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# Sensitivity of Types 1 and 2 Herpes Simplex Virus to 5-Iodo-2′-Deoxyuridine and 9-β-D-Arabinofuranosyladenine

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The sensitivities of 21 strains of herpes simplex virus (HSV), 13 type 1 strains and 8 type 2 strains, to 5-iodo-2′-deoxyuridine (IUdR) and 9-β-D-arabinofuranosyladenine (ara-A) were evaluated by the plaque-suppression test in chick embryo fibroblast (CEF), WI-38, and HeLa cell cultures. In CEF, type 1 strains were considerably more sensitive to the inhibitors than were the type 2 strains. In WI-38, the type 1 strains were more sensitive than the type 2 strains to IUdR; however, the two serotypes were equally sensitive to ara-A. In HeLa cells, the differences in sensitivity to IUdR between the two serotypes were less. Eight HeLa-adapted strains (four type 1 and four type 2) evaluated in HeLa cell cultures were equally sensitive to IUdR; the type 2 strains were slightly more sensitive than type 1 strains to ara-A. These results demonstrate the wide variation in sensitivity of HSV types 1 and 2 to antiviral agents which results from differences in the cell culture system and passage history of the strains.

The antigenic, biological, and biochemical differences between types 1 (oral) and 2 (genital) herpes simplex virus (HSV) have been studied in considerable detail (1, 16, 21, 22, 27). It has been known for some time that HSV is sensitive to inhibitors of deoxyribonucleic acid (DNA) synthesis in cell cultures, in animals, and in man (4, 9, 13, 15, 19, 26, 28, 29), but it was not until recently that it was reported that type 1 HSV was more sensitive to such inhibitors than was type 2 (16, 32; S. P. Lowry and W. E. Rawls, Bacteriol. Proc., p. 171, 1969). It was the purpose of this study to investigate further and to quantify the sensitivity of 21 recent isolates of HSV (from human infections) to 5-iodo-2′-deoxyuridine (IUdR) and 9-β-D-arabinofuranosyladenine (ara-A) in various cell culture systems. It will be shown that the differences in sensitivity to these DNA inhibitors can vary depending on the cell culture system used and, at times, on the prior passage history of the virus. In some instances, there is slight or no difference in the sensitivities of types 1 and 2 HSV to DNA inhibitors.

## MATERIALS AND METHODS

**Virus.** Twenty-one isolates of HSV from various sites in humans were randomly selected from hundreds of available virus isolations. All had been isolated originally from clinical specimens in human fetal diploid fibroblast cultures (WI-38 strain, Flow Laboratories, Rockville, Md.; reference 3), and they had been passaged from one to five times in these cells to produce pools that contained $10^5$ to $10^8$ 50% tissue culture infectious doses ($TCID_{50}$) per ml.

**Cell cultures.** Monolayer cultures of chick embryo fibroblasts (CEF), HeLa cells (originally from Flow Laboratories, adapted to bovine serum), and WI-38 cells were grown in 60- and 100-mm plastic petri plates (tissue culture type, Falcon Plastics, Los Angeles, Calif.). The CEF were obtained by trypsinization of 9- and 11-day-old chicken embryos (8), and the resultant cells then were grown in Melnick's lactalbumin hydrolysate-calf serum medium (14) with Earle's balanced salt solution (BSS) and tris-(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.6 (Calbiochem, Los Angeles, Calif.; 1.6 ml of a 1 M solution/100 ml of medium; reference 4). The HeLa cell medium was Eagle's basal medium (BME, powdered type; Grand Island Biological Co., Grand Island, N.Y.) with 10% (v/v) fetal bovine serum, sodium bicarbonate (1.75 ml of 7.5% solution/100 ml), 1 M Tris buffer, *p*H 7.6 (1 ml/100 ml), Tryptose Phosphate Broth (Difco; 10 ml/100 ml), penicillin G (100 units/ml), dihydrostreptomycin (Calbiochem; 134 μg/ml), and chlortetracycline (Lederle Laboratories, Pearl River, N.Y.; 25 μg/ml). The growth medium for WI-38 cells was the same as that used for the HeLa cells except that it contained no Tryptose Phosphate Broth.

Cell culture petri plates were seeded in the following manner: $2 \times 10^6$ to $2.5 \times 10^6$ CEF cells/ml, 14 ml/

100-mm plate; $1.4 \times 10^5$ HeLa cells/ml, 20 ml/100-mm plate; and $5 \times 10^4$ to $10^5$ WI-38 cells/ml, 7 ml/60-mm plate. The 100-mm plates (18 to 20 plates) or 60-mm plates (25 to 50 plates) were then sealed, airtight, in Pyrex baking dishes with Saran Wrap (Dow Chemical Co., Midland, Mich.) and were incubated at 36 C. Confluent cell monolayers developed in 48 to 72 hr with CEF and HeLa cells and in 5 to 7 days with WI-38 cells.

The growth and maintenance of WI-38 cells in culture tubes and the isolation and identification procedures for HSV have been described (5).

The cell cultures were infected with HSV by making an appropriate dilution of the stock virus in Tryptose Phosphate Broth and depositing 0.2 ml of the dilution into the 60-mm plates or 0.5 ml into the 100-mm plates. HeLa and CEF monolayers were infected with $10^2$ to $5 \times 10^2$ plaque-forming units (PFU); WI-38 cultures were infected with $10^2$ to $2 \times 10^2$ PFU of each isolate. Virus was permitted to adsorb to the monolayer for 2 hr at room temperature. At the end of this time, agar overlay medium (6) was added (the same overlay medium was used for all three cell culture types). This medium was prepared twofold concentrated by mixing 200 ml of Earle's BSS (10 times concentrated), 20 ml of BME amino acid mixture (Microbiological Associates, Bethesda, Md.), 20 ml of BME vitamin mixture (Microbiological Associates), 15 ml of 0.2 N sodium hydroxide, 200 ml of fetal bovine serum, 20 ml of Tris buffer (*p*H 7.6), 30 ml of 7.5% sodium bicarbonate, and 20 ml of an antibiotic stock solution per liter of medium (no phenol red was used). To each 100 ml of medium, 20 ml of Tryptose Phosphate Broth was added; after it was made up to volume, the solution was warmed to 45 C and then mixed with an equal volume of melted 1.3% Ionagar (No. 2, Colab Laboratories, Inc., Chicago Heights, Ill.) that had been cooled to 45 C. To this mixture was added BME glutamine (Microbiological Associates) at the rate of 1 ml/100 ml of overlay medium. The following quantities of overlay medium were then added to the infected cell monolayers: 7 ml for WI-38 cells in 60-mm plates; 14 ml for CEF cells and 20 ml for HeLa cells, both in 100-mm plates.

When the overlay medium had solidified, discs impregnated with various concentrations of the test compounds, prepared as described previously (4, 6, 8), were placed on the surface of the agar. The culture plates were returned to the baking dishes, sealed airtight, and incubated at 36 C. CEF cells were incubated for 6 days, and HeLa and WI-38 cells were incubated for 8 days. At the end of the incubation periods, the cultures were vitally stained by adding a mixture of equal volumes of 1.3% Ionagar and INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride; Aldrich Chemical Co., Milwaukee, Wis.; 2.1 g/liter in distilled water] at the rate of 7 ml for the 100-mm plates and 3 ml for the 60-mm plates. The overlay cultures were further incubated for 24 hr at 36 C until the living cells took on a deep red-purple color. The dead cells within virus plaques remained colorless, as described previously (4, 6, 8).

**Antiviral compounds.** Stock solutions of IUdR (International Chemical and Nuclear Co., City of Industry, Calif.). and ara-A (Parke, Davis & Co., Detroit, Mich.) at 10 mg/ml were prepared by use of dimethylsulfoxide (DMSO; J. T. Baker Chemical Co., Phillipsburg, N.J., or Mallinckrodt Chemical Works, St. Louis, Mo.). Further dilutions were made in DMSO, and 6-mm (diameter) paper discs, punched from paper strips (grade 470-C, Schleicher & Schuell, Inc., Keene, N.H.), were impregnated with 0.02 ml as previously described (4, 6, 8); the DMSO was permitted to evaporate at 36 C [as discussed in a prior publication (6), these compounds are stable under these conditions].

**Serological methods.** Serotyping was not done until all the other studies had been completed. Immune ascites (similar to blood plasma) were induced in mice by multiple injections of HSV and Freund's adjuvant (Bacto Adjuvant, complete Freund; Difco) as described elsewhere (7, 11). Preliminary neutralization of 100 $TCID_{50}$ of each of the 21 HSV isolates was performed in tube cultures of WI-38 cells with undiluted HSV-specific immune ascites. Further serotyping of the isolates was done by using the modified kinetics neutralization (12). The procedure involved mixing equal volumes of immune ascites specific for type 1 HSV with a virus suspension diluted in Tryptose Phosphate Broth to contain $10^3$ PFU/0.2 ml. This mixture was incubated in a water bath at 37 C, and 0.1-ml portions were removed at 0, 2, and 8 min, immediately diluted in 10 ml of Tryptose Phosphate Broth, and chilled in an ice bath. Plaque counts were then performed on the diluted samples by use of agar overlay cultures of WI-38 cells as described above. The percentage of plaque reduction of each virus isolate was determined by comparing the samples taken at 2 and 8 min with those taken at zero time.

**Chorioallantoic membrane (CAM) inoculation.** Standard methods were utilized for the "dropped membrane" method of infecting the CAM of 12-day-old chicken eggs with HSV (30). Virus suspensions were inoculated, 0.1 to 0.2 ml/egg, either undiluted or diluted 1:10 in Tryptose Phosphate Broth. Control eggs were inoculated with diluent only. The CAM was harvested after 72 hr of incubation at 36 C, floated in 0.15 M sodium chloride, and examined by indirect light against a black background for measurement of the pock size.

**Measurement of antiviral effect.** The drug sensitivity of the HSV isolates was determined by the plaque-suppression test (4, 6, 8), which involves measuring the plaque-free zone that surrounds a test disc impregnated with the antiviral compound. On occasion, the so-called plaque-free zones do in fact have from one to five plaques well within the zone. From prior work (10), it is clear that these represent a few drug-resistant particles and are not representative of the virus population. Therefore, for assay purposes they were ignored.

## RESULTS

Of the 21 HSV strains investigated, the 9 isolated from oral sites (401, 403, 404, 413, and 414 from the throat; 402, 406, and 407 from lip lesions; and 405 from the buccal mucosa) all produced small (0.25 to 0.5 mm) pocks on the CAM and all were of serotype 1 (Fig. 1). Of the 12 isolates from genital sites (387, 390, 391, 392, and 393 from lesions of the penis; 388, 394, 395, and 396 from vulvar lesions; 385 from the vagina; 386 from a lesion on the buttock; and 387 from the perineum), 4 strains (389, 390, 394, and 395) produced small pocks on the CAM and behaved in the kinetics neutralization test as typical type 1 strains (Fig. 1), and 8 strains produced large pocks on the CAM (0.5 to 1.5 mm) and reacted as type 2 strains in the kinetics neutralization test (Fig. 1). These results emphasize that the site of the lesion cannot be relied on to identify the type without exception (16).

The size of the pock on the CAM seemed to be a useful criterion for separating HSV isolates into types 1 and 2 (17). Unfortunately, about 25% of the eggs receiving virus showed no pocks, and the observations were further complicated by the occurrence of nonspecific lesions, no doubt caused by trauma to the membrane during inoculation, as described previously (20).

Before antiviral tests were undertaken, it was necessary to determine the ability of the various HSV isolates to form plaques. No plaques occurred under Noble agar (Difco) with 2 of 13 strains of type 1 HSV in CEF cultures and with 3 type 2 strains in HeLa cell cultures. All isolates produced plaques in all three cell lines when Ionagar was used; hence, this medium was used throughout these studies.

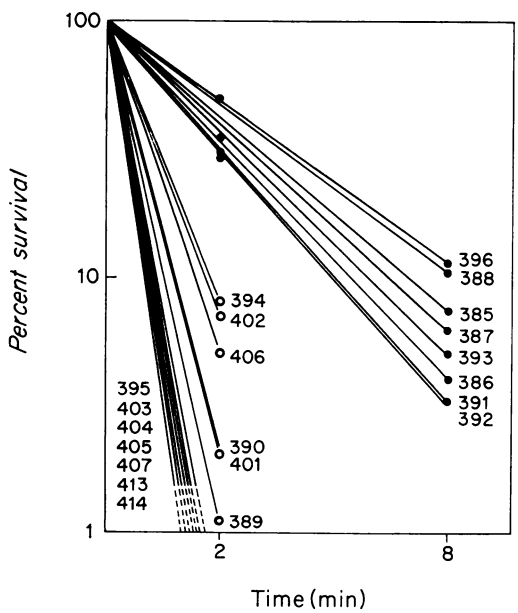


FIG. 1. *Kinetics of neutralization of HSV type 1 (○) and type 2 (●) strains by immune ascites prepared against a type 1 HSV strain.*

In CEF cultures, type 1 strains produced small (0.5 to 1.0 mm), hazy plaques, whereas type 2 strains produced large (2 to 3 mm), distinct, clear plaques. The nature of the plaques produced by type 1 HSV suggested that these strains do not multiply as well as type 2 strains in CEF, in agreement with the results of egg inoculations. It has been reported (1) that type 1 strains produce no plaques in CEF cells; this is not the case if Ionagar is used. In WI-38 cells, both types produced large, clear, irregular-shaped plaques that were surrounded by a hazy halo. It again was apparent that type 1 strains produced the smaller plaques (3.5 to 5.9 mm compared with 4.6 to 7.3 mm). The plaques formed in HeLa cells were pinpoint in size with both types. Because the small size of these plaques might cause problems in the plaque-suppression test, eight isolates of HSV were further subpassaged in HeLa cell liquid cultures in an attempt to increase the plaque size. After four such passages, larger (0.5 to 2.0 mm), more distinct plaques were produced, but again there was no detectable difference between types 1 and 2.

Prior to use, all virus stocks were titrated in all cell systems. The mean titration values suggested that there was little difference between types 1 and 2 in their ability to reach high titer in WI-38 liquid culture ($10^{7.0}$ and $10^{6.5}$ $TCID_{50}$/ml, respectively) or in their plaquing efficiency in WI-38 overlay culture ($4.4 \times 10^6$ and $6.2 \times 10^5$ PFU/ml, respectively). In CEF overlay cultures, the plaquing efficiency of type 2 strains was superior ($3.9 \times 10^7$ PFU/ml) to that of type 1 strains ($5.6 \times 10^5$ PFU/ml), whereas the reverse was true in HeLa cells ($1.8 \times 10^6$ PFU/ml for type 1 and $1.5 \times 10^4$ PFU/ml for type 2). Despite the fact that isolates subpassaged in HeLa cells four times produced larger plaques in these cells, the plaquing efficiency of the two strains was little affected ($2.2 \times 10^6$ PFU/ml for type 1 and $7.8 \times 10^3$ PFU/ml for type 2).

In CEF cultures, the type 1 strains were more sensitive to IUdR than were the type 2 strains (Fig. 2A). However, when WI-38 cells were used, these differences were not so great (Fig. 2B). In HeLa cell overlay cultures, type 1 strains were only slightly more sensitive to IUdR than type

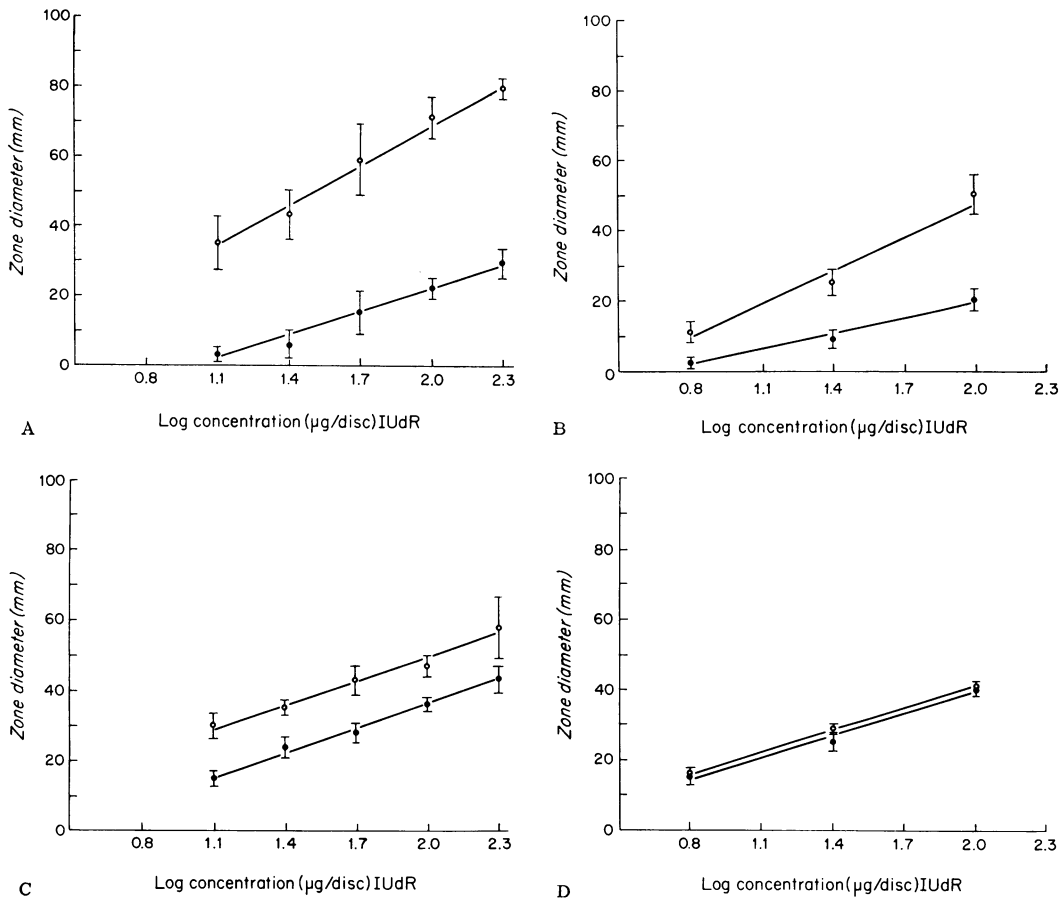


FIG. 2. *Sensitivities of HSV strains plotted as mean ± 1 SD. (A) Of 12 type 1 (○) and 8 type 2 (●) to IUdR in CEF cell cultures. (B) Of 13 type 1 (○) and 8 type 2 (●) to IUdR in WI-38 cell cultures. (C) Of 8 type 1 (○) and 3 type 2 (●) to IUdR in HeLa cell cultures. (D) Of 4 HeLa-adapted type 1 (○) and 4 HeLa-adapted type 2 (●) to IUdR in HeLa cell cultures.*

TABLE 1. *Inhibition of HSV by ara-A*

| Cell | Serotype | Zone of inhibition, mean ± SD (mm) | |
|---|---|---|---|
| | | 100 μg | 200 μg |
| CEF..... | 1 | 26 ± 3.3 | 33 ± 9.9 |
| | 2 | 3 ± 3.7 | 3 ± 5.1 |
| WI-38..... | 1 | 24 ± 4.0 | 36 ± 3.2 |
| | 2 | 21 ± 2.8 | 31 ± 1.4 |
| HeLa..... | 1[a] | 20 ± 1.4 | 26 ± 1.4 |
| | 2[a] | 26 ± 1.7 | 35 ± 6.2 |

[a] HeLa-adapted strains.

2 strains (Fig. 2C), and, if the isolates had been subpassaged in HeLa cells at least four times, there was no detectable difference in their sensitivity to this inhibitor (Fig. 2D).

The data in Table 1 show that, at the concentrations of ara-A used, there was a distinct difference in the sensitivities of type 1 and 2 HSV in CEF cultures. In WI-38 cultures, however, there was virtually no difference. With those strains subpassaged in HeLa cells, the type 2 strains were somewhat more sensitive to ara-A when tested in HeLa cultures. Although it is not known as yet what the sensitivity of these strains might be if tested in cells other than HeLa cells, there is nonetheless a suggestion that subpassage in HeLa cells may well alter sensitivity to antiviral agents.

## DISCUSSION

A number of investigations have detailed how the type 1 (so-called oral) strain of HSV differs from the type 2 (genital) strain in the site of infection, pock size on the CAM, cytopathic effect in various cell cultures, neurovirulence in

laboratory animals, heat stability, percentage of enveloped particles, antigenic protein structure, and base ratios of the DNA (1, 16, 21–23, 27). This study has confirmed that, under certain conditions, the type 2 strains are more resistant to certain DNA inhibitors than are type 1 strains (16, 32; Lowry and Rawls, Bacteriol. Proc., p. 171, 1969). From the data presented here, it also is clear that these differences in sensitivity to antiviral agents can vary greatly, depending on the cell culture system used and even on the passage history of the virus strains. In HeLa cultures there was not the striking resistance of the type 2 strains seen in CEF cultures and, with strains further subpassaged in HeLa cells, no differences in resistance could be detected in HeLa cell overlay cultures. With ara-A, there was even more variation because, even though type 1 strains were sensitive to this drug in CEF cultures, both types had similar sensitivities when tested in either HeLa or WI-38 cultures.

It has long been known that microorganisms can vary in their susceptibility to inhibitors depending on the medium in which they are cultivated and tested (2, 18, 31, 33). What has still to be resolved is the relationship these cell culture results have to the sensitivity of various strains of HSV as they exist in human disease states. It would seem presumptuous to assume, at this time, that the results in CEF cells establish that neither IUdR nor ara-A has any use in the treatment of infections caused by type 2 HSV. This also raises the question of the significance of a number of reports of development of drug resistance under laboratory conditions and what relationship, if any, this may have in the development of drug resistance under clinical conditions of treatment (24, 25). Furthermore, when a virus infection seems not to respond to adequate treatment, which cell culture system should be used to establish that the virus is resistant as opposed to other possible explanations? At present, it would seem wise not to extrapolate laboratory data to the extent of drawing conclusions regarding the entire problem of drug resistance in viral infections.

No explanation is readily apparent for the wide variation in the drug sensitivity of strains of HSV in different cell culture systems. It might be suggested, however, that the substantial differences observed in CEF cells may be a direct result of the inability of type 1 strains to multiply well in such cells so that the DNA inhibitors appear to be more effective. In HeLa cells, on the other hand, both types produced plaques of the same size in the same incubation time, possibly suggesting that they multiply equally well (or poorly) in these cells and so there are no differences in sensitivity.

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