Inhibition of Herpes Simplex Virus Strains Isolated from Herpetic Keratitis by Polyinosinic Acid-Polycytidylic Acid

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Received for publication 13 December 1976

Fifty strains of herpes simplex virus, isolated from patients with herpetic keratitis, were examined *in vitro* for susceptibility to polyinosinic acid-polycytidylic acid [poly(I:C)] in the presence of a constant concentration of diethylaminoethyl-dextran. The minimal inhibitory concentration of poly(I:C) for 44 of these strains ranged from 0.0001 to 0.1 $\mu\text{g/ml}$; for the remaining six strains, the minimal inhibitory concentration stood at 1 to 2 $\mu\text{g/ml}$. Fifteen isolates from primary infections were more susceptible to poly(I:C) than 35 isolates from recurrent infections. Isolates acquired at different points of a given clinical episode showed similar susceptibilities to poly(I:C). In two patients, isolates from consecutive recurrences of infection exhibited reduced susceptibilities. The implications of the above observations for the therapeutic use of poly(I:C) are discussed.

It is known that polyinosinic acid-polycytidylic acid [poly(I:C)], a double-stranded ribonucleic acid complex, is highly active in inducing interferon as well as in resistance to viral infections in animals and in cell cultures (2).

Herpes simplex virus (HSV), the widespread virus of ocular inflammations, has been reported to be only moderately susceptible to the antiviral action of interferon (5). However, Park and Baron (9) found that poly(I:C)-induced interferon was effective in treating herpetic keratoconjunctivitis in rabbits. Guerra et al. (3) reported success in a preliminary study on treatment of human herpetic conjunctivitis by topical poly(I:C). Oosterhuis et al. (8) recently described favorable results in patients with stromal keratitis treated with a combination of poly(I:C) and iododeoxyuridine (IUdR).

Several investigators have evaluated the effect of poly(I:C) *in vitro* in the hope of using it as an interferon inducer in human ocular herpetic keratitis (4, 6, 11, 14). One such study (14) investigated the ability of poly(I:C)-induced interferon to protect rabbit eye tissue cultures prepared from cornea, conjunctiva, and iris against HSV infection: the degree of protection inversely proportional to the virus challenge was significantly enhanced by addition of neomycin. Other *in vitro* studies tested susceptibility of HSV to poly(I:C)-induced interferon in different tissue cultures, including HEP-2, rabbit kidney cells, and mouse embryonic cells (4, 6, 11).

In the present study, the effect of poly(I:C) on

different HSV strains from human herpetic keratitis was investigated in an attempt to determine the value of this interferon inducer in the treatment of such ocular infections. We selected 50 HSV strains isolated during different stages of the disease from primary or recurrent infections in humans, from recurrences in the same individual, and from different days of the same recurrence.

MATERIALS AND METHODS

Cell cultures. Primary rabbit kidney (PRK) cell cultures were prepared from 3-week-old rabbit kidneys according to the method of Smith and Wagner (13). The cells were dispersed in tissue culture tubes in M-199 medium containing 10% calf serum at a density of 6×10^5 to 8×10^5 cells per ml. Confluent monolayers were obtained after 7 to 10 days of incubation at 37°C. For preparation of Vero cells, a continuous line of African Green Monkey kidney cells was grown in tissue culture tubes in M-199 medium containing 5% calf serum at a cell density of 1.5×10^5 to 2×10^5 cells per ml. The tissue cultures were used after 1 to 2 days, when a confluent monolayer was obtained. M-199 medium contained: penicillin, 200 U/ml; streptomycin, 200 mg/ml; and mycostatin, 50 U/ml. The maintenance media used was M-199 containing 2% calf serum.

Viruses. The 50 HSV strains were isolated from patients with herpetic keratitis from the Department of Ophthalmology of the Haim Sheba Medical Center. The viruses were isolated at various stages of the disease from untreated patients and from patients treated with IUdR, corticosteroids, or poly(I:C). The standard HSV strains used were HSV type 1, VR₂ McIntyre strain, and HSV type 2, Rapp

strain. The challenge viruses for interferon determination were vesicular stomatitis virus, Indiana strain, and vaccinia virus.

Isolation and identification of HSV strains. Material for HSV isolation was obtained by gentle swabbing of the corneal epithelium with a sterile cotton swab moistened with M-199 medium containing: 5% fetal calf serum; penicillin, 500 U/ml; streptomycin, 500 mg/ml; and mycostatin, 50 U/ml. The swabs were agitated, inoculated immediately into PRK tissue cultures, and incubated at 37°C until appearance of cytopathogenic effect (CPE). After CPE detection and specific neutralization with anti-HSV serum, the virus stocks were held at -70°C. Before each experiment, the virus titer was determined in PRK cells, and the mean tissue culture infective dose was calculated for each virus stock. All 50 HSV strains isolated from the eyes were found to belong to the type 1 HSV group.

Reagents. Poly(I:C) was obtained from Miles Chemical Co., Elkhart, Ind., as a lyophilized preparation. The stock solution was prepared in a concentration of 1,000 µg/ml in phosphate buffer, pH 7 (2), and stored at -70°C. The working solutions were made in M-199 just before the experiments and held in an ice bath until used.

Diethylaminoethyl (DEAE)-dextran was obtained from the Pharmacia Co., Uppsala, Sweden, at a molecular weight of 2×10^6 . The stock solution was prepared in phosphate buffer, pH 7, to a concentration of 400 µg/ml and held at 4°C. The working solution was diluted in M-199 to 100 µg/ml.

Trypsin (crystallized two times) and soya bean trypsin inhibitor (crystallized three times) were obtained from Worthington Biochemicals Corp., Freehold, N.J. Actinomycin D was obtained from Sigma Chemical Co., St. Louis, Mo.

Procedure for HSV inhibition by poly(I:C). PRK tissue cultures were incubated for 2 h with 100 µg of DEAE-dextran per ml. The DEAE-dextran was removed, the tissue cultures were washed three times with phosphate-buffered saline, and poly(I:C) was added in dilutions of 2 to 0.0001 µg/ml. Control tissue cultures were incubated with 1 ml of M-199 with 2% calf serum. After 18 to 24 h of incubation at 37°C, the poly(I:C)-treated and control cultures were challenged with 100 mean tissue culture infective doses of each HSV strain tested. The tissue cultures were reincubated at 37°C until the CPE in the virus control tubes was observed, usually after 2 days. Then the poly(I:C)-induced cellular resistance was measured as the percent reduction of the CPE in test cultures as compared with the control.

A 50% CPE reduction was taken as the minimal inhibitory concentration (MIC) of poly(I:C) required by every HSV strain tested.

RESULTS

Comparison of the MICs of poly(I:C) with and without DEAE-dextran. In a preliminary experiment, it was found that DEAE-dextran used alone does not induce interferon or antiviral resistance in PRK tissue cultures treated for 18 to 24 h with 50 to 200 µg of DEAE-dex-

tran per ml (and washed three times with phosphate-buffered saline). When 10 HSV strains were tested to compare the MICs of poly(I:C) required with and without DEAE-dextran (Table 1), the MIC of poly(I:C) with DEAE-dextran was 5 to 20,000 times lower than that without it. Note in Table 1 that HSV strains inhibited by a low MIC of poly(I:C) with DEAE-dextran show a very high ratio of poly(I:C) with DEAE-dextran to poly(I:C) without DEAE-dextran. The results show that for better inhibition of HSV strains, it is important to use poly(I:C) together with DEAE-dextran.

MIC of poly(I:C) with DEAE-dextran required against 50 HSV strains. Once it was established that poly(I:C) together with DEAE-dextran was a better inducer for HSV inhibition, 50 HSV strains were tested for determination of the MIC of poly(I:C) with DEAE-dextran required for inhibition of each strain. The HSV strains were tested against different dilutions of poly(I:C) from 2 to 0.0001 µg/ml. The DEAE-dextran concentration was 100 µg/ml in all the experiments. The results (Fig. 1) show that the 50 HSV strains were distributed into six groups in accordance with the concentration of poly(I:C) required for inhibition of each strain: 1 of the HSV strains was inhibited by the smallest dose of poly(I:C) used, 0.0001 µg/ml; 13 were inhibited by 0.001 µg/ml; nearly half (24) were inhibited by 0.01 µg/ml; and 6 were inhibited by 0.1 µg/ml. The remaining six HSV strains were inhibited by 1 to 2 µg of poly(I:C) per ml, the highest dose used.

To state the data in another way, 88% of the HSV strains were inhibited by a low MIC of poly(I:C), ranging from 0.1 to 0.0001 µg/ml, and only 12% were inhibited by a higher MIC, 1 to 2

TABLE 1. MIC of poly(I:C) compared to that of poly(I:C) with DEAE-dextran

HSV strain	MIC of poly(I:C) (µg/ml)	MIC of poly(I:C) with DEAE-dextran* (µg/ml)	MIC of poly(I:C)/MIC of poly(I:C) + DEAE-dextran
1	5.0	1.0	5
2	5.0	0.1	50
3	50.0	2.0	25
4	50.0	1.0	50
5	100.0	2.0	50
6	100.0	1.0	100
7	20.0	0.01	2,000
8	5.0	0.001	5,000
9	10.0	0.001	10,000
10	2.0	0.0001	20,000

* The concentration of DEAE-dextran was 100 µg/ml.

FIG. 1. Inhibitory effect of poly(I:C) on HSV strains in PRK tissue cultures. The indicated dilutions of poly(I:C) were added to PRK cultures after treatment with 100 µg of DEAE-dextran per ml.

µg/ml. The MICs of poly(I:C) required for the standard HSV strains were found to be 0.01 µg of poly(I:C) per ml for inhibition of HSV type 1 strain McIntyre and 0.001 µg of poly(I:C) per ml for inhibition of HSV type 2 strain Rapp.

Comparative MIC of poly(I:C) used against HSV strains isolated from primary and recurrent infections. Of the 50 HSV strains tested, 15 were isolated from primary ocular infections and 35 from recurrent infections. A comparison of the resistance of these two groups to poly(I:C) in vitro (Fig. 2) showed that more of the HSV strains isolated from primary infections were inhibited by a MIC of poly(I:C) ranging from 0.001 to 0.1 µg/ml. Under the same conditions, fewer of the HSV strains from recurrent infections were inhibited by this low MIC of poly(I:C). HSV strains isolated from primary infections were slightly more susceptible to poly(I:C) than those isolated from recurrent infections.

Susceptibility to poly(I:C) of HSV strains isolated from the same patient on different days of the same recurrence. Of the 50 HSV strains tested, 4 were isolated more than once in the same patient on different days during the same recurrence: 2 were isolated twice, and 2

were isolated three times (Table 2). These viruses were isolated despite treatment with either IUdR or poly(I:C), which caused a decrease in virus titer but did not affect reisolation. No changes were found in the MIC of poly(I:C) required against the HSV strains isolated from the same patient on different days of the same recurrence. The results were the same in untreated patients and in those who had received IUdR or poly(I:C).

Susceptibility to poly(I:C) of HSV strains isolated from recurrences in the same patient. It was important to determine whether HSV strains isolated from recurrences in the same patient retained their original susceptibility to poly(I:C). This issue was examined in seven patients: in five there were two recurrences; in two cases there were three consecutive recurrences. The HSV isolations were made from recurrences that occurred at 0.5- to 2-year intervals despite the treatment given between and before isolations [IUdR, corticosteroids, or poly(I:C), the latter administered only when IUdR treatment proved unsuccessful]. The seven HSV strains were tested for their resistance to poly(I:C) in vitro (Table 3). No differences were found among five HSV strains isolated in different recurrences. However, for the inhibition of two HSV strains (no. 270 and 195), 10 times the MIC of poly(I:C) was required. HSV 270 was reisolated 1 year after IUdR treat-

FIG. 2. Inhibitory effect of poly(I:C) on HSV strains isolated from primary and recurrent infections. The experimental procedures used were the same as in Fig. 1. The comparison of the HSV strains isolated from primary infections and those from recurrent infections are presented as percentages.

TABLE 2. Susceptibility to poly(I:C) of HSV strains isolated from patients during different days of same recurrence

HSV strain	Treatment before virus isolation	Time intervals between virus isolations	HSV titer ^a	MIC of poly(I:C) (μ g/ml)
696 ^b	None	1st isolation	RE 8.0 LE 5.0	0.1
	IUDr (2 days)	2nd isolation, 2 days later	RE 4.0 LE 2.0	0.1
1000	None	1st isolation	RE 6.5	0.0001
	IUDr (4 days)	2nd isolation, 4 days later	RE 5.0	0.0001
1097	IUDr (3 days)	1st isolation	RE 8.5	0.1
	None	2nd isolation, 1 day later	RE 7.5	0.1
	Poly (I:C) (6,200 μ g/ml)	3rd isolation, 7 days later	RE 5.0	0.1
1162	None	1st isolation	LE 7.5	0.01
	None	2nd isolation, 2 days later	LE 7.0	0.01
	Poly(I:C) (2,000 μ g/ml)	3rd isolation, 4 days later	LE 4.0	0.01

^a HSV titer = log₁₀ mean tissue culture infective doses per milliliter. RE, Right eye; LE, left eye.^b Bilateral herpetic keratitis.

ment (no change in MIC) and 0.5 year after poly(I:C) treatment, at which time the MIC had risen 10-fold. HSV 195 was isolated from a primary infection and 2 years later from the first recurrence, never having been treated by poly(I:C): the MIC required for the latter was 10-fold that required for the primary strain (Table 3).

It is important to note that this change in MIC of poly(I:C) for strain 195 occurred between the HSV isolated from a primary infection and the HSV isolated from the first recurrence 2 years later.

DISCUSSION

Of the interferon inducers, poly(I:C) was found to be one of the best inhibitors of HSV (11), and the inhibition could be enhanced by a polybasic substance such as DEAE-dextran (1) or neomycin (14).

The results obtained in our study revealed differences in susceptibility to poly(I:C) among the various HSV strains tested; some differences were found between HSV strains from primary and recurrent infections and from recurrences in the same patient. The 50 HSV strains could be divided into six groups accord-

ing to the MIC of poly(I:C) required for inhibition. Standard HSV strains tested also required a different MIC of poly(I:C): HSV type 1 strain VR₂ McIntyre was inhibited by 0.01 μ g/ml, and HSV type 2 strain Rapp was inhibited by 0.001 μ g/ml. Our data are in accordance with the findings of Oh (7) and Munk and Frick (6), who showed that genital HSV was better inhibited *in vitro* by poly(I:C) than oral or corneal HSV strains. It is known that HSV is resistant to inhibition by interferon (5, 7, 11), but there are interstrain differences in addition to intertype differences (7). It is important to note that most of the HSV strains tested (88%) were inhibited by a low MIC of poly(I:C) (0.0001 to 0.1 μ g/ml), and few strains (12%) were inhibited by a higher MIC of poly(I:C) (1 to 2 μ g/ml). We assumed that this low MIC of poly(I:C) was obtained by the use of DEAE-dextran, which increased the permeability of the infected cells to the inducer (1).

Another reason for this low MIC of poly(I:C) required may be the tissue cultures: PRK cultures are known for their high sensitivity to induction and testing of rabbit interferon. The importance of the appropriate tissue culture system was demonstrated by Person et al. (10),

TABLE 3. *Susceptibility to poly(I:C) of HSV strains isolated from recurrences in the same patient*

HSV strain	Treatment between virus isolations	Time intervals between virus isolations	Treatment before each isolation	HSV titer ^a	MIC of poly(I:C) (μ g/ml)
166	IUdR	1st isolation	IUdR	3.7	0.1
	IUdR	2nd isolation, 2 yr	None	3.5	0.1
163	IUdR	1st isolation	IUdR	5.0	0.001
	c.st. ^b	2nd isolation, 1 yr	None	5.2	0.001
139	IUdR + c.st.	1st isolation	c.st.	5.0	1.0
	Poly(I:C) (10,000 μ g/ml)	2nd isolation, 2 yr	c.st.	5.7	1.0
525	IUdR	1st isolation	c.st.	6.7	0.01
	Poly(I:C) (5,200 μ g/ml)	2nd isolation, 2 yr	c.st.	4.7	0.01
170	IUdR	1st isolation	IUdR	6.5	0.01
	Poly(I:C) (5,000 μ g/ml)	2nd isolation, 1 yr	None	6.0	0.01
	IUdR	3rd isolation, 2 yr	None	7.0	0.01
270	IUdR	1st isolation	None	5.5	0.1
	Poly(I:C) (5,300 μ g/ml)	2nd isolation, 1 yr	None	4.5	0.1
	Poly(I:C) (5,600 μ g/ml)	3rd isolation, 0.5 yr	IUdR	4.5	1.0
195	None	1st isolation	None	4.5	0.001
	IUdR	2nd isolation, 2 yr	None	6.7	0.01

^a HSV titer from the eye = log₁₀ mean tissue culture infective doses per milliliter.^b c.st., Corticosteroids.

who concluded that the variation in susceptibility of different HSV types to antiviral agents resulted from differences in the cell culture system used. We found no significant differences in susceptibility to poly(I:C) among HSV strains isolated from primary and recurrent infections; however, the HSV strains isolated from primary infections were somewhat more susceptible than "recurrent strains." The same results were obtained by testing HSV strains isolated from recurrences in the same patient. In one case a rise in MIC of poly(I:C) was found between the virus strain isolated from the primary infection and the strain isolated 2 years later from the first recurrence of the infection. In another study (12) a high correlation was found between the severity of the patient's herpetic keratitis, the resistance of the isolated HSV in vitro (MIC) to IUdR and poly(I:C), and the virulence of the same virus strain for laboratory animals. In view of our results in vitro and those of the previous study, it is suggested that each HSV strain isolated from patients with herpetic keratitis, especially in those "treatment resistant" to IUdR, may be tested in vitro for the MIC of poly(I:C) required, to deter-

mine the efficacy of poly(I:C) treatment of this ocular infection.

ACKNOWLEDGMENTS

We thank A. Romano from the Department of Ophthalmology, Haim Sheba Medical Center, for generously providing us with clinical material used in this study.

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