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Development of Clinical Resistance to Acyclovir in Herpes Simplex Virus-Infected Mice Receiving Oral Therapy

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Mice inoculated in the ear pinna with herpes simplex virus were treated effectively by including 1 mg of acyclovir per ml in the drinking water. During a 5-day course of treatment the development of resistance was not readily apparent. However, when a suboptimal therapeutic dose was used and virus was repeatedly inoculated into further mice undergoing therapy, the infection became completely refractory to treatment by passage 4. Some of the viruses isolated exhibited reduced ability to induce thymidine kinase, and this appeared to account at least in part for the development of resistance. However, the viruses isolated from the tissues of such mice comprised complex mixtures of strains with widely differing in vitro susceptibilities to acyclovir. The properties of these virus yields gave an indication of the likely nature of resistance to nucleoside analogs in humans and suggested some difficulties which may be encountered when clinical specimens are analyzed.

Resistance to nucleoside analog inhibitors of herpes simplex virus (HSV) can arise extremely readily in tissue culture. For example, a single passage of HSV in BHK cells in the presence of 10 times the 50% effective dose (ED_{50}) of acyclovir results in a reduced yield of virus which subsequently can be shown to be resistant (6). However, previous work has suggested that resistant mutants cannot be detected readily in infected tissues from acyclovir-treated mice (4). The explanation for this marked difference between in vivo and in vitro observations may lie in the biological properties of different kinds of resistant viruses. Two virus-induced enzymes are involved in the mode of action of acyclovir. The nucleoside is first converted to the monophosphate form by HSV thymidine kinase. Subsequently, acyclovir-triphosphate interacts with HSV DNA polymerase. My co-workers and I have devised a series of simple tests which enable the likely nature of particular resistant mutants to be identified (3, 6). The majority of acyclovir-resistant mutants selected in vitro were found to be defective in the induction of thymidine kinase activity. However, animal experiments have suggested that these thymidine kinase-deficient (TK^-) mutants fail to thrive in vivo (5, 9); in particular, these mutants multiplied poorly in the skin and in nervous tissue and established latent infections only with difficulty. This suggests that a selective pressure against TK^- viruses operates in vivo and explains why resistance was not readily observed. In contrast to the TK^- mutants, viruses whose resistance depends on an altered thymidine kinase (2a) or DNA polymerase (1a, 5, 8, 15) are fully pathogenic, although these mutants may arise more rarely.

Recently, there have been several preliminary reports of resistance to acyclovir in humans (1, 2; C. D. Sibrack et al., Pediatr. Res. **15**:621, abstr. no. 1072, 1981; H. Shiota, Excerpta Med. Int. Congr. Ser. in press). Therefore, I returned to the mouse model and pursued the question of in vivo resistance by repeated passage of virus in mice undergoing suboptimal therapy. In this study, viruses isolated from mouse infections completely resistant to acyclovir treatment were analyzed. I identified the likely nature of one type of clinical resistance which may be encountered in humans, as well as some difficulties which may confront diagnostic virologists.

MATERIALS AND METHODS

Virus infection. Acute HSV infections were produced in 3- to 4-week-old BALB/c mice by inoculating 10^5 PFU suspended in 20 μl of Eagle minimal essential medium into the skin of the left ear. The virus strains used were a clone (obtained by one single-plaque isolation) of HSV type 1 strain SC16 (11) and the TK^- mutant SC16 R5C1, which was derived from strain SC16 by one passage in tissue culture in the presence of acyclovir (6). Virus was also inoculated intracerebrally into similar mice. Groups of eight mice were inoculated with 20-μl portions of different virus dilutions to a depth of approximately 1 mm into the left cerebral hemisphere. The deaths that occurred between days 2 and 28 postinfection were scored, and the 50% lethal dose was calculated by the Spearman-Kärber method (10).

Measurement of inflammation. Ear thickness was

measured with an engineer's screw gauge micrometer, as described previously (4). Results are expressed as the difference in thickness between the left ear and the right (uninoculated) ear. This has been shown to be a useful measure of cell-mediated infiltration into infected ears (13), which does not occur in nude (athymic) mice. This method was used in this study as a simple and reliable procedure to measure the clinical severity of infection.

Isolation of virus from ears and passage in mice. The left pinnae were removed on day 3 or 5 and were tested individually. The tissue was homogenized, and the virus titers were determined by plaque titration in BHK cell monolayers. The monolayers were fixed and stained after 48 h of incubation, and the plaques were enumerated at the appropriate dilution; titrations were performed in duplicate. The possibility that residual acyclovir in the tissue might influence the in vitro results was considered. The following tests were performed: (i) three washes with phosphate-buffered saline after the dilutions of tissue homogenate were adsorbed onto the cell monolayer; (ii) 1 mg of thymidine per ml was added to the medium to compete with residual acyclovir; and (iii) ear tissue homogenates from separate acyclovir-treated mice were added to virus suspensions of known concentration. None of these treatments influenced the plaque counts, and I concluded that there was not sufficient residual acyclovir in the tissue to affect the development of plaques in vitro.

For further mouse passages, the 10^{-1} or 10^{-2} dilutions of ear homogenates obtained on day 5 were selected, and the infected cells were harvested and passaged once more in BHK cells to produce working stocks. The virus was then titrated and stored in small samples for biochemical tests or additional mouse inoculations. These viruses were designated MP1, MP2, etc., corresponding to the passage number.

Acyclovir therapy. Acyclovir was dissolved in the drinking water to give a final concentration of 0.25 or 1 mg/ml. The cages contained groups of five mice provided with 250 ml of drinking water in graduated bottles. Daily consumption of the drug was recorded. This was found to be remarkably constant at approximately 3 ml/day per mouse, with no marked reduction in drinking rate as the infection progressed. Acyclovir is stable under the conditions used, but the drug-containing drinking water was replaced at 48-h intervals. The 1-mg/ml oral dose was shown to be superior in treating local HSV infections to 50 mg/kg per day given in two daily intraperitoneal inoculations (7). Each mouse regularly drank approximately 3 ml of water per day; this corresponded to daily intakes of 167 ± 56 and approximately 40 mg of acyclovir per kg for the 1- and 0.25-mg/ml doses, respectively.

Thymidine kinase assay. Preformed monolayers of bromodeoxyuridine (BUdR)-resistant BHK cells were infected at a standard multiplicity of 5 PFU/cell. The cells were checked visually and harvested after 18 h of incubation, and cell extracts were prepared. Thymidine kinase activity was determined by the method of Klemperer et al. (12).

Plaque reduction assay. Each virus suspension tested was diluted to give approximately 200 PFU/0.2 ml. The virus was allowed to adsorb for 1 h onto preformed BHK cell monolayers in 5-cm petri dishes. Then, 5 ml of Eagle minimal essential medium containing 1% calf serum, carboxymethyl cellulose, and various concentrations of acyclovir was added, and the cultures were incubated for 48 h at 37°C. The monolayers were fixed and stained, and the plaques were counted. The results were expressed as percentages of the values for control cultures incubated without acyclovir and were plotted versus $\log_{10}$ acyclovir concentration, and the ED_{50} values were determined directly from the graph. Similar plaque titrations were also carried out in TK^+-transformed BUdR-resistant BHK cells. These cultures had a resident virus-specific thymidine kinase and thus could reveal types of resistance independent of a loss or change in the test virus thymidine kinase (3).

Purification of mixtures by single-plaque isolation. Virus suspensions obtained from infected mice were found to be mixtures of virus strains. Single plaques were selected by inoculating BHK monolayers preformed in 26-well plastic trays (Linbro Plastics). The virus suspension was ultrasonically vibrated for 1 min to disperse clumps and then diluted to give approximately one plaque per five wells. When a single plaque was observed, the well containing it was marked and checked for 3 or 4 days to be sure that no further plaques appeared (carboxymethyl cellulose was included in the medium to reduce secondary plaque formation). The infected cells from the single well were then harvested and used to produce a small working stock, as described above.

RESULTS

Development of clinical resistance in mice receiving acyclovir therapy. Each mouse was inoculated intradermally with 10^5 PFU of strain SC16 into the left pinna. The mice either were not treated or were given drinking water containing 0.25 or 1 mg of acyclovir per ml from the time of virus inoculation. After 5 days the increased ear thickness was measured as an indication of the clinical severity of the disease; the mice were killed, and the amounts of virus present in the pinnae were determined.

The mean ear thickness was reduced to 25% of the control untreated pinna thickness by treatment with 1 mg of acyclovir per ml, and the virus titer in the ear was reduced to 0.005% of the control value (Table 1). To encourage the development of resistance, a suboptimal dose of the drug (0.25 mg of acyclovir per ml) was used. In this case more virus replication occurred (0.3%), and the ear thickness was 57% of the thickness in the untreated group.

In five individual mice treated with the lower level of acyclovir, the $\log_{10}$ ear virus titer on day 5 ranged from 1.0 to 3.0. The mouse ear with the highest titer was considered the most likely to show resistance, and accordingly the virus from this ear was inoculated into 10 additional mice, using the same dose (10^5 PFU/mouse). Five mice were not treated, and five were given 0.25 mg of acyclovir per ml in the drinking water. There was a similar reduction in ear thickness in

TABLE 1. Development of clinical resistance in mice inoculated with HSV type 1 strain SC16 and treated orally with acyclovir[a]

Virus inoculum[b]	Passage no.	Virus yield from ear[c]			Cell-mediated inflammation[d]		
		Untreated	Treated with 0.25 mg of acyclovir per ml[e]	Treated with 1.0 mg of acyclovir per ml[e]	Untreated	Treated with 0.25 mg of acyclovir per ml[e]	Treated with 1.0 mg of acyclovir per ml[e]
SC16	1	4.3 ± 0.7	1.8 ± 0.5 (0.3)	0.0 (0.005)	27.3 ± 5.7	15.5 ± 5.8 (57)	6.7 ± 2.5 (25)
SC16 MP1	2	4.8 ± 0.9	2.9 ± 0.4 (1.3)	ND[f]	33 ± 3.9	19.5 ± 4.2 (59)	ND
SC16 MP2	3	5.0 ± 1.6	3.1 ± 0.7 (1.3)	ND	34.8 ± 1.6	25.2 ± 4.3 (72)	ND
SC16 MP3	4	4.4 ± 0.7	ND	3.2 ± 0.5 (6.3)	29.3 ± 6.4	ND	23.0 ± 3.2 (78)
SC16 MP3	4	4.5 ± 0.9	4.0 ± 0.7 (32)	ND	26.6 ± 6.8	26.6 ± 2.4 (100)	ND
SC16 MP4	5	3.9 ± 0.6	4.0 ± 0.9 (>100)	3.3 ± 0.3 (25)	23 ± 3.0	20.0 ± 3.5 (87)	14.0 ± 11.3 (61)

[a] All values were determined 5 days after virus inoculation.
[b] A total of 10^5 PFU was inoculated into each left ear pinna. SC16 MP1, SC16 MP2, SC16 MP3, and SC16 MP4 were viruses from individual mice treated with 0.25 mg of acyclovir per ml, obtained from the previous passage.
[c] Geometric mean titer ($\log_{10}$ PFU/ear) ± standard deviation obtained from five mice independently. The numbers in parentheses are percentages compared with untreated mice.
[d] Mean ear thickness difference (left ear thickness minus right ear thickness [in millimeters], $\times 10^{-2}$); five mice were measured at each time.
[e] Treatment with acyclovir in the drinking water from the time of virus inoculation.
[f] ND, Not determined.

the treated group (59% compared with untreated mice) but a smaller reduction in virus titer (1.3%) on day 5. Additional serial passages were made; in each case the virus from the mouse which showed the highest virus titer on day 5 of treatment was used. Table 1 shows that through the series there was a gradual increase in the level of infectious virus in mice treated with 0.25 mg of acyclovir per ml and that successively the disease became less responsive to treatment. Indeed, by mouse passages 4 and 5 (MP4 and MP5, respectively) the infection appeared to be completely resistant to this therapy and moreover was significantly resistant to the higher dose (1 mg of acyclovir per ml) (Table 1).

Evidence for biochemical resistance in virus from acyclovir-treated mice. The working stocks of virus obtained from individual mice at each passage level were tested for in vitro susceptibility to acyclovir. Table 2 shows that there was a small increase in the ED_{50} over the first three passages (0.02 μg/ml, increasing to 0.06 μg/ml) and then a further sixfold increase after passage 4. Overall, a 17.5-fold increase in the ED_{50} was observed. However, marked changes in the shape of the plaque inhibition curve occurred at successive passage levels (Fig. 1). Although there was a minimal change in the ED_{50} after a single mouse passage, the long tail on the curve at higher drug concentrations (Fig. 1) suggested a heterogeneous population containing viruses with at least two different levels of susceptibility to acyclovir. This effect increased markedly throughout the series, and the proportion of virus resistant to 0.1 μg of acyclovir per ml in the test rose from <1 to 100%. However, when virus was injected in passage 4 in the absence of chemotherapy, the virus isolated (designated MP4ut4) contained a reduced proportion of resistant virus.

TABLE 2. Evidence for resistance to acyclovir in vitro of viruses isolated from HSV-infected mice undergoing therapy

Virus[a]	Passage no.	ED_{50} in BHK cells (μg/ml)	% Plaques at 0.1 μg of acyclovir per ml	Thymidine kinase induction (% of SC16 value)
SC16	0	0.02	<1	100
SC16 MP1	1	0.03	4	54
SC16 MP2	2	0.04	21	55
SC16 MP3	3	0.06	28	16
SC16 MP4	4	0.35	100	5
SC16 MP4ut4	4	0.13	55	35

[a] SC16 was the original inoculum virus; SC16 MP1, SC16 MP2, SC16 MP3, and SC16 MP4 were uncloned viruses from individual mice during each of four successive passages. SC16 MP4 was obtained from a treated mouse, and SC16 MP4ut4 was obtained from an untreated mouse. The inoculum was the previous virus immediately above in the column (except SC16 MP4ut4).

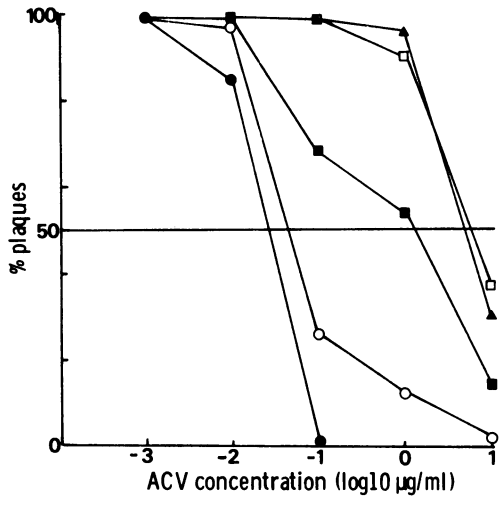

FIG. 1. In vitro susceptibilities of virus isolates from treated mice to acyclovir (ACV), as determined by plaque reduction in BHK cells. Symbols: ●, HSV type 1 SC16; ○, SC16 MP1; ■, SC16 MP2; □, SC16 MP3; ▲, SC16 MP4ut4; △, SC16 MP4.

The results of the thymidine kinase assays on the same viruses (Table 2) showed a steady decline in the amount of enzyme induced to 5% by passage 4 compared with strain SC16. The thymidine kinase was markedly increased (35%) in the virus sample from the group from which treatment was withheld. It is notable that after a single passage in treated mice (MP1), although the increase in the ED_{50} of acyclovir was minimal, the amount of thymidine kinase induced was 50% of the original inoculum.

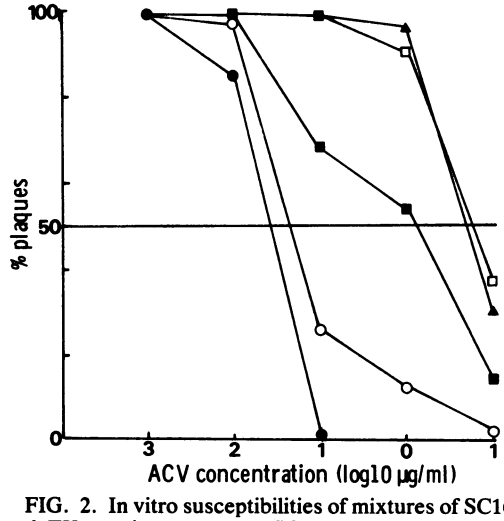

FIG. 2. In vitro susceptibilities of mixtures of SC16 and TK^- resistant mutant SC16 R5C1 to acyclovir (ACV), as determined by plaque reduction in BHK cells. Symbols: ●, SC16; ○, 90:10 SC16-SC16 R5C1 mixture; ■, 50:50 SC16-SC16 R5C1 mixture; □, 10:90 SC16-SC16 R5C1 mixture; ▲, SC16 R5C1.

The results of the ED_{50} titrations and thymidine kinase assays with the viruses from mouse ear homogenates suggested that these homogenates contained mixtures of resistant and susceptible virus particles. Accordingly, several artificial mixtures were constructed by adding a mutant derived from strain SC16 to the starting virus; both the parental virus (strain SC16) and the mutant were derived from single-plaque isolates. These mixtures were compared with the uncloned viruses isolated from treated mice by using similar tests.

In vitro analysis of artificial mixtures of resistant and susceptible viruses and comparison with viruses isolated from mice. Mutant SC16 R5C1 was chosen for these tests since previous work (6) had shown that its resistance to acyclovir (5 μg/ml in BHK cells) could be fully accounted for by its complete lack of detectable thymidine kinase. Mixtures were prepared by combining suspensions of strains SC16 and SC16 R5C1 in different proportions between 1:1 and 99:1. The parental strain, the mutant, and the mixtures were then tested for susceptibility to acyclovir in BHK cells and for thymidine kinase induction. The shapes of the acyclovir inhibition curves (Fig. 2) showed some similarity to the shapes of the curves for the viruses isolated from mice (Fig. 1), although the value obtained with strain SC16 R5C1 alone suggested that this virus may be about 10-fold more resistant than the most resistant mouse virus. The mixture containing 50% SC16 had an intermediate ED_{50} (1 μg/ml). For estimates of thymidine kinase induction BUdR-resistant BHK cells were infected with various mixtures at a multiplicity of infection of 5 PFU per cell. The thymidine kinase levels obtained, expressed as percentages (Table 3), were numerically very similar to the actual proportions of strain SC16 present. Thus, a mixture containing 50% strain SC16 gave 42% thymidine kinase, and a mixture containing 10% SC16 gave 13% thymidine kinase compared with a preparation containing SC16 alone. This background information was particularly useful when I analyzed the individual plaque-purified viruses (see below).

Analysis of plaque-purified "clones" of viruses obtained from acyclovir-treated mice. Since the viruses obtained from homogenized mouse tissues appeared to be mixtures of resistant and susceptible viruses, single plaques were isolated from the mouse yield after three passages (SC16MP3). Nine clones were derived from single plaques, and each was grown in BHK cells to produce a working stock (this necessitated two cell passages). Each clone was then tested for acyclovir susceptibility and thymidine kinase

TABLE 3. Thymidine kinase induction by mixtures of SC16 and SC16 R5C1 and by mouse isolates and the "neurovirulence" for mice after intracerebral inoculation

Virus(es)	Thymidine kinase induction in BHK cells (% of SC16 value)	PFU/LD_{50} ratio after intracerebral inoculation of BALB/c mice[a]
SC16	100	7×10^0
SC16 + SC16 R5C1 (99:1)	87	ND[b]
SC16 + SC16 R5C1 (90:10)	68	3×10^1
SC16 + SC16 R5C1 (50:50)	42	ND
SC16 + SC16 R5C1 (10:90)	13	3×10^2
SC16 + SC16 R5C1 (1:99)	<1	$>10^3$
SC16 R5C1	<1	$>10^5$
SC16 MP4t1	5	3×10^2
SC16 MP3 C5	<1	$\geq 10^4$
SC16 MP3 C9	57	5×10^1

[a] LD_{50}, 50% Lethal dose.
[b] ND, Not determined.

induction, as described above. Table 4 shows that there was considerable variation among the individual clones. Three of the nine clones appeared to be thymidine kinase defective and resistant to approximately 2 to 3 μg of acyclovir per ml. The other six clones induced intermediate levels of thymidine kinase, and three (clones MP3 C6, MP3 C7, and MP3 C9) were fairly susceptible to acyclovir (0.06 μg/ml), inducing about 40 to 60% thymidine kinase. Two additional isolates were obtained from clone MP3 C8 (which induced 7% thymidine kinase compared with strain SC16). Subclones MP3 C8.1 and MP3 C8.2 induced less thymidine kinase than MP3 C8 (<1 and 2%, respectively). MP3 C9 itself very much resembled a mixture (e.g., 90% SC16 R5C1 and 10% SC16). Six subclones were isolated from MP3 C9, and these were also tested. The results (Table 4) showed a surprisingly heterogeneous population. Subclone MP3 C9.1 (inducing 16% thymidine kinase) was cloned further to produce MP3 C9.1.3 and MP3 C9.1.4, and these subclones induced 45 and <1%, respectively.

In summary, the analysis of the single-plaque isolates confirmed the idea that the virus yield from each treated mouse comprised a heterogeneous virus population. It was not possible to purify completely the mixture by one or two single-plaque isolations. It was notable that no single-plaque isolate induced wild-type levels of thymidine kinase; the enzyme levels were lower in all cases, and the majority of the thymidine kinase-inducing strains showed slightly increased resistance to acyclovir in vitro compared with strain SC16. Both the slight resistance and the depressed thymidine kinase levels could be attributed to contamination with TK^- (resistant) virus, as demonstrated by the artificial mixtures described above. However, this did not appear to be the full explanation.

The MP4 yield, MP3 C9, and subclones MP3 C9.1.3 and MP3 C9.1.4 were also tested in TK^+-transformed BUdR-resistant BHK cells. Subclone MP3 C9.1.4 (which was TK^-) was completely susceptible in these cells (Fig. 3),

TABLE 4. Thymidine kinase induction by SC16 MP3 and various clones and subclones isolated from it and resistance to acyclovir, as determined by plaque reduction in BHK cells

Virus[a]	Thymidine kinase induction (% of SC16 value)	ED_{50} in BHK cells (μg/ml)	% Plaques at 0.1 μg of acyclovir per ml
SC16 MP3	16	0.06	28
SC16 MP3 C1	<1	3.2	85
SC16 MP3 C2	<1	3.5	100
SC16 MP3 C3	3.2	1.6	75
SC16 MP3 C4	2.7	2.5	95
SC16 MP3 C5	<1	4.0	96
SC16 MP3 C6	43	0.06	41
SC16 MP3 C7	64	0.06	45
SC16 MP3 C8	7	3.5	100
SC16 MP3 C8.1	<1	1.8	100
SC16 MP3 C8.2	2	2.5	100
SC16 MP3 C9	57	0.06	44
SC16 MP3 C9.1	16	6.3	100
SC16 MP3 C9.1.3	45	0.1	100
SC16 MP3 C9.1.4	<1	>10	100
SC16 MP3 C9.2	29	2.8	65
SC16 MP3 C9.3	56	0.05	25
SC16 MP3 C9.4	<1	1.3	100
SC16 MP3 C9.5	77	0.03	4
SC16 MP3 C9.6	78	0.02	4

[a] SC16 MP3 (see Table 2) was the uncloned virus from a single mouse obtained after three successive passages and treatment with 0.25 mg of acyclovir per ml in the drinking water. SC16 MP3 C1 to SC16 MP3 C9 were viruses obtained from single isolated plaques of SC16 MP3. SC16 MP3 C8.1 and SC16 MP3 C8.2 were subclones of SC16 MP3 C8. Similarly, SC16 MP3 C9.1 to SC16 MP3 C9.6 were subclones of SC16 MP3 C9. SC16 MP3 C9.1.3 and SC16 MP3 C9.1.4 were further single-plaque isolates derived from SC16 MP3 C9.1.

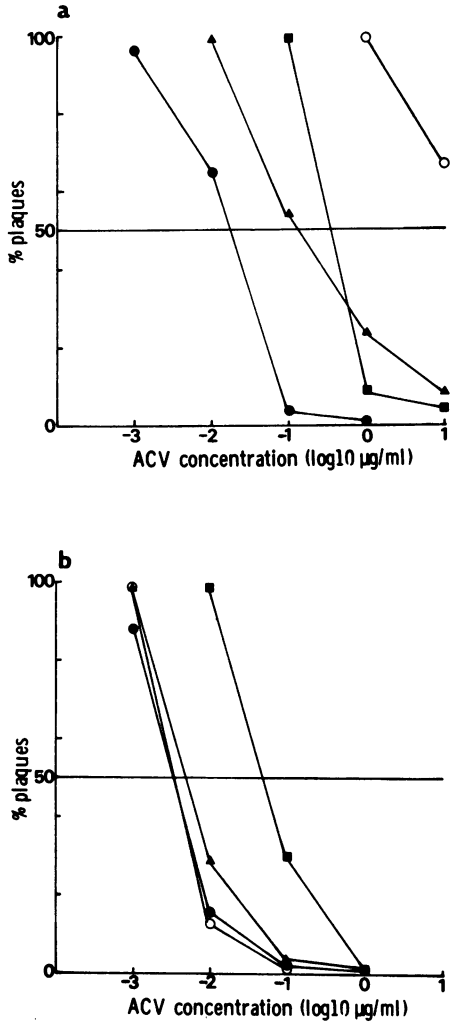

FIG. 3. In vitro susceptibilities of several viruses to acyclovir (ACV), as determined by plaque reduction in BHK cells (a) and TK^+ BUdR-resistant BHK cells (b). Symbols: ●, SC16; ■, SC16 MP4; ▲, SC16 Mp3 C9.1.3; ○, SC16 MP3 C9.1.4.

implying that its resistance may be explained by a lack of thymidine kinase. However, subclone MP3 C9.1.3 (45% thymidine kinase) was somewhat resistant, suggesting that its resistance (which was more obvious in BHK cells) may be caused by a change at a different site (e.g., in the DNA polymerase). It was notable that uncloned virus MP4 was quite resistant in TK^+ cells. These observations suggest further heterogeneity among the resistant virus population and will be pursued in a future study.

Pathogenicities and acyclovir resistance of several viruses and artificial mixtures in mice. Previous work has shown that mutants of HSV with defective induction of thymidine kinase have a reduced ability to produce disease in mice (5, 9). This was particularly marked when the mutant viruses were inoculated intracerebrally; the TK^- viruses showed at least a 200-fold increase in the ratio of plaque-forming units to 50% lethal dose. These strains also grew poorly in the skin and did not readily establish latent infections.

Mouse isolate MP4 (inducing 5% thymidine kinase) was inoculated intracerebrally into mice and compared with TK^+ and TK^- single-plaque isolates obtained from the third passage in mice. Several SC16-SC16 R5C1 mixtures were also inoculated for comparison. The 50% lethal doses obtained (Table 3) showed a remarkably consistent trend of decreasing virulence with decreasing thymidine kinase level. The mixtures containing 90, 50, 10, and 1% SC16 behaved as if only the wild-type portions of the mixtures were inoculated. The presence of the avirulent TK^- virus (SC16 R5C1) seemed to have little influence. MP4 resembled very closely the 10:90 SC16-SC16 R5C1 mixture. MP3 C5, which is TK^-, was avirulent, but MP3 C9 (57% thymidine kinase) was relatively virulent in this test and most closely resembled the 90:10 SC16-SC16 R5C1 mixture.

Several of the viruses were also inoculated into the ears of mice by using the standard inoculum of 10^5 PFU per mouse, as described above. Mice either were given 0.25 or 1.0 mg of acyclovir per ml in the drinking water or were not treated. On days 3 and 5 postinfection the mice were tested for ear inflammation, and the levels of virus in the inoculated ears were determined. The results confirmed (Fig. 4a and f) that parental virus SC16 was susceptible to treatment, particularly on day 5 when the higher dose of acyclovir was used. Strain SC16 MP4 (Fig. 4b and g) grew with characteristics similar to those of strain SC16 but was completely resistant to treatment with 0.25 mg of acyclovir per ml. However, when the higher dose (1 mg of acyclovir per ml) was used, there was some reduction in the virus yield. Previous work had shown that SC16 R5C1 replicates poorly in mouse ears. Reduced virus replication in this experiment (Fig. 4c and h) was only marked at day 5, and the inflammation produced was similar to that produced by strain SC16. This probably reflected the higher inoculum (10^5 PFU) used in this study. However, strain SC16 R5C1 appeared to be completely resistant to acyclovir. (The 1-mg/ml samples were lost due to contamination, but the ear thickness was not reduced by the higher dose.) A mixture containing strains SC16 and SC16 R5C1 (10:90) resembled closely strain SC16 alone (Fig. 4a, d, f, and i) and was somewhat susceptible to treatment, but less so than strain SC16 (a 2-$\log_{10}$ reduction in virus titer on day 5, compared with a reduction of >4 $\log_{10}$ for

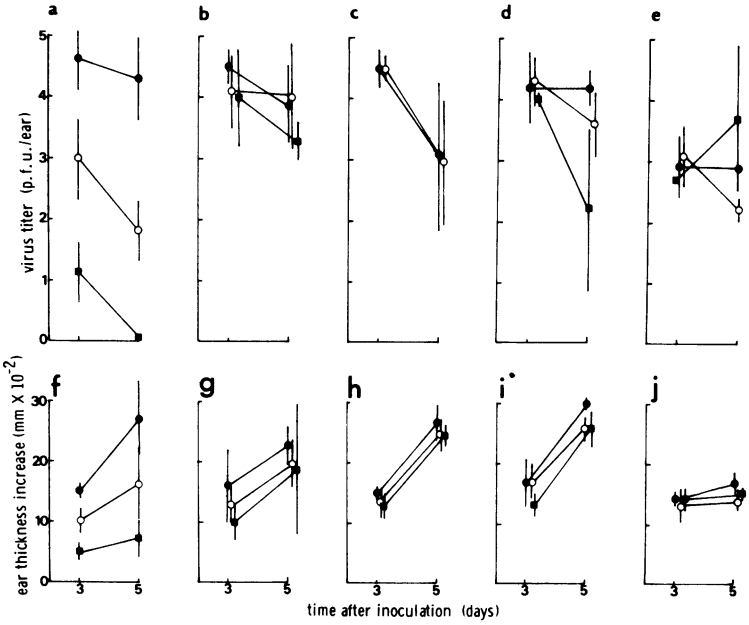

FIG. 4. Growth of virus in the ear pinnae (a through e) and inflammation of ears (f through j) in acyclovir-treated mice inoculated with SC16 (a and f), SC16 MP4 (b and g), SC16 R5C1 (c and h), a 90:10 SC16-SC16 R5C1 mixture (d and i), and SC16 MP3 C5 (e and j). Symbols: ●, untreated; ○, treated with 0.25 mg of acyclovir per ml in the drinking water from the time of inoculation; ■, treated with 1.0 mg of acyclovir per ml in the drinking water from the time of inoculation.

strain SC16). The TK^- clone isolated from MP3 C5 (Fig. 4e and j) produced little virus growth. The titers on day 5 showed rather wide variation but appeared to be affected little by treatment. Thus, the pathogenicity of strain SC16 MP4 (derived from passage in acyclovir-treated mice) was consistent with the hypothesis that it was a mixture of virulent and avirulent strains; similar effects were reproduced with deliberate mixtures. However, the acyclovir resistance observed in the "natural" virus was not observed in such mixtures. The former appeared to comprise a more complex variety of virus strains, which blended to produce an infection that was refractory to treatment.

DISCUSSION

The development of resistance to acyclovir occurs readily in tissue culture, and the nature of a number of mutants obtained in this way has been defined (6). The work of Smith et al. (16) demonstrated the importance of resistant virus in the inoculum in HSV infections of rabbit eyes, but in this case the biochemical nature of the resistance was unknown. The aims of the present study were to demonstrate that resistance to acyclovir could arise in HSV-infected mice undergoing therapy and to analyze the nature of the resistant virus by comparison with mutants which had been studied previously.

When the technique of repeated inoculation from mouse to mouse to amplify the trend to resistance was used, a virus population which produced an intractable infection emerged rapidly. However, defining the precise nature of the virus proved to be difficult. Each successive mouse isolate was tested directly for the induction of thymidine kinase (the virus enzyme which activates acyclovir), and over the passage series a trend to reduced induction of this enzyme was observed. However, the virus yields from the mouse pinnae were clearly heterogeneous and subsequently were shown to contain mixtures of viruses which individually induced different levels of thymidine kinase and varied in susceptibility to acyclovir. Using simple mixtures containing two viruses, one susceptible and one TK^- resistant, I obtained similar effects in vitro and in vivo. However, the natural isolates were clearly more complex. For example, there was evidence from examining resistance in thymidine kinase-transformed cells for further heterogeneity among the resistant population. Furthermore, it appeared that slight general resistance occurred; however, it was not possible to show whether this was due to changes (from the original inoculum) in the quality of

either thymidine kinase or DNA polymerase, the enzymes involved in the mode of action of acyclovir. Further experiments are now under way to investigate directly the ability of virus isolates to phosphorylate acyclovir to look for changes in the thymidine kinase; alternatively, evidence is being sought for alterations in the virus-induced DNA polymerase, which could also result in resistance to the drug. Although the mechanisms are not yet clear, the small change leading to increased resistance was clinically significant in the context of this experimental system.

A problem in pursuing the individual single-plaque isolates obtained from the mixed population was that these isolates also appeared to be heterogeneous. I propose the following two explanations for this: (i) the technique for single-plaque isolation was not adequate due to the presence in the wells of infected cells which did not produce visible plaques; (ii) the clones were unstable, and further mutation occurred at a high frequency. It may be that both of these possibilities occurred; the question has not been elucidated so far.

As for the uncloned virus specimens obtained from mice, it was notable that the level of resistance which developed was not high compared with mutants selected in vitro (the latter were selected by using 1 to 10 μg of acyclovir per ml) and probably reflected the relatively low levels of acyclovir achieved in the mouse tissue (14). Thus, it seems that the virus underwent minimal changes to adapt to the unfavorable environment caused by the presence of the inhibitor. This suggests that suboptimal therapy predisposes the virus to the more rapid emergence of resistance. There was some evidence from this study that when treatment was withheld, a return to a more susceptible virus population occurred.

No thorough investigation of the establishment of latent infections in the peripheral nervous system was undertaken. However, HSV was reactivated from explant cultures of cervical dorsal root ganglia of mice several weeks after the treatment was terminated at passage level 3. Different individual ganglia yielded either resistant or susceptible viruses. This is now being studied in more detail since the implication is that if further neurons become latently infected during an episode of HSV under treatment, subsequent recurrence of the infection may have acquired resistance to therapy.

Little clinical information is available regarding the nature of recurrent HSV infections in acyclovir-treated patients. At least one case has been described (Sibrack et al., Pediatr. Res. **15**:621, 1981) in which an immunosuppressed patient underwent a prolonged course of acyclovir treatment and the virus isolated from the persistent HSV infection became resistant to the drug, coupled with defective thymidine kinase induction. It remains to be seen whether such viruses are heterogeneous mixtures of strains, similar to those observed in the present study, and, if so, whether such mixtures are more pathogenic than the cloned TK^- mutant viruses studied to date.

Although the mouse experimental system described here is highly artificial, it does provide a useful method for the study of resistance to nucleoside analogs. The results obtained so far highlight two difficulties which may be encountered in the analysis of HSV specimens from humans. First, a relatively small change in resistance can be overlooked easily, yet be clinically important. The results from the plaque reduction test (or similar tests involving cytotoxicity) may be misleading, and great care should be taken to establish the shape of the dose-response curve for evidence of mixtures. Second, the amount of thymidine kinase induction by particular virus specimens is important since it relates to their ability to phosphorylate and thus to be susceptible to acyclovir or similar inhibitors. Reduced thymidine kinase induction may reflect a general change (either qualitative or quantitative) in enzyme induction by the virus population and, as described above, may result from mixtures containing both high and low thymidine kinase producers. The presence of small amounts of either extreme type (TK^+ or TK^-) is difficult to detect in a mixture without examining many clones. These results suggest that analyses of clinical specimens for drug resistance will be complex and that we should not expect, at first, a ready correlation between the clinical response in individuals and the biochemical characteristics of the virus isolates.

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