Mixed Infection With Herpes Simplex Virus Type 1 Generates Recombinants With Increased Ocular and Neurovirulence

Curtis R. Brandt*† and Dorene R. Grau*

The authors used the method of mixed ocular infection and subsequent in vivo selection to isolate Herpes simplex virus type 1 intratypic recombinants with increased ocular virulence and neurovirulence. Four recombinants were studied in some detail (DRG1A3, DRG2A2, DRG3A3, and DRG4A1). The recombinants had lethal doses in 50% of animals tested (LD50) at least 2-3 log units lower than either parent virus (OD4 and CJ394) and caused significantly more severe stromal keratitis, vascularization of the cornea, and blepharitis than either parent. Studies on the ability of DRG1A3 and DRG4A1 to replicate in the eye, trigeminal ganglia, and brain showed that these recombinants replicated to higher titers (1-3.5 log units) than the parents in all three tissues. One of the parents, OD4, spread to the central nervous system with the same kinetics as CJ394, DRG1A3, and DRG4A1 but had a restricted ability to replicate in all tissues, which may account for its lack of virulence. The other parent, CJ394, was nonneurovirulent but replicated to titers which were only 1-1.5 log units lower than the neurovirulent recombinants. These recombinants should be useful in studying virulence determinants in herpetic ocular infections. Invest Ophthalmol Vis Sci 31:2214–2223, 1990

Herpes simplex virus (HSV) infections of the eye range in severity from asymptomatic shedding to mild conjunctivitis to necrotizing stromal keratitis. Studies using animal models show that three factors contribute to the severity of an ocular HSV infection. The first is the genetic makeup of the host. Metcalf and Michaelis1 found that strains of inbred mice differed in their susceptibility to stromal keratitis. The C57 BL/6 mice were the most resistant, BALB/c and C3H mice had intermediate resistance, and DBA/2 mice were the least resistant. This order of resistance to stromal keratitis parallels the resistance to latency2 and death from HSV encephalitis.3 The ability of HSV to replicate in primary murine fibroblast cultures also parallels the resistance to stromal keratitis.4 Genetic studies recently showed that host resistance also correlates with the Igh-1 phenotype of the mouse.5

The second factor is the host immune system. Animals that have either recovered from an HSV infec-

From the Departments of *Ophthalmology and †Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, Wisconsin. Supported by a grant (EY07336) from the National Institutes of Health. Reprint requests: Curtis R. Brandt, PhD, Departments of Ophthalmology and Medical Microbiology and Immunology, Room 6630 Medical Sciences Center, 1300 University Avenue, Madison, WI 53706.
al reported that mutants lacking TK activity were unable to cause stromal keratitis. However, the role of TK in ocular virulence is not clear since the authors did not isolate a TK+ revertant and show that virulence was restored. Hendricks et al suggested that glycoprotein C (gC) plays a role in the development of severe stromal keratitis. Mutant viruses unable to express gC did not induce severe stromal keratitis in mice. However, revertants which expressed gC were not tested. Centifanto-Fitzgerald et al suggested that gC does not influence ocular disease in the rabbit. These authors implicated a gene or genes in the region mapping from 0.7-0.83 map units. Nine known genes map in this region: UL 50–56, latency-associated transcript, and ICPO, but the specific gene or genes involved in ocular virulence have not been identified. Genes potentially involved in neurovirulence also map in this region of the HSV genome.

We recently characterized the ocular disease and mortality caused by ten recent low-passage clinical isolates of HSV type 1 (HSV-1) and the laboratory strains HSV-1 KOS and HSV-2 33. We found that the ability to cause severe stromal keratitis did not necessarily correlate with neurovirulence. This result suggested that the two virulence traits may involve separate genetic determinants but did not exclude the presence of determinants that affect both. Using the technique of mixed in vivo infection, we isolated a series of HSV-1 intratypic recombinants which have significantly increased ocular virulence and are neurovirulent. In this report, we describe the isolation and characterization of four of these recombinants. These recombinants should be useful in identifying virulence determinants in ocular HSV infections.

Materials and Methods

Cell Culture

African green monkey kidney (Vero) cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% serum [a 1:1 mixture of fetal bovine serum and defined calf serum; HyClone, Ogden, UT], 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. Cells infected with virus were grown in DME containing 2.0% serum with antibiotics as described above.

Viruses

The two HSV-1 strains used as the parents to generate recombinants have been previously characterized. Briefly, strain OD4 causes little noticeable disease, and strain CJ394 causes severe stromal keratitis in BALB/c mice. Neither OD4 nor CJ394 are lethal for mice, and they are therefore nonneurovirulent. High-titer stocks were prepared by infecting Vero cells at a multiplicity of infection (MOI) of 0.01 and harvesting the infected monolayers when the cytopathic effect (CPE) was 90–100%. The infected cells then underwent three freeze–thaw cycles using a dry ice–ethanol bath, centrifuged at 100 × g for 10 min to remove debris, and stored at −80°C. Viral titers were determined by plaque assay on Vero cells using 2% methylcellulose.

Animal Inoculation and Disease Scoring

Procedures for inoculation of animals and scoring of ocular disease (stromal keratitis, vascularization of the cornea, and blepharitis) have been described previously. Briefly, mice were anesthetized with 2.5% halothane inhalation. The cornea was then scratched with a 30-G needle. A suspension containing the desired inoculum in 5 μl of DME with 2% serum was placed on the cornea. After 30 sec, the excess fluid was removed with a sterile swab and the eyelid closed.

Ocular disease scoring was based on the percentage of the cornea involved, and it was done by microscopic examination of the cornea. All studies were done on BALB/c mice (Harlan-Sprague Dawley, Indianapolis, IN). Before infection, mice were inspected microscopically, and any animals showing corneal defects were excluded. For the mortality studies, only those mice that died 6–12 days postinfection (PI) with evidence of central nervous system (CNS) involvement before death were used for scoring. We previously showed that there is no increase in mortality beyond 12–14 days PI in our ocular infection model. All experiments in this study were done in accordance with the ARVO Resolution on the Use of Animals in Research and National Institutes of Health guidelines.

Measurement of Viral Titers in Tissues

At various times after infection, whole eyes, trigeminal ganglia (TG), and brains were aseptically removed and placed in DME with 2% serum. Tissues were disrupted using a Tekmar STD homogenizer (Tekmar Co., Cincinnati, OH) with a S25N86 generator. Tissues were disrupted for 30 sec, underwent three freeze–thaw cycles in a dry ice–ethanol bath, and their titers were assayed in six-well tissue culture plates. Tissues were resuspended in as small a volume as possible to maximize sensitivity. Eyes and TG were suspended in 600 μl, and the entire sample was assayed. The brains were resuspended in 2 ml, and 600 μl of the suspension was assayed. Because we assayed the titers of the entire eye and TG samples, we report...
the results as total plaque-forming units (PFU) and not PFU/g. Three mice were used for each data point.

**Determination of Lethality**

Groups of five mice each were infected, using the eye scratch method, with amounts of virus varying by 1 log unit (1 × 10^2–1 × 10^4 or 1 × 10^7 PFU) and scored for mortality. The lethal dose for 50% of the animals tested (LD_{50}) values for each virus were calculated using the method of Reed and Muench.

**Isolation of Infected Cell DNA and Southern Blot Analysis**

Infected cell DNA was prepared by infecting Vero cells at an MOI of 1.0, harvesting the infected cells when the CPE reached 80–100%, lysing the cells in SET buffer (10 mm Tris HCl, pH 7.4, 1 mm ethylenediaminetetraacetic acid, and 1% sodium dodecyl sulfate), digesting overnight with proteinase K (50 μg/ml) at 37°C, and purifying the total infected cell DNA by phenol/chloroform extraction. Restriction site analysis of parental and recombinant viral genomes was done by southern blot analysis of total infected cell DNA using cloned HSV-1 probes. The DNAs were digested with restriction enzymes as described by the manufacturers (Promega Biotech, Madison, WI, or Boehringer Mannheim, Indianapolis, IN), underwent electrophoresis in 0.7% agarose gels, and were transferred to either nitrocellulose (Biorad, Richmond, CA) or Gene Screen Plus (New England Nuclear, Boston, MA). Procedures for transfer of the DNA, labeling of DNA probes by nick translation, and hybridization have been described elsewhere. We used BamH I, Bgl II, EcoR I, Hind III, Pst I, and Xba I to analyze the DNAs. In some regions of the genome, none of these enzymes were able to distinguish between OD4 and CJ394. These regions were screened with Bcl I, BstE II, Mlu I, Pvu II, Sal I, Sca I, Sma I, Sac I, and Stu I. The enzymes Pvu II and Sca I were useful in analyzing these regions.

**Statistical Analysis**

Analysis of variance was done on a Macintosh II computer using Statview 512+ (Brainpower, Inc., Calabasas, CA) programs. Statistically significant differences between the recombinant and parent viruses for ocular disease were calculated at the 95% significance level using Fisher's protected least significant difference test. The statistical significance of mortality was calculated using the chi-square test on data obtained using 4–6-week-old mice at a dose of 1 × 10^5 PFU/mouse.

**Results**

**Isolation of Recombinant Viruses with Increased Neurovirulence**

In the course of studying the titer dependence of stromal keratitis, we mixed an avirulent strain, OD4, and a strain that caused severe stromal keratitis, CJ394, at ratios of 10:1, 100:1, and 1000:1 and used these mixtures to infect mice. We chose to use OD4 and CJ394 because neither virus appeared to be neurovirulent. However, all of the mice in the 10:1 mixture died of HSV encephalitis on days 8–12 PI. The two most likely explanations for this result were synergistic infection as a result of (1) complementation or (2) generation of neurovirulent strains through recombination. Therefore, we attempted to isolate recombinants between OD4 and CJ394.

We aseptically removed the brains and purified the virus from four of the mice. We then passed the virus two additional times (1 × 10^7 PFU per mouse) in vivo. One virus preparation representing each of the four originally infected animals was then subjected to two rounds of plaque purification on Vero cells and high-titer stocks of one plaque from each preparation were prepared. In total, the recombinants described below were passed three times in vivo and then plaque purified twice. Each recombinant was isolated from a separate animal in the initial isolation to avoid selection of siblings. The recombinants are named with alternating numbers and letters denoting the animal from which it was isolated at each passage in vivo. The plaque-purified recombinants killed 100% of the mice when infected with 1 × 10^5 PFU.

**The Four Neurovirulent Isolates Were Recombinants**

To determine if the isolates were recombinants, we analyzed the restriction-enzyme digestion patterns of the viral DNA. Total DNA isolated from infected cells was digested with several restriction enzymes that recognize six base-pair recognition sequences, underwent electrophoresis in 0.7% agarose gels, and were transferred to a solid support. The filters were then hybridized sequentially with 32P-labeled EcoR I fragments from a cloned library of HSV-1 KOS and autoradiographed. Representative examples of the southern blots across the HSV-1 genome are shown in Figure 1. Digestion with Bgl II, EcoR I, Hind III, and Xba I did not distinguish between the parents, OD4 and CJ394, and were not useful for this analysis. The digestion patterns produced by BamH I proved to be the most useful. Differences were found in the regions corresponding to the EcoR I JK, JK, D, G, M, O, and L fragments. BamH I was not useful in the regions corresponding to the EcoR I A, I, and F frag-
Fig. 1. Southern blot analysis of strains OD4, CJ394, and recombinants DRG1A3, DRG2A2, DRG3A3, and DRG4A1. Total infected cell DNA (0.1-1.0 μg) was digested with various restriction enzymes, electrophoresed in 0.7% agarose gels, and blotted to either Gene Screen Plus or nitrocellulose. The filters were then sequentially hybridized with probes from an EcoR I library of HSV-1 KOS and autoradiographed. The probes were removed by immersing the filters in boiling water. Probe removal between hybridizations was monitored by exposing the filters to x-ray film. The map coordinates appear on the top line. The second line shows a schematic diagram of the HSV genome. The EcoR I restriction fragments of HSV-1 KOS are shown in the third line and representative examples of the Southern blots appear at the bottom. The fragments used to probe each blot are shown by the lines connecting the blots and the EcoR I map. The restriction enzymes used are shown below each blot. The size markers (Xciss? Hind III digest) were not aligned between blots. Lane 1, OD4; lane 2, 1A3; lane 3, 2A2; lane 4, 3A3; lane 5, 4A1; lane 6, CJ394.

ments. To map the regions corresponding to EcoR I A, I, and F, we tested the restriction-fragment patterns generated by Pvu II, Sca I, and Pst I.

Four classes of restriction patterns were found. Since this analysis generated a large amount of data, we will describe a representative southern blot from each class to illustrate how we proved the isolates were recombinants. With some enzyme-probe combinations, there were no detectable differences between the viruses. When this occurred, we attempted to find other enzymes that allowed us to distinguish between the parents. The only part of the genome where we could not detect a difference between the parents with any enzyme tested was the EcoR I N region (Fig. 1). This represents the first class of fragment patterns.

Restriction-fragment patterns clearly indicating the parental origin of the DNA represent the second class of restriction-fragment patterns. A representative blot is shown in Figure 2A. When we digested viral DNA with Pst I and probed the blots with the EcoR I F fragment, we could clearly differentiate between OD4 and CJ394. OD4 (lane 1) contains a 3.5-kb Pst I fragment (•), and CJ394 (lane 6) has a 1.7-kb Pst I fragment (>). OD4 does not have the 1.7-kb fragment and CJ394 does not have the 3.5-kb Pst I fragment. The recombinants (Fig. 2A, lanes 2-5) all have the 3.5-kb Pst I fragment and lack the 1.75-kb Pst I fragment, indicating the DNA in this region of the recombinants originated from the OD4 parent.

In some regions of the genome, we found restriction fragments characteristic of both parents were present in the same recombinant, indicating that a recombination event or events occurred in this region. This represents the third class of fragment patterns. Figure 2B shows an example of this. When we digested the DNA with Pvu II and probed with the EcoR I I fragment, we found that OD4 had a 4.1-kb Pvu II fragment (•, lane 1) and CJ394 had a 2.5-kb Pvu II fragment (>, lane 6). The 4.1-kb Pvu II fragment was missing in CJ394 and the 2.5-kb Pvu II fragment was missing in OD4. Examination of the Pvu II fragment patterns in the recombinants (lanes 2-5) shows that 1A3 has the 4.1-kb Pvu II fragment. Thus, DNA in this region of the 1A3 genome originated from the OD4 parent. The recombinants 2A2, 3A3, and 4A1, however, have both Pvu II fragments, indicating that both parents contributed to the DNA in this region of the recombinants, and therefore a recombination event occurred somewhere in EcoR I I. The digestion patterns do not result from mixing of DNA samples for the following reasons. First, the 4.1-kb and 2.5-kb fragments are equimolar. It would be very unlikely to have an equimolar mixture resulting from an accident. A second more convincing argument is that all of the fragments were stripped and sequentially hybridized with other HSV-1-specific probes. If the samples had been accidently mixed, we would not have been able to distinguish between the recombinants in other regions of the genome.

The fourth class of digestion patterns was observed only in the terminal and joint regions. Digestion with several restriction enzymes generated fragment patterns in the terminal and joint regions unique to each virus (parent and recombinant) and some fragments common between some recombinants and each par-
ent (Fig. 2C). These regions of the genome contain repeated sequences. The number of copies of each repeat can vary between isolates. In the absence of restriction fragment-pattern differences in any other region of the genome with these enzymes, we assumed the differences were due to variations in the repeats.

A schematic diagram summarizing our restriction-mapping results is shown in Figure 3. Our analysis clearly showed that all four neurovirulent isolates were recombinants and that each isolate was the result of multiple recombination events.

**Neurovirulence of the Recombinants**

All of the neurovirulent recombinants were isolated after serial inoculation of $1 \times 10^5$ PFU. All of the mice died in these experiments, suggesting the LD$_{50}$ values for the recombinants was less than $1 \times 10^3$. To quantitate neurovirulence, we inoculated $1 \times 10^3$, $1 \times 10^4$, $1 \times 10^5$, and $1 \times 10^6$ PFU of the neurovirulent recombinants and $1 \times 10^3$, $1 \times 10^4$, $1 \times 10^5$, and $1 \times 10^6$ PFU of the parents using our eye scratch method and scored for death due to encephalitis. We could not prepare a stock of OD4 with a high enough titer to test $1 \times 10^7$ PFU, so we used $1 \times 10^6$ PFU as the maximum titer tested for the two parents. The LD$_{50}$ values are shown in Table 1.

The LD$_{50}$ values for the recombinants varied from just over $1 \times 10^3$ to just over $1 \times 10^4$. Since none of the mice infected with either parent (OD4 or CJ394) died, we could not determine an LD$_{50}$ value, and the increases in neurovirulence are minimum estimates.

We found that the LD$_{50}$ values for recombinants 2A2 and 3A3 were approximately 2 log units lower than for the parents, and the LD$_{50}$ values for recombinants...
Table 1. LD50 values for parental and recombinant viruses

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>LD50 (PFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG1A3</td>
<td>10^4.17</td>
</tr>
<tr>
<td>DRG2A2</td>
<td>10^4.17</td>
</tr>
<tr>
<td>DRG3A3</td>
<td>10^4.17</td>
</tr>
<tr>
<td>DRG4A1</td>
<td>10^4.18</td>
</tr>
<tr>
<td>CJ394</td>
<td>≥10^6</td>
</tr>
<tr>
<td>OD4</td>
<td>≥10^6</td>
</tr>
</tbody>
</table>

A3 and 4A1 were approximately 3 log units lower than for the parents. The mortality of all four recombinant viruses was significantly increased over the two parental strains (P < 0.001).

Ocular Disease Severity

To determine if the recombinants caused more severe eye disease than the parents, we infected mice using the eye scratch method with 1 × 10^5 PFU and scored the severity of stromal keratitis, vascularization of the cornea, and blepharitis for 2 weeks after infection. Viruses that are neurovirulent can complicate the scoring of severe eye disease because the animals die before eye disease peaks, artificially lowering the disease scores. To avoid this problem, we used slightly older mice (7–8 weeks versus 4–6 weeks of age) that are more resistant to HSV encephalitis.

Figure 4 shows the time course for the development of stromal keratitis (Fig. 4A), vascularization of the cornea (Fig. 4B), and blepharitis (Fig. 4C). Stromal keratitis appeared from days 4–6 with all of the neurovirulent isolates and the CJ394 parent. Stromal disease continued to increase in severity until it peaked on day 10 or 11 and remained at peak levels until the experiment was halted. We could not score DRG2A2-infected animals beyond day 9 because none of the animals survived. Vascularization of the cornea became visible from days 3–7 and continued to increase for all of the recombinants. The vascularization caused by CJ394 increased until day 10 or 11 and then remained steady until the end of the experiment. The parent OD4 did not cause stromal keratitis or vascularization of the cornea. Blepharitis was visible as early as day 3 in all of the recombinants with the exception of DRG2A2 which became visible on day 5. Blepharitis peaked at days 6–7 for DRG4A1, DRG2A2, and DRG3A3 but continued to rise throughout the experiment for DRG1A3. A small amount of blepharitis was noticed with OD4.

To compare the ocular disease severity of the HSV strains better, we developed the mean peak disease score (MPDS). There were statistically significant differences between the stromal keratitis caused by the strains (F = 27.8, df = 65, P < 0.01). The stromal disease caused by DRG4A1, DRG1A3, DRG3A3, and DRG2A2 was significantly greater than CJ394-, OD4-, and mock-infected animals; strain CJ394 was significantly greater than both OD4- and mock-infected animals. Thus, the strains separate into three groups: those causing very severe stromal disease (DRG4A1, DRG1A3, DRG2A2, and DRG3A3), severe stromal disease (CJ394), and no stromal disease (OD4).

There were significant differences also between the vascularization scores of the viruses (F = 14.88, df = 65, P < 0.01). Statistically, the viruses separated into three groups: those causing very severe vascularization (DRG4A1 and DRG1A3), severe vascularization (DRG3A3, DRG2A2, and CJ394), and no vascularization (OD4). The effects of strain OD4 were not significantly different from those of mock-infected animals.

The severity of blepharitis was also significantly different (F = 33.1, df = 65, P < 0.01). Statistically, the viruses could be separated into three groups: those
that cause very severe blepharitis (DRG4A1, DRG1A3, DRG2A2, and DRG3A3), severe blepharitis (CJ394), and very little blepharitis (OD4). Strain OD4 did not cause effects that were significantly different from those of mock-infected animals.

These differences in ocular virulence were also reflected in the percentage of the infected animals with disease (Table 2). We found that 90–100% of the animals infected with the recombinants had evidence of stromal disease, vascularization, and blepharitis; only 60% of the animals infected with CJ394 had evidence of disease. With OD4, none of the animals developed stromal disease or vascularization, and only 30% developed blepharitis.

Replication In Vivo

To determine if the increased virulence was due to enhanced replication of the recombinants in the animal, we measured the amount of infectious virus at various times after infection. Mice (4–6 weeks of age) were infected by the ocular route with 1 × 10^5 PFU of OD4, CJ394, DRG1A3, and DRG4A1. At days 1, 2, 3, 6, 8, and 10 PI, we aseptically removed the eyes, TG, and brains, and assayed the amount of infectious virus in each tissue. Peak titers occurred on days 1–3 in the eye and declined thereafter (Fig. 6). There was no detectable OD4 in the eyes on day 6; titers of CJ394 fell to undetectable levels by day 8. Peak titers were achieved on day 3 in the TG and declined thereafter. The amount of OD4 virus was at background levels by day 8. There was no detectable CJ394 in the TG on day 8. Infectious virus was detectable in the TG of DRG1A3- and DRG4A1-infected animals even on day 8. Similar results were obtained in the brain except the peak titers occurred on day 6. Titers of OD4 declined to undetectable levels by day 8. Infectious virus was still detectable in the brains of CJ394-, DRG1A3-, and DRG4A1-infected animals even on days 8–10. The titers of CJ394 dropped after day 6, although the titers of the neurovirulent recombinants remained high until the animals died. Table 3 shows the number of tissues that were positive for virus on each of the days that were sampled. As further confirmation of the nonneurovirulence of OD4, we injected 1 × 10^5 PFU intracranially into 4–6-week-old mice. The mice had ruffled fur for 2–3 days after the injection, but all of the injected mice survived.

Discussion

We previously suggested that neurovirulence and ocular virulence may be controlled by separate genetic determinants. This suggestion was based on the characterization of virulence properties of 12 viral strains, some of which caused severe ocular disease but were not neurovirulent. Several strains, however, were neurovirulent and caused significant stromal

Table 2. Percentage of animals showing ocular disease

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of animals</th>
<th>Stromal keratitis</th>
<th>Vascularization</th>
<th>Blepharitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG4A1</td>
<td>10</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>DRG1A3</td>
<td>10</td>
<td>90.0</td>
<td>90.0</td>
<td>100.0</td>
</tr>
<tr>
<td>DRG2A2</td>
<td>12</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>DRG3A3</td>
<td>10</td>
<td>100.0</td>
<td>90.0</td>
<td>100.0</td>
</tr>
<tr>
<td>CJ394</td>
<td>10</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>OD4</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>30.0</td>
</tr>
<tr>
<td>Mock</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are no. positive/no. sampled.

*All mice in these groups were dead by day 10.
keratitis, suggesting some genetic determinants may influence both phenotypes. In this report, we described the isolation and characterization of HSV-1 intratypic recombinants that are both neurovirulent and cause more severe ocular infections than either parental virus.

Restriction mapping of recombinants can be used to identify the location of the gene or genes involved in a phenotypic difference between two viruses. The DNA from one parent present in all of the recombinants indicates the possible location of the gene. Our preliminary mapping results suggested a gene or genes contributed by the parent OD4 map in the regions 0.29–0.4, 0.47–0.62, or 0.84–0.94 map units since OD4 DNA is present in these regions in all four recombinants. The gene or genes contributed by the parent CJ394 presumably map between 0.47–0.62 since this is the only region of the genome where all four recombinants have CJ394 sequences. The region from 0.47–0.62 is listed as a potential map location for genes contributed by both OD4 and CJ394 because DNA from both parents was present in this region of all four recombinants as a result of multiple recombination events (Fig. 2B). A gene or genes from each parent also could map in 0.29–0.31 map units since we could not differentiate the parental genomes.

We urge caution in the interpretation of our results, however, for several reasons. First both of our parental viruses were HSV-1 and have similar restriction maps with several enzymes. This results in large imprecision in the identification of recombination boundaries. We, therefore, cannot exclude the possibility that the gene or genes involved map to the left or right of the boundaries described above. Second we analyzed a small number of recombinants which limits the precision of the mapping data. Third because of the limits on the precision of our mapping data, we could have missed a recombination event in a region scored as parental in the recombinants. Finally, we cannot exclude completely the possibility of a mutation in another region of the genome which affects virulence. Construction of a more detailed restriction map will not eliminate these uncertainties in mapping the genes involved. We are currently cloning the genomes of the parental viruses and using marker transfer with specific restriction fragments to identify the gene or genes involved in virulence.

Several regions or individual genes in the HSV-1 genome have been implicated in virulence. A region involved in ocular virulence in the rabbit maps between 0.7–0.83 map units. Genes potentially involved in neurovirulence have been mapped in the regions from 0.71–0.74, 0.079–0.143 and 0.25–0.53 map units. Genes involved in tissue invasiveness have been located between 0.32–0.42 and 0.49–0.64 map units. Several individual genes have also been implicated in virulence. These include DNA polymerase, TK, RR and ICPO (IE-128). Deletion of all of the genes encoded in the U3 region, with the exceptions of gD and gE, reduces the neurovirulence of the mutants when they are injected intracranially. However, most of these regions do not overlap with the regions identified in our preliminary mapping studies.

Some of the previously identified virulence determinants do map to the regions we identified. The region from 0.84–0.94 map units contains most of the U genes studied by Meignier et al. The TK gene maps in the region 0.31–0.4, and the RR gene maps in the region 0.48–0.62. We are presently testing the recombinants for their TK activity. Two genes potentially involved in virulence map near the boundaries of the regions we identified and, given the imprecision of our mapping, could be involved. These are DNA polymerase (near 0.4) and gC (near 0.62). The regions denoted INV1 and INVII by Goodman et al also overlap the regions we identified.

When we infected mice by the ocular route and assayed the viral titer in the eye, TG, and brain, we found that the peak titers of the avirulent parent OD4 were only 1 log unit above background in all three tissues. Clearly, OD4 does not replicate well in the mouse. We also consistently found our OD4 stocks were 1–2 log units lower in titer than either CJ394 or the recombinants. Since our stocks were prepared in Vero cells, it would appear that OD4 may have a general defect in replication in several cell types. This could account for its lack of virulence. The other parent, CJ394, replicates well, reaching titers of approximately 1 × 10^5, 4 × 10^5, and 1 × 10^5 in the eye, TG, and brain, respectively. Strain CJ394 causes more severe ocular disease than OD4 and the higher eye titers could be related to the increased virulence. Further studies of the growth properties of these viruses in different cell types are in progress. The recombinants DRG1A3 and DRG4A1 replicate to higher titers (1.0–1.5 log units) than CJ394 in all three tissues. This raises the possibility that DRG4A1 and DRG1A3 are more virulent because they replicate better than the parents. However, further studies are needed to confirm this, since the cell tropism of the recombinants could be different from the parents and might account for the differences in neurovirulence, particularly between CJ394, DRG4A1, and DRG1A3.

The quantitation of the tissue titers also show the sequential spread of the viruses from the eye to the TG and then to the brain. The viral titers peak on days 2–3, 3, and 6 in the eye, TG, and brain, respec-
the ability to form syncytia. 43 Syncytia formation is a property that has been related to increased virulence and was not lethal (data not shown). Another biologic gene involved and showing that a single gene can enhance spread of the virus in the host. None of our recombinants, however, were efficient at inducing cell fusion (data not shown).

The ambient temperature of the mouse is 38.5°C and a temperature-related restriction of growth may explain the lack of neurovirulence in some HSV isolates. 16,21,34 Although we have not yet tested the replication of these viruses in vitro at different temperatures, our in vivo replication results suggest that the inability of OD4 to replicate in the mouse is not related to temperature. Our infection protocol involves inoculation and initial replication of the virus in the cornea which has an ambient temperature 2-4°C cooler than internal body temperature. Our results demonstrate that OD4 does not replicate any better in the eye than in the TG or the brain.

In summary, we used mixed infections in vivo to isolate recombinant HSV-1 that have both increased neurovirulence and ocular virulence. This raises the possibility that there may be genetic determinants which influence neurovirulence and ocular virulence. Formal proof, however, will require the cloning of a gene involved and showing that a single gene can influence both traits. The continued biologic and genetic characterization of these recombinants should contribute important information on the genetics of ocular virulence and neurovirulence in HSV-1.

Key words: Herpes simplex virus, stromal keratitis, neurovirulence, recombinant virus

Acknowledgments

The authors thank Dr. M. Levine, University of Michigan, Ann Arbor, MI, for providing the cloned HSV-1 KOS library and Bernadette Bull for preparing the manuscript.

References


33. Taha MY, Clements GB, and Brown SM: A variant of herpes simplex virus type 2 strain HG52 with a 1.5 kb deletion in R1 between 0.002 and 0.81–0.83 map units is non-neurovirulent for mice. J Gen Virol 70:705, 1989.


