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Pathogenicity in Mice of Strains of Herpes Simplex Virus Which Are Resistant to Acyclovir In Vitro and In Vivo

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Mice infected with three different isolates of herpes simplex virus (HSV) and treated with acyclovir (acycloguanosine; ACV) showed low levels of virus replication during the acute phase of infection. However, virus isolated from such treated mice did not show increased resistance to ACV. In contrast, resistant virus was readily isolated in vitro by passaging HSV in the presence of the drug. The degree of resistance was determined, in part, by the nature of the cells used to test the virus. The majority of ACV-resistant strains induced low or undetectable levels of HSV-specified thymidine kinase (TK), the enzyme responsible for phosphorylating ACV in infected cells. The TK^--resistant strains were attenuated when injected into mice as indicated by reductions in virus replication, inflammation, and establishment of latent infections in sensory ganglia. The reduced virulence of the TK^- strains was most marked after intracerebral inoculation, where the lethal dose was increased more than 100-fold compared with the parental isolates. However, one mutant is described which induced high levels of TK but was highly resistant to ACV and retained virulence for mice.

Acyclovir [9-(2-hydroxyethoxymethyl) guanine or acycloguanosine; ACV] behaves as a thymidine analog which is selectively phosphorylated by herpes simplex virus (HSV)-specified thymidine kinase (TK) (4, 7). The monophosphate form of ACV is then converted to the triphosphate which appears to be a potent inhibitor of HSV-specified deoxyribonucleic acid (DNA) polymerase (7). On this basis, at least two kinds of mutation would be anticipated which could lead to the virus acquiring resistance to the drug: (i) loss or change in virus-specified TK, an enzyme which appears not to be essential when the virus is replicating in actively dividing tissue culture cells (12), or (ii) change in the sensitivity of the HSV-specified DNA polymerase to ACV triphosphate. The latter, in contrast to TK, is an essential function, and its complete loss would prevent virus replication.

Natural isolates of HSV of both oral and genital origin have been shown to differ quantitatively in their ability to induce virus-specific TK in a standard tissue culture assay (3), and this has led others to speculate (16) that strains of HSV which are poor TK inducers may reflect this in resistance to drugs such as idoxuridine (IDU) which depend on phosphorylation by HSV TK. A survey of 27 isolates of HSV made by Crumpacker et al. (1) revealed 10-fold differences in susceptibility to ACV among the isolates, but it has not yet been reported whether this reflects differences in expression of virus TK. Three of the isolates described in the present study also differ significantly from one another in their susceptibility to ACV as measured by plaque reduction in vitro. Again, it is not yet known whether this reflects qualitative differences in virus TK or DNA polymerase, or a difference at some yet undefined level.

Mutants resistant to ACV were derived from these isolates. Two were obtained by selection in the presence of bromodeoxyuridine (BUdR) and induce no detectable TK. These independently derived strains are innately resistant to ACV and have been useful for comparison with a number of resistant mutants obtained by passage of the parental viruses in vitro in the presence of ACV.

While examining the various ACV-resistant mutants in vitro, we found that the cell type used to measure resistance determined, in part, the degree of resistance of that particular virus strain. Comparisons of resistance of HSV strains to ACV in different cell types has given important clues to the nature of resistance mechanisms, and this fact should be considered when comparisons of resistance are made in different laboratories. The emphasis of this paper is on the behavior in mice of several strains of HSV with widely differing susceptibilities to ACV.

Previously, we showed that doses of ACV which effectively ablated clinical signs in mice inoculated in the skin of the ear pinna with a susceptible strain of HSV-1 failed to eradicate the infection completely (5); low levels of virus

replication were shown to persist in tissue near the inoculation site throughout the period of treatment. However, in contrast to the ready isolation of resistant virus from tissue cultures in the presence of ACV, ACV-resistant virus was not isolated from such mice during treatment. The reasons or this apparent paradox will be illuminated by the behavior of the artificially obtained resistant mutants in mice described here.

Our results suggest that the most frequent mutation leading to high resistance involves loss or reduction of HSV TK, and that this is also associated with loss of virulence in vivo. However, resistance of a different kind may occur, and this resistance probably reflects changes in the specificity of the virus TK or DNA polymerase.

MATERIALS AND METHODS

Viruses. HSV-1 SC16 is an oral isolate which produces a well-characterized infection in mice (5, 10).

HSV-1 Cl(101) and its BUdR-selected TK-deficient mutant Cl(101)TK$^-$ (also known as B2006 or MDK) were originally isolated by Dubbs and Kit (2).

HSV-2 Bry and its BUdR-selected TK-deficient mutant Bry TK$^-$ were isolated by Skinner and Thouless (18). The pathogenicity of Cl(101) and Bry and of their TK$^-$ derivatives was recently studied in mice (6).

A number of virus strains were derived from these parental isolates by passage in BHK-21 cells at low multiplicity of infection (approximately 0.1 plaque forming units [PFU]/cell) in the presence of ACV. After a preliminary determination of resistance by plaque inhibition was performed on a particular yield, the virus was diluted to produce single plaques in BHK-21 cell monolayers prepared in a multiwell tissue culture plate. Infected cells from a well containing an isolated single plaque were then used to prepare working stocks of the virus. The particular strains described in this study were prepared as follows: HSV-1 SC16 R_1C_1—three passages of SC16 in 7 μg of ACV per ml, one in 10 μg/ml, and then one in 1 μg/ml; HSV-1 SC16 R_5C_1—a single passage in 5 μg of ACV per ml; HSV-1 SC16 R_6C_2—two passages in 1 μg/ml, two in 5 μg/ml, and six in 10 μg/ml; HSV-1 Cl(101)TK$^-$ P7—derived from Cl(101)TK$^-$ after seven passages in 10 μg of ACV per ml; HSV-1 Cl(101) P_2C_5 and Cl(101) P_2C_6—both from Cl(101) after two passages in 10 μg of ACV per ml (two separate clones from the same virus yield); HSV-2 Bry P_3C_1 and Bry P_3C_2—three passages of Bry in 10 μg/ml. HSV-1 H29 and its ACV-resistant mutant H29R were a gift from P. Collins of Burroughs Wellcome Co. Ltd., Langley Court, Beckenham, Kent, England; the mutant was obtained by 10 successive passages in Vero cells in increasing concentrations of ACV and was not cloned.

Cell lines. BHK-21 cells and African green monkey (Vero) cells were grown in Eagle medium supplemented with 10% (vol/vol) tryptose phosphate broth and 10% calf serum (ETC medium). For plaque reduction assays, serum was reduced to 1% and the tryptose phosphate broth was excluded.

Determination of in vitro resistance to ACV and induction of TK. Resistance was measured by inhibition of plaque formation in cell monolayers after 48 h of incubation with a range of concentrations of ACV. The percentage of inhibition was plotted against $\log_{10}$ ACV concentration, and the 50% effective dose (ED_{50}) was thus determined. A sigmoid curve was normally obtained; the 50% value was read from the approximately linear portion of the curve (1). The results are expressed in micrograms of ACV per milliliter (1 μg/ml = 4.4 μmol). Thymidine kinase induction was determined on cell extracts, using TK$^-$ BHK cells 17 h after infection (multiplicity of infection = 5 PFU/cell) (14). The in vitro characteristics of the virus mutants will be the subject of a further publication where these techniques will be described in more detail (H. J. Field, G. Darby, and P. Wildy, J. Gen. Virol., in press).

Mice. Three-week-old female BALB/c mice were obtained from Bantin & Kingman Ltd. (Grimstone, Aldbrough, Hull, England) and used when they were 4 weeks old.

Inoculation of mice and virus growth in mouse ear. A 20-μl volume of virus suspension was inoculated intradermally into the left pinnae of anesthetized 4-week-old mice (10). A total of 50 mice were inoculated; 25 were treated with ACV, and 25 were untreated. To study virus replication, the pinnae were cut off from groups of three mice; the tissue from each mouse was then homogenized, and the amount of infectious virus was determined by plaque assay using BHK-21 cells.

Measurement of ear thickness. Ear thickness was measured with a micrometer screw gauge; the technique has previously been described in more detail (5). Ear thickness has been shown to be a sensitive measure of the development of cell-mediated inflammation in the ear (Nash, A. A., H. J. Field, and R. Quartey-Papafio, J. Gen. Virol., in press) and reflects the host response to infection which in magnitude depends mainly on the antigen load in the infected ear tissue

Reactivation of latent virus from dorsal root ganglia. Four to six weeks after inoculation, all mice remaining from the acute-phase experiments (groups of 6 to 13) were sacrificed. The second, third and fourth cervical dorsal root ganglia were removed, explanted into 0.5 ml of ETC (with serum reduced to 2%), and incubated at 37°C for 6 days in closed bottles. The ganglia were then homogenized, and the amount of infectious virus was determined by plaque formation, using BHK-21 cells (5).

Testing virus isolated from mouse tissues for resistance to ACV. The homogenized pinnae were stored at −70°C. When the virus titer had been determined, the ED_{50} of ACV was measured by using replicate samples of the appropriate dilution of the tissue suspension. This test was carried out with BHK-21 cells by the method described above. Since a small proportion of resistant virus could have been present in the virus population, a further test was carried out. A 0.1-ml sample of the undiluted tissue homogenate was plated with BHK-21 cells in the presence of 10 times the ED_{50} of ACV for that particular isolate. The monolayers were examined after 3 days for the presence of virus plaques.

Intracerebral (i.c.) inoculation of virus. Twenty microliters of serial 10-fold dilutions of virus suspen-

sion was inoculated into the left cerebral hemisphere of groups of 10 anesthetized 4-week-old mice. The 50% lethal dose (LD_{50}) was determined by the Spearman-Kärber method from the number of mice dying between the 2nd and 14th days after inoculation.

Preparation and administration of ACV. ACV, a gift from G. B. Elion of Burroughts Wellcome Co., Research Triangle Park, N.C., was suspended in water at a concentration of 3.2 mg/ml and finely dispersed by ultrasonic vibration. Mice were treated by intraperitoneal inoculation twice daily (9 a.m. and 6 p.m.), with a total dose of 50 mg/kg per day.

RESULTS

In vitro properties of ACV-resistant mutants. The four parental viruses [HSV-1 strains SC16, Cl(101), and H29 and HSV-2 strain Bry] from which resistant mutants were derived differed by a factor of 20 in their susceptibility to ACV as measured by a plaque reduction assay (Table 1). It is notable that Cl(101), the least susceptible isolate, was outside the range of susceptibilities in Vero cells reported by Crumpacker and his associates (1) in their survey of 27 isolates of HSV-1 and HSV-2. (Crumpacker's results are expressed in micromoles of ACV; 1 μg/ml = 4.4 μmol).

Mutants derived from the parental isolates by passage in the presence of ACV, and also the independently derived TK^- mutants, all showed increased resistance to the drug.

The ability of the mutants to induce TK in vitro was investigated in BUdR-resistant (TK^-) cells (Table 1). Cl(101)TK^- and Bry TK^- induced no detectable TK and are thought to have deletions in the TK gene. SC16 R_5C_1 was similar to the TK^- mutants. The other ACV-derived strains [except Cl(101) P_2C_5] induced low but significant levels of TK. However, we have no evidence that these low levels of enzyme induction are virus specified.

Cl(101) P_2C_5 was exceptional in that it induced levels of enzyme comparable with those induced by the parent strains, suggesting that this virus has a lesion in function(s) other than TK.

Since Cl(101)TK^- P7 was more resistant to ACV in BHK-21 cells than Cl(101)TK^-, it must have acquired resistance at other levels in addition to TK. The same may be true of SC16 R_9C_2, which was more resistant than R_5C_1, a TK^- derivative of SC16. Further support for this assumption has been obtained by comparing the resistance of these viruses to ACV in other cell lines, including $D2_1$ cells, a biochemically transformed mouse cell line which expresses an HSV-2 TK gene. In this line, defects in the virus TK are complemented by the resident HSV TK, and alterations in virus functions other than TK are revealed (G. Darby, B. Larder, K. F. Bastow, and H. J. Field, J. Gen. Virol., in press).

Susceptibility of parental virus strains in mice. Half of the mice were treated intraperitoneally with ACV, 25 mg/kg twice daily, begin-

TABLE 1. *Strains of HSV which are resistant to ACV*

Virus strain	ED_{50} by plaque reduction (μg/ml)		Induction of TK in TK^- cells (% SC16)	i.c. inoculation of mice		Established latency in ganglia after inoculation with 10^4 PFU[b]
	BHK-21	Vero		Log_{10} ED_{50}/0.02 ml ± SD[a]	PFU/LD_{50}	
HSV-1						
SC16	0.05[c]	0.15	100	0.8 ± 0.4	7	11/13
SC16 R_1C_1	3	2.8	1.5[c]	3.5 ± 0	3×10^3	1/10[d]
SC16 R_5C_1	7	6.5	0.2	4.7 ± 0.2	5×10^4	0/10
SC16 R_9C_2	>50	>50	2.4	>5.0	$>10^5$	0/10
Cl(101)	0.3	0.8	37.5	0.0 ± 0.2	1	6/6
Cl(101)TK^-	7	28	0.1	2.3 ± 0.2	2×10^2	0/10
Cl(101)TK^- P7	25	ND		4.2 ± 0.3	2×10^4	ND[e]
Cl(101) P_2C_5	25	>50	78	0.9 ± 0.3	8	7/9
Cl(101) P_2C_6	40	>50	0.6	2.1 ± 0.2	1×10^2	5/9
HSV-2						
Bry	0.15	0.6	73.5	0.07 ± 0.2	1	5/6[f]
Bry TK^-	6	11.3	0.2	>5.0	$>10^5$	0/10
Bry P_3C_1	50	40	0.5	>2.0	$>10^2$	ND
Bry P_3C_2	20	8	0.4	>2.0	$>10^2$	ND
HSV-1						
H29	0.07	0.04	>100	0.3 ± 0.2	2	ND
H29R	22	13	2.3[g]	>2.2	>130	ND
TK^- BHK cells			0.2			

[a] SD, Standard deviation.
[b] Number mice latently infected/number tested after inoculation into ear.
[c] Mean result from up to three independent determinations.
[d] This isolate was tested and found not to have altered resistance to ACV compared with R_1C_1.
[e] ND, Not done.
[f] These are surviving mice, since 10^4 PFU of Bry exceeds the LD_{50} by ear inoculation.
[g] 1.9% of parent strain H29.

ning at virus inoculation. This dose produced significant reductions in virus replication in the pinna and in production of cell-mediated inflammation as measured by increase in ear thickness. However, as previously reported for HSV-1 SC16 (5), despite the effective reduction of clinical signs, virus replication in the ear persisted and reached a peak 4 to 6 days after virus inoculation (Fig. 1). The observation of increased virus replication was reproducible. This increase could reflect the changed environment of infected cells due to the infiltration of mononuclear cells which occurs at this time, for example, resulting in the excretion of pyrimidine nucleosides which compete with ACV in infected cells. Bry and Cl(101), which are, respectively, four- and fivefold less susceptible than SC16 to ACV in vitro, were susceptible to ACV in mice, although the persistence of virus replication was more pronounced (Fig. 1).

The virus yields from three treated mice infected with each of the three viruses were examined at the time of peak titer for increased resistance of the virus population to ACV in vitro; none was found. When the undiluted tissue suspension was plated with cells in the presence of 10 ED_{50} of ACV, sporadic single plaques were observed in samples derived from both treated and untreated animals. These were of doubtful significance, since spontaneous resistance to ACV occurred so readily in vitro and also occasional plaques appeared when stock virus suspensions were similarly tested. Despite less effective control of virus replication of the isolates Bry and Cl(101), the inflammation measured by ear thickness was almost completely prevented. Bry is a more neurovirulent virus than either SC16 or Cl(101), and the dose of 10^4 PFU is approximately the LD_{50}. However, none of the ACV-treated Bry-infected mice died, although all six of a group of treated mice remaining at the end of the experiment were subsequently found to be latently infected. Similar establishment of latency despite treatment has been reported for SC16 (5).

Susceptibility in mice of ACV-resistant strains of HSV. Of the resistant strains of virus inoculated into BALB/c mice (10^4 PFU into the pinna) and treated with ACV, only SC16 R_1C_1 showed significant response to treatment, with reduced virus titers in the pinnae and greatly reduced inflammation (Fig. 2). SC16 R_1C_1 was one of the least resistant strains in vitro, having an ED_{50} of 3 μg/ml in BHK-21 and Vero cells. SC16 R_5C_1, which was slightly more resistant in vitro, appeared to have marginally reduced virus titers in treated mice. Yields of virus from groups of three mice treated or untreated and infected with the various strains were examined for resistance to ACV in vitro. In no case was this significantly greater or less than that of the original inoculum.

Most mutant strains tested appeared to retain resistance to ACV after ear inoculation (Fig. 2 and 3). However, it was noted that cell-mediated inflammation was usually slightly reduced. This was particularly noticeable with Cl(101) P_2C_5 and Cl(101) P_2C_6; although there was no reduction in virus titer in the ear pinna (Fig. 3), at several time points a small but significant reduction of ear thickness occurred in treated mice. This effect was also noted when mice were inoculated with large doses of Cl(101)TK^- (data not shown).

Pathogenicity in mice of ACV-resistant strains of HSV. ACV-selected resistant virus strains showed a tendency to reduced virus replication in the ear and reduced ear thickening compared with their parent strains (Fig. 1 and 2). Except for R_9C_2, this effect was much less marked than previously described for Cl(101)TK^- or Bry TK^- (6). No clinical signs suggesting neurological involvement were observed.

Several weeks after the acute phase of the infection had subsided, mice were tested by explanting the cervical dorsal root ganglia (relating to the sensory nerve supply of the ear) to see whether a latent infection had been established. Among the SC16-derived mutants, an extremely low incidence of latency was recorded (Table 1); only 1 of 10 mice infected with SC16 R_1C_1 was latently infected. This virus, reactivated from the ganglia, was subsequently tested and found not to have changed its resistance to ACV compared with R_1C_1 used for the original inoculum. In contrast, most of the Cl(101) P_2C_5- and P_2C_6-infected mice harbored the virus in latent form (seven of nine and five of nine, respectively). This observation was consistent with the relatively more vigorous growth of these viruses in the pinna (Fig. 3) and the idea that the establishment of latency reflects the amount of virus growth in peripheral tissue (5, 21).

For a more direct test of neurovirulence, all the viruses were inoculated i.c. into mice, and this produced a more clear-cut distinction between the parent viruses and the TK-deficient mutant strains. All mutants [except Cl(101) P_2C_5, which was TK^+] showed a marked decrease in lethality (Table 1). Most strains were >100-fold less virulent by this route of inoculation, and Cl(101)TK^- P7 was less virulent than Cl(101)TK^-, from which it was derived (Table 1). By comparison, the parent strain SC16, passaged 10 times in BHK-21 cells and cloned similarly to the most-passaged ACV-resistant strains, showed no reduction in neurovirulence when given i.c.

The virus strain Cl(101) P_2C_5, which showed

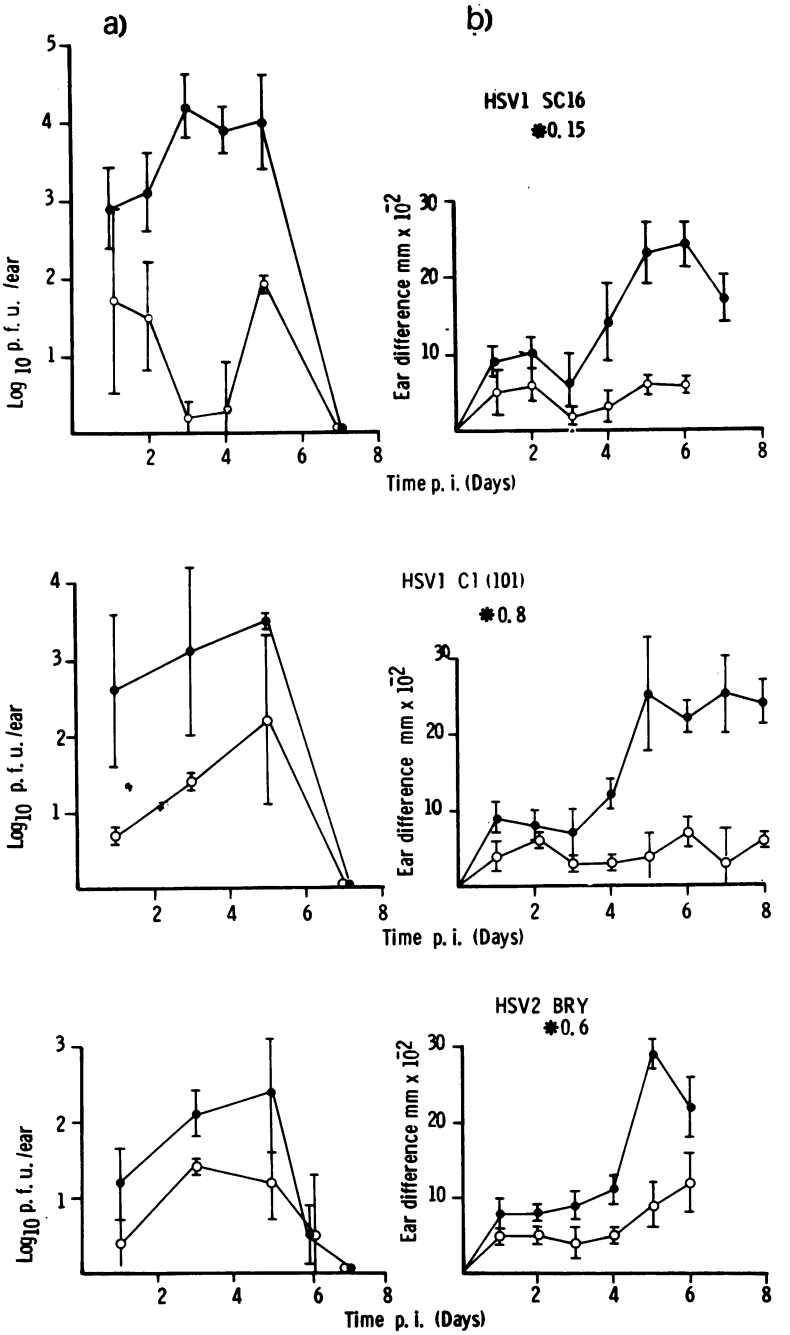

FIG. 1. *Susceptibility of parent virus isolates to ACV. 10^4 PFU of virus was inoculated into the left pinna. Symbols: (●) Untreated; (○) ACV, 50 mg/kg in twice daily doses from the time of virus inoculation for 10 days thereafter. (*) in vitro susceptibility to ACV (micrograms per milliliter) measured in Vero cells. (a) Virus growth in ear, geometric mean virus titer with standard deviation; (b) cell-mediated inflammation determined by ear thickness (left-right) with standard deviation.*

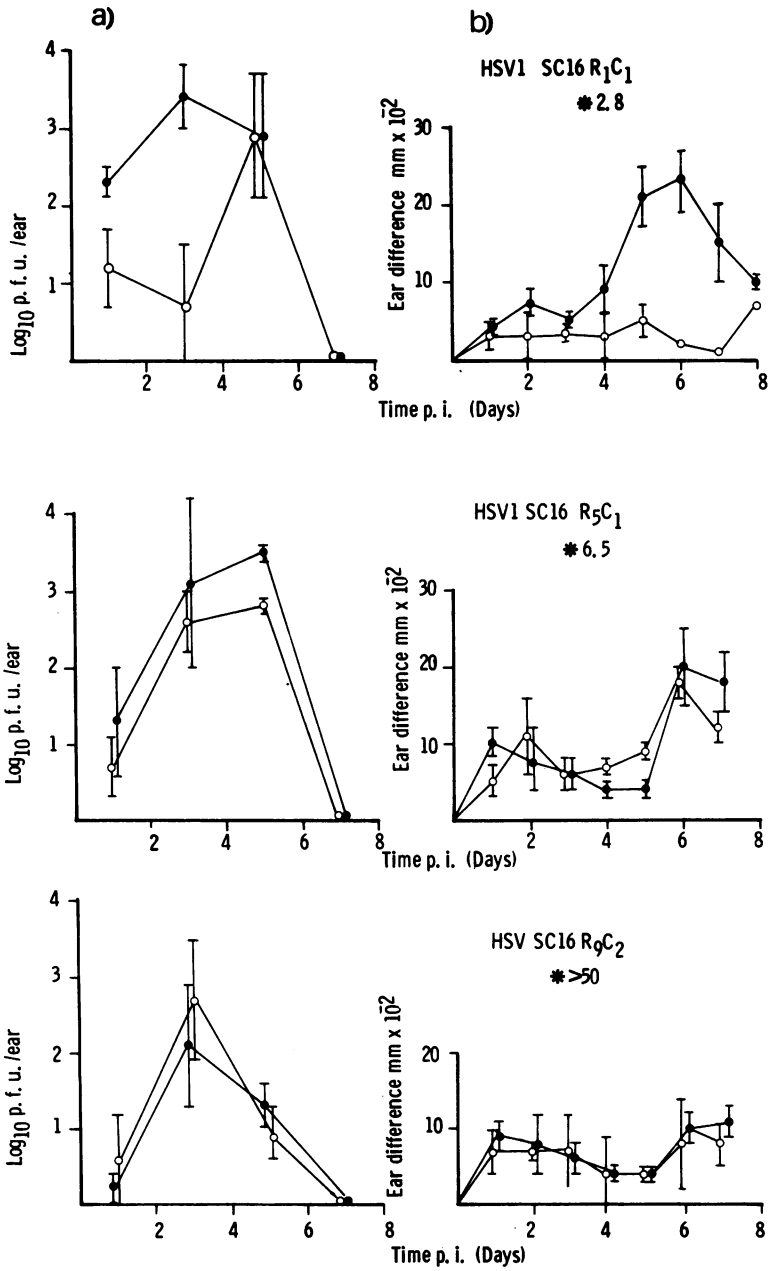

FIG. 2. *Pathogenicity and resistance of SC16-derived strains of HSV in mice. 10^4 PFU of virus was inoculated into the left pinna. Symbols and panels are as in Fig. 1.*

least reduction in neurovirulence compared with its parent strain Cl(101), was also the strain which induced high levels of TK in vitro (Table 1).

DISCUSSION

Development of resistance by herpesviruses to chemotherapeutic agents in vivo is a controversial topic, with few well-documented comparisons of the in vitro resistance of virus isolates before and after treatment (8, 9). "Virus resistance" can be confused with "treatment failure." Thus, it has been reported that most human isolates from patients undergoing IDU treatment were still susceptible to IDU in vitro (13). Nevertheless, there are several reports of true IDU resistance developing in both humans and lower animals (11, 20). The recent findings of

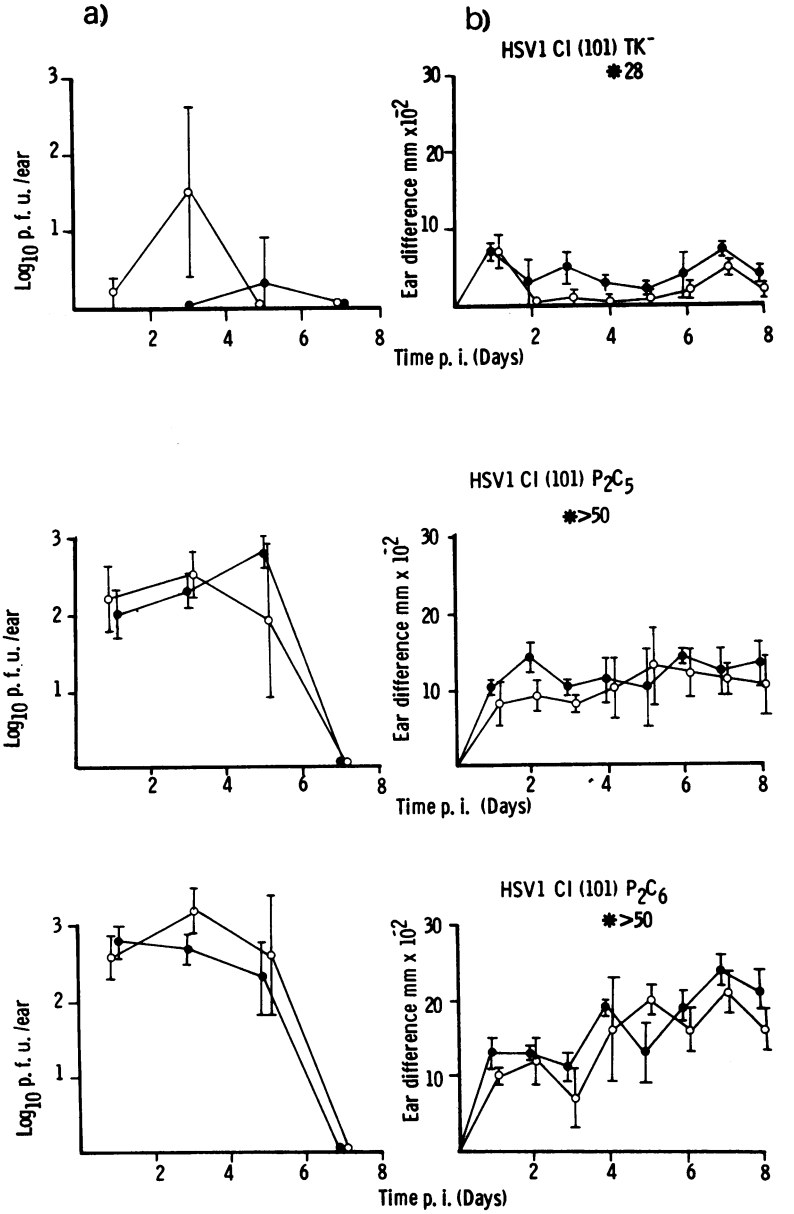

FIG. 3. *Pathogenicity and resistance of Cl(101)-derived strains of HSV in mice. 10^4 PFU of virus was inoculated into the left pinna. Symbols and panels are as in Fig. 1.*

Hirano and co-workers (11) are of particular interest. In these studies, isolates from IDU-treated patients were obtained which were highly resistant but retained the ability to induce TK. Since IDU is phosphorylated by HSV TK (although less selectively than ACV), resistance to IDU is likely to reflect changes at levels other than TK, for example, in the virus DNA polymerase.

In this study, the majority of artificially selected resistant isolates exhibited attenuation of virulence for mice. This suggests that mutations leading to resistance involve modifications of functions important to the virus in vivo, a suggestion supported by failure to isolate resistant virus from treated animals. It has already been established that TK^- HSV replicates poorly in serum-starved cells (12) and in skin and neural tissue (6, 15). This requirement for an effective TK in vivo may explain why resistant virus is difficult to isolate in vivo whereas it is readily obtained by passaging virus in the presence of ACV in vitro.

Several ACV-resistant strains do induce low

but detectable levels of TK. Similar low TK producers have been reported by others (2, 3, 17). It is not yet clear whether it is this low level of TK function which enables our resistant strains to replicate in the mouse ear more effectively than the two BUdR-selected viruses Cl(101)TK$^-$ and Bry TK$^-$, both of which appear to lack completely the TK function (2, 17, 19).

It has been reported that clinical isolates of HSV differ quantitatively in induction of TK (3) but that clinical isolates of TK$^-$ virus are extremely rare (and presumably in these rare cases TK loss may have occurred spontaneously during isolation from clinical material).

Although our parent strains differed markedly in their resistance to ACV, it is unclear whether this reflects qualitative or quantitative differences in virus TK induction or some other function. A study of substrate specificity of the isolated enzymes TK and DNA polymerase should establish at which level differences reside. The most resistant parent strain, Cl(101), has a long passage history in mouse brain and mouse L cells (2), which may have led to some change or evolution of the virus TK, that makes it less susceptible to ACV.

The in vitro isolation of a resistant strain derived from Cl(101) which induced high levels of TK was of particular interest. This virus strain shows some attenuation in mice but was the most virulent of the resistant strains when inoculated i.c. Thus, resistance to ACV can arise entirely independently of loss of virus TK induction.

Only when ACV becomes widely used will it become clear whether resistance will be a problem. The evidence obtained here in mice suggests that development of resistance may not be a common occurrence, but perhaps particular attention should be given to looking for viruses which are low TK inducers or have resistance at levels other than TK.

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