Construction of a herpes simplex virus/varicella-zoster virus (HSV/VZV) thymidine kinase recombinant with the pathogenic potential of HSV and a drug sensitivity profile resembling that of VZV

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A recombinant of herpes simplex virus (HSV) was constructed in which the HSV thymidine kinase (TK) gene was deleted and the varicella-zoster virus (VZV) TK gene was introduced into the US5 region under the control of the human cytomegalovirus IE promoter. Infection with the recombinant (R18) led to the induction of TK although the kinetics of synthesis resembled those of a ‘late’ gene product. The recombinant was virulent in the zosteriform mouse model with the pattern of pathogenesis similar to that of wild-type HSV-1. The sensitivity of the recombinant to several nucleoside analogues was assessed and in most cases (BVaraU, ACV and GCV) it resembled VZV rather than HSV. The enhanced sensitivity of the recombinant to BVaraU compared with wild-type HSV resulted in a far greater response to treatment with BVaraU as assessed in the mouse model.

Introduction

The development of antiviral drugs is greatly facilitated if animal models of disease are available. In systems where infection with a human virus leads to disease resembling that seen in humans, assessment of potential clinical efficacy can be made with some confidence. Alternatively, related viruses which naturally infect animal species may be used, providing the animal virus has a drug sensitivity similar to the human virus and produces a similar disease.

There is no ideal small animal model for the human herpesvirus varicella-zoster virus (VZV). Currently, the two most popular models are a guinea-pig model that employs strains of VZV adapted to grow in guinea-pig cells (Myers et al., 1980, 1991) and a model using simian varicella virus in the African green monkey (Soike et al., 1992). Both systems have major limitations. The hairless guinea-pig develops minimal symptoms, and experiments with non-human primates are expensive and the pathogenesis of the infection is quite different from the human disease.

Herpes simplex virus (HSV), another member of the α-herpesvirus subfamily, grows in a number of small animal systems and one model in particular, the ‘zosteriform’ model in the mouse, has several characteristics resembling human zoster. Following infection on the skin, a primary lesion develops and virus invades the peripheral nervous system infecting the local sensory ganglion. From there it spreads back along sensory nerves to the skin where it infects the whole of the dermatome causing an extensive secondary lesion (Simmons & Nash, 1984). It is the latter part of this process, infection of the skin via the sensory nerves from the local sensory ganglion, which appears to parallel closely events which occur in zoster.

Unfortunately, many nucleoside analogues active against VZV are inactive or only weakly active against HSV and so cannot be tested in this system. E-5-bromovinyl-1-β-D-arabinofuranosyluracil (BVaraU; Yokota, 1989) and 5-propynyl-1-β-D-arabinofuranosyluracil (PYaraU, 882C; Rahim et al., 1992) are examples of such compounds. The triphosphates of these analogues are effective inhibitors of the DNA polymerases.
encoded by both HSV-1 and VZV, but the drugs must first be activated to triphosphates. Although the thymidine kinases (TK) encoded by both HSV-1 and VZV are capable of phosphorylating the analogues to their monophosphates, only the VZV enzyme can further phosphorylate them to diphosphates. Since no cellular kinase can perform this function, neither drug is active against HSV-1 (Yokota et al., 1989; Rahim et al., 1992).

The objective of the current work was to create a recombinant of HSV-1 which expresses VZV TK rather than the normal HSV enzyme. It was hoped that the resulting recombinant would resemble HSV in its pathogenesis but exhibit a sensitivity profile to nucleoside analogues similar to that of VZV. Such a variant could then be useful in assessing the treatment potential of nucleoside analogues active against VZV in vitro.

Methods

Viruses and cells. The HSV-1 strains used were SC16 (Hill et al., 1975), and a TK deletion mutant, DM21, derived from it (Efstathiou et al., 1989). The VZV strain G31 (Dumas et al., 1981) was used for comparative purposes.

High titre stocks of HSV-1 were grown in Vero cells using a low multiplicity of infection. Infected cell stocks of VZV were prepared in the human embryonic diploid fibroblast line MRC 5 as described by Bron & Elion (1980). TK+ variants of HSV were selected in the TK-human osteosarcoma cell line 143B, obtained from the American Type Culture Collection.

All cell lines were grown in the Glasgow modification of Eagle’s medium (GMEM, Gibco) supplemented with 2 mM-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. In the case of Vero cells 10% newborn calf serum (Gibco) was added and for other lines 10% fetal calf serum was used.

Antiviral compounds. E-5-bromovinyl-1-β-d-arabinofuranosyluracil (BVaraU) was kindly provided by Dr H. Machida, Yamasa Shoyu Co Ltd, Choshi, Japan. Other compounds [9-(2-hydroxyethyl)guanine (acyclovir, ACV); 9[2-hydroxy-1-(hydroxymethyl)-ethoxy]-methylguanine (ganciclovir, GCV); and 5-propynyl-1-β-d-arabinofuranosyluracil (pYaraU or 882C87)] were synthesized at Wellcome Chemical Works, Dartford, UK.

Isolation of the VZV TK gene. VZV DNA was obtained from infected cells as follows. An infected cell stock (500 μl containing approximately 10^8 p.f.u.) was incubated for 4 h at 37°C in 1 ml of TE buffer (10 mM-Tris–HCl pH 8, 5 mM-EDTA), 0.1% SDS and 1 mg/ml protease K (Boehringer-Mannheim). The DNA was extracted twice with phenol–chloroform and precipitated with ethanol. After centrifugation, the DNA was dried and dissolved in 10 μl of distilled water. The VZV TK gene was amplified by PCR under standard conditions (Saiki et al., 1988). The primers used (5’ GTCCCTTGCCGAGATATAACCGATGTA 3’ and 5’ TCTTCACCTCGCGACAG CGTGCAGG 3’) were based on the published VZV sequence (Davison & Scott, 1986) and were synthesized using an Applied Biosystems 394 DNA Synthesizer. They were designed to introduce blunt Ncol sites (underlined) into both the upstream and downstream regions, immediately preceding the third codon of the original VZV TK gene and following the poly(A) signal.

Plasmid constructs. The 1.15 kb product from the PCR reaction was purified by electrophoresis in a 1% Tris-acetate–EDTA (TAE) gel and ligated into M13mp18 which had been linearized with SmaI. The insert was sequenced by the dideoxy method of Sanger et al., (1977) using the M13 universal primer (Amersham) and a series of 25-mer primers described elsewhere (Lacey et al., 1991). Once it had been confirmed that the sequence of the gene was correct, it was cleaved from the phage with NcoI, purified and ligated with pUC118 linearized pIEP/3FATG. [The latter plasmid was derived from pMV10 (Forrester et al., 1992) initially by insertion of a synthetic polynucleotide (GGATCCGGGTACCCATATGAGATCC BamHI, Smal, KpnI, EcoRV, XbaI) into the unique BamHI site located downstream of the human cytomegalovirus immediate early (HCMV IE) promoter. Recombination led to incorporation of the TK gene into US5.]
Infectious virus titres were determined at each stage and the proportion of TK- virus estimated by [3H]thymidine autoradiography on 143 TK- cells as described by Tenser et al. (1983).

After three passages, infected cell DNA was prepared and analysed for the presence of the insert and Southern blot analysis was performed to determine the approximate proportions of wild-type and recombinant viruses (data not shown). The recombinant was then subjected to three rounds of plaque purification in Vero cells and the clonal isolate (R18) was then further characterized.

Southern blot analysis of the genome of HSV-1 R18. A working stock of the recombinant was grown in Vero cells infected at low multiplicity and this was then used to prepare infected cell DNA for Southern blot analysis. In addition, high molecular mass DNA was prepared from SC16 and DM21 for use as controls. Samples of DNA (10 μg) were digested with BamHI and the fragments were separated on a 0.8% agarose TAE gel and transferred to nitrocellulose (Schleicher and Schuell). The blots were probed with purified radiolabelled DNA fragments using Southern analysis. In addition, high molecular mass DNA was prepared from Vero cells infected with HSV-1 strain SC16, the recombinant R18, or DM21 as a negative control. At appropriate times post-infection, cells were harvested and TK activities were measured as described previously (Bevilacqua et al., 1991).

Immunoprecipitation. To prepare samples for immunoprecipitation, confluent monolayers of 143 TK- cells were infected with 10 p.f.u. per cell of HSV-1 strain SC16, the recombinant R18, or DM21 as a negative control. At appropriate times post-infection, cells were harvested and TK activities were measured as described previously (Bevilacqua et al., 1991).

HSV/VZV TK recombinant

Results

Construction of an HSV-1 recombinant expressing VZV TK under the control of the HCMV IE-1 promoter

Vero cells were co-transfected with DNA derived from cells infected with the TK deletion mutant of HSV-1, DM21, and with the plasmid, pUS5/VZV TK, which contains the VZV TK gene under the control of the HCMV IE-1 promoter inserted into the US5 coding region of HSV-1 in the same orientation as US5 (Fig. 2a). The progeny from this co-transfection were expected to include recombinants carrying the VZV TK gene under the control of the HCMV IE-1 promoter integrated into the US5 coding region. In order to select such recombinants from the population the virus yield was passaged in 143 TK- cells in the presence of methotrexate. After two passages the proportion of TK+ virus in the population as judged by plaque autoradiography had increased from approximately 1% to 95%. Three further rounds of plaque purification were performed and a TK+ recombinant, R18, was selected for further study.

Initially, a restriction analysis of the recombinant genome was performed using Southern blotting to identify relevant fragments. DNA from Vero cells infected with R18 was digested with BamHI and the DNA fragments were separated on agarose gels and transferred to nitrocellulose filters. The blots were then probed with the appropriate 32P-labelled fragments (probes 1 and 2; Fig. 2a). The controls in these experiments were DNA derived from Vero cells infected with HSV-1 strain SC16, and the linearized plasmids pUS5 and pUS5/VZV TK.

As predicted, using probe 1 which contains US5 sequences, a band corresponding to the 6.6 kb BamHI fragment encompassing the intact HSV-1 US5 gene was detected in the digest of wild-type DNA (Fig. 2b). However, a similar band was not detected in the digest of R18 DNA, but instead two smaller bands of approximately 2 and 5 kb were detected. This is the pattern expected if the IEP/VZV cassette is integrated into the US5 gene in the same orientation as US5, since this

The 'zosteriform' model. BALB/c mice were infected with the appropriate viral strain by scarification according to the procedure described previously by Simmons & Nash (1984). Mice were infected with 5 x 10^6 p.f.u. and at various times after infection animals were killed, samples of skin from the primary and secondary inoculation sites were removed and virus was titrated. Where required drugs were administered subcutaneously twice daily using an appropriate concentration in a volume of 0.1 ml.

Latency and reactivation. Female BALB/c mice were infected with 10^6 p.f.u. of R18. Three weeks after infection sensory dorsal root ganglia were removed, cultured in GMEM containing 2% newborn calf serum for 1 week, and then homogenized and virus titrated (Simmons & Nash, 1984; Speck & Simmons, 1991).
would interrupt the US5 sequence and introduce additional BamHI sites. To confirm this interpretation the blots were analysed using probe 2 which contained both HCMV IE promoter and VZV TK sequences. As expected, this probe detected the 2 kb fragment identified using probe 1, but it also detected a 1 kb fragment corresponding to VZV TK sequences. Thus it appeared from this analysis that the recombinant R18 had acquired the VZV coding sequences and the HCMV promoter inserted into the US5 region.

**Growth characteristics of the recombinant R18**

Previous work had shown that the growth characteristics of SC16 and DM21 were indistinguishable under single-step growth conditions (Efstathiou et al., 1989).
Similarly, the growth characteristics of the recombinant R18 in Vero cells under single-step conditions at an m.o.i. of 5 p.f.u./cell were indistinguishable from those of the wild-type virus (data not shown).

**Kinetics of TK expression**

We next tested the ability of the recombinant to express TK. 143 TK− cells were infected with either wild-type virus, SC16, the TK deletion mutant, DM21, or the recombinant, R18, at a multiplicity of 10 p.f.u./cell and cell extracts were assayed at various times for TK activity (Fig. 3a). No induction of TK was seen with DM21 but similar high levels of activity were seen with both the wild-type virus and the recombinant, R18. However, in the case of the recombinant, induction appeared to be significantly delayed (approximately 5 h), and there was no evidence of the down regulation of TK synthesis observed with wild-type HSV at late times.

Further evidence that the TK observed in R18 infected cells was derived from VZV was obtained using immunoprecipitation. Vero cells infected with wild-type HSV-1 or the recombinant R18 were labelled with [35S]methionine and cell extracts were prepared at various times. Immunoprecipitation was carried out using polyclonal anti-VZV TK serum, the products were separated by PAGE and detected by autoradiography. The results are shown in Fig. 3(b). A specific product of the expected molecular mass was detected at 4 h post-infection. Increased amounts were detected at 8 h and there appeared to be a further increase at 24 h. The major product was accompanied by a minor band of slightly lower molecular mass possibly resulting from internal initiation (Haar et al., 1985). It thus appeared that the recombinant, R18, expressed active VZV thymidine kinase, but with delayed kinetics similar to those expected for a ‘late’ protein.

**Virulence and pathogenesis**

It was hoped that replacement of HSV TK with the VZV TK gene would not alter the in vivo growth characteristics of the virus. However, it was possible that the altered kinetics of synthesis of VZV TK could modulate in vivo behaviour. To test this, the growth of the recombinant was investigated in the mouse ‘zosteriform’ model again using wild-type HSV-1 (strain SC16) and the TK deletion mutant DM21 as controls.

In the case of wild-type HSV, small lesions developed at the primary sites of inoculation of the skin, and later, large secondary lesions developed in bands around the flanks of the animals. In contrast, infection with the TK deletion mutant, DM21, was entirely restricted to the primary site. Infection with the recombinant, R18,
resembled that of wild-type HSV in that extensive secondary lesions developed. The virus titres in primary and secondary sites (Fig. 4) paralleled the clinical signs. Significant virus titres at both primary (Fig. 4a) and secondary (Fig. 4b) sites were detected with wild-type virus and R18, but DM21 infection was detected only at the primary site. Thus the data suggest that the recombinant virus is unimpaired in its ability to invade the peripheral nervous system and to generate secondary lesions. One apparent difference between R18 and wild-type HSV-1 was its decreased virulence. Animals infected with R18 survived the acute infection whereas all wild-type infected animals suffered severe symptoms and had to be killed on day 6. This phenomenon was not investigated further.

**R18 reactivates from latent infection**

Previous work had shown that the TK deletion mutant, DM21, cannot reactivate from latently infected peripheral ganglia in the mouse, and suggested that a functional TK gene is required for reactivation (Efstatthiou et al., 1989). We therefore investigated the ability of the recombinant to establish latency and reactivate. Five mice were infected with 10^5 p.f.u. of R18 and sensory dorsal root ganglia were removed 3 weeks post-infection as described by Speck & Simmons (1991). The ganglia were cultured and the virus yield was titrated in Vero cells. Virus was recovered from the ganglia of all five mice, thus demonstrating the ability of R18 to reactivate from a latent state in the ganglia.

**Sensitivity to antiviral compounds**

One of the major aims of this work was to produce an HSV recombinant with a sensitivity profile to nucleoside analogue inhibitors similar to that of VZV. The sensitivity of R18 to a series of anti-herpesvirus nucleoside analogues was therefore investigated. The analogues chosen were: BVaraU and PYaraU, potent inhibitors of VZV but significantly weaker inhibitors of HSV-1; and ACV and GCV, potent inhibitors of HSV-1, but less
Table 1. Drug sensitivities of HSV-1, VZV and recombinant R18

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<th>HSV-1</th>
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<td>ED₅₀ (µM)</td>
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<td>BVaraU</td>
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<td>PYaraU</td>
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A recombinant virus (R18) was constructed in which the VZV TK gene under the control of the potent HCMV IE promoter was introduced into the non-essential glycoprotein gene US5 (Weber et al., 1987) in the HSV-1 background of the TK deletion mutant SC16 DM21. This strategy was employed since direct replacement of the HSV TK gene by the VZV homologue would have disrupted the UL24 gene which overlaps HSV TK (Jacobsen et al., 1989). Furthermore, the product of the US5 gene (gJ) appears to have no role in pathogenesis in the mouse (Balan et al., 1994) and so was felt to be an appropriate insertion site.

The structure of the recombinant was confirmed by Southern blot analysis and its growth characteristics in culture were shown to be similar to those of wild-type HSV-1. Furthermore, it was shown that infection with the recombinant resulted in the expression of VZV TK, levels of activity late in infection being similar to those seen in cells infected with HSV-1. However, although the TK gene was under the control of the strong, constitutive HCMV IE promoter, synthesis of the product appeared to be delayed by about 5 h compared to authentic HSV TK and there appeared to be no down regulation of TK expression at late times. Although the explanation is unclear, one possibility is that expression of VZV TK in R18 is influenced by the local environment of the gene. Other groups have reported that the site of integration can influence the kinetics of expression of a gene irrespective of the nature of the promoter associated with it (Roemer et al., 1991; Goodart et al., 1992). Unlike authentic TK which is located in UL, the TK in R18 is inserted into the U₅ region in US5, a ‘late’ glycoprotein gene.

A concern was that the altered kinetics of synthesis of TK might modulate the in vivo characteristics of the virus. Our main interest was in the ability of the virus, after infection at the primary site, to invade the peripheral nervous system and return to the skin via retrograde axonal spread to infect the whole of the dermatome and to establish a secondary lesion. The development of this secondary lesion mimics quite closely the eruption of the zosteriform rash in patients suffering from herpes zoster.

In fact, the recombinant appeared to be unimpaired in its ability to infect both primary and secondary sites in the mouse zosteriform model. Furthermore, additional evidence for the effective functional replacement of the HSV TK gene by VZV TK and the need for TK in reactivation was the recovery of the recombinant from latent infections in sensory ganglia, a property lacking in the parental TK⁻ variant DM21 (Efstatheiu et al., 1989). Although in the zosteriform model the pattern of pathogenesis with the recombinant was similar to that with HSV-1, virulence appeared impaired. No mice
infected with the wild-type virus survived beyond day 5 whereas all animals infected with a similar dose of the recombinant survived the acute infection. This difference in virulence could be due to the altered kinetics of synthesis of TK, but an alternative explanation is that disruption of US5 itself attenuates the virus. This possibility has not been investigated further.

Having established that the recombinant could be used in the zosteriform model, we next assessed its drug sensitivity profile. Four nucleoside analogues were chosen which differed markedly in their relative efficacies against HSV-1 and VZV. Two of the compounds (BVaraU and PYaraU) are potent inhibitors of VZV with efficacy against HSV-1 several orders of magnitude lower. The others (ACV and GCV) are significantly more potent against HSV-1 than against VZV.

For three of the inhibitors tested (BVaraU, ACV and GCV) the sensitivity profile of the recombinant resembled that of VZV. In the case of the fourth compound, PYaraU, the sensitivity of the recombinant was intermediate between that of HSV-1 and VZV. The target for all of these drugs is the virus DNA polymerase and so a possible explanation for the reduced sensitivity of the recombinant to PYaraU relative to VZV could have been a difference in the relative sensitivities of the HSV and VZV polymerases. However, in vitro experiments have suggested a similar sensitivity of the two polymerases to inhibition by PYaraU triphosphate (Purifoy et al., 1993). An alternative explanation is that sensitivity is influenced by the altered kinetics of synthesis of TK, since relatively small amounts of enzyme are present prior to DNA synthesis when drug activation is required. This then raises the question as to why the recombinant is sensitive to BVaraU which also requires activation. The explanation to this apparent paradox may lie in the relative efficiencies with which TK handles the different substrates. It is far more efficient at phosphorylating BVaraU than PYaraU and so the low concentrations present prior to DNA synthesis may be sufficient to activate BVaraU but insufficient to activate PYaraU (Roberts et al., 1993).

Whatever the reason for the differences in sensitivity, BVaraU was clearly a good candidate for testing in the zosteriform model with the recombinant since the surrogate virus (R18) had the same sensitivity to the drug as the human virus of interest (VZV).

Infection with the recombinant R18 responded dramatically to treatment with both high and low dose BVaraU. Importantly, zosteriform spread of the virus was blocked completely and no virus was detected at the secondary site. In contrast, high dose BVaraU had no effect on the secondary spread of the HSV-1 parent, SC16.

These results suggest that this surrogate virus model could be useful for assessing the in vivo potential of anti-VZV nucleoside analogues. A limitation is the lack of sensitivity of the recombinant to all anti-VZV compounds illustrated by its relatively poor sensitivity to PYaraU. This situation could possibly be improved if the kinetics of expression of VZV TK by the recombinant were more similar to the kinetics of the authentic HSV-1 enzyme. Attempts are now underway to construct recombinants with these characteristics.

References


kinase gene is important for viral growth in cell culture. *Journal of Virology* 63, 1839–1843.


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