Restoration of Wild-type Pathogenicity to an Attenuated DNA Polymerase Mutant of Herpes Simplex Virus Type 1

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(Accepted 10 July 1986)

SUMMARY

The drug-resistant variant, RSC-26, which was derived from the herpes simplex virus type 1 wild-type strain SC16, expresses an altered DNA polymerase and has reduced pathogenicity in animal models. To determine whether the attenuation in pathogenicity was due solely to mutation in the polymerase gene, a fragment of the wild-type gene was cloned, transferred into the genome of RSC-26 and recombinants were isolated. Three recombinants examined had similar properties to wild-type virus with respect to their sensitivity to antiviral drugs, DNA polymerase activities and their pathogenicity for mice. These results strongly suggest that expression of the altered polymerase of RSC-26 results in attenuated pathogenicity.

There has been considerable interest recently in attempting to define those genes in herpes simplex virus (HSV) which influence the pathogenicity or virulence of the virus (e.g. Thompson et al., 1983, 1985; Kumel et al., 1985; Sears et al., 1985; Stanberry et al., 1985). Any alteration in an essential virus gene which impairs virus multiplication would also affect the performance of the virus in vivo, and so all essential genes could be considered 'virulence' genes. In addition, it is now clear that the virus possesses genes whose expression in culture is not required for multiplication but those same genes may have important roles in vivo. For example, the thymidine kinase (TK) gene has long been known to be inessential for replication in actively growing tissue culture cells, but in contrast failure to express the gene in vivo results in a dramatic reduction in neurovirulence (Field & Wildy, 1978; Field & Darby, 1980; Tenser et al., 1981). In this paper, we demonstrate that a virus may acquire a lesion in an essential gene which results in a marked reduction in pathogenicity, while having no significant effect on multiplication in culture. The implication of this observation is that there may be more stringent demands on gene function in vivo than there are in tissue culture systems, and that at least some of the variation in pathogenicity that we observe between different wild-type (wt) strains might be due to subtle differences in essential gene functions.

We have described previously the properties of the variant RSC-26 which was derived from the HSV type 1 (HSV-1) strain SC16 (Darby et al., 1984). Briefly, RSC-26 carries a mutant DNA polymerase gene which confers resistance to ACV [acyclovir, or 9-(2-hydroxyethoxymethyl)guanine] and phosphonoacetic acid (PAA), and hypersensitivity to aphidicolin. Examination of preparations of highly purified RSC-26 DNA polymerase confirmed that the enzyme itself exhibited decreased sensitivity to PAA and ACV-triphosphate (ACV-TP) (Darby et al., 1984).

The peculiar feature of this virus is its reduced pathogenicity for mice, a property which could not be explained by reduced growth potential in culture or by a temperature-sensitive phenotype (Darby et al., 1984). In fact, one-step growth curves in both BHK and L cells were very similar for both the wt virus and RSC-26 (Fig. 1 and Darby et al., 1984). There were, however, two other
possible explanations for the observed reduction in pathogenicity; either the lesion in DNA polymerase was having an effect on virus growth in vivo but not in vitro, or the virus had acquired fortuitously a lesion (or lesions) in other genes which modified the pathogenicity. The latter explanation could not be discounted since the virus had been derived by a complex process including extensive passage in the presence of ACV and also recombination between a double mutant and the wild-type virus (Field et al., 1980; Darby et al., 1984). However, we have recently observed that a number of TK + DNA polymerase virus mutants, selected for resistance to ACV, tended to show reduced pathogenicity in a mouse model system (Larder & Darby, 1985), and this prompted us to ask whether the lesion in the DNA polymerase gene in RSC-26 could account for its reduced pathogenicity. The approach adopted was to restore the wt polymerase phenotype by marker rescue using a cloned fragment of the polymerase gene, and to assess the effect of this on the pathogenic phenotype of the virus. The recombinants expressing wt polymerase could be isolated by taking advantage of the aphidicolin hypersensitivity of the parent mutant RSC-26. Clearly, in any recombinant all genes apart from polymerase would be derived from RSC-26 and so if any were affecting the pathogenicity of the virus we would not expect to restore full pathogenic potential.

Initially, the BamHI r fragment of SC16 was cloned into the plasmid pBR322 at the unique BamHI site. This fragment contains about 87% of the DNA polymerase coding region and no other known coding sequences (Gibbs et al., 1985; Quinn & McGeoch, 1985) (Fig. 2). The recombinant plasmid was mixed with RSC-26 genomic DNA and co-transfected into baby hamster kidney cells (BHK-21) by the calcium phosphate precipitation method (Graham & Van der Eb, 1973; Stow & Wilkie, 1976). In order to enrich for recombinants expressing wt DNA polymerase the yield from the co-transfection experiment was passaged once in the presence of 0.3 μM-aphidicolin. Single plaque isolates were obtained from this population and screened for sensitivity to PAA since this could clearly distinguish the sensitive wt from the PAA-resistant RSC-26. Of 20 clones isolated, six were sensitive to PAA, suggesting a wt polymerase phenotype and three were chosen for further investigation, 26R7, 26R8 and 26R13.

The objective of the first series of experiments was to investigate the phenotypes of the recombinants in more detail, particularly with respect to DNA polymerase but also with respect to TK. The sensitivity of the recombinants to inhibition by ACV, PAA and aphidicolin was
Fig. 2. SC16 BamHI r fragment cloned into pBR322. The r fragment extends from map units 0.413 to 0.434, including 87% of the DNA polymerase gene coding region. SC16 BamHI r was purified from BamHI-digested SC16 DNA and ligated into pBR322 cleaved with BamHI to create pDP(SC16), the insert running anticlockwise. HSV DNA sequences are represented in the plasmid as heavy block. Restriction endonuclease recognition sites shown are: B, BamHI; Bg, BglII; K, KpnI; E, EcoRI; P, PstI. Amp', Ampicillin resistance gene.

Table 1. Properties of SC16, RSC-26 and viruses derived by marker transfer using SC16 BamHI r fragment

<table>
<thead>
<tr>
<th></th>
<th>ED50 (μM)*</th>
<th>TK activity†</th>
<th>ID50 (μM)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACV</td>
<td>PAA</td>
<td>Aphidicolin</td>
</tr>
<tr>
<td>SC16</td>
<td>0.22</td>
<td>88</td>
<td>0.62</td>
</tr>
<tr>
<td>RSC-26</td>
<td>2.22</td>
<td>730</td>
<td>0.03</td>
</tr>
<tr>
<td>26R7</td>
<td>0.13</td>
<td>124</td>
<td>1.23</td>
</tr>
<tr>
<td>26R8</td>
<td>0.13</td>
<td>117</td>
<td>0.92</td>
</tr>
<tr>
<td>26R13</td>
<td>0.18</td>
<td>73</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* ED50 values were obtained from plaque reduction assays in BHK cells as described previously (Darby et al., 1980).
† Extracts for TK and DNA polymerase assays were prepared from TK− BHK cells (BU-BHK) as described previously (Larder & Darby, 1982). TK activity was measured using the method of Klemperer et al. (1967), with [14C]thymidine (Amersham) as substrate at 30 μM and 1.6 μCi/ml. Uninfected cell controls gave <0.2% TK activity compared to SC16-infected extracts.
‡ DNA polymerase activity was determined with [3H]dGTP (Amersham) in the assay mixture at 1 μM and 11.7 μCi/ml and 'activated' calf thymus DNA as template, as described previously (Larder et al., 1983).

assessed by plaque reduction assay and compared with both wt and RSC-26, and then crude extracts were prepared from infected cells 18 h post-infection to investigate directly the DNA polymerase and TK activities. The levels of inhibition of the polymerase activities by ACV-TP and PAA were compared with similar parameters for wt enzyme and RSC-26 preparations and finally the level of TK activity in all extracts was measured. The results of these experiments are collated in Table 1 and they demonstrate clearly that by these criteria all three recombinants resembled the wt virus and were thus likely to have been derived by recombination between the small wt polymerase fragment and the RSC-26 genome. It is possible that one or more may have been derived by reversion to the wt phenotype without recombination but this would seem
E50

I

I

40

x

g 30

20

.9

10 14

0

Time after inoculation (days)

Fig. 3. Pathogenicity of recombinant HSV strains. (a) Groups of 10 female 6- to 7-week-old BALB/c mice were inoculated in the left flank with $5 \times 10^6$ p.f.u. of virus and mortality was recorded over the next 14 days. Cumulative mortality is plotted as the percentage of mice surviving against the number of days post-inoculation. (b) Groups of 10 female 5-week-old BALB/c mice were inoculated in the left ear pinna with $10^5$ p.f.u. of virus, and the difference in ear swelling between the inoculated and uninfected ears was recorded over the next 6 days. ●, SC16; ○, RSC-26; △, 26R7; ▲, 26R8; □, 26R13.

unlikely since in a control transfection where no wt fragment was added all 20 cloned isolates investigated had the phenotype of RSC-26 (being PAA-resistant). In any case, wt polymerase revertants could potentially answer the question as well as wt recombinants and so this was not considered a serious problem.

Our next objective was to investigate the pathogenicity of the recombinants in the mouse and for these experiments we employed two different model systems, either flank inoculation (Simmons & Nash, 1984) or ear inoculation (Nash et al., 1980).

Six-week-old BALB/c mice (groups of 10) were inoculated in the flank with similar doses of either wt virus, RSC-26, 26R7, 26R8 or 26R13. The dose was chosen so that the wt virus killed all mice within 2 weeks. The cumulative mortality curves are shown in Fig. 3(a) and they demonstrate clearly the difference between RSC-26 and wt, with only 50% mortality in the RSC-26 group and 100% mortality in the wt group. Two recombinants were almost identical to wt both in terms of the overall mortality (100%) and in terms of the rate with which they killed the animals. The third recombinant, 26R13, killed 90% of the mice and so it too appeared to resemble the wt rather than the parent, RSC-26.

In the flank model it is assumed that mortality is due to invasion of the central nervous system and so is a marker of neurovirulence. To assess the ability of the recombinants to grow at a peripheral site in the skin, an ear inoculation model was used and ear swelling was measured as a marker of virus replication (Nash et al., 1980). Again similar doses of all five viruses were used ($10^5$ p.f.u. into the left ear pinna). The results (Fig. 3b) showed once more that the recombinants had a wt phenotype which could readily be distinguished from the phenotype of RSC-26. These data were entirely consistent with earlier experiments using RSC-26 and SC16 in the mouse ear inoculation model (Darby et al., 1984).

Thus, we have been able to establish that the expression of an altered DNA polymerase was responsible for the attenuated pathogenicity observed in the HSV-1 SC16 variant RSC-26. This approach (the restoration of the wt phenotype by marker rescue with a wt DNA fragment) is
clearly a powerful tool which is able to yield unequivocal results and should thus be the method of choice when attempting to identify pathogenicity or virulence genes using attenuated virus strains. Perhaps the most significant observation is that a subtle change in an essential virus gene can have a dramatic effect on the growth of virus in vivo and its pathogenicity without significantly affecting its potential to grow in tissue culture systems.

We thank Miss J. Phelan for her expert assistance with the mouse ear inoculation experiments and Mr M. J. Churcher for providing the virus growth curve data. B.A.L. was in receipt of a Junior Research Fellowship at Churchill College, Cambridge University. This work was supported by the Medical Research Council, U.K.

Note added in proof: We have recently determined the nucleotide sequence of the SC16 and RSC-26 DNA polymerase genes. RSC-26 polymerase contains a single nucleotide change with respect to the wt, at position 1790 within the BamHI r fragment and this leads to a predicted amino acid substitution (Glu→Asp) at residue 597 in the mutant polymerase polypeptide. This single amino acid substitution is therefore responsible for the attenuation observed in the mutant RSC-26.

REFERENCES


(Received 8 April 1986)