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NOTES

Pathogenicity of Herpes Simplex Virus Mutants Containing Drug Resistance Mutations in the Viral DNA Polymerase Gene

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Three herpes simplex virus mutants that contain drug resistance mutations in the DNA polymerase gene exhibited no significant reduction in replication in the ears of mice compared with the wild type after inoculation at that site but were attenuated for pathogenicity after intracerebral inoculation. Cataracts were common sequelae in mice that survived mutant infections.

The development of drugs such as vidarabine (araA) and acyclovir (ACV) has made it possible to treat many of the manifestations of herpes simplex virus (HSV), which range from lesions at peripheral sites to rarer life-threatening encephalitis (25).

HSV mutants that are resistant to antiviral drugs arise in the laboratory at a frequency approaching 10^{-3} (5, 7, 23, 26, 32). Most laboratory-derived ACV-resistant mutants exhibit decreased viral thymidine kinase (tk) activity (6, 16, 33). In general, *tk*-defective mutants exhibit reduced pathogenicity in animal models at most peripheral sites and are particularly attenuated for neuropathogenicity (for reviews, see references 13 and 29). Other ACV-resistant mutants specify tk activities with altered substrate specificity; these mutants replicate well at peripheral sites, but several exhibit little if any reduction in neuropathogenicity (13, 29). However, certain ACV-resistant mutants and all known araA-resistant mutants owe their resistance exclusively to mutations in the viral DNA polymerase (*pol*) gene (3, 5–10, 20, 21). Drug-resistant *pol* mutants can exhibit wide differences in sensitivity to various antiviral drugs and in physical map location (3–5, 10, 21–23). Certain *pol* mutants exhibit the antimutator phenotype, with much lower spontaneous mutation frequencies to *tk* deficiency (23). There have been fewer studies (10, 15, 27, 28, 30) of the pathogenicity of presumed *pol* mutants than of *tk* mutants, and to our knowledge, there have been no studies of *pol* mutants whose mutations have been definitively located in the *pol* gene by physical mapping or whose antimutator phenotypes are known. Studies of the replication of antimutator derivatives in experimental animals may help address why the virus has evolved to exhibit such a high mutation frequency (23).

We chose to study the pathogenicity of three distinct *pol* mutants, PAA^{r}5, PAA^{r}C, and AraA^{r}9. All three mutants were derived from wild-type HSV type 1 strain KOS and are resistant to ACV and araA. AraA^{r}9, unlike the other two mutants, is sensitive to pyrophosphate analogs such as phosphonoformic acid (4), and its drug resistance mutation maps to a different portion of the *pol* gene than do the mutations of the other two mutants (22). PAA^{r}5 and AraA^{r}9 are antimutator derivatives, whereas PAA^{r}C exhibits wild type or higher mutation frequencies (23) (Table 1).

Replication in the pinna. PFU (5×10^5) of each mutant or wild-type KOS were inoculated into the left ear pinnae of 4-week-old female BALB/c mice (Bantin and Kingman, Grimston, United Kingdom), and HSV replication in the ears was determined by removing pinnae, homogenizing the tissue, and measuring the amount of infectious virus by plaque titration (15, 24). The ears of three mice were sampled at each time point for each of the four virus inocula. The results (Fig. 1A) show that the ability of the mutant viruses to replicate in pinnae was very similar to that of the wild-type parent.

Ear inflammation caused by the viruses was examined by measuring increases in ear thickness relative to the uninfected right ear (15) after inoculation as described above. Previous work has shown that ear swelling is a cell-mediated response to virus antigens that correlates well with virulence (31). Mutant PAA^{r}5 induced increases in ear thickness similar to those induced by wild-type virus, except perhaps on day 4 (Fig. 1B). The other two mutants induced increases in ear thickness indistinguishable from those induced by wild-type virus (data not shown). Thus, *pol* mutations did not necessarily decrease replication in the pinna.

These results contrast with those obtained with most *tk*-defective mutants, which replicate poorly at peripheral sites, including pinnae (10, 12, 13, 15, 17, 19, 29). The data are in accord with those obtained with most other presumed drug-resistant *pol* mutants (15, 27, 28, 30). One presumed *pol* mutant has exhibited attenuation in replication in pinnae (10); however, additional mutations may have been present. Evidently, drug resistance mutations in the *pol* gene generally do not attenuate pathogenicity at peripheral sites.

Central nervous system virulence. No clinical signs suggesting neurological involvement were noted in any mice inoculated in the pinna with either the *pol* mutants or wild-type strain KOS. To investigate neuropathogenicity, the viruses were introduced directly into the central nervous system. Groups of eight 3-week-old BALB/c mice were inoculated intracerebrally (i.c.) as previously described (1) with 10-fold serial dilutions of each virus such that each group received inocula ranging from 10^2 to 10^6 PFU per mouse. The number of deaths was recorded after 2 weeks. All three mutants were less virulent than their wild-type

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TABLE 1. Properties of HSV drug-resistant *pol* mutants

Strain or mutant	Phenotype[a]				Mutation location[b]	PFU/LD_{50}[c]	Sequelae[d]
	PFA	ACV	araA	Mut			
KOS	S	S	S	+	N/A[e]	4.7×10^2	0/7
PAA^{r}C	R	R	R	+	696–924	2.0×10^5	5/14[f]
PAA^{r}5	R	R	R	Anti	696–924	$>1 \times 10^{6}$[g]	2/15[h]
AraA^{r}9	S	R	R	Anti	924–1235	$\geq 1 \times 10^{6}$[i]	2/17[h]

[a] Phenotypes listed are only a subset of those that have been examined (3–6, 22, 23; H. Chiou and D. Coen, unpublished data). PFA, phosphonoformic acid; Mut, frequency of mutation to iododeoxycytidine resistance; S, sensitive; R, resistant; +, wild-type mutation frequency; anti, antimutator phenotype (low mutation frequency).

[b] Location of mutations as determined by marker rescue in terms of amino acid position in the long open reading frame in the *pol* gene (22; Chiou and Coen, unpublished data).

[c] LD_{50}, 50% lethal dose.

[d] Sequelae were scored 2 months postinfection. Only mice which survived the dose of virus that killed at least one of eight mice were scored. The survivors of KOS infection came from groups of eight mice in which at least five died.

[e] N/A, Not applicable.

[f] Four mice had cataracts, and one exhibited shaking.

[g] Only two of eight mice died when each was inoculated with 10^6 PFU.

[h] Two mice had cataracts.

[i] Four of eight mice died at the highest dose (10^6 PFU).

parent (Table 1). The dose of mutant PAA^{r}C required to kill 50% of the mice was roughly 400 times that of KOS; the 50% lethal doses of PAA^{r}5 and AraA^{r}9 were at least 2,000 times that of KOS. The greater virulence of PAA^{r}C relative to the other two mutants may be due to greater reversion to wild type since AraA^{5}9 and PAA^{r}5 are antimutators and PAA^{r}C is not (23). Alternatively, PAA^{r}C may be intrinsically less virulent than the other mutants. Regardless, a change in the polymerase conferring drug resistance without decreasing mutation frequency appears, at least qualitatively, to decrease replication in nervous tissue. This finding may make it difficult to assign a role in vivo to the high mutation frequency of HSV.

These data and those from previous studies (10, 15, 30) suggest that *pol* drug resistance mutations can attenuate central nervous system pathogenicity. Further studies with recombinants derived from marker rescue of such mutants are required to establish this principle firmly. We speculate that the decrease in neuropathogenicity is due to decreased affinities of mutant polymerases for deoxynucleoside triphosphates (10, 11, 34) that are presumed to be in lower concentrations in the nondividing cells of nervous tissues than in peripheral tissues.

Sequelae after i.c. inoculation. A number of mice that survived i.c. inoculation with mutant virus exhibited sequelae, especially cataracts (Table 1). Of 7 mice surviving infection with KOS, none exhibited sequelae, whereas 9 of 46 surviving infections with the mutants did. Mice surviving infection with PAA^{r}C exhibited this effect most prominently, with 4 of 14 mice exhibiting cataracts and 1 exhibiting shaking. There is a low probability that the differences in sequelae after infection with mutant or wild-type viruses could be due to chance ($P = 0.1$ for the pair PAA^{r}C and KOS, and $P = 0.2$ for all mutants versus KOS by Fisher's exact test). However, in experiments with six other wild-type HSV strains, cataracts have occurred in fewer than 2 of 100 mice and neurological sequelae of any kind have occurred in fewer than 5 of 100 (H. J. Field, unpublished data). Interestingly, cataracts due to virus replication in the eye without substantial replication in the brain were common sequelae following i.c. inoculation of mice with an ACV-resistant, partially *tk*-defective *pol* double mutant (1, 10, 14). In contrast, four ACV-resistant mutants that contain only *tk* mutations did not produce such sequelae (14). Further experiments are necessary to determine whether *pol* mutations play a role in this phenomenon. Perhaps *pol* mutants that are attenuated for mouse encephalitis remain competent for replication in the optic nerve and retina while most *tk* mutants do not.

Clinical implications. The ready isolation of mutants resistant to ACV and araA in the laboratory has raised concerns that resistant strains arising in clinical settings will vitiate the use of these drugs. To date, surveys of viruses isolated from patients before, during, and after drug treatment have not shown a general trend to resistance; however, several examples of ACV-resistant mutants have been isolated from immunocompromised patients after ACV treatment. Thus far, these mutants have specified decreased or altered *tk* activities; polymerase alterations have not been reported, although they have not been excluded rigorously (for reviews, see references 13 and 29). Clinical experience with anti-HSV drugs is still limited; if drug-resistant *pol* mutants

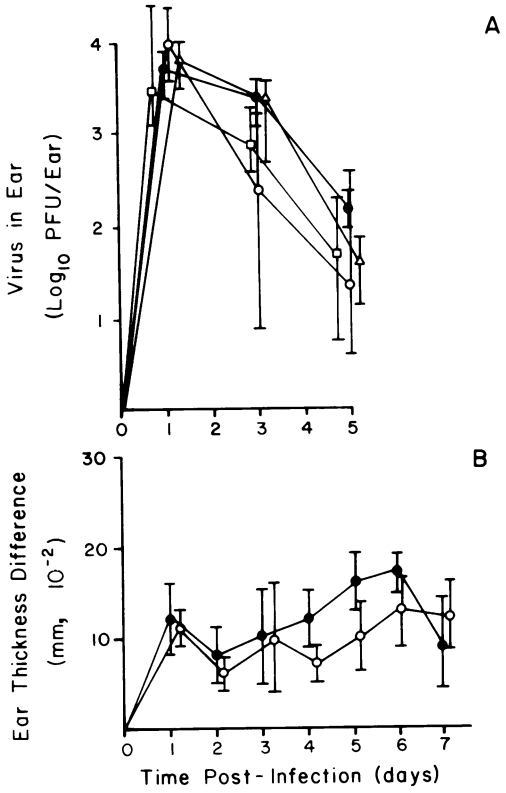

FIG. 1. Pathogenicity of *pol* mutants in pinnae. (A) Replication of wild-type strain KOS (●) and *pol* mutants PAA^{r}C (△), PAA^{r}5 (○), and AraA^{r}9 (□) was measured (15) in the left pinnae of three mice per virus per time point. Data are presented as the geometric mean, with the bars indicating the ranges of values obtained. (B) Inflammation of ears produced by wild-type strain KOS (●) and mutant PAA^{r}5 (○) measured as described previously (15). Data are presented as means of measurements of five mice ± one standard deviation. In both panels A and B, all measurements were made at the same time on each day; however, the data points are offset horizontally for easier visualization.

have not yet been isolated in clinical settings, they may well arise in the future.

Although extrapolating from laboratory studies is risky, our results indicate features of drug-resistant *pol* mutants that might have clinical importance. Such mutants appear to replicate well in the periphery (15, 27, 28; this report). Data suggest that *pol* mutants are capable of establishing latency (13, 28–30; Field, unpublished data). Thus, should these mutants arise in the clinic, they may well cause peripheral disease. Nevertheless, thus far, such mutants have been attenuated in animal models of encephalitis, although the degree of attenuation has not always been great (15; this report). Some of these mutants also are only modestly resistant to ACV or araA and are susceptible to several as yet unlicensed anti-HSV agents (4, 10, 18). We predict that such mutants are less likely to cause encephalitis and, if they do, will more likely be treatable.

Finally, the occurrence of cataracts in mutant-inoculated mice is interesting. We emphasize that this phenomenon has been observed only after i.c. inoculation and not when infection has been established by other routes (2). It may be a laboratory artifact. On the other hand, the phenomenon may reflect subtle changes in pathogenicity resulting from changes in DNA polymerase and, if so, suggests that viruses with novel patterns of pathogenicity may arise under the pressure of selection by inhibitors such as ACV and araA.

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