Study on the Apparent Resistant Strains of Herpes Simplex Virus Type 1 against 9- β -D-Arabinofuranosyladenine

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YOSHIDA, T., SUZUTANI, T. and AZUMA, M. *Study on the Apparent Resistant Strains of Herpes Simplex Virus Type 1 against 9- β -D-Arabinofuranosyladenine.* Tohoku J. exp. Med., 1988, **156** (3), 279-290 — The appearance of drug resistant strains of herpes simplex virus type 1 (HSV-1) against 9- β -D-arabinofuranosyladenine (araA), in comparison with 5-iodo-2'-deoxyuridine (IUdR) and 9-(2-hydroxyethoxymethyl)guanine (acycloguanosine, ACG), has been investigated in green monkey kidney cell cultures (Vero) and human embryo lung cell cultures (HEL). Growth curves of HSV-1 in Vero cells and HEL cells in the presence or absence of 20 μ g/ml of araA showed that not only the viruses produced by these cells in the presence of araA but also the viruses produced in the absence of araA were resistant to the same concentration of araA. However, both viruses were unable to grow in the presence of a combination of 20 μ g/ml of araA and 3 μ g/ml of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) which is an inhibitor of adenosine deaminase. On the other hand, the viruses produced only in the presence of IUdR or ACG were resistant to each drug. All virus clones obtained from five serial clonings in the presence of araA alone or araA and EHNA in combination were not resistant to araA. These results show that the strain of HSV-1 resistant to araA is an apparently resistant strain, and a truly resistant strain to araA may not be established as easily as the truly resistant strains to IUdR and ACG. — herpes simplex virus type 1; anti-herpes compound; drug resistant strains; adenosine deaminase

Recently, many compounds which have a potent anti-herpes virus activity in cell cultures and in experimental animals have been reported (De Clercq 1984; Prusoff et al. 1985; Machida 1986). 9- β -D-arabinofuranosyladenine (araA), 5-iodo-2'-deoxyuridine (IUdR) and 9-(2-hydroxyethoxymethyl)guanine (acycloguanosine, ACG) are widely used in treating herpes simplex virus (HSV) infection (Hirano et al. 1979; Whitley et al. 1981; McLaren et al. 1983, 1985), and possible mechanisms of action have been analyzed in relation to the mecha-

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nism of replication of HSV (Pelling et al. 1981; De Clercq 1984; Prusoff et al. 1986).

On the other hand, many studies have reported the appearance of resistant strains of HSV against these drugs in the clinical field and in the laboratory, (Hirano et al. 1979; Smith et al. 1980; Coen et al. 1982; McLaren et al. 1983, 1985), and the mechanisms of resistance of HSV have been extensively investigated (Darby et al. 1981; Crumpacker et al. 1982; Coen et al. 1984, 1985; Fleming and Coen 1984; Chiou et al. 1985). This, however, still leaves open the question as to how such resistance against these drugs appears. Furthermore, Suzutani et al. (1988a, b) has shown that the sensitivity of HSV type 1 (HSV-1) to drugs is markedly different between cell strains used in drug-sensitivity assay.

The purpose of this study was to investigate the appearance of resistant strains of HSV-1 to araA, IUdR and ACG in two cell strains, green monkey kidney (Vero) cells and human embryo lung (HEL) cells.

MATERIALS AND METHODS

Compounds. AraA and IUdR were purchased from Sigma Chemical Co., St. Louis, MO, USA, and Wako Fine Chemical Products, Ltd., Osaka, respectively. ACG was kindly supplied by Japan Wellcome Co., Osaka. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was generously provided by Dr. H. Schaeffer, Wellcome Research Laboratories, Research Triangle Park, NC, USA. (Methyl-³H)thymidine (specific activity of 83 Ci/mmol) was purchased from the Radiochemical Centre, Amersham Japan, Tokyo.

Cells and virus. Vero cells were obtained from Flow Laboratories, Inc., VA, USA. HEL cells, strain HAIN-55, were kindly supplied by H. Okumura, National Institute of Health, Tokyo. These cells were cultivated in Eagle's minimum essential medium supplemented with 10% heat-inactivated calf serum (MEM-CS-10). After infection, the cells were maintained in MEM supplemented with 2% of heat-inactivated CS (MEM-CS-2).

The VR-3 strain of HSV-1 was cloned three times in HEL cells and grown in HEL cells for less than three passages. Stock virus was stored in small aliquots at -80°C .

Assay for virus growth. Nearly confluent monolayers of Vero cells and HEL cells grown in culture bottles (3×10^6 and 2×10^6 cells per $3.5 \times 6 \text{ cm}^2$, respectively) were infected with HSV-1 at a multiplicity of infection (MOI) of 0.01 PFU/cell. After adsorption of the virus for 1 hr at 37°C , cell sheets were washed twice with MEM, and then replenished with MEM-CS-2 or MEM-CS-2 containing araA at a concentration of $20 \mu\text{g/ml}$, IUdR at a concentration of $20 \mu\text{g/ml}$ for HEL cells or $40 \mu\text{g/ml}$ for Vero cells, or ACG at a concentration of $4 \mu\text{g/ml}$. The infected cells were incubated at 37°C in humidified 5% CO_2 -incubator, sampled daily for 7 days, and frozen at -80°C . The virus was released from the cells by freezing and thawing three times and separated from cell debris by centrifugation at 1,500 rpm for 5 min.

The infectivity titer of virus samples was assayed by the plaque-forming method using MEM-CS-2 containing 0.5% methyl-cellulose (overlay medium) on confluent monolayers of Vero cells in a 24-well culture plate (Corning Glass Works, Corning, NY, USA). Assay for drug sensitivity of the virus was performed on the Vero cell monolayers by a plaque reduction method using an overlay medium containing a serially diluted drug. The plaque count was carried out on the 4th day after infection. There was no difference in the size of the plaque, if it appeared, between the wells overlayed with overlay medium containing araA, IUdR or ACG and the wells with drug-free overlay medium, except that the plaque size in the wells overlayed with the medium containing araA and EHNA was very small

similar to a needle point.

Cloning of drug-resistant strain. HEL cells were infected with HSV-1, strain VR-3, at MOI of 0.1 PFU/cell. After adsorption for 1 hr, the cells were replenished with MEM-CS-2 containing 50 $\mu\text{g/ml}$ of araA, and incubated at 37°C until 100% cytopathic effect (CPE) was observed. The virus harvested was then serially passaged 5 times at an estimated MOI of 0.1 PFU/cell in HEL cells with MEM-CS-2 containing an increasing concentration of araA, up to 100 $\mu\text{g/ml}$. Subsequently, this virus was passaged twice as follows; HEL cells infected with this virus at MOI of 0.1 PFU/cell were cultured with MEM-CS-2 containing 30 $\mu\text{g/ml}$ araA and 3 $\mu\text{g/ml}$ EHNA. After 6 days, the infected cell cultures, which showed a few tiny plaque, were harvested and treated 3 times using the freezing-thawing procedures. New HEL cell cultures were inoculated with this supernatant fluid (2×10 PFU/ml), replenished with drug-free MEM-CS-2, and incubated for 5 days until 90% CPE was observed. The virus was harvested and processed once more, as above (thereafter, referred to as A-E-VR-3 strain).

Cloning of drug-resistant clones from the A-E-VR-3 strain was carried out as follows: the confluent monolayers of Vero cells in a 24-well culture plate were infected with the A-E-VR-3 strain at 30 PFU per well, and then overlaid with overlay medium containing serial concentrations of araA alone or araA plus 3 $\mu\text{g/ml}$ of EHNA. After 3 to 5 days, four clones were harvested from each well containing 20 $\mu\text{g/ml}$ araA, 5 $\mu\text{g/ml}$ araA plus 3 $\mu\text{g/ml}$ EHNA and 20 $\mu\text{g/ml}$ araA plus 3 $\mu\text{g/ml}$ EHNA. Cloning from the cultures overlaid with overlay medium containing araA plus EHNA was frequently unsuccessful, since the plaques that appeared were so few and tiny as to be undistinguishable from deformation of the monolayer cell sheet. Each clone obtained was grown in HEL cells with MEM-CS-2. This cloning procedure was repeated five times with 3 $\mu\text{g/ml}$ of EHNA and an increasing concentration of araA, and 30 clones (10 clones each from the 3rd, 4th, and 5th cloning) were finally obtained. These clones were then tested for susceptibility to araA alone or araA plus EHNA by the plaque reduction assay.

Pulse-labeling experiment for analysis of drug effects. Nearly confluent monolayers of Vero cells in 96-well culture plates were replenished with MEM-CS-2 containing 10 $\mu\text{g/ml}$ of araA, 3 $\mu\text{g/ml}$ of EHNA, or 10 $\mu\text{g/ml}$ of araA plus 3 $\mu\text{g/ml}$ of EHNA, and incubated for 6 or 24 hr prior to the addition of (methyl- ^3H)thymidine. MEM-CS-2 containing 25 mM HEPES and 10 $\mu\text{Ci/ml}$ of (methyl- ^3H)thymidine was added to the cultures, and incubated for 1 hr at 37°C. After pulse-labeling, the cells were scraped off the well using a Teflon scraper in an ice bath, and then washed three times with cold PBS, twice with cold 5% (wt/vol) trichloroacetic acid and finally twice with cold ethyl alcohol on glass fiber filter paper (Whatman, GF/C, Whatman Inc., Clifton, NJ, USA). The acid-insoluble radioactivity retained on the filter paper was measured in a liquid scintillation spectrometer.

RESULTS

Growth of HSV-1 in cell cultures in the presence or absence of drugs. Growth patterns of HSV-1 in Vero cells and HEL cells in the presence or absence of araA, IUdR and ACG, and the drug sensitivity of the produced viruses were examined. The growth curves of HSV-1 in Vero cells and HEL cells in the presence or absence of 20 $\mu\text{g/ml}$ of araA were almost the same (Fig. 1). In the presence of araA, the growth of the virus was inhibited in the early period, while in the later period, the titer of virus yield in the presence of the drug was close to that in the absence of the drug. Then, the sensitivity of the virus produced by these cells to araA was investigated by assaying the virus titer in the presence of araA. As shown in Fig. 1A and 1B, the viruses produced in either the presence

Fig. 1. Growth curve of HSV-1 in Vero cells and HEL cells in the presence or absence of drugs. Vero cell (A) and HEL cell (B) monolayers were infected with HSV-1, VR-3 strain, at MOI of 0.01 PFU/cell, and incubated with MEM-CS-2 (control) or MEM-CS-2 containing 20 $\mu\text{g/ml}$ of araA. The infectivity titer of the virus produced at the indicated periods in the presence (■—■, ▲—▲) or absence (□—□, △—△) of the drug was measured in Vero cell monolayers by the plaque assay using an overlay medium containing (▲—▲, △—△) or not containing (■—■, □—□) 20 $\mu\text{g/ml}$ of araA.

or absence of 20 $\mu\text{g/ml}$ of araA were unexpectedly able to grow in the same concentration of araA. AraA completely suppressed the growth of HSV-1 in both Vero cells and HEL cells at a concentration of 40 $\mu\text{g/ml}$ (data not shown). In contrast with the above results, viruses produced in Vero cells and HEL cells in the absence of IUdR or ACG were sensitive to these drugs, however viruses produced in the presence of 40 $\mu\text{g/ml}$ of IUdR or 4 $\mu\text{g/ml}$ of ACG were markedly resistant to the respective drug at the above mentioned concentrations (data not shown), which was in agreement with an earlier report (Smith et al. 1980).

Growth of HSV-1 passaged serially in cell cultures in the presence or absence of araA. The results presented above showed that the susceptibility to araA of each virus produced in the presence or absence of araA was similar and all virus samples could grow at a concentration of 20 $\mu\text{g/ml}$ of araA. Therefore, the growth curve of HSV-1 passaged serially in the presence or absence of araA was analyzed in Vero cells and HEL cells. Figs. 2 and 3 show that 1st (A), 2nd (B) and 3rd (C) passaged viruses have the same level of susceptibility to araA. We could not detect any significant difference in the growth pattern and the sensitivity to araA between these serially passaged viruses.

Effect of araA alone or araA and EHNA combination on cellular DNA synthesis, cell growth, and virus growth. Earlier reports have indicated that araA was converted to 9- β -D-arabinofuranosylhypoxanthine (araHx), a substance with less anti-herpes virus activity, by cellular adenosine deaminase (Schwartz et al. 1976, 1984; Shipman et al. 1976; Gephart and Lerner 1981). Schaeffer and Schwender (1974), and Plunkett and Cohen (1975, 1977) have shown that EHNA

Fig. 2. Growth curve of HSV-1 passed serially in Vero cells in the presence or absence of araA. The virus produced in Vero cell monolayer at 5 days after infection in the presence of 20 $\mu\text{g/ml}$ of araA (arrow-marked in column A) was secondarily passed under the same conditions (column B), and again the virus produced (arrow-marked in column B) was tertiarily passed (column C). The infectivity titer of the virus produced at the indicated periods in the presence (■—■, ▲—▲) or absence (□—□, △—△) of araA was measured in Vero cell monolayers by plaque assay using an overlay medium containing (▲—▲, △—△) or not containing (■—■, □—□) 20 $\mu\text{g/ml}$ of araA.

Fig. 3. Growth curve of HSV-1 passed serially in HEL cells in the presence or absence of araA. Conditions and symbols as in Fig. 2, except that HEL cell monolayers were used for virus production.

inhibited the activity of adenosine deaminase, and increased the activity of araA. To obtain information on the effect of EHNA on DNA synthesis, the growth of Vero cells and the growth of HSV-1, more experiments were carried out. Table 1 shows that in agreement with an earlier report (Shewach and Plunkett 1986), cellular DNA synthesis was markedly reduced by 10 $\mu\text{g/ml}$ of araA at 6 hr, but recovered to 80% of control at 24 hr. On the other hand, when araA was combined with EHNA, cellular DNA synthesis was strongly inhibited at both 6 hr

TABLE 1. *Effect of EHNA on the inhibition of DNA synthesis of Vero cells by araA*

Treatment of Vero cells with	Incorporation (%) of ^3H -thymidine after treatment for*	
	6 hr	24 hr
None	2,650** (100.0)	3,744 (100.0)
EHNA 3 $\mu\text{g/ml}$	3,831 (144.6)	6,024 (160.9)
AraA 10 $\mu\text{g/ml}$	410 (15.5)	3,013 (80.5)
AraA 10 $\mu\text{g/ml}$ + EHNA 3 $\mu\text{g/ml}$	187 (7.1)	825 (22.0)

*Vero cell monolayers were treated with EHNA, araA, or araA plus EHNA. At the indicated period, the cells were pulse-labeled with 10 $\mu\text{Ci/ml}$ of ^3H -thymidine for 1 hr, and acid-insoluble radioactivity was measured.

**CPM.

and 24 hr.

The effect of araA alone or the combination of araA and 3 $\mu\text{g/ml}$ of EHNA on the growth of Vero cells was also examined. Both patterns of cell growth in the presence of araA or araA and EHNA at the indicated concentration were indistinguishable (Fig. 4).

The anti-herpes activity of EHNA was next examined. EHNA alone did not exhibit this activity, whereas in the case of the araA and EHNA combination, the extent of antiviral activity depended on the concentration of araA (Fig. 5), indicating the inhibition of degradation of araA to araHx by EHNA.

Sensitivity of HSV-1 produced in the presence of araA to araA and EHNA combination. The results shown in Figs. 2 and 3 showed that each virus produced

Fig. 4. Effect of araA alone or araA and EHNA combination on growth of Vero cells. Non-confluent Vero cell sheets (9×10^4 cells/ 2 cm^2 well) were cultivated with MEM-CS-2 (control) or MEM-CS-2 containing the indicated concentration of araA alone (●—●) or araA plus 3 $\mu\text{g/ml}$ of EHNA (■—■). The cell sheets were trypsinized at 3 days and viable cells were counted.

Fig. 5. Anti-herpesvirus activity of EHNA in Vero cells. Plaque forming titer of HSV-1, VR-3 strain, was measured by using the overlay medium containing the indicated concentration of EHNA alone (●—●) or the combination of EHNA and 1 $\mu\text{g/ml}$ of araA (▲—▲) and 4 $\mu\text{g/ml}$ of araA (■—■).

in the presence or absence araA could grow in the same concentration of araA. Therefore, we measured the sensitivity of HSV-1 harvested in the presence or absence of araA in the 1st and 3rd passage (Fig. 2A and 2C, and Fig. 3A and 3C) to a combination of araA and EHNA. As shown in Fig. 6, none of the virus samples could grow in the medium containing a combination of 20 $\mu\text{g/ml}$ of araA and 3 $\mu\text{g/ml}$ of EHNA.

AraA sensitivity of HSV-1 cloned in the presence of araA or the combination of araA and EHNA. Thirty clones, 10 clones each from the 3rd, 4th, and 5th cloning were examined for their sensitivity to araA or the araA and EHNA combination. Fig. 7A and 7B show that the parent VR-3 strain, A-E-VR-3 strain and 30 clones present similar plaque-reduction curves. The average 50% effective dose (ED_{50} ; the concentration of drug which inhibits plaque formation by 50%) for the VR-3 strain, A-E-VR-3 strain, 3rd clones, 4th clones, and 5th clones was 6.7 ± 1.1 , 10.0 ± 0.9 , 11.0 ± 2.4 , 10.6 ± 2.0 and 10.2 ± 1.4 $\mu\text{g/ml}$ of araA alone, respectively (Fig. 7A). The sensitivity of VR-3, A-E-VR-3 and clones to the combination of araA and EHNA was examined (Fig. 7B). The ED_{50} of a combination of araA and 3 $\mu\text{g/ml}$ of EHNA for VR-3, A-E-VR-3, 3rd, 4th and 5th clones was 3.3 ± 0.1 , 4.6 ± 0.3 , 6.1 ± 0.4 , 5.8 ± 0.4 and 5.6 ± 0.8 $\mu\text{g/ml}$, respectively. In this case, the ED_{50} for A-E-VR-3 and all clones were less than twofold different when compared to the ED_{50} for the parent VR-3 strain, and the ED_{50} of araA plus EHNA for all viruses was twofold lower than that of araA alone.

DISCUSSION

A large number of attempts have been made to determine the mechanism of resistance of HSV to araA, IUdR and ACG which are widely used in the clinical

Fig. 6. Sensitivity of HSV-1 produced in Vero cells and HEL cells in the presence or absence of araA alone against the araA and EHNA combination. The viruses produced in Vero cells (A and B) and in HEL cells (C and D) in the presence (■—■, ▲.....▲) or absence (□—□, △.....△) of 20 µg/ml of araA alone, which were shown in Fig. 1A and 1B, were re-assayed by the plaque forming method with an overlay medium containing a combination of 20 µg/ml of araA and 3 µg/ml of EHNA (▲.....▲, △.....△).

field. Coen and colleagues have reported that the major mechanism of resistance to araA is a mutation in the DNA polymerase locus of the HSV genome (Coen et al. 1982, 1984, 1985; Fleming and Coen 1984; Chiou et al. 1985). Many reports have shown that a mutation in the viral thymidine kinase gene results in resistance to IUdR and ACG (Darby et al. 1981; McLaren et al. 1985).

However, there are few reports which show the comparative degree of appearance of resistant strains using these drugs. In this study, we have investigated the appearance patterns of strain of HSV-1 resistant to araA, in comparison with an appearance of resistant strains to IUdR and ACG. The present results have shown that both the viruses produced in the presence or absence of araA showed araA-resistant patterns (Figs. 1, 2 and 3). On the other hand, the virus produced only in the presence of IUdR or ACG was highly resistant to each drug (data not

Fig. 7. Sensitivity of HSV-1 clones obtained by serial clonings in the presence of araA or araA and 3 $\mu\text{g/ml}$ EHNA combination against 20 $\mu\text{g/ml}$ of araA alone (A) or 20 $\mu\text{g/ml}$ of araA and 3 $\mu\text{g/ml}$ of EHNA combination (B) in HEL cells. Symbols: $\circ$ — $\circ$, parent VR-3 strain; $\square$ — $\square$, A-E-VR-3 strain; $\bullet$ — $\bullet$, 3rd clones; $\blacksquare$ — $\blacksquare$, 4th clones; $\blacktriangle$ — $\blacktriangle$, 5th clones.

shown), which is in agreement with an earlier report (Smith et al. 1980). Earlier reports showed a metabolic conversion of araA to araHx, which has a lower anti-herpes virus activity, by cellular adenosine deaminase, which might be involved in the regulation of the intracellular adenosine levels (Schwartz et al. 1976, 1984; Plunkett and Cehen 1977). Furthermore, several kinds of inhibitor of adenosine deaminase were found (Agarwal 1982), and the effect of these inhibitors on the efficacy of araA was extensively investigated (Plunkett and Cohen 1977; Shannon et al. 1980; North and O'Connor 1983); EHNA increased antiviral activity and cytotoxic activity of araA. Shannon et al. (1980) and North and O'Connor (1983) showed that EHNA itself has an anti-herpesvirus activity, whereas De Clercq et al. (1980) suggested that antiviral activity of EHNA was very low, and that this discrepancy might be due to the cell culture, but not the virus strains used. Therefore, we have determined a suitable concentration of EHNA which potentiates the inhibition of DNA synthesis of araA without causing cytotoxicity of the confluent monolayer of Vero cells, and also without the antiviral activity of EHNA alone (Table 1, Figs. 4 and 5). Then, we investigated the sensitivity of HSV, which was shown in Fig. 2A and 2C, and in Fig. 3A and 3C, against a combination of araA and EHNA at a concentration determined as above. All virus samples were sensitive to araA plus EHNA (Fig. 6).

These results indicate that growth of HSV-1 in the presence of araA alone was inhibited in an earlier period of post-infection, whereas the virus entered into

growth processes and produced plaques in a later period, since araA was rapidly converted to araHx which has less anti-herpes virus activity. In this connection, it is pertinent to assume that the araA-resistant HSV-1 produced in the presence of araA was apparently araA-resistant, but not truly araA-resistant. However, these findings do not provide a direct clue to the apparent resistance of HSV to araA. In order to better define the above phenomena, experiments were then performed to determine whether virus clones obtained from five serial clonings in the presence of araA alone or araA and EHNA combination were resistant to araA or not. Coen et al. (1985) presented a criteria for drug resistance; when the ED_{50} for a mutant strain is more than twofold greater than the ED_{50} for parent strain, this mutant strain is designated as a resistant strain. The present results (Fig. 7) show that the ED_{50} of araA alone or araA plus EHNA for all clones was less than twofold greater than the ED_{50} for the parent VR-3 strain, confirming the apparent resistance of HSV to araA, and suggesting that truly araA-resistant HSV-1 might not appear easily in the growth of the virus in the presence of not only araA but also the araA and EHNA combination.

The comparative study of the frequency of isolation of drug-resistant mutants in the laboratory may help to predict the frequency of appearance of resistant mutants in a clinical setting, and can aid the elucidation of the mechanisms of action of antiviral drugs. These findings also can aid the development of derivatives of antiviral drugs. The development of any derivatives of araA, which do not convert to araHx by cellular adenosine deaminase, and which have a specific affinity for viral DNA polymerase, and which also do not induce a drug resistant virus, will be important.

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