Experimental Herpes Simplex Virus Type 1 Encephalitis: Treatment with 5-Trifluoromethyl-2'-Deoxyuridine

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5-Trifluoromethyl-2'-deoxyuridine (F_3dThd) was evaluated for its neurotoxicity and for its ability to increase the life span of mice injected intracerebrally with herpes simplex virus type 1 (HSV-1) and F_3dThd simultaneously. F_3dThd showed no neurotoxicity at the highest concentration tested (100 mg/kg). Mice injected intracerebrally with HSV-1 died within 5 days postinfection. However, all mice injected concurrently with HSV-1 and 100 mg of F_3dThd per kg lived through the termination of the experiment (60 days). Protection of mice from HSV-1 encephalitis by F_3dThd has been shown to be dose dependent, with 100, 75, 50, and 25 mg of F_3dThd per kg yielding a survival rate of 100, 90, 50, and 10%, respectively. HSV-1 titers in mouse brains receiving HSV-1 and 100 mg of F_3dThd per kg concurrently were 100- to 1,000-fold lower at 2 to 4 days postinfection than control mice receiving HSV-1 alone. F_3dThd was shown not to stimulate interferon production. Encephalitis caused by a ribonucleic acid virus, encephalomyocarditis virus, was not modified by F_3dThd treatment.

5-Trifluoromethyl-2'-deoxyuridine (F_3dThd) was first synthesized in 1964 as a thymidine analogue for the treatment of neoplasias (8). Until recently, it had met with limited success in vivo (L. Helson, A. Yagoda, M. McCarthy, M. L. Murphy, and I. H. Krakoff, *Proc. Am. Assoc. Cancer Res.* 11:136, 1970) (3). However, more recent evaluations in the treatment of childhood neuroblastoma have shown it to be effective in combination therapy with cyclophosphamide, vincristine, and papaverine (L. Helson, *Proc. 67th Annu. Meet. Am. Assoc. Cancer Res.* 17:236, 1976). It inhibits the growth of various mammalian cells in culture (16, 21) and some tumors in mice (7). F_3dThd is also active against herpes simplex and vaccinia viral keratitis in rabbits (11, 12). It has been shown to be highly effective in the treatment of human ocular herpetic keratitis (22).

In vitro studies have demonstrated that deoxythymidine kinase is inhibited by F_3dThd (4) and thymidylate synthetase is inhibited by the nucleotide $F_3dThd-5'-P$ (19). Studies with deoxyribonucleic acid (DNA) polymerase from HeLa cells and from vaccinia-infected HeLa cells indicate that $F_3dThd-5'-PPP$ exhibits competitive inhibition with $dThd-5'-PPP$ and is incorporated into DNA (20).

A major problem encountered when using nucleoside analogues as antiviral agents is the problem of toxicity due to inhibition of host cell DNA biosynthetic functions. Since DNA in neurons of the cerebral cortex replicates at very

low levels after birth (14), viral infections of the brain are logical candidates for target-directed antiviral chemotherapy. To effect this target-directed approach, intracerebral (IC) inoculation was used. This technique eliminated any effects mediated by the blood-brain barrier and delivered a high concentration of F_3dThd in close proximity to infected cells. This communication describes the results of an investigation into the effects of simultaneous administration of F_3dThd and herpes simplex virus type 1 (HSV-1) on the course and outcome of experimentally induced HSV-1 encephalitis in mice.

MATERIALS AND METHODS

Animals. Male Swiss albino HA/ICR mice (ARS/Sprague-Dawley, Madison, Wis.) weighing 18 to 22 g were used throughout this study.

Cell culture. Eagle minimum essential medium (GIBCO) supplemented with 10% heat-inactivated calf serum (GIBCO), 50 U of penicillin per ml, and 50 μ g of streptomycin per ml was used for growth of HEP-2 monolayer cultures, L_{929} cultures, and virus propagation. Eagle minimum essential medium for Spinner culture (GIBCO) supplemented with 10% heat-inactivated calf serum (GIBCO), 50 U of penicillin per ml, 50 μ g of streptomycin per ml, and 0.1% Pluronic F68 (BASF Wyandotte, Wyandotte, Mich.) was used for growth of stock HEP-2 cultures.

Virus. The MacIntyre strain of HSV-1 (ATCC VR-539) was propagated in HEP-2 cells. Confluent HEP-2 monolayers were used to assay infectious HSV-1 (9).

Drug. F_3dThd was generously supplied by Charles Heidelberger of the McArdle Laboratory for

Cancer Research, University of Wisconsin, Madison, Wis.

Virus and drug administration. Mice were injected IC (1) with 30 μ l of one of the following preparations: 26 plaque-forming units (PFU) of HSV-1; 26 PFU of HSV-1 with 100, 75, 50, 25, or 0 mg of F₃dThd per kg; drug alone at 100, 75, 50, or 25 mg of F₃dThd per kg; 1.0×10^2 freeze-thawed HEP-2 cells; or saline. Mice were observed for 30 to 70 days, and deaths were recorded daily.

Lethal effects of HSV-1. Various dilutions of HSV-1 in saline were injected IC into mice. Mice were observed for symptoms of encephalitis and length of time until death. It was determined that 26 PFU of HSV-1 was sufficient to kill 100% of the mice injected within 5 days. This inoculum was used throughout this study.

Brain titers of HSV-1. Groups of 20 mice were inoculated with 26 PFU of HSV-1, 26 PFU of HSV-1 with various drug concentrations, or saline. Three mice from each group were sacrificed at 0, 24, 48, 72, and 96 h and 70 days after injection. Three untreated mice were similarly sacrificed for controls. Brains were aseptically removed, disrupted in a Dounce homogenizer, centrifuged at 1,500 rpm for 15 min to remove cellular debris, freeze-thawed once, and assayed for PFU on confluent HEP-2 monolayers.

Inactivation of HSV-1 in the presence and absence of F₃dThd. Dilutions of HSV-1 were made into sterile saline or into F₃dThd. These incubation mixtures contained 1.0×10^7 PFU of HSV-1 per ml and 2.5×10^{-3} M F₃dThd. The samples were incubated at 4°C. Aliquots were removed hourly for dilution and plaque assay. Plaque assays were performed on confluent HEP-2 monolayers (9).

Induction of interferon by F₃dThd. HEP-2 cells were grown to near confluency in 10-ounce (300 ml) bottles. Cells were exposed to 5.0×10^{-6} M, 5.0×10^{-7} M, or 5.0×10^{-8} M F₃dThd. An aliquot of the supernatant was removed at 8, 25, and 49 h after exposure to drug and assayed for interferon by the encephalomyocarditis (EMC) hemagglutinin yield reduction assay method of Grossberg (6). L₉₂₉ cells were grown to near confluency in culture tubes and exposed to 5.0×10^{-6} M, 5.0×10^{-7} M, and 5.0×10^{-8} M F₃dThd for 24 h. The supernatant was removed and assayed for interferon by the GDVII hemagglutinin yield reduction assay (15). Drug toxicity to L₉₂₉ cells was investigated by determining the hemagglutination yield of GDVII virus. Cells were treated with the same concentrations of F₃dThd as was used in the interferon assays and compared with untreated controls (15).

RESULTS

IC toxicity of F₃dThd. To determine the mean lethal dose of F₃dThd in mice, animals were injected IC with either 100, 75, 50, 25, or 0 mg of F₃dThd per kg in a volume of 30 μ l and observed for 30 days. No deaths occurred in any of the drug-treated or control mice. Therefore, F₃dThd is not neurotoxic at the highest concentration of drug tested. Concentrations of F₃dThd greater than 0.225 M (drug concentra-

tion required to give 100 mg/kg in 0.03 ml) formed precipitates at room temperature; therefore, injections were limited to 100 mg of F₃dThd per kg.

Inactivation of HSV-1 in the presence and absence of F₃dThd. To rule out the possibility that HSV-1 may be inactivated by F₃dThd before IC injection, the following experiment was performed. Duplicate dilutions of HSV-1 were made into sterile saline or into F₃dThd. These incubation mixtures contained 1.0×10^7 PFU of HSV-1 per ml and 2.5×10^{-3} M F₃dThd or saline. The samples were incubated at 4°C. Aliquots were removed hourly for dilution and plaque assay. Figure 1 indicates that the rate of virus inactivation was very similar for both samples. Therefore, preincubation of HSV-1 with F₃dThd does not result in inactivation of the virus beyond that observed in the saline control. This established that mice receiving F₃dThd with HSV-1 in fact receive the same number of PFU of HSV-1 and that these preparations are equally infective. This experiment was repeated at 25 and 37°C with similar results.

Demonstration of a dose-response effect with F₃dThd. To determine the drug concentration necessary to protect mice from fatal encephalitis, the following experiments were conducted. HSV-1 was prepared for injection by

FIG. 1. *In vitro* inactivation of HSV-1 in the presence of F₃dThd. HSV-1 + saline (○) and HSV-1 + 2.5×10^{-3} M F₃dThd (●) were incubated at 4°C. Samples were removed hourly, diluted, and assayed for PFU on confluent HEP-2 monolayers.

dilution into saline or into F₃dThd. One group of mice also received freeze-thawed HEP-2 cells as controls. A dose-response effect was observed when either 100, 75, 50, or 25 mg of F₃dThd per kg was administered concurrently with HSV-1, resulting in a survival rate of 100, 90, 50, and 10%, respectively (Fig. 2). This experiment was performed in duplicate with 10 mice in each group. These results indicate that F₃dThd does in fact modify the course of HSV-1 encephalitis and that this response is dose dependent.

HSV-1 titers in mouse brains after administration of F₃dThd. For an antiviral agent to be effective, it must be capable of inhibiting the replication of the virus in the target tissue (2). In these experiments, we evaluated the effectiveness of F₃dThd in inhibiting the replication of HSV-1 in mouse brains. Mice were injected with either HSV-1 alone or with HSV-1 and 100 mg of F₃dThd per kg concurrently. Three mice each from control and drug-treated groups were sacrificed at 0, 1, 2, 3, 4, and 70 days postinfection. Brains were aseptically removed, disrupted with a Dounce homogenizer, and centrifuged, and the supernatant was assayed for PFU on confluent HEP-2 monolayers. Figure 3 shows the data obtained. Mice receiving 100 mg of F₃dThd per kg had brain titers of HSV-1 that were 100- to 1,000-fold lower at 2 to 4 days postinfection than control mice receiving HSV-1 alone. At 70 days postinfection, no virus was

FIG. 3. Brain titers of HSV-1 in mice at various times postinfection. Groups of mice received 26 PFU of HSV-1 alone (○) or 26 PFU of HSV-1 concurrently with 100 mg of F₃dThd (●) per kg. Three mice from each group were sacrificed daily, and brains were aseptically removed, pooled, disrupted with a Dounce homogenizer, and centrifuged at $1500 \times g$ for 15 min. The supernatant fluid was assayed for PFU on confluent HEP-2 monolayers.

FIG. 2. Dose-response effect of F₃dThd on the outcome of HSV-1 encephalitis. Groups of 10 mice each were infected IC with 26 PFU of HSV-1 alone (○) or concurrently with 100 (●), 75 (△), 50 (▲) or 25 (□) mg of F₃dThd per kg and observed for 30 days or until death. Mortalities were recorded daily.

detected using the plaque assay, indicating that productive infection had ceased in these brains.

Interferon induction by F₃dThd. HEP-2 cells were exposed to 5.0×10^{-6} M, 5.0×10^{-7} M, and 5.0×10^{-8} M F₃dThd for 8, 25, or 49 h, and the supernatant was assayed for interferon by the method of Grossberg et al. (6). There was no stimulation of interferon by F₃dThd as compared with untreated control cells. Supernatant fluid from L₉₂₉ cells exposed to the same concentrations of F₃dThd as above was assayed after 24 h for interferon. This system also showed no stimulation of interferon production as compared with control cells (15). Yield of GDVII virus from cells treated with the same concentrations of F₃dThd also showed no difference in hemagglutination yield as compared with untreated controls.

Effect of F₃dThd on EMC-induced encephalitis. To eliminate the possibility that the antiviral activity of F₃dThd is nonspecific, EMC virus, a ribonucleic acid virus, was used to induce fatal encephalitis in mice. F₃dThd, whose

mechanism of action involves the inhibition of DNA synthesis, should not affect the outcome of this disease. Groups of 10 mice received 300 PFU of EMC virus in saline or 300 PFU of EMC virus concurrently with 100 mg of F₃dThd per kg. Both groups of mice died within 3 days postinjection, indicating that F₃dThd did not modify the outcome of EMC virus-caused encephalitis.

DISCUSSION

HSV encephalitis is one of the most common causes of sporadic fatal encephalitis in the United States (5). The characteristic presentations of this disease range from a subacute meningitis-like disease to a severe, necrotizing, fatal encephalitis. The majority of the severe cases result in death or permanent neurological sequelae including hemiparesis, aphasia, and memory deficit (13). Untreated cases of this disease have shown a mortality rate of 50 to 60%.

A few antiviral agents that have shown promise in the treatment of other viral diseases have also been evaluated for their effectiveness in the management of HSV-1 encephalitis. The three compounds most thoroughly studied have been 5-iodo-2'-deoxyuridine (IdUrd), 1- β -D-arabinofuranosylcytosine (ara-C), and 9- β -D-arabinofuranosyladenine (ara-A). IdUrd has been shown to be ineffective in the management of human HSV-1 encephalitis, with a high percentage of treatment failures and marked toxicity to the compound (18). Based on the recent clinical trials using IdUrd in the treatment of HSV-1 encephalitis, any further use of this drug in the management of this disease should be seriously questioned (18). Ara-C is not as toxic as IdUrd; however, current reports indicate that this drug is less than satisfactory in the management of herpes encephalitis (5). Ara-A has recently shown promise in the treatment of herpes encephalitis in animal model systems. One study of ara-A treatment of HSV-1 encephalitis has shown that 1,000 mg of ara-A per kg given intraperitoneally gives a 21-day survivor percentage of 50%, while increasing the mean survival time from 5.6 to 8.1 days (17). Experiments of this nature, however, have little bearing on the comparative effectiveness of F₃dThd to ara-A in the treatment of this disease. For completeness, both drugs should be simultaneously tested in identical model systems to clearly establish their comparative effectiveness. Ara-A and F₃dThd have been tested side by side in clinical trials for the treatment of herpetic keratitis. These studies show F₃dThd to be superior in terms of shorter heal-

ing time, fewer treatment failures, and the ability to heal more extensively involved disease, at lower drug concentrations, with no significant toxicity (17).

Studies with F₃dThd indicate that in a mouse model system of HSV-1 encephalitis, this antiviral agent is effective by two criteria. First, at doses of 100 mg/kg there is 100% protection against fatal encephalitis. Second, mice treated with 100 mg of F₃dThd per kg have brain titers of HSV-1 that are 100- to 1,000-fold lower than untreated mice between 2 and 4 days postinfection (all control mice succumb to HSV-1 encephalitis by 5 days postinfection). Drug-treated mice brain titers return to zero shortly thereafter. When F₃dThd was tested for neurotoxicity, there was no indication of toxicity at the highest concentration tested, 100 mg/kg. Evidence indicates that HSV-1 is not inactivated by incubation with F₃dThd before injection. Attempts to demonstrate induction of interferon activity by F₃dThd have been unsuccessful. A nonspecific antiviral effect has been eliminated by the failure of F₃dThd to protect mice from EMC-caused encephalitis. Thus, F₃dThd appears to be capable of fulfilling several important criteria of an effective antiviral agent: increased survival time and potent antiviral activity at a nontoxic concentration.

Considering the demonstrated toxicity of F₃dThd to bone marrow and to the gastrointestinal tract (7), a route of administration that bypasses the systemic circulation would be of considerable benefit in the management of HSV-1-caused encephalitis. For instance, administration of F₃dThd into the cerebrospinal fluid could be from one of several anatomical viewpoints: intrathecal, intraventricular, or intracisternal. Current evidence in the treatment of bacterial meningitis indicates that intracisternal and intraventricular routes of administration are beneficial in the management of this disease (10). Further studies into the effectiveness of these routes of administration of F₃dThd may prove to be valuable.

These data have provided sufficient evidence to warrant further investigation of F₃dThd as a potential agent in the treatment of HSV-1 encephalitis.

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