Susceptibility of Vaccine Strains of Varicella-Zoster Virus to Antiviral Compounds

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Using a plaque reduction assay, we determined the 50% effective doses of six antiviral compounds against low- and high-passage viruses of the KMCC and Oka strains of varicella-zoster virus vaccine. The potency, as indicated by the ranges of 50% effective doses (micrograms per milliliter) of the antiviral compounds, in decreasing order was as follows: (*E*)-5-(2-bromovinyl)-2'-deoxyuridine, 0.0007 to 0.0035; 1-(2'-fluoro-2-deoxy- β -D-arabinofuranosyl)-5-iodocytosine, 0.0063 to 0.0091; aphidicolin, 0.092 to 0.180; acyclovir, 0.79 to 1.81; vidarabine, 0.62 to 2.10; and phosphonoformic acid, 8.18 to 16.4. Susceptibility to the various antiviral compounds was independent of passage level or strain. These data, along with the available *in vivo* data, indicate that varicella-zoster virus vaccine infections requiring antiviral therapy most probably would be treated as effectively as would natural varicella infections.

The success of Japanese investigators in immunizing large numbers of normal and immunosuppressed children and adults with a live attenuated varicella-zoster (V-Z) virus vaccine over the past decade (31, 32) has led to vaccine field trials in this country (1, 2, 7, 18, 24). Two vaccine strains have been used: the KMCC strain, passed 10 to 60 times in WI-38 cells (1, 24), and the Japanese or Oka strain, passed 11 times in human embryo fibroblasts, 12 times in guinea pig embryo cells, and an additional 1 to 21 times in human diploid cells (2, 7, 18, 31, 32). To date the vaccines have been shown to be safe, immunogenic, and effective. However, with the immunization of increasing numbers of leukemics, severe infections due to vaccine may necessitate treatment with an antiviral drug, as discussed at a recent workshop on V-Z virus vaccine held at the National Institutes of Health in September 1983. The current experience in treating wild-type V-Z virus infections with arabinosyladenine (9- β -D-arabinofuranosyladenine, vidarabine, or Ara-A) (35, 36), acyclovir [9-[(2-hydroxyethoxy)methyl]guanine, acycloguanosine, or ACV] (26, 29), and *E*-5-(2-bromovinyl)-2'-deoxyuridine (bromovinyldeoxyuridine, or BVDU) (14), as well as the available data on the *in vitro* susceptibility of the Oka vaccine strain (15, 22, 30), suggests that these three compounds would be therapeutically effective. Favorable results with ACV were discussed at the National Institutes of Health workshop. There are, however, no published data on the *in vitro* susceptibility of the KMCC strain of vaccine virus.

We determined the 50 and 90% effective doses (ED₅₀s and ED₉₀s, respectively) of six antiviral drugs against low- and high-passage KMCC strain viruses with a plaque reduction assay (16, 27). Aphidicolin, a tetracyclic diterpene-tetraol produced by *Cephalosporium aphidicola* (10, 25), was included in this group. This compound is a specific α -polymerase inhibitor and is effective *in vitro* against herpes simplex virus (3, 10, 25) and cytomegalovirus (E. Gonczol, personal communication). The activity of aphidicolin against V-Z virus is, to the best of our knowledge, untested.

To control for experiment-to-experiment variation in results (22), we performed antiviral titrations for the two passages in parallel. Similarly, we determined the susceptibility of a low-passage (pre-guinea pig) and vaccine-level-passage Oka strain virus.

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MATERIALS AND METHODS

Cell culture. Human fetal lung diploid fibroblasts (MRC-5), a gift from J. P. Jacobs, Medical Research Council Laboratory, Holly Hills, Hampstead, London, England, were used in these studies. The growth medium consisted of Eagle minimal essential medium (MEM) with Earle salts (Flow Laboratories, Inc., McLean Va.) supplemented with 0.35 mg of L-glutamate per ml, 0.02 ml of 100 $\times$ vitamins per ml, 20 μ g of vancomycin per ml, 0.5 μ g of gentamicin per ml, 0.5 μ g of amphotericin B per ml, and 7.5% fetal bovine serum. The serum content of the maintenance medium was reduced to 2.0%.

Cell cultures for virus propagation and harvest of cell-free virus (see below) were grown in 80-cm², 260-ml tissue culture flasks (Nunc, Delta, A/S Nunc, Roskilde, Denmark). These uninfected cells were used also in a 1:9 split to make monolayers for the petri plates used in the plaque reduction assays (see below).

Since antimicrobial agents were added to growth and maintenance media, virus pools and cells were screened for the presence of mycoplasma.

Virus strains. The KMCC strain of V-Z virus, passages 13 and 50, was provided by B. J. Neff, Merck Institute for Therapeutic Research, West Point, Pa. The Oka strain, passages 5 (in human tissue culture only) and 30 (i.e., 12 passages in guinea pig embryo cells and 18 passages in human diploid cells), was provided by M. Takahashi, Osaka University, Suita, Osaka, Japan. Both strains were isolated from the vesicle fluid of patients with chickenpox, KMCC virus in 1968 (24) and Oka virus in 1974 (32), and serially passed. They were provided as cell-free preparations and were further propagated in our laboratory by passage of trypsin-dispersed infected MRC-5 cells.

Preparation of cell-free virus. Cell-free virus was prepared by the freeze-thaw method described by Saito (28). When

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more than 70% of the cell monolayer showed typical cytopathic effect, the tissue culture fluid was removed and the cells were washed once with Tris-Mg-Cl₂-FCS (0.0025 M Tris-hydrochloride buffer [pH 7.4], 0.15 M NaCl, 0.001 M MgCl₂, 10% fetal calf serum). SPGA-FCS (0.218 M sucrose, 0.0038 M KH₂PO₄, 0.0072 M K₂HPO₄, 0.005 M sodium glutamate, 1% bovine serum albumin, 10% fetal calf serum) was then added, and the cell monolayer was dislodged with a rubber policeman. The cell suspension was centrifuged at 1,200 rpm for 15 min in an HL-8 rotor of a Sorvall RC-3 centrifuge, and the resultant supernatant was discarded. The cell pellet was suspended in SPGA-FCS, and the cell suspension was freeze-thawed for 6 cycles. The mixture was clarified by low-speed centrifugation, and the supernatant was harvested and stored in aliquots at -70°C.

One cell-free virus pool of each of the four viruses, stored in SPGA at -70°C, was used for all of the antiviral drug titration experiments. The final passage levels were 18 and 108 for the KMcc strain and 13 and 33 for the Oka strain.

Antiviral compounds. The antiviral compounds and their sources were as follows: ACV, a gift from K. K. Biron, Burroughs Wellcome Co., Research Triangle Park, N.C.; aphidicolin, a gift from A. H. Todd, Imperial Chemical Industries, Ltd., Cheshire, England; Ara-A, purchased from Warner-Lambert, Morris Plain, N.J.; BVDU, a gift from T. B. Martinez, G. D. Searle & Co., Chicago, Ill.; 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (fluoridoaracytosine, or FIAC), a gift from C. McLaren, Bristol-Meyers Co., Syracuse, N.Y.; and phosphonoformic acid (PFA), purchased from Richmond Organics, Inc., Ashland, Va.

Ara-A, provided as a 5-ml sterile suspension (200 mg/ml), was stored at room temperature. A volume of 0.1 ml was withdrawn after vigorous shaking and diluted appropriately just before each titration experiment.

The other antiviral drugs were provided in powder form. Each was dissolved in maintenance MEM, brought up to a working concentration, filtered, and stored in aliquots. Enough of each antiviral agent was prepared so that only one batch was used for all of the experiments. Aphidicolin was diluted in dimethyl sulfoxide and stored at 4°C in aliquots of 5,000 µg/ml. The remaining compounds were diluted in maintenance MEM and stored at -70°C (PFA, 5,000 µg/ml; ACV, 1,000 µg/ml; and BVDU and FIAC, 500 µg/ml each).

Each of the antiviral compounds was further diluted in maintenance MEM to achieve concentrations used in the plaque reduction assays.

Plaque reduction assay. Plaque reduction assays were used to determine the potency of each of the antiviral compounds. Low- and high-passage viruses of each strain were used in parallel for each experiment. On one occasion PFA was tested against all four viruses in parallel.

The assays were carried out using 4- to 6-day-old confluent MRC-5 monolayers (passages 26 to 34) grown in 60-mm-wide petri plates which had a grid (2 by 2 mm) to facilitate plaque counting (Lux Scientific Corp., Newbury Park, Calif.). The monolayers were infected with 50 to 100 PFU of virus in 0.1 ml of maintenance MEM. Virus was allowed to adsorb to the cells by incubating the plates for 50 min in 5% CO₂ at 37°C (the plates were shaken every 10 min to disperse the virus). Thereafter, 6 ml of each antiviral compound in at least four 10-fold dilutions (with concentrations from 0.0001 to 100 µg/ml) was added to the infected cell cultures. Three monolayers were used for each drug concentration, as well as for the virus control (virus and maintenance MEM) and the tissue culture control (only maintenance MEM). Aphidi-

colin controls included maintenance MEM with the highest concentration of dimethyl sulfoxide used in the titration.

After 6 days of incubation in 5% CO₂ at 37°C, all plates were fixed with methanol and stained with a 5% May-Grunwald-Giemsa solution (Accra-Lab, Inc., Bridgeport, N.J.). Plaques were then counted on an inverted microscope at a magnification of ×40. The mean plaque count of the three plates used for each concentration of drug was used to calculate the percent plaque reduction relative to the virus control plates.

To quantitate the potency of each antiviral compound, we determined both the ED₅₀s and ED₉₀s, the drug concentrations necessary to reduce the number of plaques to 50 and 90%, respectively, of those in the virus control culture. ED₉₀s were determined so as to identify the slope of the drug resistance-response curve. ED₅₀s and ED₉₀s were derived from linear regression analysis of the observed percent plaque reduction for each concentration of drug used in the titration experiment. Only the linear portion of the raw datum titration curve was used in the analysis. The lowest possible concentration of drug producing 100% plaque reduction (i.e., total inhibition of virus replication) was defined so as to avoid factitious elevations of the calculated EDs. The ACV and Ara-A drug titrations were done in triplicate against the low- and high-passage KMcc strain of virus; all other titrations were done in duplicate.

Preliminary experiments indicated that the percent plaque reduction by any given drug concentration was unaffected if the viral inoculum was not washed off before the antiviral compound was added. Since the highest concentration of each of the antiviral agents used was shown to be nontoxic to uninfected cells, an antiviral control plate was not incorporated into each titration experiment.

RESULTS

Each of the antiviral drugs tested inhibited virus replication and affected plaque size and appearance. Whereas the plaques in the absence of drug and in the presence of low drug concentrations were large and fully lytic, plaques in the presence of high concentrations of drug, if present, were frequently small and almost totally devoid of a lytic center. In the latter circumstance, there were small, elliptical, darkly stained areas of pyknotic nuclei which were never observed in control plates. These foci were counted as plaques and might have evolved into lytic areas if the plates had been allowed to incubate for a longer period of time. On the other hand, they might not have been noted at all had the plates been fixed and stained after fewer than 6 days.

Figure 1 presents the titration curves for the six antiviral compounds against the low- and high-passage KMcc and Oka viruses. The curves were drawn by using the mean ED₅₀s and ED₉₀s presented in Table 1. It is quite apparent that BVDU and FIAC were the most potent antiviral compounds, whereas PFA was the least potent against all of the viruses tested. Aphidicolin was ca. 10 times more potent than ACV and Ara-A, which in turn were of comparable potency. Although there was little difference in the mean ED₅₀s for ACV (1.08 to 1.40 µg/ml) and Ara-A (1.07 to 1.58 µg/ml), ACV did have a somewhat lower range of mean ED₉₀s than did Ara-A (7.83 to 9.04 versus 10.5 to 12.4 µg/ml). Complete plaque reduction was achieved consistently by the same concentration of drug for all viruses (BVDU, 0.075 µg/ml; FIAC, 0.125 µg/ml; aphidicolin, 1 µg/ml; ACV, 15 µg/ml; Ara-A, 20 µg/ml; and PFA, 100 µg/ml).

There were no consistent differences in EDs by passage

FIG. 1. Susceptibility of the KMCC and Oka vaccine strains of V-Z virus, by passage level, to six antiviral compounds. (A) KMCC strain, passages 18(---) and 108 (—); (B) Oka strain, passages 13 (---) and 33 (—). APH, Aphidicolin.

level or strain. Since there was up to a threefold difference in EDs determined in repeat experiments, none of the differences in EDs between passage levels or between strains could be considered significant. Results obtained in parallel tended to vary less than independently derived data. For example, in one experiment the ED₅₀s of PFA against the low- and high-passage KMCC viruses were 16.4 and 15.2 $\mu\text{g/ml}$, respectively (Table 1). In another experiment, the ED₅₀s for the Oka strain viruses were approximately one-half those noted for the KMCC strain: 8.18 and 9.60 $\mu\text{g/ml}$, respectively. However, the ED₅₀s for the single titration done with the four viruses in parallel ranged from 12.8 to 14.1 $\mu\text{g/ml}$.

DISCUSSION

Our data substantiate the observation that the attenuation of wild V-Z virus isolates by multiple passages in tissue culture (even >100) in and of itself does not necessarily lead to altered susceptibility to the antiherpesvirus drugs tested (4, 15, 22, 30). Since there are no data suggesting that simple tissue culture passage results in the development of thymidine kinase-deficient mutants, it is not surprising that the high-passage KMCC virus and the Oka vaccine virus were no

less susceptible than their low-passage counterparts were to ACV (6, 15, 30), BVDU (15, 30), and FIAC (21). The activity of DNA polymerase was also not affected, as evidenced by the equal susceptibility of viruses of different passage levels to Ara-A (11, 17), PFA (19, 33), and aphidicolin (3, 10, 25), which specifically inhibits α -polymerase. Our data indicate also that there is no strain-dependent difference in antiviral drug susceptibility between the KMCC and Oka strains of V-Z vaccine virus. Since our EDs are generally consistent with those of other authors who tested either lower-passaged virus (15, 16, 22, 30) or a larger number of different strains (4, 8, 9, 12, 15, 17, 22, 30), a general conclusion appears to be that susceptibility of V-Z virus to the antiviral compounds tested is not significantly altered by changes in passage level or strain. With respect to other strains of V-Z virus, one would expect BVDU and FIAC or ACV and Ara-A to be of comparable *in vitro* potency, the former two, however, being far more active than the latter two. Aphidicolin would be somewhat more potent than both ACV and Ara-A, and PFA would be the least active, like phosphonoacetic acid, a well-studied related compound (23, 30, 34).

By testing in parallel the susceptibility of the low- and high-passage viruses (and in one case the two strains) to the test drugs, we attempted to control for inherent biological and internal technical variation in the plaque reduction assay and to minimize the possible misinterpretation of any observed difference in ED₅₀ and ED₉₀ results. This fact is important, since a number of other authors have also noted wide ranges in EDs (4, 9, 12, 15, 22, 30). Some have noted as much as 10-fold differences in EDs either among different V-Z virus strains (22, 30) or between repeat experiments involving the same strain (22). Without an internal control (i.e., one reference virus tested in parallel with each virus) any differences must be evaluated carefully before any significance can be ascribed to the finding (30).

Such an approach, however, will not control for differences in ED results reported by different laboratories (i.e., an ED₅₀ for ACV of 4.6 $\mu\text{g/ml}$ [30] versus a range of values of 0.5 to 1.5 $\mu\text{g/ml}$ [4]). The true significance of such discrepant results is confounded by the lack of uniformity in the plaque assay technique, including factors such as the cell line used (13), the cell-free (15, 30) or cell-associated (4, 15) nature of the input virus, the size of the inoculum (8, 12, 15, 30), and the procedure for incubating the virus in the presence of the antiviral agent (4, 5, 9, 30). Furthermore, EDs have been determined with either pooled data (21) or a single experiment (22) and have been derived either off a raw datum plot (12, 20) or from linear regression analysis (21). As noted above, our EDs are in general agreement with the published data. However, there are some exceptions. For example, the ACV concentration necessary to inhibit viral replication by 90 to 100% (7.83 to 15.0 $\mu\text{g/ml}$) was approximately 4 to 6 times that reported by Biron and Elion (4), Crumpacker et al. (12), and Levin and Leary (20). On the other hand, Bryson and Hebblewaite observed a range of values up to 12.5 $\mu\text{g/ml}$ (9). This difference may be related to some of the factors just cited.

An important point concerns the correlation of the *in vitro* ED data and the expected *in vivo* clinical response (30). It is not at all clear what the ED₅₀ and ED₉₀ mean clinically. As noted by Shigeta et al. (30), whereas the plasma level-to-ED₅₀ ratio is 400:1 for BVDU, this ratio is only 8:1 for ACV (2:1 with the data of Shigeta et al.), yet the latter is quite effective *in vivo* (26, 29). Whether BVDU will prove to proportionately more clinically efficacious remains to be seen. Furthermore, whereas drug concentrations equal to or above the

TABLE 1. Susceptibility of the KMCC and Oka vaccine strains of V-Z virus to six antiviral drugs, by passage level

Antiviral drug	Strain and passage level ^a	ED ₅₀ (μ g/ml)		ED ₉₀ (μ g/ml)	
		Avg	Individual determinations ^b	Avg	Individual determinations ^b
BVDU	K-18	0.0012	0.0007, 0.0016	0.028	0.023, 0.033
	K-108	0.0024	0.0013, 0.0035	0.032	0.033, 0.030
	O-13	0.0016	0.0011, 0.0021	0.040	0.035, 0.045
	O-33	0.0024	0.0019, 0.0028	0.040	0.038, 0.041
FIAC	K-18	0.0081	0.0078, 0.0084	0.080	0.073, 0.086
	K-108	0.0075	0.0063, 0.0087	0.078	0.076, 0.081
	O-13	0.0068	0.0064, 0.0071	0.065	0.059, 0.071
	O-33	0.0087	0.0083, 0.0091	0.078	0.076, 0.081
APH ^c	K-18	0.136	0.092, 0.180	0.722	0.602, 0.841
	K-108	0.128	0.100, 0.156	0.796	0.700, 0.892
	O-13	0.141	0.131, 0.150	0.706	0.632, 0.780
	O-33	0.170	0.160, 0.180	0.795	0.751, 0.838
ACV	K-18	1.40	0.79, 1.60, 1.81	7.83	6.91, 7.60, 8.99
	K-108	1.22	1.25, 0.80, 1.58	8.25	6.50, 7.84, 10.4
	O-13	1.18	1.00, 1.36	9.04	9.01, 9.07
	O-33	1.08	0.90, 1.27	8.76	8.31, 9.21
Ara-A	K-18	1.11	1.15, 0.80, 1.39	10.5	8.89, 9.82, 12.7
	K-108	1.28	1.07, 1.40, 1.39	11.3	10.4, 12.0, 11.6
	O-13	1.58	1.06, 2.10	12.4	10.6, 14.2
	O-33	1.07	0.62, 1.52	12.3	12.2, 12.5
PFA	K-18	14.4	12.4 ^d , 16.4	73.7	71.1 ^d , 76.3
	K-108	14.6	14.1 ^d , 15.2	73.2	68.6, 77.9 ^d
	O-13	10.7	8.18, 13.2 ^d	70.1	65.0, 75.1 ^d
	O-33	11.2	9.60, 12.8 ^d	71.8	67.2, 76.5 ^d

^a O, Oka; K, KMCC; 13, 18, 33, and 108, passage levels.

^b Parallel results by passage level as given unless otherwise indicated.

^c APH, Aphidicolin.

^d Parallel results (one PFA experiment done with all four viruses in parallel).

ED₅₀ have been reported to be viricidal as opposed to being only virostatic (4, 9), even these findings should not be taken as prima facie evidence of clinical applicability or superiority.

Taken together, however, both our in vitro data, as well as those of others, and the available in vivo data indicate that either Ara-A or ACV would currently be the antiviral drug of choice and should be effective in treating any severe V-Z vaccine virus infection, regardless of the vaccine strain. As more clinical data are collected, BVDU (14), as well as many other antiherpesvirus compounds, may be added to the list of effective drugs. Available clinical data on FIAC, which is of a potency relatively similar to that of BVDU, indicate that this drug may be efficacious for V-Z virus infections (37). PFA has in vivo activity against herpes simplex virus (33). The clinical utility of aphidicolin has yet to be explored but may be limited by the necessity of using dimethyl sulfoxide as a solvent (3, 25).

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