Susceptibility to Other Antiherpes Drugs of Pathogenic Variants of Herpes Simplex Virus Selected for Resistance to Acyclovir

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Cross-resistance data for a group of nine acyclovir-resistant variants of herpes simplex virus type 1 are reported. These mutants, which express either altered thymidine kinase (TK) or DNA polymerase, were all derived from the same wild-type (wt) strain after exposure to acyclovir in tissue culture. Furthermore, all variants have pathogenic properties similar to the wt parental strain as assessed using mouse model systems (G. Darby, H. J. Field, and S. A. Salisbury, Nature (London) 289:81–83, 1981; B. A. Larder and G. Darby, Virology 146:262–271, 1985). Two groups of antiviral compounds were used: those requiring activation by TK and those whose action is independent of that enzyme. The TK substrate-specificity mutants were generally resistant to the TK-activated drugs but showed wt susceptibility to phosphonoacetic acid, 9-β-d-arabinofuranosyladenine, and aphidicolin. The DNA polymerase mutants were relatively susceptible to most TK-activated drugs, although two were resistant to 5-(trifluoromethyl)-2'-deoxyuridine. The polymerase variants showed a more complex pattern of susceptibility, however, to those compounds whose mode of action is independent of TK. In general, these variants showed similar responses to phosphonoacetic acid, phosphonoformate, and 9-β-d-arabinofuranosyladenine, a particular variant being either resistant, susceptible, or hypersensitive to all three. The response of each variant to aphidicolin, however, appeared to be the inverse of its response to the other three drugs. The cross-resistance patterns are discussed, and their implications for combined or successive therapies are considered.

The newly developed antiviral agent acyclovir (ACV; 9-[2-hydroxyethoxy)methyl]guanine; 9) is now in extensive clinical use for the treatment of herpes simplex virus (HSV) infections. However, recent reports have warned that resistant strains may emerge during therapy and thus pose a potential threat to future treatment (2, 25, 28, 31). In view of these observations, it is important to assess the susceptibility of ACV-resistant mutants to other antivirals. Any patterns that are recognized may then be exploited both in therapy and in the future design of antivirals compounds.

Selection for ACV resistance in tissue culture most frequently results in the isolation of variants defective in the expression of thymidine kinase (TK) activity (5, 11, 27), a virus-coded enzyme required to initiate activation of ACV by phosphorylation (19). Although TK+ mutants replicate in culture under normal conditions, they are considerably attenuated in animal model systems (10, 14, 30), suggesting they are unlikely to be of major clinical importance in normal individuals. In contrast, some HSV variants selected with ACV retain the ability to induce TK activity, and these often show little change in pathogenic properties compared with wild-type (wt) virus (7, 10, 13). Variants of this latter type, although less common than TK− mutants, cause greater concern. They fall into two groups: firstly those which induce TK with altered substrate specificity such that the variant enzyme phosphorylates thymidine but not ACV (24) and, secondly, mutants which induce altered DNA polymerase with reduced sensitivity to inhibition of ACV-triphosphate (6, 16), the active form of the drug (8, 17, 18). Because both groups of TK− variants have proved more difficult to isolate in culture than TK− mutants, little information is available regarding the spectrum of susceptibility of such ACV-resistant strains to other drugs.

We report here cross-resistance data for a group of DNA polymerase and TK mutants, all of which were derived from the same HSV type 1 (HSV-1) wt strain after exposure to ACV in culture. A number of interesting patterns of cross-resistance and susceptibility were observed. The implications of these findings with regard to treatment of ACV-resistant strains and to the possibility of preventing their emergence are discussed.

MATERIALS AND METHODS

Cells and tissue culture. Baby hamster kidney-21 (BHK) cells and 5-bromo-2'-deoxyuridine-resistant BHK (BU-BHK) cells were the cell lines used in this study. BU-BHK cells express very low levels of cellular TK activity. Both cell lines were free of mycoplasma contamination.

Cells were maintained in Glasgow modified Eagle medium supplemented with 10% newborn calf serum and 10% trypsin phosphate broth. In plaque reduction assays, trypsin phosphate broth was omitted from the medium and 1% carboxymethyl cellulose (CMC) was included in the overlay (ECm-CMC medium).

Viruses. The wt HSV-1 strain SC16 (20) and ACV-resistant mutants derived from it were used. The isolation and properties of the TK substrate-specificity mutants SC16 S1 (S1) and SC16 Tr7 (Tr7) were described previously (7, 24). S1 induces about 30% TK activity, and Tr7 induces about 100% TK activity compared with the wt (7, 24). The DNA polymerase mutants (transformed cell passage series) were isolated recently following passage of SC16 in TK-transformed BU-BHK cells (BHK TK1) in the presence of increasing concentrations of ACV (23). All of these mutants induce TK activity levels similar to that of the wt SC16 (23).

Virus stocks, derived from single-plaque isolates of each
strain, were made by infection of BHK cells at low multiplicity.

**Plaque reduction assays.** The susceptibility of viruses to antiviruses compounds was assessed by plaque reduction assay using monolayers of BHK or BU-BHK cells as described previously (11). Briefly, dishes (6 cm) of cells were inoculated with 100 to 250 PFU of virus per dish in 0.2 ml of EC10. The dishes were incubated at 37°C for 30 to 60 min to allow virus adsorption, after which the inoculum was removed and 5 ml of EC10-CMC containing the required drug concentration was added. Plaques were allowed to develop for 2 days, and the cells were then fixed and stained. The ED50, defined as the dose required for 50% plaque reduction, was obtained from a plot of the reduction in plaque number (relative to the no-drug control) against drug concentration. Plaque reduction assays with each drug were performed at least twice.

**Antiviral drugs.** The following compounds were gifts: ACV and 9(2-hydroxy-1-((hydroxymethyl)ethoxy)methylguanine (alternatively known as BIOLF-62, 2'-NDG, BW759, or DHPG) from P. Collins, The Wellcome Research Laboratories, Beckenham, Kent, United Kingdom; (E)-5-2-bromovinyl-2'-deoxyuridine (BVdU) from E. De Clercq, Rega Institute, Louvain, Belgium; phosphonoacetic acid (PAA) and aphidicolin from R. W. Honess, National Institute for Medical Research, Mill Hill, London, United Kingdom; and phosphonoformate (PFA) from B. Oberg, Astra Lakemedel, Söderstalje, Sweden. 5-Iodo-2'-deoxyuridine (IUdR), 5-trifluoromethyl-2'-deoxyuridine (TFT), and 9-beta-D-arabinofuranosyladenine (araA) were purchased from the Sigma Chemical Co., Poole, Dorset, United Kingdom.

**RESULTS**

The wt strain (SC16) and nine ACV-resistant TK+ mutants were tested for susceptibility to a number of different antiviral compounds by plaque reduction assay. Two of the variants used were TK substrate-specificity mutants (Tr7 and S1), and the remainder were resistant at the level of DNA replication (transformed cell passage series).

The compounds we used can be broadly divided into two groups, those which require activation by TK (DHPG, BVdU, IUdR, and TFT) and those whose action is independent of TK (PAA, PFA, araA, and aphidicolin). It is believed that the inhibitory effects of these compounds or their active derivatives result from their interaction with viral DNA polymerase, causing inhibition of the enzyme or producing deleterious effects after incorporation into viral DNA (3, 15, 29; reviewed in reference 22).

**ED50s were obtained directly from the inhibition curves derived from plaque reduction data.**

**Susceptibility of DNA polymerase mutants to PAA, PFA, araA, and aphidicolin.** The seven ACV-resistant DNA polymerase mutants have been classified in three groups according to their susceptibility to PAA (23). These groups are defined as follows: PAA resistant (PAA1) with an ED50 greater than threefold that of the wt, PAA hypersensitive (PAA2) with an ED50 less than one-third-fold that of the wt, and PAA susceptible (PAA3), the remainder. The resistance of these variants to drugs whose action is independent of TK is shown in Table 1. Several points emerge. Firstly, no correlation was seen between the degree of ACV resistance and sensitivity or susceptibility to PAA. For example, TP2.5 and TP3.2 showed a similar reduction in susceptibility to ACV (ED50 40- and 50-fold higher, respectively, than wt values), although TP2.5 was PAA1 and TP3.2 was hypersensitive to the drug. Secondly, all mutants showed very similar patterns of susceptibility to PAA, PFA, and araA. Hence, the PAA1 mutants (TP2.4 and TP2.5) were both PFA1 and araA1. Thirdly, an inverse relationship was found between PAA and aphidicolin susceptibility. Thus, the PAA4 variants showed increased susceptibility to aphidicolin compared with the wt, and the PAA5 strains were slightly resistant. Interestingly, the PAA6 variant, TP4.4, had some characteristics of the PAA5 strains, because it was significantly resistant to aphidicolin (with an ED50 fourfold that of the wt) and showed hypersensitivity to araA (giving an ED50 fivefold lower than that of the wt).

**Susceptibility of DNA polymerase mutants to DHPG, BVdU, TFT, and IUdR.** The polymerase mutants were also tested for susceptibility to a number of TK-activated nucleoside analogs; the ED50 data are shown in Table 2. The mutants showed the least change in susceptibility to BVdU, since the greatest increase in ED50 (seen with TP4.1) was less than twofold that of the wt. The situation with DHPG and IUdR was different in that most of the variants showed marginal but significant resistance to these compounds. For example, the increases in ED50 for DHPG were between two- and fivefold compared to that of the wt, and for IUdR they were up to sixfold. Most of the mutants were significantly less susceptible to TFT, the most resistant being the PAA1 mutants (TP2.4 and TP2.5), with ED50s nearly 50-fold that of the wt.

**Susceptibility of TK substrate-specificity mutants to antiviral agents.** The TK substrate-specificity mutants S1 and Tr7 were also tested for their susceptibility to the drugs used as

**TABLE 1. Susceptibility of HSV polymerase mutants to TK-independent drugs**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Phenotype</th>
<th>ED50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ACV</td>
</tr>
<tr>
<td>SC16</td>
<td>wt</td>
<td>0.15</td>
</tr>
<tr>
<td>TP2.4</td>
<td>PAA1</td>
<td>2.9</td>
</tr>
<tr>
<td>TP2.5</td>
<td></td>
<td>7.1</td>
</tr>
<tr>
<td>TP1.3</td>
<td>PAA2</td>
<td>0.5</td>
</tr>
<tr>
<td>TP1.7</td>
<td></td>
<td>4.4</td>
</tr>
<tr>
<td>TP4.4</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>TP3.2</td>
<td>PAA5</td>
<td>8.8</td>
</tr>
<tr>
<td>TP4.1</td>
<td></td>
<td>28</td>
</tr>
</tbody>
</table>

**TABLE 2. Susceptibility of HSV polymerase mutants to TK-activated drugs**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Phenotype</th>
<th>ED50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ACV</td>
</tr>
<tr>
<td>SC16</td>
<td>wt</td>
<td>0.15</td>
</tr>
<tr>
<td>TP2.4</td>
<td>PAA1</td>
<td>2.9</td>
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<td>TP2.5</td>
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<td>7.1</td>
</tr>
<tr>
<td>TP1.3</td>
<td>PAA2</td>
<td>0.5</td>
</tr>
<tr>
<td>TP2.7</td>
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<td>4.4</td>
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<tr>
<td>TP4.4</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>TP3.2</td>
<td>PAA5</td>
<td>8.8</td>
</tr>
<tr>
<td>TP4.1</td>
<td></td>
<td>28</td>
</tr>
</tbody>
</table>

* Plaque reduction assays were performed in BU-BHK cells.
TABLE 3. Susceptibility of TK substrate-specificity HSV mutants to other antiherpes drugs

<table>
<thead>
<tr>
<th>Virus</th>
<th>ACV (µM)</th>
<th>PAA</th>
<th>araA</th>
<th>Aphidicolin</th>
<th>BVdU</th>
<th>DHPG</th>
<th>IUdR</th>
<th>TFT *</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC16</td>
<td>0.15</td>
<td>55</td>
<td>6.7</td>
<td>0.4</td>
<td>0.02</td>
<td>0.17</td>
<td>0.45</td>
<td>0.57</td>
</tr>
<tr>
<td>S1</td>
<td>11.1</td>
<td>65</td>
<td>15</td>
<td>0.34</td>
<td>0.54</td>
<td>3.0</td>
<td>1.3</td>
<td>20</td>
</tr>
<tr>
<td>Tr7</td>
<td>22.2</td>
<td>65</td>
<td>13</td>
<td>0.55</td>
<td>0.36</td>
<td>21</td>
<td>0.7</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Plaque reduction assays were performed in BU-BHK cells.

Predictably, these variants showed normal susceptibility to inhibitors that do not require prior activation by TK. Thus, S1 and Tr7 showed no significant changes in susceptibility to PAA, araA, or aphidicolin.

The picture with TK-activated compounds, however, was different, because both mutants were less susceptible to these inhibitors. The degrees of resistance observed varied considerably. For example, Tr7 was significantly resistant to...
DHPG (ED$_{50}$, 124-fold that of the wt) and BVdU (ED$_{50}$, 20-fold that of the wt) showed only slightly decreased susceptibility to TFT and IUdR. The mutant S1 gave a different pattern, as it was considerably less resistant to DHPG (ED$_{50}$, 6-fold that of the wt) but more resistant to BVdU and TFT (ED$_{50}$, about 30- and 35-fold, respectively, that of the wt). Like Tr7, this variant showed only a marginal decrease in susceptibility to IUdR.

The data for all drugs and mutants are summarized in Fig. 1.

DISCUSSION

The major interest in cross resistance is that it may provide information which will aid the design of suitable therapy for infections caused by drug-resistant variants of HSV. It may also suggest drug combinations which would allow us to minimize the frequency of appearance of resistant isolates. We chose ACV-resistant isolates for this study as ACV is now the most widely used and successful of the antiviral drugs. Furthermore, ACV-resistant strains have already been isolated from patients undergoing therapy (2, 25, 28, 31). We specifically excluded viruses deficient in TK expression, because variants of this type exhibit markedly reduced virulence in vivo (10, 14, 30). Although we are clearly not yet in the situation in which the clinician has at his disposal a selection of antiviral drugs with different modes of action, there are many compounds with considerable clinical potential, and it is appropriate that these compounds be examined in relation to their effects on ACV-resistant mutants. Such studies may provide guidance for the future design and use of antiviral drugs.

The picture which emerged with the TK substrate-specificity mutants (S1 and Tr7) was fairly clear. As expected, these viruses were as susceptible as the wt to drugs whose action is independent of the virus-induced TK. Thus, replication of these strains was inhibited by PAA, araA, and aphidicolin, and we expect that in vivo the response of the TK substrate-specificity mutants to these drugs would resemble the response of the wt virus. Furthermore, combined therapy with ACV and any drug in this group would be expected to prevent the appearance of TK substrate-specificity mutants, because a virus would require multiple lesions if it is to exhibit resistance to such a combination. The TK substrate-specificity mutants were in general resistant to other nucleoside analogs, such as DHPG, BVdU, and TFT, although they showed only marginal resistance to IUdR.

The situation with DNA polymerase mutants is more complex. All drugs used in this survey are believed to interact ultimately with the virus-coded DNA polymerase, and so we might expect altered susceptibility to any of these compounds. Surprisingly, all seven polymerase mutants were susceptible to BVdU and relatively susceptible to DHPG and IUdR, showing smaller than 10-fold changes in the ED$_{50}$ relative to that of the wt virus. Thus, alterations at the DNA polymerase locus which confer ACV resistance resulted in relatively little change in susceptibility to the other nucleoside analogs. TF1 was exceptional in that several polymerase mutants (TP2.4, TP2.5, TP2.7, and TP4.4) showed changes of 10-fold or greater in their response to this compound. Again, these results indicate that there may be advantages in using drugs in combination rather than individually in the treatment of herpes infections.

A more intriguing observation was the response of polymerase mutants to those compounds whose action is independent of TK (PAA, PFA, araA, and aphidicolin). With these compounds, we observed not only increases in resistance but also, with almost equal frequency, significant decreases. The observation that polymerase mutants isolated by using one compound may be hypersensitive to others is not new (1, 4, 12, 21, 26); however, the pattern was striking. Each mutant appeared to respond similarly to PAA, PFA, and araA. In general, a variant was either resistant to all three, hypersensitive to all three, or susceptible to all three. This suggests that the interactions of these compounds with the polymerase might be analogous, not a surprising conclusion for PFA and PAA, which are both pyrophosphate analogs, and certain surprising for the nucleoside analog araA. In contrast, the response of these variants to aphidicolin appeared to be the inverse of their responses to PAA, PFA, and araA. Viruses resistant to the latter were hypersensitive to aphidicolin, and those hypersensitive to PAA, PFA, and araA were resistant to aphidicolin. These viruses similar to the wt in susceptibility to PAA, PFA, and araA remained susceptible to aphidicolin.

It is inevitable that reliance on a single compound, however effective, for the treatment of HSV infections will lead to the establishment of resistant virus strains in the population. Alternatives to ACV will be required in the future, and it appears from the present study that none of the drugs whose mode of action involves TK or DNA polymerase will provide the ideal alternative. Although it may be possible to develop cocktails of drugs able to deal with ACV-resistant variants (e.g., PFA plus aphidicolin), there is clearly a need to direct our efforts toward developing drugs targeted to other virus-specific functions.

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LITERATURE CITED