Antiviral Research, 15 (1991) 287–300
© 1991 Elsevier Science Publishers B V / 0166-3542/91/$03 50
ADONIS 0166354291001521

AVR 00483

Mini-Review

The implications of resistance to antiviral agents for herpesvirus drug targets and drug therapy

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(Received 20 November 1990, accepted 5 February 1991)

Summary

Antiviral drug resistance is an area of increasing clinical importance in treatment of a number of viruses including herpes simplex virus (HSV) and human cytomegalovirus (CMV). Work with these herpesviruses illustrates the value of studies of drug resistance. Novel aspects of drug mechanisms, such as a CMV gene product that contributes to ganciclovir phosphorylation, can be identified via drug resistance mutations. Drug targets such as the HSV DNA polymerase that are involved in drug recognition can be dissected by sequencing of drug-resistance mutations, which can point to alternate therapeutic strategies. Analysis of virus mutants in animal models and in patient populations can help assess the value of viral proteins such as the HSV thymidine kinase and ribonucleotide reductase as drug targets and the pathogenic potential of drug resistant mutants. Such studies reveal a broad spectrum of alterations conferring resistance and emphasize the importance of heterogeneous populations of virus in resistance and pathogenesis and the need to develop alternate therapies.

Antiviral drug; Herpesvirus; Drug resistance; Nucleoside analog

Introduction

Antiviral drugs are now enough of a success that we have considerable knowledge about how viruses can become resistant to them. There are several good reas-

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ons to focus a discussion of antiviral drug resistance on agents that have been successful in treating herpesvirus infections We know the most about resistance to these agents in part because the most widely prescribed antivirals are those against the herpesviruses, particularly herpes simplex virus (HSV) Resistance to antiherpesvirus drugs has become an important clinical problem in immunocompromised patients (Hirsch and Schooley, 1989). The lessons that we have learned in studying resistance to antiherpesvirus drugs are quite germane to drug resistance of other viruses such as human immunodeficiency virus (Larder et al , 1989) This review will emphasize the use of drug resistant mutants and genetic approaches to understanding targets of antiviral drugs and the features of drug resistance that may be important in the clinic.

Classes of antiherpesvirus drugs

There are two major classes of antiherpesvirus drugs The first class, which has had the most clinical success, comprises a wide variety of nucleoside analogs These include idoxuridine, vidarabine (araA), acyclovir (ACV), which is the most successful and widely used antiviral drug with indications against many herpesvirus diseases, and ganciclovir (DHPG), which is seeing considerable use against CMV retinitis These nucleoside analogs are converted to mono-, di-, and triphosphorylated forms by cellular and/or viral kinases and it is these phosphorylated forms that are active intracellularly For example, in HSV-infected cells, araA is converted to araA-triphosphate via cellular enzymes (Bennet et al., 1978) and then goes on to inhibit HSV DNA polymerase (Coen et al , 1982) while ACV is converted to ACV-monophosphate almost exclusively by the HSV thymidine kinase (TK) (Fyfe et al , 1978) and then primarily, if not exclusively by cellular enzymes to the triphosphate (Miller and Miller, 1980, 1982), which is a potent inhibitor of HSV DNA polymerase (Furman et al , 1979)

The second major class of antiherpesvirus drugs are analogs of pyrophosphate These include phosphonoacetic acid and phosphonoformic acid (foscarnet, PFA) The latter is beginning to see some clinical use These drugs do not require activation; rather they inhibit viral DNA polymerases directly, evidently by binding to the site involved in releasing the pyrophosphate product of DNA synthesis (Leinbach et al , 1976)

Drug resistance and drug mechanism

Because viruses are obligate intracellular parasites, the detection of resistance to an antiviral implies a certain level of 'selectivity' in the action of the antiviral. Put another way, virus replication can be inhibited either by interfering directly with virus-specific processes or by incapacitating the host The isolation of virus mutants resistant to a drug strongly implies that the drug acts at least in part by the former mechanism This idea was probably first put into print by Herrmann and Herrmann (1977). Once a drug-resistant virus mutant is isolated, a drug target can be identified by defining the gene in which mutation to drug resistance has

occurred. It can be inferred that this drug target contributes to the selectivity of the antiviral. Herpesvirus drug resistance mutations can be assigned to specific genes by genetic analyses, including complementation and recombination analyses (e g. Coen and Schaffer, 1980) and more precise marker rescue and DNA sequencing methods (e.g Gibbs et al., 1988)

Mechanisms of ganciclovir action and resistance in CMV This review will cite just one of several examples where studies of drug resistance have been invaluable for understanding drug mechanism.DHPG resistance in CMV. In HSV-infected cells, DHPG, like ACV, is activated by conversion to its monophosphate by viral TK. However, CMV is not known to encode or express a TK like that of HSV. Despite that, CMV induces an increase in DHPG-triphosphate upon infection (Biron et al., 1985; Freitas et al., 1985). Because CMV also induces numerous cellular nucleoside kinases, including one with weak activity towards DHPG (Freitas et al., 1985), it was widely assumed that DHPG is activated by a cellular kinase that is induced upon CMV infection.

However, the analysis of a CMV mutant that is resistant to DHPG challenged this assumption (Biron et al , 1986). Strikingly, compared to wild type, this mutant exhibited both a ten-fold increase in the dose of DHPG that inhibits virus growth 50% and a ten-fold decrease in the level of phosphorylated DHPG This decrease was not due to decreased stability of phosphorylated drug or to decreased induction of cellular nucleoside kinases or nucleotide pools. Moreover, the mutant is not deficient in induction of the weak cellular DHPG-kinase activity (K. Biron and J. Fyfe, personal communication).

These data demonstrated that DHPG is a selective anti-CMV drug and provided substantial evidence that selectivity is due, at least in part, to specific phosphorylation of drug More importantly, the results demonstrate that CMV encodes a gene product that contributes to DHPG-phosphorylation. Although this product could induce very specifically a hitherto-unknown cellular kinase, a much simpler interpretation is that it is a viral enzyme that phosphorylates DHPG or possibly its mono- or diphosphate Thus, a genetic approach provided evidence for a unique virus drug target, which was not forthcoming from biochemical studies Genetic studies to identify the CMV gene product that contributes to DHPG-phosphorylation are ongoing.

Pitfalls of biochemical approaches Biochemical approaches can be limited not only in assessing mechanisms of drug action, but also in assessing how a given virus mutant becomes resistant to an antiviral agent Such understanding is critical when dealing with issues of pathogenicity and resistance in the clinic. Two examples from HSV illustrate the pitfalls of a strict biochemical approach to resistance. The first example is an HSV mutant that contains a *tk* mutation leading to very low levels of TK polypeptide, which is unstable (Irmiere et al , 1989). As a result, a standard TK enzyme assay on extracts of cells infected with this mutant gives the result that the mutant is TK-negative (Coen et al., 1980). The standard interpretation would be that the mutant was resistant to a variety of drugs due to a

TK defect In fact, the mutant's TK defect has little effect on its sensitivity to many of these agents (Coen et al , 1989a) Instead, the mutant is a recombinant virus containing a DNA polymerase mutation that confers the drug resistance (unpublished results) Sorting this out would be very difficult without genetic approaches, which by definition measure resistance in the authentic context of the virus-infected cell

The second example is a mutant that specifies a DNA polymerase with an increased K_i value for bromovinyldeoxyuridine triphosphate; yet, this mutant is more sensitive to bromovinyldeoxyuridine than its wild-type parent (Darby et al , 1984). Thus, the biochemical analysis did not match the situation in virus-infected cells. Similar pitfalls have recurred in analysis of HIV mutants (e g Larder et al , 1989).

Antiviral drug resistance and functional dissection of drug targets

HSV DNA polymerase Once drug resistance mutations identify a gene product, they can then be used to dissect it functionally They can be particularly useful in studies of essential gene products since the resistance mutation does not abrogate protein function, but rather alters specific functional features In the example of the HSV DNA polymerase, mutations conferring altered sensitivity to pyrophosphate analogs are expected to alter amino acids involved in pyrophosphate recognition Mutations conferring altered sensitivity to aphidicolin, which inhibits competitively with deoxynucleoside triphosphate (dNTP) substrates, and mutations conferring altered sensitivity to nucleoside analogs are expected to alter amino acids involved in dNTP recognition For structure-function studies, it is important to study many different drug resistance mutations, because any one could be due to mutations at a position removed from a substrate binding site, exerting its effects by changing protein folding

Sequence analyses of eighteen different HSV polymerase drug-resistant mutants have been reported (Fig. 1). In the most extensive study (Gibbs et al , 1988), nine different mutations were found in four distinct clusters within about 1/4 of the polymerase, starting from about halfway in from the N-terminus. The majority of these nine mutations and five of the six other spontaneous mutations (Knopf, 1986, Tsurumi et al., 1987, Larder et al., 1987, Hall et al , 1989) lie in or near two regions of sequence similarity with diverse other DNA polymerases including eukaryotic cellular replicative DNA polymerases, termed regions II and III. This result led to the proposal that regions II and III directly participate in drug and substrate recognition (Gibbs et al , 1988) The other regions where spontaneous drug resistance mutations map, regions A and V, also are involved in substrate recognition, but too few mutations have been mapped in these to propose direct participation More recently, three mutant viruses engineered to contain mutations in region I, the most highly conserved region of sequence similarity shared among diverse polymerases, were shown to be drug resistant Thus, this region too is involved in drug and substrate recognition (Marcy et al , 1990) No one region seems solely involved in pyrophosphate recognition or dNTP recognition Rather, it seems likely that folding brings the various amino acids together to form substrate recognition sites The

REGIONS OF SEQUENCE SIMILARITY OF HSV pol WITH OTHER DNA POLYMERASES

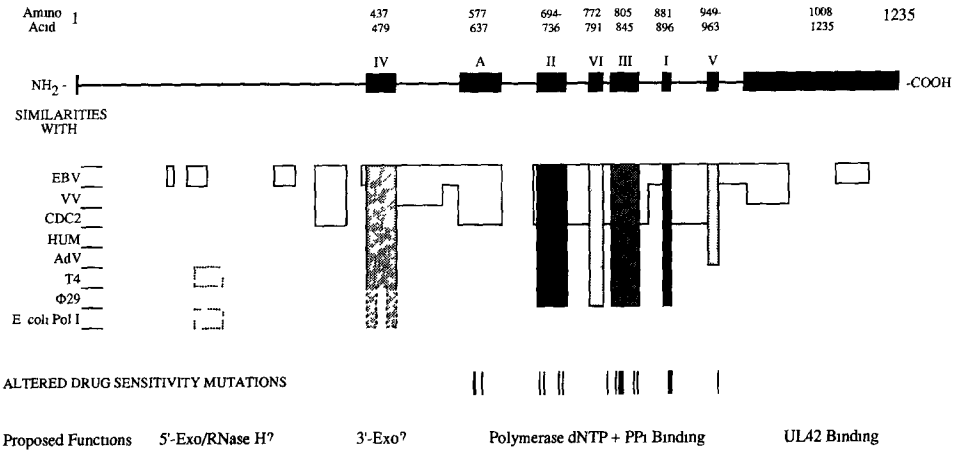

Fig 1 Locations of functional sites on the HSV DNA polymerase relative to regions of sequence similarity A linear representation of the HSV polypeptide is shown, with the six regions of sequence similarity shared among various DNA polymerases, region A, and the UL42-binding region indicated as solid boxes Boxed areas below delineate the sequence similarities between HSV and the indicated DNA polymerases with the intensity of shading corresponding roughly to the degree of sequence conservation Abbreviations EBV, Epstein-Barr virus, VV, vaccinia virus, CDC2, yeast DNA polymerase delta, HUM, human DNA polymerase α, T4, bacteriophage T4, ϕ29, bacteriophage ϕ29 Also indicated are the location of point mutations that result in altered drug sensitivity of the HSV enzyme and the proposed functions of various regions of the protein See text and Digard and Coen (1990) for more detailed discussion

mapping of acyclovir-resistance mutations to regions conserved among viral and cellular polymerases argues that acyclovir-triphosphate acts selectively by exploiting relatively subtle differences between viral and cellular polymerases.

Recent results suggest a strategy for targeting antiviral drugs to regions of the polymerase that are not shared with cellular polymerases In this study (Digard and Coen, 1990), a C-terminal domain that does not overlap regions of sequence similarity with cellular polymerases (Fig 1) was shown to be necessary and sufficient for interaction with the HSV UL42 protein, a polymerase processivity factor that is essential for viral DNA replication. This raises the possibility that peptides and non-peptide analogs that mimic either UL42 or this portion of the polymerase could interrupt this interaction and interfere with HSV replication.

In summary, combining molecular genetic analyses such as those cited above with structural studies should lead to detailed information about the functional sites of this enzyme, which may permit the design of highly specific antiviral drugs.

Studies of the frequency and properties of drug-resistant mutants

Frequency of drug resistant mutants Drug resistance mutations arise frequently in laboratory stocks of HSV. This is true not only for mutations in the TK gene,

which is non-essential in cell culture, but for mutations in the polymerase A compilation of the available data (Coen, 1986) gives an estimate of mutation frequency for either ACV-, araA-, or PAA-resistance of $10^{-4} - 10^{-3}$ One source of this high frequency of mutation to drug resistance is the HSV DNA polymerase (Hall et al , 1984, 1985) There have been no published reports on the frequency of CMV mutants arising in the laboratory, but they appear to arise less frequently than do HSV mutants (unpublished results) Evidently, mutation frequencies in both viruses are high enough to give rise to drug resistance in the clinic (see below)

Sensitivities to other antiviral agents Experience with bacterial pathogens has highlighted the importance of agents that can combat organisms that are resistant to other useful drugs Table 1 summarizes the results from studies too numerous to cite here that have been obtained with HSV mutants resistant to acyclovir There are four classes of single mutations that confer acyclovir-resistance Three of these affect TK mutations that completely abolish enzyme activity (TK-negative), mutations that decrease activity, for example by reducing the amount of activity or by decreasing affinity for substrates (TK-partial), and mutations that have little if any effect on activity towards the natural substrate, but substantial effect on the ability to phosphorylate acyclovir (TK-altered) The remaining class of single mutations results in altered DNA polymerase, which is less able to be inhibited by acyclovir-triphosphate. Double mutants with mutations in both the TK and DNA polymerase genes can also be found

TK mutants of all three classes remain sensitive to drugs that inhibit viral polymerase without requiring phosphorylation by TK The TK-negative mutants are generally resistant to any drug such as DHPG that requires TK for activation while TK-partial or -altered mutants are sometimes sensitive to certain of these drugs depending on their relative affinity for TK and the nature of the mutation. It is much harder to generalize with polymerase mutants, which can vary widely in cross-resistance Many acyclovir-resistant polymerase mutants, but not all, are resistant to PFA, and many, but not all, are sensitive to DHPG One can imagine a double mutant that could be resistant to all clinically useful anti-HSV drugs As will be discussed subsequently, one can also imagine a double mutant that would be highly resistant to acyclovir and retain considerable pathogenicity This is an argument for continuing development of new agents Even if such agents are too toxic for widespread systemic use, they should be kept available in the event of life threatening infections by HSV mutants resistant to all drugs that are currently available for clinical use

TABLE 1

Cross-resistance and sensitivities of HSV acyclovir-resistant mutants

Type of mutant	Resistant to	Sensitive to
TK-negative	DHPG	PFA, araA
TK-altered or -partial	Depends	PFA, araA
Polymerase-altered	Often PFA	Often DHPG
TK, polymerase double	Possibly all	Possibly none

TABLE 2

Behavior of acyclovir-resistant mutants in mouse models of HSV disease

Type of mutant	Replication at periphery	Reactivation from latency	Neurovirulence upon I C inoculation
Wild-type	++++	++++	++++
TK-negative	++−++++	0	0
TK-partial	++−++++	+−++++	+
TK-altered	++++	++−+++	+++
Pol-altered	++++	++++	0−+++

++++ = Wild-type levels of activity, 0 = little or no activity, +,++,+++ refer to intermediate levels of activity with +++ being nearly wild-type and + being nearly no activity

Sensitivities of drug-resistant CMV A DHPG-resistant CMV isolated in the laboratory proved sensitive to every other compound tested, except for a slight decrease in sensitivity to acyclovir (Biron et al , 1986). Two PFA-resistant CMV mutants are sensitive to DHPG, but fairly resistant to acyclovir (Sullivan and Coen, manuscript in preparation).

Pathogenicities of HSV drug-resistant mutants Table 2 summarizes the results of studies, too numerous to cite here, of the pathogenesis of single acyclovir-resistant mutants in mouse models of HSV disease. Of course, caution must be used in extrapolating these results to humans All of the classes of mutants are generally capable of replication at peripheral sites of inoculation. TK-negative and TK-partial mutants are the most impaired in this category, but such mutants replicate to wild-type titers after corneal inoculation (Tenser and Dunstan, 1979; Coen et al., 1989b) TK mutants are more impaired in their ability to reactivate from latent infections upon explant of ganglia, with truly TK-negative viruses being completely reactivation-incompetent (Tenser and Dunstan, 1979, Coen et al , 1989) In general, polymerase mutants have no difficulty reactivating from latency The assay that is most sensitive to drug-resistance mutation is the ability of the virus to kill mice after intracerebral inoculation Even in this assay, TK-altered mutants and some polymerase mutants are only modestly impaired.

Why are certain drug-resistant mutants altered in pathogenicity? This question appears easy to answer for TK-deficient viruses, which account for the majority of ACV-resistant mutants isolated in the laboratory. Presumably, since HSV is neurotropic and avoids immune surveillance in the nervous system, it must build up its own nucleotide pools in non-replicating nerve cells. Thus, HSV TK would be much more important in this setting than in cell culture. This would also explain why many ACV-resistant, TK-deficient clinical isolates have come from immunocompromised patients where the virus can replicate more readily in non-nervous tissue.

Less understood is why polymerase mutants would be less pathogenic, as most have proven to be One possible explanation would be that these mutants generally

display lower affinities for normal dNTPs (Derse et al., 1982, St Clair et al., 1984, Hall et al., 1985) Thus, because dNTP pools may be lower in nerve cells than in cell culture, these mutants, like TK mutants, may be at a disadvantage Nevertheless, as described above one can isolate drug-resistant mutants that exhibit substantial pathogenicity in several assays

Mixtures of acyclovir-sensitive and resistant HSV Field and Ellis and colleagues have explored how mixtures of acyclovir-resistant and sensitive viruses behave in infections of acyclovir-treated mice (Field, 1982, Field and Lay, 1984, Ellis et al., 1989) Mice infected with defined mixtures of acyclovir-resistant and -sensitive viruses caused disease that was less responsive to acyclovir therapy Mixtures of pathogenic TK-altered and wild type viruses were particularly more difficult to treat. After passage of either defined mixtures or wild-type virus in mice treated with acyclovir, highly heterogeneous mixtures of virus were derived that retained pathogenicity and were more resistant than the input virus. These viruses caused the most serious acyclovir-resistant disease These results suggest that sensitive viruses can complement resistant viruses for pathogenicity and resistant viruses can complement sensitive ones for resistance In view of the latter idea, one can speculate that the particularly pathogenic and resistant highly heterogeneous mixtures contained some DNA polymerase mutants as these mutants are known to complement sensitive viruses for resistance (Coen and Schaffer, 1980) Such heterogeneity may already have reared its head in resistant isolates from patients suffering from severe, progressive HSV infections that do not respond to acyclovir (see subsequent section)

TK-negative mutants establish latent infections Even though it is not possible to recover virus upon explant of ganglia from mice infected with TK-negative and certain TK-deficient mutants, recent results argue that these mutants establish latent infections Viral DNA in sufficient quantities can be found in these ganglia (Leist et al., 1989; Katz et al., 1990) to account for latency The mutant viruses express the latency associated transcript (Coen et al., 1989, Tenser et al., 1989; Leist et al., 1989) and can be rescued from ganglia upon superinfection with a TK-competent virus (Coen et al., 1989, Efstathiou et al., 1989) These results help distinguish establishment of latency from reactivation They also have a number of clinical implications, assuming that the mouse studies can be extrapolated to humans (see below) Acyclovir-resistant, TK-deficient virus would be present in ganglia of patients who have developed resistant infections This might be reactivated by TK-positive virus present in the same neurons giving rise to mixed populations of virus that retain both pathogenicity and resistance Whether this occurs in either patients or animal models remains to be seen The finding by Erlich et al (1989a) of recurrence of an acyclovir-resistant virus in an AIDS patient may be of interest in this regard

An additional implication of the results with TK-negative viruses is that drugs targeted to inhibit HSV TK might be useful prophylactically during immunosuppression, but would be unlikely to prevent establishment of latency during acute

infection. Indeed, two such drugs do significantly decrease the reactivation of wild-type HSV from explanted ganglia (Leib et al., 1990).

Thus, it seems possible to target drugs against viral proteins that are not essential for replication in cell culture because these proteins are important for pathogenesis in the mammalian host. A key issue is how important such proteins will be in human infections. Clinical experience with acyclovir-resistant mutants indicates that TK-deficient mutants do not arise frequently or cause disease in immunocompetent patients and they have only rarely been associated with recurrences after acyclovir-resistant infections have resolved in immunocompromised patients. Thus, it appears TK is important for pathogenicity in humans much as it is in mice.

There has been considerable effort to develop drugs that inhibit another viral enzyme that is not essential for replication in cell culture, the HSV ribonucleotide reductase (RR). RR-negative mutants are even less pathogenic than TK-negative mutants in mice (Cameron et al., 1988; Jacobson et al., 1989). However, these mutants are severely impaired for replication in mouse cells at 38°C (Jacobson et al , 1989), which is considered mouse body temperature, whereas they replicate relatively efficiently in human cells at 37°C, human body temperature. Thus, it cannot be concluded from experiments in mice that RR is a valid antiviral drug target.

Summary of pathogenicity studies in mice Based on these studies, among single mutants, TK-altered and DNA polymerase mutants have the greatest potential for causing dangerous drug-resistant infections. Infections caused by mixed infections may be even more worrisome, especially if they contain polymerase mutants Studies of virus mutants can shed light on mechanisms of latency and pathogenesis and can help determine the value of viral proteins as drug targets

Drug-resistant herpesvirus infections in immunocompromised patients

The first several reports of acyclovir-resistant HSV isolated from patients in the early 1980s were not convincingly shown to contribute to disease and in some cases acyclovir-treatment did not seem to be impeded (reviewed by Larder and Darby, 1984). More recently, however, there have been several reports, especially from AIDS patients, of severe, progressive disease in the face of appropriate acyclovir therapy (e.g. Norris et al., 1988, Erlich et al., 1989b; Chatis et al , 1989; Sacks et al., 1989) One report indicates that acyclovir resistance occurs in about 5% of immunocompromised patients from whom HSV can be isolated (Englund et al , 1990) Nevertheless, there has been tremendous variation in the severity of disease ranging from mere shedding, through 'indolent' lesions, to life-threatening illness

The few cases of ganciclovir-resistant CMV infections reported thus far were associated with disease that progressed and was refractory to DHPG treatment (Erice et al , 1989)

Types of mutations found in clinical isolates As yet there have been few if any virological correlates with clinical outcome. All of the acyclovir-resistant clinical

HSV isolates except two have exhibited TK deficiency, mainly as assayed by in vitro enzyme assays It should be stressed, however, that most of the TK-deficient isolates have not been examined adequately for whether they were TK-partial as opposed to TK-negative Neither have most of these been examined for the heterogeneity of the virus population Two isolates have been predominantly TK-positive, altered DNA polymerase (Sacks et al , 1989, Parker et al , 1987, Collins et al , 1989). Both types of isolates – predominantly TK-deficient and predominantly TK-positive – have been associated with severe disease

In the few cases when heterogeneity has been examined, both relatively uniform populations and highly heterogeneous mixtures have been found Interestingly, in one case, homogeneity was associated with an indolent infection (Collins et al , 1989) and in a second, heterogeneity was associated with a severe, progressive infection (Sacks et al , 1989) Both populations contained DNA polymerase mutants It is tempting to speculate that the polymerase mutations combined with heterogeneity led to severe disease in the latter case and not the former

There are no published data yet on the nature of the alterations in the ganciclovir-resistant CMV isolates, however, preliminary studies indicate that these isolates, like the original laboratory isolate, are deficient in phosphorylation of drug (K Biron, personal communication)

Pathogenicity studies in mice There has been some examination of the pathogenicity of acyclovir-resistant HSV isolates from patients in mouse models, however, in many cases the resistant isolates have not been compared appropriately with sensitive, pre-treatment isolates This is in part due to the difficulties of obtaining pre-treatment isolates from AIDS patients who often receive ACV treatment before virus isolation Nevertheless, there seems to be insufficient recognition that mouse models of pathogenicity are not absolute measures of pathogenic potential in humans Different acyclovir-sensitive HSV isolates from patients with severe HSV disease require very different titers of virus to establish reactivatable latent infections in mice or to kill mice after various routes of inoculation Thus, finding that 10^5 PFU cause 100% mortality after intranasal inoculation does not imply that this isolate is 'fully neurovirulent' (Erlich et al , 1989b). It may well be orders of magnitude less virulent than its acyclovir-sensitive parent

Only a few acyclovir-resistant isolates from patients have been compared appropriately with a sensitive, pre-treatment isolate that can be inferred to be the parent of the resistant isolate by restriction enzyme fingerprinting (Ellis et al , 1987, Sacks et al , 1989; Collins et al , 1989, Oliver et al , 1989) Of these, some have exhibited decreased pathogenicity and some have exhibited substantial pathogenicity in mice. As yet, no obvious correlation has emerged between behavior in mice and clinical course

Treatment of drug-resistant infections in humans Treatment of ACV-resistant HSV infection with PFA has been associated with clinical improvement (Vinckier et al , 1987, Youle et al , 1988; Chatis et al., 1989, Sacks et al , 1989, Erlich et al , 1989a) Although this has been interpreted in some cases as reflecting successful

treatment, in the absence of controlled studies it is difficult to be sure that clinical improvement was not the result of improvement in immune status or some other factor At this time of writing, there are no published reports yet on treatment of ganciclovir-resistant CMV; PFA would be a likely candidate for such treatment in such situations, too. For both viruses, the increasing number of cases of resistance warrant redoubled efforts to find safe, effective alternative therapies.

Acknowledgements

Grant support from the National Institutes of Health (R01 AI19838, R01 AI26126, P01 AI24010, U01 AI26077, S07 RR05381) is acknowledged. I thank many colleagues for helpful discussions.

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