals had approximately 40-, 150-, and 200-fold less virus per gram of tissue in

![Fig. 2. Effect of 5-ethyl-dUrd on survival time of immunosuppressed mice subcutaneously infected with HSV-1 or HSV-2. There were 10 mice per group. 5-ethyl-dUrd was administered at a dose of 500 mg/kg per day by an i.m. route 3 h postinfection for 5 days. Symbols: (A) •, HSV-1, untreated controls; ○, HSV-1, 5-ethyl-dUrd treated. (B) ▲, HSV-2, untreated controls; △, HSV-2, 5-ethyl-dUrd treated.]
Table 2. Titer of HSV-1 in tissues obtained from 5-ethyl-dUrd-treated and untreated immunosuppressed mice

<table>
<thead>
<tr>
<th>Source* of tissue</th>
<th>5-ethyl-dUrd treated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Footpad</td>
<td>$6.0 \times 10^4$</td>
<td>$1.9 \times 10^3$</td>
</tr>
<tr>
<td>Sciatic nerve</td>
<td>$1.0 \times 10^5$</td>
<td>$4.0 \times 10^4$</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>$1.8 \times 10^4$</td>
<td>$2.8 \times 10^4$</td>
</tr>
<tr>
<td>Brain</td>
<td>$9.0 \times 10^5$</td>
<td>$2.0 \times 10^2$</td>
</tr>
</tbody>
</table>

* Tissues were removed from respective animal groups on day 4 after subcutaneous infection and assayed for virus as described in the text. Control animals were near death, whereas 5-ethyl-dUrd-treated animals showed no apparent signs of morbidity.

† Average of two mice from each.

‡ Sensitivity, ≥10 PFU/g of tissue.

The sciatric nerve, spinal cord, and brain, respectively, untreant controls near death. These data indicate that the significant increase in mean survival time of the treated animals may be attributed to inhibition of virus replication by 5-ethyl-dUrd therapy.

DISCUSSION

The major finding of this study was that systemic therapy with 5-ethyl-dUrd was highly effective in preventing a fatal HSV-2 encephalitis developing from non-immunosuppressed mice after infection by a subcutaneous route. In addition, 5-ethyl-dUrd was shown to be as effective as ara-A in reducing mortality when administered i.p. or i.v. at doses of 250 mg/kg per day for 10 days.

In the present investigation, the ability of systemic therapy with 5-ethyl-dUrd to prevent HSV-1 and HSV-2 encephalitides was evaluated by utilizing a peripheral route of infection, simulating that for the natural pathogenesis of the disease. Subcutaneous inoculation of HSV into the footpads of young adult mice results in a fatal encephalitis due to progression of the virus from the site of inoculation to the brain via the sciatic nerve and spinal cord (18). When 5-ethyl-dUrd was administered at 250 mg/kg per day for 10 days by an i.v. or i.p. route, a high survival rate (50%) was observed in HSV-2-infected, non-immunosuppressed mice. ara-A given by an i.v. or i.p. route at 250 mg/kg per day was comparable with 5-ethyl-dUrd in reducing mortalities. However, i.m. injection of 5-ethyl-dUrd at 500 mg/kg per day provided a 60% survivor rate and 2,000 mg/kg per day gave a striking 100% survival value with no apparent toxicity. Although ara-A was not tested at these levels, it has been reported that doses of 2,000 mg/kg per day for 5 days is lethal for mice (13).

Replication, and thus spread, of HSV-2 from the site of infection in non-immunosuppressed mice were inhibited by 5-ethyl-dUrd as demonstrated by the observation that HSV-2 was not detectable in the sciatic nerves, spinal cords, or brains of mice surviving at 15 days. Because 5-ethyl-dUrd-treated animals surviving infection were found to be resistant to rechallenge with 100 LD50 of homologous virus, whereas mice of a similar age not previously infected were susceptible, it is concluded that a virus infection sufficient to establish immunity had occurred. Furthermore, these findings indicate that treatment did not impair the ability of the HSV-2-infected animals to acquire an adequate immune response.

Contrary to the findings for uncompromised animals, it was shown that in mice immunosuppressed by irradiation, passage of virus through the central nervous system was delayed in 5-ethyl-dUrd-treated animals; however, the eventual outcome was death of the animal. These data indicate that the significant increase in life span observed for 5-ethyl-dUrd-treated immunosuppressed mice infected with HSV-1 or HSV-2 may be attributed to the ability of the drug to reduce virus replication. Eventual death of these animals could be due to the absence of a radiotherapy-sensitive component of the immune system. In this regard, ara-A has also been reported to require an uncompromised host defense mechanism for manifestation of its full antiviral activity (26), although Worthington et al. (30) have reported a therapeutic cure with ara-A in immunosuppressed mice infected with HSV-1. Unlike the studies presented here, the route of virus inoculation was i.p. and the mechanism of immunosuppression was by the use of antithymocyte serum.

It is acknowledged that the predictive value of an animal model in extrapolating therapeutic success to the human disease requires many considerations, such as the route of infection, size of the virus inoculum, and route and timing of drug administration. Therefore, comparison of 5-ethyl-dUrd with ara-A as a therapeutic agent in the clinic is relative and tenuous. However, based on these data it appears that 5-ethyl-dUrd when given systemically is a highly effective antiviral compound which offers promise as an added means of treatment for a variety of HSV diseases in humans. In addition, it has the important advantages of high solubility and low toxicity. Along with the findings presented here, these properties suggest that clinical efficacy studies with 5-ethyl-dUrd are warranted.

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