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"The End of Innocence" Revisited: Resistance of Herpesviruses to Antiviral Drugs

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INTRODUCTION

In the 1989 *New England Journal of Medicine* editorial "Resistance to Antiviral Drugs: the End of Innocence," Martin Hirsch and Robert Schooley highlighted the importance and ramifications of progressive herpetic disease resulting from drug-resistant herpesviruses in AIDS patients (64). The emergence of herpes simplex viruses (HSV) resistant to antiviral drugs was not a new observation, for selection for resistance has generally not been difficult in cell culture (29). Indeed, resistant HSV had also been recovered from patients (26, 42, 104, 105), but these viruses were not associated with, nor did they cause, progressive disease in the immunocompetent patient (7). However, the emergence of drug-resistant herpesviruses in the immunosuppressed patient did result in progressive disease, as described in the articles accompanying the editorial (15, 46, 47), as well as elsewhere (90). In the intervening years since the Hirsch and Schooley editorial, greater attention has been paid to rapid detection of drug-resistant clinical isolates and the clinical importance of infections associated with drug-refractory herpesviruses in immunosuppressed patients. Indeed, the frequency of encountering acyclovir (ACV)-resistant HSV and ganciclovir (GCV)-resistant human cytomegalovirus (HCMV) has been increasing as the population of AIDS patients has mushroomed. As a result, increasing attention is being focused on defining the mechanisms involved in antiviral drug activities and resistance and on developing antiviral drugs with novel modes of action. The resistance problem has also heightened interest in evaluating the efficacy of combination therapy with antiviral agents having different targets for inhibition.

In this article, we summarize the recent progress in dealing with clinically important drug resistance among the herpesviruses and in understanding the mechanisms of drug action with the aid of drug-resistant isolates. The important benefit of this understanding should be progress in developing more effective therapeutic modalities. Other recent reviews (14, 20, 51) further emphasize the importance of this growing drug resistance problem.

HSV

HSV types 1 and 2 (HSV-1 and HSV-2) are alpha herpesviruses which are generally associated with primary and recurrent mucocutaneous facial, ophthalmic, or genital lesions. The lesions are self-healing and localized and reflect a host-parasite relationship in which the virus establishes a latent ganglionic infection in the neurons that innervate the area of the primary lesion. Virus is periodically reactivated in the immune host and may cause repeated self-limiting cutaneous lesions in the same innervated region. Following the introduction and use of ACV (discussed below), recurrences could be minimized in the immunocompetent host. However, in the immunocompromised host, as particularly noted with the emergence of AIDS in a growing segment of the population, recurrence of HSV-1 and -2 may cause progressive fulminant lesions that are refractory to repeated antiviral treatment.

Antiviral Drugs

There are two categories of antiviral drugs that are clinically useful against herpesvirus infections. Representatives of the first category are nucleoside analogs such as ACV,

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GCV, and adenine arabinoside (ara-A), prodrugs that require phosphorylation to the respective triphosphates that are the active inhibitors of viral DNA polymerase (DNA pol), with resultant inhibition of viral DNA synthesis. In the second category are direct HSV DNA pol (HSV pol) inhibitors such as phosphonoformate (PFA; foscarnet) and phosphonoacetate. However, for primary and recurrent HSV, only ara-A (vidarabine), ACV (Zovirax), and PFA (Foscovir) are approved therapeutics in the United States.

ACV has by far gained the widest acceptance for clinical use. ACV is approved for topical application as an ointment for initial herpes genitalis and for limited non-life-threatening mucocutaneous herpes in immunocompromised patients; for oral administration in the treatment of primary and recurrent genital herpes and for herpes zoster; and for intravenous administration for mucosal and cutaneous HSV and herpes zoster infections in immunocompromised patients, as well as for HSV central nervous system infections (110). The mechanism of antiviral activity of ACV involves two viral enzymes: one for activation of the drug, and the second as the target for inhibition of virus replication. In infected cells, ACV is selectively converted to ACV triphosphate (ACVTP), the viral DNA pol inhibitor, by phosphorylation to the 5′-monophosphate (ACVMP), using HSV-encoded deoxypyrimidine (thymidine) kinase (TK^+ virus). ACVMP is then progressively converted to the 5′-di- and -triphosphates by cellular kinases. Finally, ACVTP inhibits viral DNA synthesis by acting as an HSV pol substrate analog which is incorporated into the nascent viral DNA chain. The resultant complex of viral polymerase and ACV-altered nascent DNA results in removal of functional HSV pol from further catalytic activity (113). Since the antiviral activity of ACV is dependent on two viral functions, the TK and the HSV pol, resistance may result from either of two mechanisms: failure to phosphorylate ACV to the ACVMP or failure of ACVTP to inhibit HSV pol.

Resistance Due to Altered or Deficient HSV TK

Resistance due to failure to phosphorylate ACV usually is the result of HSV mutants that express little or no functional TK (TK^-). For instance, Palu et al. recently described a series of HSV-2 isolates obtained from an AIDS patient who had received extensive ACV treatment (103a). The isolates were resistant to ACV because of a deficiency in TK production, the consequence of a chain-terminating mutation resulting in synthesis of a truncated TK polypeptide. Alternatively, TK-altered mutants in which the enzyme is capable of phosphorylating thymidine, the usual substrate, but selectively does not phosphorylate ACV, have also been described (13, 29, 42). The existence of these TK-altered mutants suggests that an understanding of the structure of the enzymatic site for TK could lead to the development of more effective antiviral substrates or inhibitors of the enzyme. Unfortunately, only limited genetic analysis has been applied to understanding the structure of the active phosphorylating site of HSV TK. However, on the basis of the phenotypes of TK mutants with defined substrate specificities, a model was proposed in 1986 by Darby et al. (28) for the active center of the enzyme. In this model, three highly conserved regions act in concert. These regions include a nucleotide binding pocket, an ATP-binding site, and a thymidine-binding site. As more mutants are defined from clinical and laboratory isolates and more structure-function studies are performed, a clearer picture of the TK amino acids essential for catalytic activity should emerge (13, 14).

In the immunocompetent host, TK^- and TK-altered mutants have generally not presented a risk, for recurrent lesions have been mainly associated with TK^+ virus. Indeed, ACV-resistant TK-deficient strains have generally been less virulent in animal model neurovirulence experiments. However, in the immunocompromised host, primarily the TK^-, but also the TK-altered, isolates may be associated with progressive disease. Ellis et al. (43) first demonstrated this experimentally by subjecting athymic (immunocompromised) mice to HSV infections. Infections with TK^+ ACV-susceptible HSV-1 resulted in heterologous mixtures of ACV-susceptible and ACV-resistant virus populations that were less responsive to ACV treatment. Herpetic infections that are refractory to ACV treatment have also been observed in the clinic. In a retrospective clinical incidence study of consecutive HSV isolates collected from 207 patients over the course of 1 year prior to 1990, Englund et al. (44) showed that 7 of 148 immunocompromised patients were hosts to ACV-resistant virus, and each of these patients had clinical disease. Five of the seven patients with ACV-resistant virus were bone marrow transplant patients or AIDS patients. By contrast, none of 59 immunocompetent patients yielded ACV-resistant HSV isolates. In more recent studies (116, 119), complete healing in response to ACV in 235 patients with HSV lesions was strongly correlated with the in vitro susceptibility of the isolates to ACV. In addition, the duration of clinical lesions and the absolute $CD4^+$ cell counts for patients were independently correlated with in vitro resistance to ACV. Ljungman et al. (84) described three bone marrow transplant patients who developed severe pneumonia due to ACV-resistant HSV that emerged during the course of ACV treatment. By contrast, Boivin et al. (12) recently showed that, for solid organ transplant patients, prophylaxis with ACV or GCV did not result in emergence of ACV-resistant HSV variants and that HSV-related disease in these patients responded to antiviral therapy without subsequent recurrences. These authors suggest that the relatively lower degree of immunosuppression in these patients compared with AIDS and bone marrow transplant patients, and the relatively high-dose ACV regimen used in prophylaxis, may explain the lack of resistance development.

Neurovirulence and the capacity of TK-deficient HSV-2 isolates from AIDS patients to establish latency have also been demonstrated. Gateley et al. (54) and Erlich et al. (47) have isolated neurovirulent virus from mouse model intracerebral infections. A survey of HSV isolates from patient lesions that failed to respond to ACV therapy revealed that 77 were either partially or totally TK^-, while three were TK altered (63). These clinical isolates, which likely consisted of mixtures of TK variants and wild-type TK^+ viruses, were further evaluated for their capacity to cause experimental mouse cutaneous and intracerebral infections and to reactivate virus from latently infected ganglia. The establishment of infections in the immunocompetent hairless mouse and reactivation from latently infected ganglia appeared similar for the ACV-resistant isolates compared with other TK^+ ACV-susceptible patient isolates. Since the original ACV-resistant isolates were not plaque purified, the ACV-susceptible virus that reactivated from ganglionic latency may have been present in the original isolate mixture. However, for the TK-deficient isolates, the capacity to cause encephalitis following intracerebral inoculation was reduced, but not eliminated. Recently, Sasadeusz and Sacks (121) reported the spontaneous reactivation of a TK-deficient, ACV-resistant HSV-2 isolate from an AIDS patient. Following plaque

purification, the isolate was inoculated into mice and shown to cause acute lesions; this was followed by a latent ganglionic infection which, when reactivated, produced a mixed population of TK^- and TK^+ viruses. Again, these observations suggest that the plaque-"purified" isolate was in reality a mixed population and that the presence of the TK^+ HSV-2 may have aided spontaneous reactivation of the TK^- HSV-2 by in vivo complementation in the patient. These experiences strongly indicate that great care must be taken in strain isolation and characterization. Determining the neurovirulence of TK-deficient HSV isolates, and their capacity to establish and reactivate from latent infections, may be complicated by the residual presence of TK^+ virus in the isolates.

Since the problem of resistance to ACV treatment continues to grow particularly in the AIDS patient population, and since the majority of those ACV-resistant isolates are TK deficient, evaluating alternative therapies has received increasing attention. The ACV-resistant isolates are usually also resistant to GCV but susceptible to ara-A and PFA (116). However, in clinical evaluation, only PFA has been successfully used to treat ACV-resistant lesions (116, 118). PFA treatment of ACV-resistant lesions has resulted in cessation of viral shedding and complete lesion healing. However, recurrent lesions also shed virus with the ACV resistance phenotype, indicating that continued alternative therapy is important (116).

The successful alternative use of PFA for therapy is expected since PFA directly inhibits HSV pol independent of phosphorylation by viral TK. An additional approach to circumventing resistance due to TK-altered virus or TK^- virus is to provide the HSV-infected cell with a prodrug of ACVMP. This approach most recently has been taken by Hostetler et al. (66) by synthesizing and evaluating ACV diphosphate dimyristoylglycerol for its capacity to inhibit ACV-resistant HSV clinical isolates in cell culture. ACV diphosphate dimyristoylglycerol is an analog of the naturally occurring phospholipid cytidine diphosphate diglyceride and is active via a TK-independent metabolism that liberates ACVMP inside the HSV-infected cell. ACV diphosphate dimyristoylglycerol is a potent inhibitor of both TK^- and TK-altered HSV strains and has a very favorable efficacy/toxicity selectivity index in cell culture, suggesting that it may be useful therapy for problematic infections due to HSV strains that do not phosphorylate ACV. Another approach is represented by 2′-nor-cGMP, a cyclic phosphate GCV analog. Field et al. demonstrated that 2′-nor-cGMP is a potent inhibitor of infections by TK-deficient HSV in vitro and in vivo (49a). Subsequently, Germershausen et al. showed that the cyclic 2′-nor-cGMP is opened to the acyclic monophosphate and subsequently converted to the triphosphate in the absence of HSV-TK, thus circumventing the requirement for the viral TK (55).

Resistance Due to Altered HSV DNA Pol

Prior to 1989, drug-resistant mutants of HSV pol had been generated only in the laboratory and used to evaluate possible pathogenicity in animals or the structure-function relationships of domains within the polymerase gene (50). The academic interests in these studies took on very practical aspects with the isolation of clinical ACV-resistant mutants (24, 67, 115). The resistance of one isolate, from an immunocompromised patient, was the result of a single base change at nucleotide residue 2521 of the HSV pol gene, resulting in a predicted amino acid substitution of serine for glycine at residue 841 of the HSV pol. The resistance of a second isolate resulted from a tyrosine-to-histidine conversion at amino acid residue 941. Since HSV pol is the ultimate target for the present clinically approved antiviral drugs ACV and PFA, we can anticipate that in time additional mutants will arise. For this reason alone, it is appropriate to briefly explore our knowledge of the structure and function of HSV pol, as has been revealed from the genetic studies of drug-resistant variants. Furthermore, by producing genetically defined drug-resistant HSV mutants with known resistance phenotypes, new inhibitors with novel modes of action may be more readily identified. A detailed analysis of HSV pol and the other required viral gene products involved in DNA replication can be found in the recent review by Matthews et al. (92).

The HSV-1 and HSV-2 DNA pols are 1,235- and 1,240-amino-acid multifunctional enzymes, respectively. Associated with the large polypeptide are a 5′ to 3′ exonuclease RNase H function, a 3′ to 5′ exonuclease editing function, and a deoxyribonucleotide polymerizing (catalytic) function, probably spanning multiple regions of the polypeptide. HSV pol belongs to a large family of alpha polymerases with six regions having somewhat conserved nucleotide sequences, suggesting that these conserved regions are critical for polymerizing functions (139). The regions are numbered I to VII on the basis of degree of conservation among the DNA pol genes, with region I being the most conserved. Regions I, II, and III are present in DNA pols obtained from bacteriophage through mammals, suggesting that these regions may participate in the most essential catalytic functions. On a linear map of HSV pol, the regions are shown by order from the N terminus of the protein as IV, II, VI, III, I, VII, and V.

As a result of observing the frequency or clustering of drug-resistant mutants within HSV pol, functional regions of the enzyme have been deduced (Fig. 1). Mutations involving alterations in susceptibility to nucleoside analogs such as ACV and GCV should reflect regions of the enzyme that are important to nucleoside triphosphate substrate recognition, while mutations conferring resistance to a pyrophosphate analog such as PFA may involve pyrophosphate recognition. The most systematic studies of HSV pol function, based on mutational analysis, have been done by Coen and colleagues (56, 67, 88, 89) and Matthews and colleagues (61, 91, 92). ACV- and foscarnet-resistant mutants have been mapped primarily to regions I, II, and III and the A region, a relatively nonconserved sequence between IV and II. Marcy et al. (89) described mutants with changes in region I. These mutants were resistant to ACVTP and to phosphonoacetate, a pyrophosphate analog, indicating that this region is probably involved in substrate recognition. Indeed, many of the mutations also in regions II and III represent single amino acid changes, indicating that these regions may also be involved in substrate recognition. Since these regions and others (e.g., regions A and VII) are identified with drug resistance, it appears that interaction of the antiviral drugs with the DNA pol requires folding of the enzyme to provide important drug contact points at noncontiguous regions of the polypeptide.

The knowledge of the specific amino acid changes that alter drug susceptibilities of the HSV pol can also be used to evaluate the potential utility of novel inhibitors against ACV-resistant mutants. Matthews and his colleagues (92) have used this technique by generating previously described single-base-mutated HSV pols (57, 80), resulting in single amino acid changes at sites within regions II and III.

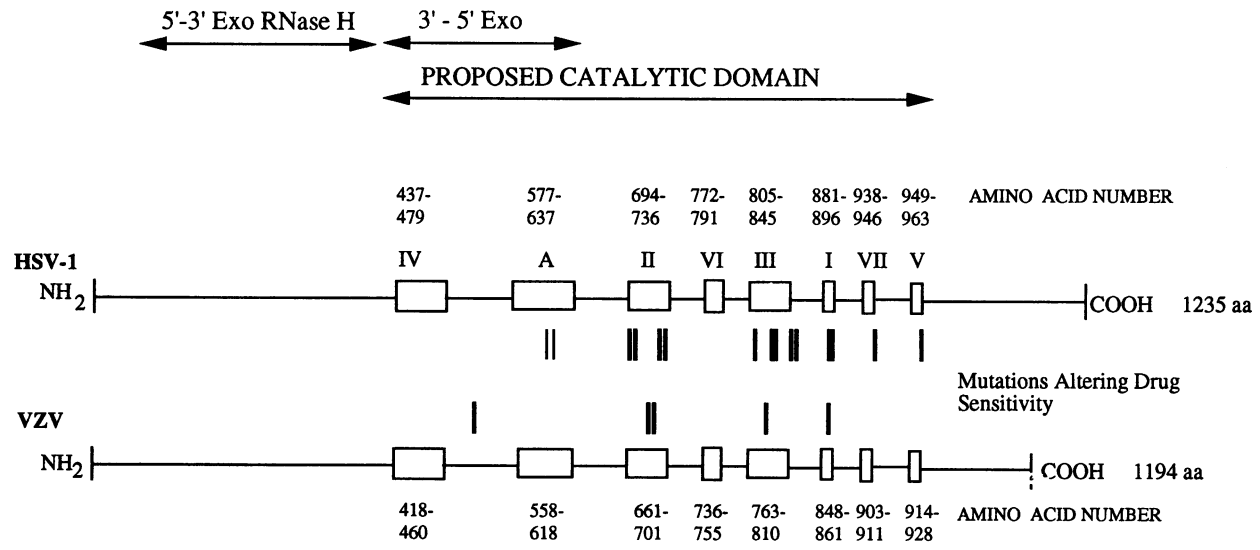

FIG. 1. Linear N-terminus to C-terminus configurations for the HSV-1 and VZV DNA pols. Conserved regions are indicated by boxes I to III and A. Laboratory and clinical drug-resistant mutants are indicated by vertical lines. Amino acid changes and point mutations and their drug susceptibility alterations are indicated in the text and references.

Following cloning and expression of the altered enzymes in yeasts, HSV pol enzymatic activities in the producer cell extracts were shown to display the same susceptibility or resistance phenotypes as those reported previously by Larder et al. (80). Other mutant enzymes with a variety of base substitutions at the same sites were also generated and observed to retain enzymatic activities, indicating that these mutational sites were tolerant of extensive amino acid variation. However, these further amino acid substitutions did result in variations in the degree of resistance or hypersusceptibility to ACVTP and phosphonoacetate, respectively. When they were used to evaluate the novel cyclobutyl nucleoside analog 9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine triphosphate and the nucleotide analog 9-[2-(phosphonomethoxy)-ethyl]guanine diphosphate, all of the altered HSV pols, as well as the wild-type HSV pol, retained identical susceptibilities (92). Thus, screening for novel and effective antiviral compounds can be done at the molecular level by employing an array of genetically engineered HSV pol enzymes with various drug resistance profiles. It is hoped that this method can speed the process of identifying potently active antiviral agents that are potentially useful against HSV strains refractory to conventional drug therapies as the result of alterations in the DNA pol target.

VZV

Varicella-zoster virus (VZV), an alpha herpesvirus, is closely related to HSV on the basis of structural and functional properties of the virus and aspects of infection and persistence in the host. Primary infection with VZV is a systemic disease manifested as varicella, or chicken pox. As with HSV infections, primary infection with VZV is followed by the establishment of latency in the sensory ganglia, with the potential for reactivation to clinical disease. Recurrent disease is usually localized to a given dermatome, resulting in zosteriform lesions (zoster or shingles). In the immunodeficient host, the recurrent disease may rarely disseminate (21), and it was recognized that herpes zoster could be an early clinical sign of the development of AIDS (52).

Antiviral Drugs

VZV infections also respond clinically to the nucleoside analog antiviral agents ara-A and ACV and to the DNA pol inhibitor PFA. ara-A has shown clinical benefit in the treatment of VZV infections in both immune competent (3) and immunocompromised patients (125, 142). However, as is the case for HSV infections, ACV has become the first-line treatment for recurrent infections caused by VZV and for both primary and recurrent disease in immunocompromised patients (4, 141). Recently, ACV has shown clinical benefit in the treatment of chicken pox in otherwise healthy children (141). PFA has shown efficacy in the treatment of ACV-resistant VZV infections in the AIDS population (117). However, the intravenous mode of administration and renal toxicity may limit the clinical utility of this drug. It is worth noting that several candidate VZV inhibitors are now in clinical development: bromovinyl uracil arabinoside, 5-propynyluracil arabinoside, and famciclovir. These compounds are selectively activated by the VZV TK and have little or no activity against HSV. They show advantages in potency and pharmacokinetic properties over ACV and PFA and are in preclinical and clinical development (bromovinyl uracil arabinoside recently was approved in Japan for the treatment of zoster).

VZV Drug Resistance: Clinical Features and Therapeutic Strategies

ACV has been the preferred drug therapy for VZV infections in the clinic, yet because of the difficulties in culturing the virus in the laboratory, virologic studies have not been a part of most clinical trials. The basic mechanisms of VZV resistance to ACV appear similar to those determined for HSV: resistance arises from mutations in either the VZV-encoded TK or DNA pol genes. Currently, there are no data regarding the natural occurrence of TK-deficient, drug-

resistant VZV or the incidence of drug resistance acquired in various patient populations treated with ACV or PFA for VZV infections. The consequences of VZV mutation to drug resistance at either the TK or the DNA pol locus in terms of virulence, pathogenicity, and latency cannot be directly examined in animal models. However, correlation of the mutations that arise in drug-treated patients with the clinical course of disease progression may indirectly provide information on the role of these genes in the disease process.

At the time of the Hirsch and Schooley editorial, which highlighted the clinical problems of drug-resistant HSV and HCMV, there were only two reports correlating the presence of ACV-resistant VZV with persistent or progressive infection (74, 102). One case involved a child congenitally infected with human immunodeficiency virus (HIV), while the other was an adult with HIV. Since then, there have been several additional reports documenting the emergence of drug-resistant VZV, but only in highly immunocompromised patients with AIDS. In this unique population, the marked T-cell depletion leads to severe chronic varicella (58, 75, 108) and to prolonged, recurrent episodes of zoster, often with cutaneous dissemination (22, 60). The lesion characteristics are quite distinct in these patients: varicelliform at onset, subsequently evolving into persistent crusted, necrotic, hyperkeratotic lesions. These lesions can often be coinfected with other opportunistic pathogens, including *Candida* species and *Mycobacterium avium* complex (60, 69, 74, 81). It is this setting, with persistent virus replication in the presence of an antiviral drug as a selective agent, that favors the emergence of drug-resistant virus.

Clinical nonresponsiveness to therapy can be attributed to the presence of drug-resistant virus only with the appropriate laboratory documentation. In the case of VZV, these studies are not readily available, and when available, test results are complicated by the variable passage history of the isolate as well as the method of assay. Accordingly, there are numerous accounts of localized or disseminated VZV disease persisting during ACV therapy in the HIV-infected patient which do not provide supporting virologic studies (1, 2, 33, 76, 81; reviewed by Cohen and Grossman [22]). However, in the absence of such laboratory documentation of resistant virus, physicians make therapeutic decisions on the basis of historical case reports. For this reason, we will briefly review several documented cases of drug resistance that illustrate key points in the chemotherapeutic management of chronic VZV disease and that highlight important features of the persisting virus.

One such case was that of a 27-year-old, HIV-seropositive patient with no previous history of varicella who presented with disseminated necrotic vesiculopustular lesions of 2 months duration (no pretherapy isolates recovered). The patient received intravenous ACV (length of treatment not stated), and VZV recovered from persistent lesions 1 year later appeared to be resistant in vitro (74). In the severely immunosuppressed patient with AIDS, the inability to completely clear virus can result in a persistent infection in which it is difficult to clinically distinguish chronic varicella from chronic disseminated zoster. A second case documenting VZV drug resistance in a prolonged infection exemplifies the clinical circumstances in which drug resistance is likely to emerge and the genetic complexity of the resulting virus isolates. This case described continuous VZV infection in a 4-year-old child congenitally infected with HIV. The progressive cutaneous infection was clearly associated with ACV-resistant VZV (102). The one isolate, recovered after 8 months of ACV therapy, was later shown phenotypically and genetically to consist of a complex mixture of TK-dysfunctional virus, DNA pol-altered virus, virus containing both of these mutations, and wild-type virus (10). The ACV-selected DNA pol mutation in this isolate conferred cross-resistance to PFA. This patient had responded to two initial courses of intravenous ACV therapy, but subsequent recurrences were poorly managed, with low, gradually escalating doses of oral therapy alternating with intravenous therapy. It is likely that inadequate drug dosing, in terms of quantity or duration, permitted the gradual evolution of this genetically heterogeneous isolate. This case illustrates a very important point: the role of suboptimal therapy in the selection of drug-resistant virus in the immunocompromised patient setting. These are the conditions used in the laboratory to select drug-resistant viruses.

The route of dosing may also be crucial in achieving optimal drug levels in plasma for the management of VZV disease. The bioavailability of ACV administered orally is more biologically variable than drug administered intravenously and may be limited in AIDS patients with gastrointestinal dysfunction due to other HIV-associated enteropathies (69). Jacobson et al. (69) described four HIV-infected patients in whom the gradual development of ACV-refractive disease could have resulted from such inadequate oral bioavailability of the drug. All four severely immunocompromised patients developed cutaneous VZV infections that initially responded to intravenous ACV induction therapy followed by oral ACV therapy. However, their disease subsequently recurred and evolved to yield ACV-resistant virus after chronic oral suppressive therapy of 1 to 5 months duration. These patients shed resistant virus from the atypical hyperkeratotic skin lesions that developed late in the chronic infection. Importantly, in only one of these four patients was initial therapy continued beyond the point of resolution of the VZV infection. This emphasizes the importance of timely, aggressive intervention to reduce the likelihood of the establishment of chronic persistent disease in AIDS patients. It also may be that monotherapy in the severely T-cell-depressed host cannot completely eliminate the burden of replicating virus, prompting consideration of combining or alternating drug therapeutic strategies for long-term suppression of persistent herpesvirus infections.

Although pretherapy isolates were not available for any of the cases described above, the initial infections were likely due to predominantly drug-susceptible VZV, since these patients responded to their early courses of therapy. Indeed, the isolation of ACV-resistant VZV in the non-HIV immunocompromised patient is a rare event (3). The gradual progression of susceptible virus to resistance was carefully documented in one patient (65, 82). Susceptible virus was present before and after 2 months of ACV therapy, but ACV-resistant virus was recovered after 5 months (total) of therapy. Both susceptible and resistant isolates were recovered from the verrucose chronic lesions, suggesting that the atypical presentation of the lesions may be more directly related to the chronic nature of the infection than to the presence of drug-resistant virus per se. This would agree with the observations of Disler and Dover (33) that such atypical lesions in their patient would respond to each course of ACV but drug cessation would again result in lesion reemergence.

The biochemical basis for ACV resistance in the five patients described by Jacobson et al. (69) and Linnemann et al. (82) was TK deficiency of 50 to 100% of the isolate subpopulations. All of these isolates were susceptible in vitro to concentrations of PFA achievable in plasma, and

those reported by Jacobson and colleagues were shown to be susceptible to ara-A in vitro. One of the patients in the Jacobson study and the child described by Pahwa et al. (102) were treated with ara-A without beneficial results, reflecting potency and pharmacokinetic limitations of this drug and the magnitude of the virus load. However, PFA was subsequently used to successfully resolve ACV-resistant chronic VZV infections in five of six HIV-infected patients treated (117, 127). The five patients in the Safrin study harbored 95 to 100% TK^- (four patients) or TK-altered (one patient) VZV. Resistance to ACV had developed within 4- and 12-week intervals during ACV therapy in two patients. Only one of these patients (with TK^- virus) failed to respond to PFA. In one of the responders, two serial VZV isolates recovered after 7 and 10 days of therapy had reduced susceptibility to PFA in vitro. Despite this, the patient healed with no recurrences. This same patient had again shed ACV-susceptible virus within 1 month after the ACV therapy was discontinued and before the PFA therapy was initiated. Two other patients again shed ACV-susceptible virus at the original lesion sites 7 and 14 days after discontinuing PFA treatment. One of these patients healed in response to intravenous ACV, while the other showed no response to 12 days of oral ACV therapy. These cases illustrate that the drug susceptibility phenotypes of VZV may rapidly change during or after antiviral therapy. The apparent instability of the TK-deficient phenotype provides a rationale for alternating therapies with PFA and the TK-dependent nucleoside analogs.

It is clear from the above discussion that in the patient with AIDS the management of frequently recurring, persistent, or chronic VZV infections requires careful clinical and laboratory monitoring coupled with appropriate strategies of alternating or combining therapies, perhaps interspersed with intervals of therapy discontinuation. The relevance of drug-resistant virus to clinical outcome will await carefully controlled clinical studies. Likewise, the impact of the drug-resistant variants on the disease process in the individual patient and in the community remains to be determined. It is encouraging that the majority of the cases of chronic VZV infection involve locally progressive disease; visceral dissemination is rare. These features may point to the impaired pathogenesis of TK variants of VZV, like those of HSV. An examination of the nature of the TK gene mutations in these drug-resistant variants may yield information on the role of this enzyme in pathogenesis and in the herpesvirus phenomenon of latency and reactivation.

VZV TK Mutations Conferring Drug Resistance

The VZV-encoded TK is a 35-kDa polypeptide that possesses both thymidine and thymidylate kinase activities. While only the initial phosphorylation of ACV is carried out by this enzyme, both viral enzyme functions are required for the intracellular activation of bromovinyl deoxyuridine and the 5-substituted arabinofuranosyl analogs of deoxyuridine (bromovinyl uracil arabinoside and 5-propynyluracil arabinoside).

Laboratory strains of VZV, like those of HSV, have generally acquired drug resistance through mutations that resulted in the loss of functional TK production (8, 79, 96, 122). Nucleotide deletions within the TK coding sequence have also been observed, and these generally cause frameshifts and premature termination of polypeptide synthesis, also resulting in the TK-negative phenotype (79). Just as with HSV, VZV drug resistance has also occurred due to mutations that alter the affinities of the enzyme for nucleoside analog substrates, with concomitant changes in the affinity for the normal deoxypyrimidine substrates (126). The distribution of laboratory TK mutant phenotypes is similar to that observed for HSV: the TK-deficient phenotype predominates.

The types of TK genetic lesions that arise in the clinic are similar to those described for HSV and laboratory-derived VZV drug-resistant mutants. However, there are not sufficient data to determine the relative frequency of the TK-deficient versus the TK-altered substrate phenotype. Correlation of the VZV TK gene sequences with enzyme function of 13 ACV-resistant strains purified from 10 different patients indicated that 4 of these strains were altered-substrate variants (114, 133). One of these encoded an amino acid substitution in the ATP-binding site of the enzyme, while another encoded a substitution between the ATP-binding site and the nucleoside substrate-binding site. The remaining two altered-substrate mutants had single base changes in the regions encoding the nucleoside-binding sites. Two additional mutants with single amino acid substitutions in this region were severely crippled in enzyme function, and five other ACV-resistant strains contained nucleotide deletions of 1 to 4 bases in their TK genes, encoding nonfunctional, truncated polypeptides. In terms of the cross-resistance patterns of these ACV-resistant variants, three of the altered-substrate variants retained various degrees of susceptibility to bromovinyl uracil arabinoside. The in vitro susceptibility of TK-altered VZV strains to penciclovir paralleled that of ACV more closely.

Resistance Due to Altered VZV DNA Pol

Mutations in the VZV DNA pol have been derived in the laboratory and recently have been recovered in the clinic. Similar to HSV, in VZV the mutations responsible for drug resistance consist of single base changes in the pol gene, which would result in single amino acid substitutions in conserved regions implicated in enzyme function (Fig. 1) (10, 53, 129). Although only six DNA pol variants of drug-resistant VZV have been characterized, four of these showed amino acid substitutions that have also been implicated in the drug resistance phenotype of HSV. Laboratory-generated resistance to ara-A mapped to conserved region II, and phosphonoacetate-selected resistance mapped to the most conserved region I of the polymerases, a region that appears to be relatively intolerant of mutation (89). Of great interest is the observation that two independently derived ACV-resistant laboratory mutants of VZV possess the identical amino acid change (53). This same asparagine-to-serine substitution had been identified previously in drug-resistant HSV DNA pol mutants (61, 80). The cross-resistance patterns of these VZV DNA pol mutants were variable and, in most cases, similar to those of their HSV counterparts. These studies offer evidence for the functional importance of these conserved regions in enzyme function and for the close functional homology between the HSV and VZV enzymes as targets for antiviral inhibitors.

Two DNA pol variants of VZV have been reported in the clinical setting; both were recovered from patients with AIDS. The first variant was recovered in the heterogeneous isolate from the patient described by Pahwa and colleagues in 1989 (102) and which represented approximately 40% of the isolated virus at analyses (10). The mutation apparently consisted of a single base change in conserved region II, one which conferred cross-resistance to PFA. The second vari-

ant was that recovered after several weeks of PFA therapy (117). Sequence comparison with the wild-type pretherapy strain showed one base change that would result in an amino acid change in a small region of shared homology with HSV pol that lies between conserved regions A and IV (129). This region has not been implicated previously in substrate recognition or polymerase folding. The role of this change in the drug resistance phenotype must now be confirmed by genetic transfer. Thus, just as for HSV, the study of these drug-resistant variants in VZV, supplemented by direct mutagenesis, will help define these enzymes for improved drug design. As newer antiviral nucleoside analogs are put into clinical use, we will extend our knowledge of the herpesvirus DNA pol structure and function.

HCMV

HCMV, one of the beta herpesviruses, has evolved different strategies of replication and persistence in the host. Infection of the developing fetus is associated with significant morbidity and mortality (140), whereas primary infection beyond the neonatal period is generally asymptomatic. HCMV infections are a major cause of life-threatening disease in patients with cell-mediated immune defects, particularly bone marrow and solid organ transplant patients, and in patients with AIDS (34, 36, 72, 93, 106). In the AIDS patient, HCMV infection can be manifested as retinitis, gastrointestinal disease, pneumonitis, and, less frequently, encephalitis (72, 136). Retinitis is the most common late-stage manifestation of HCMV infection in patients with AIDS who show severe depression of $CD4^+$ lymphocyte counts (generally <100 per mm^3), attesting to the importance of the cell-mediated immune response in the restriction of HCMV in latency. HCMV also establishes lifelong latency or persistence in the host, but the exact nature of this persistence is still unknown. Reactivation of HCMV in the immunocompromised host proceeds through a viremic phase and can ultimately be manifested as organ disease.

Antiviral Drugs and Resistance in HCMV Diseases

HCMV is susceptible to the nucleoside analogs GCV and ACV, although the potency of ACV against this herpesvirus is considerably less than that against HSV or VZV. ACV has shown benefit in the prophylactic suppression of symptomatic disease in both renal allograft and allogeneic marrow transplant recipients (5, 94), although these effects have not been consistent (40, 59). The strategy of prophylactic intervention with a safe, but modest inhibitor may be facilitated by an oral prodrug of ACV, currently in clinical trials. ACV is not effective for the treatment of established clinical disease (reviewed by Whitley and Gnann [141] and Chou [18]).

GCV has shown clear benefit as a preventive therapy, alone or in combination with immunoglobulin, in transplant recipients (48, 59, 123, 143). GCV has clinical benefit in the treatment of retinitis, gastroenteritis, and liver disease, but the efficacy in pneumonitis has been questionable (93). GCV is presently the drug of choice in the treatment of HCMV retinitis in AIDS patients and can effectively slow or halt the progressive retinal necrosis. However, the disease recurs rapidly when drug treatment is stopped (95, 106). Maintenance therapy is therefore required, setting up conditions that favor the emergence of GCV-resistant virus (35). The situation is complicated further by the presence of multiple strains of HCMV that can reactivate simultaneously or sequentially and that could potentially recombine (17, 38, 46).

PFA is also an effective inhibitor of HCMV in vitro and recently has been approved for the treatment of CMV retinitis in immunocompromised patients (19, 68, 73, 103). PFA appears to be comparable to GCV in controlling retinitis when given as either induction or maintenance therapy (reviewed by Balfour et al. [6]). As with GCV suppression, PFA maintenance therapy must be continuous in patients with advanced AIDS (70, 72). The toxicity profiles and the modes of action are different for these two antiviral agents, which allows strategic flexibility in accommodating the toxicities of adjunct therapies and in dealing with drug-resistant virus infections (39, 71, 78). There is one case report of an HIV-positive patient whose HCMV encephalitis was successfully resolved by the addition of PFA to a regimen of GCV, which was ineffective as monotherapy (109).

It has been difficult to define HCMV drug resistance in vitro, since there is no standardized assay. In published studies, HCMV shows a broad range of susceptibility to GCV, with 50% inhibitory concentration (IC_{50}) values ranging from 0.5 to 8 μM (23, 49, 111, 124, 135). Data have been derived by different assays in various fibroblast cells, and pretherapy isolates are not always available for direct comparisons. Moreover, the isolates may consist of complex mixtures of phenotypes and strains with changing drug susceptibilities. As published for the characterization of both HSV and VZV clinical isolates, the drug IC_{90} to IC_{95} values may provide the most sensitive indicators of the presence of drug-resistant virus. Importantly, the GCV or PFA susceptibilities measured in vitro for resistant viruses may be only 5- to 10-fold higher than those measured for susceptible strains. On the basis of cumulative GCV susceptibility studies performed to date, Drew et al. have proposed 12 μM as an approximate cutoff value for the IC_{50} value of drug-susceptible strains (37). However, because of the variability in assay systems, it is prudent to compare single isolates with several GCV-susceptible reference strains tested simultaneously (107). It is important to review the evidence for GCV resistance in the clinic with these complexities in mind.

Before the Hirsch and Schooley editorial, there was little evidence that drug resistance of HCMV correlated with poor clinical outcome in the transplant setting (23, 124). However, as pointed out by Hirsch and Schooley (64), in the more immunocompromised patient setting, the evidence for GCV resistance has clearly been demonstrated and correlated with the clinical outcome. The report by Erice et al. in 1989 (46), which prompted editorial comment, documented resistance in blood isolates recovered from three severely immunocompromised patients (one with chronic lymphocytic leukemia and two with AIDS). These case reports illustrated the familiar scenario in which early courses of therapy produced some transient clinical benefit but viremia never totally cleared, and the infections relentlessly progressed. Resistant isolates were subsequently recovered after 80 to 130 days of GCV therapy. The GCV IC_{50} values for these resistant isolates were only three- to sevenfold higher than those isolates obtained early in the course of therapy or the reference strain, while the IC_{90} values showed a more convincing elevation of 17- to 43-fold compared with the controls. One of these patients shed two different HCMV strains, on the basis of the DNA restriction enzyme profile, and both were GCV resistant (128). All three patients described in this report harbored a heavy burden of replicating virus, as evidenced by the recovery of multiple isolates from many sites throughout the protracted clinical course.

The recovery of virus with in vitro resistance to GCV was clearly correlated with clinical failure of the drug and disease progression.

Since this initial report documenting GCV-resistant HCMV, others have also reported the recovery of GCV-resistant virus from AIDS patients following prolonged therapy (37, 55a). There have been two reports of AIDS patients who developed HCMV polyradiculomyelitis while receiving GCV for retinitis (30, 41). In vitro, GCV resistance was documented for an isolate recovered from the cerebral spinal fluid of one of these two patients after several months of GCV treatment (41). The GCV IC_{50} value of this isolate was 18.5 μM; there was no mention of earlier isolates for comparison.

The incidence of GCV resistance in AIDS patients who receive GCV maintenance therapy for HCMV retinitis has been estimated at 7.6% by Drew et al. (37). In a prospective study of 72 patients, no resistant strains were identified in 31 patients before GCV therapy or in 7 culture-positive patients treated for less than 3 months. However, 5 of 13 patients who shed virus after more than 3 months of therapy showed evidence of GCV resistance in their isolates (IC_{50}, >12 μM; IC_{90}, >30 μM). Three additional patients shed isolates with intermediate resistance (defined by an IC_{50} of >4 to 12 μM). These isolates could consist of evolving mixtures of wild-type and drug-resistant viruses. IC_{50} values for the susceptible isolates ranged from 0.5 to 3 μM in a plaque reduction assay. However, the progression of HCMV retinitis could not be clearly correlated with the presence of resistant virus in urinary HCMV isolates. The overall response rate was 80% in this study of 72 patients after 1 to 2 months of therapy, and it remained at 80% for survivors on therapy after 7 months. The impact of drug-resistant virus on patient outcome may reflect the management of underlying HIV disease in this patient population. Clearly, the pathogenesis of HCMV is not well understood, and the relationship of viruria and even viremia to virus load and disease in various organs remains to be determined.

GCV Resistance Due to Altered UL97-Encoded Phosphotransferase

The basis for GCV resistance in 10 HCMV strains recovered from eight immunocompromised patients who failed long-term GCV therapy was the inability of these viruses to induce GCV phosphorylation in virus-infected cells (128). These strains included five strains that were plaque purified from resistant isolates recovered from the three patients described by Erice and colleagues (46) and five resistant isolates reported in the prevalence study by Drew et al. (37). By contrast, the available pretherapy GCV-susceptible virus isolates were competent for the induction of GCV anabolism in cells. The DNA pols of these purified strains did not appear to play a role in this GCV resistance on the basis of the overall drug susceptibility profile to other DNA pol inhibitors [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [HPMPC] and PFA]. The DNA pol functions were comparable for the enzymes induced by one clinical pair of GCV-susceptible and GCV-resistant strains.

The phenotype of these GCV-resistant isolates is similar to that of TK-deficient, ACV-resistant HSV or VZV. However, genetic and biochemical studies have shown that HCMV lacks a TK analogous to the HSV or VZV TK. Yet the selective anabolism of GCV in HCMV-infected cells is clearly required for the antiviral action of the drug (9, 11). There is a direct correlation between the in vitro susceptibility of laboratory and clinical HCMV strains to GCV and the intracellular levels of GCV nucleotides (Fig. 2). The mechanism of selective induction of GCV phosphates by HCMV remained a puzzle until the availability of a GCV-resistant laboratory mutant, 759^{r}D100, with the phosphorylation-deficient phenotype (9). This laboratory mutant was used to map the resistance phenotype to the HCMV UL97 open reading frame of the HCMV genome (132). The 78-kDa protein encoded by UL97 shares homology with several protein kinases, guanylyl cyclase, and bacterial aminoglycoside phosphotransferases (16). The GCV resistance mutation in this laboratory-derived strain consisted of a 12-bp deletion that would result in a 4-amino-acid deletion in a region of the polypeptide conserved among protein kinases and which in cyclic AMP-dependent protein kinase plays a role in substrate recognition. Supporting evidence for the direct phosphorylation of GCV by the UL97 gene product was provided by Littler and colleagues (83), who expressed active GCV phosphorylating activity of the UL97 gene in a bacterial system.

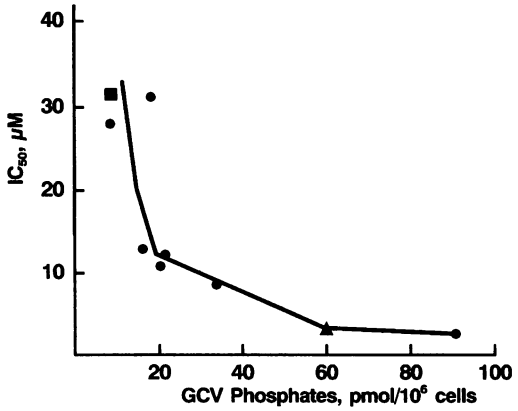

FIG. 2. Susceptibility of HCMV strains to GCV compared with the intracellular anabolism of GCV. Infected and uninfected cells were pulse-labelled on day 4 following infection with 25 μM [^{14}C]GCV for 16 h. Anabolites were measured with a cation-exchange column (128). Susceptibilities of strains to GCV were determined by a DNA hybridization assay (Hybriwix; Diagnostic Hybrids, Inc., Athens, Ohio). Virus strains included the laboratory strain AD169 (▲) and its GCV-resistant laboratory-derived mutant 759rD100 (■). Seven clinical isolates (●) were obtained from L. Drew, Mt. Zion Hospital.

Therefore, the apparently predominant GCV resistance mechanism observed in the clinic is associated with the GCV anabolism-deficient phenotype, which apparently results from mutations in the UL97 gene. The role of this gene in HCMV replication and disease pathogenesis is not known. Some information on the essential nature of this gene product in disease may come from analyses of protein expression in cells infected with the GCV-resistant clinical HCMV strains. Sequence alterations associated with GCV resistance should provide information on the amino acid residues required for functional recognition of this nucleoside analog as a substrate. These sequence studies will also indicate whether it is feasible to quickly assess GCV resistance in clinical virus samples by PCR technology, as has proven so useful in the rapid monitoring of HIV resistance to nucleoside analog reverse transcriptase inhibitors. There are homologs of the HCMV UL97 gene in HSV (UL13), VZV (gene 47), Epstein-Barr virus (EBV; BGLF4), and human herpesvirus 6 (15R), suggesting conservation of function.

Recently, the UL13 gene of HSV-1 was shown to have protein kinase activity, to control the phosphorylation of the regulatory gene alpha 22 (111a), and, by deletion mutagenesis, to play a role in the pathogenesis of HSV infection in a mouse model (25, 98). The UL13 polypeptide immunoprecipitated from virus-infected cells was shown to have protein kinase activity (25). In addition, the homolog of VZV recently has been reported to phosphorylate the assembly protein of VZV (97). However, the full function of these protein kinase homologs in virus replication and infection remains to be determined.

Drug Resistance Due to Altered HCMV DNA Pol

The HCMV DNA pol is the ultimate target for a number of nucleoside analog triphosphates and for PFA (87). In addition to the major mechanism of UL97 mutation, GCV resistance in HCMV can also result from DNA pol mutations (85, 130). One laboratory-derived, GCV-resistant mutant of AD169 possessed both a UL97 mutation (132) and a separate but unrelated mutation in the DNA pol gene. This consisted of a single base change encoding an amino acid change in the highly conserved region V of the DNA pol polypeptide (130). This particular mutation resulted in cross-resistance to candidate HCMV inhibitors [HPMPC and 1-(dihydroxy-2-propoxymethyl)cytosine]. Recently, a clinical strain of HCMV that was recovered from the bronchial brushing of a heart transplant recipient contained a mixture of GCV-susceptible and GCV-resistant viruses. The resistant virus was cross-resistant to PFA and to HPMPC, suggesting that the GCV resistance resulted from a DNA pol alteration (134). Since this GCV-resistant virus was competent for GCV phosphorylation, it is likely that a DNA pol alteration was solely responsible for the drug resistance phenotype.

PFA resistance of HCMV has been described in the laboratory setting (27, 131) and more recently in the clinic (78). The mutations associated with this resistance phenotype were not identified in these cases, nor were the confirmatory genetic experiments performed. However, on the basis of the experience with HSV and VZV, HCMV should be expected to develop resistance to PFA in the clinical setting, with direct PFA selection pressure, or potentially under GCV, ACV, or HPMPC therapy.

EBV

As with other herpesviruses, EBV can cause productive viral infections leading to cell lysis and persistent latent infection. Unlike the other human herpesviruses described above, EBV is associated with a wide array of diseases that reflect pathology resulting either from direct cytolysis enhanced by the host immune response to viral antigens or from the capacity of the latent virus to immortalize cells, resulting in neoplasia. Perhaps because of this clinically diverse pathology, which is still undergoing characterization, antiviral therapy has not had a major role in the treatment of EBV-associated diseases. More detailed discussions of the virology and diseases associated with EBV are presented superbly by Pagano in a recent series of articles (99–101). For the purposes of this review, we will summarize these diseases and the therapeutic approaches. Only meager information is available concerning drug resistance, reflecting the difficulties in readily screening for resistance with the available in vitro systems (101).

Clinical Diseases and Antiviral Therapy

EBV was first discovered by Epstein et al. in 1964 (45) in association with Burkitt's lymphoma in Africa, where EBV infection precedes the development of the B-cell lymphoma (32). The lymphoma results from translocations of the c-*myc* oncogene that result in independent B-cell multiplication. EBV is a triggering factor that is most frequently associated with the African disease, but EBV is not associated with all Burkitt's lymphoma (31). Preferred treatment of Burkitt's lymphoma utilizes cancer chemotherapeutic agents, not antiviral drugs (101). EBV is also the cause of the great majority of cases of infectious mononucleosis, the symptoms of which result from cytolytic virus replication in the oropharynx, immunopathology of other organs, and a proliferation of cytolytic T cells in response to antigens shed from virus-infected cells (101). Although ACV can be effective in halting the replication of EBV in cell culture and has been used for intramuscular treatment, the results have not been encouraging (137), and problems of drug resistance have not emerged. In nasopharyngeal carcinoma, EBV DNA is detected in episomal form and the virus does not replicate in the preneoplastic lesions or the carcinoma cells (112). Nasopharyngeal carcinoma is a neoplasm that has a very high prevalence in Southern China. In the early stages of disease, immunoglobulin A antibodies to EBV replicative antigens appear, suggesting that at this stage of disease cytolytic virus replication may be important and that conventional antiviral agents might be effective (101). To date, antiviral drugs have not been used against nasopharyngeal carcinoma, and effective means to block the expression of transforming genes of episomal EBV are not at hand. The disease pattern described for nasopharyngeal carcinoma seems similar to that now seen in a rising incidence of B-cell tumors in immunocompromised hosts, especially AIDS patients (86). The B-cell lymphomas often carry episomal EBV DNA and are probably the result of EBV transformation. However, there is also viral reactivation resulting in productive infection, leaving open the opportunity for antiviral chemotherapy (77, 101).

The most recent disease associated with EBV is hairy leukoplakia, a disease recognized in AIDS patients and characterized by tongue lesions composed of proliferating and productively infected epithelial cells (120). Hairy leukoplakia responds to ACV treatment, as might be expected since the disease is not associated with latently EBV-infected proliferating cells (62). However, the disease recurs, perhaps as a result of development of resistant virus. Hairy leukoplakia lesions are infected with multiple strains of EBV (138), with the potential for generating recombinants with altered drug susceptibilities. ACV-resistant strains of EBV have not yet been isolated and characterized, but hairy leukoplakia would appear to be the best candidate EBV-associated disease for which antiviral chemotherapy could be effectively applied and for which drug-resistant EBV variants would arise.

CONCLUSIONS

It is clear that since the 1989 editorial by Hirsch and Schooley, antiviral drug resistance has been repeatedly observed in clinical herpesvirus isolates. It is equally clear that as the immunocompromised patient population increases, in large measure because of the inevitable progression of disease in HIV-infected individuals, the need for treatment of herpesvirus infections will also increase and

will be accompanied by the emergence of new drug-resistant virus strains. As Hirsch and Schooley appropriately indicated, "the public health implications of emerging resistance to antiviral agents are considerable" (64). Will we see the spread of drug-resistant strains from patient to patient and from immunocompromised patients to immunocompetent, healthy individuals? Will virus strains emerge that have enhanced virulence? Will the pipeline of new drug development be primed to produce novel therapeutically effective agents against the resistant virus strains? And will the judicious use of drugs in combination and at appropriate intervals effectively keep the resistant strains suppressed and not provide the selective pressure favorable for drug-resistant virus replication? The bad news is that the problem of resistance can be expected to grow, but the good news is that clinicians and virologists are increasingly aware and challenged toward monitoring for resistance, characterizing the mechanisms of resistance, and responding with the most appropriate therapeutic modalities available.

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