The Quantity of Latent Viral DNA Correlates with the Relative Rates at Which Herpes Simplex Virus Types 1 and 2 Cause Recurrent Genital Herpes Outbreaks

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Herpes simplex virus types 1 (HSV-1) and HSV-2 are remarkably similar in their abilities to infect mucosal surfaces and to latently infect and reactivate from sensory nerve ganglia, despite their well-characterized genomic and antigenic differences (22). It cannot be coincidence, however, that has segregated the majority of HSV-1 infections to the oral-labial region in humans and HSV-2 to the genital region. HSV-1 and HSV-2 display distinct phenotypic patterns with regard to their rates of symptomatic reactivation at each anatomical site (8, 18, 20). By some estimates, patients with concurrent primary oral-labial and genital HSV-1 infections are nearly sixfold more likely to develop oral-labial rather than genital recurrences. Conversely, those with simultaneous oral-labial and genital HSV-2 infections are about 400-fold more likely to experience genital rather than oral recurrences (11).

Few of the viral factors that could be associated with this anatomic predilection have been compared directly in parallel studies of HSV-1 and HSV-2. Work with animal models has shown that HSV-1 and HSV-2 are equally adept at causing acute infection (12, 20). Both are transported axonally from peripheral sites to infect the central nervous system, although HSV-2 is clearly more neurovirulent than HSV-1 (6, 7, 15, 19). A comparison of HSV-1 and HSV-2 in the mouse vaginal model has shown that both viruses establish latency (1, 20). Both viruses can reactivate from facial and genital sites of inoculation, although in humans, the rates of reactivation vary according to sites of infection and virus type (11).

Recent work suggests that tissue-specific rates of virus reactivation are influenced by sequences in an HSV gene that is expressed during latency (23). In latently infected animal oractivation are influenced by sequences in an HSV gene that is expressed during latency (23). In latently infected animal or

Materials and Methods

Cells and viruses. Vero cells were grown in Dulbecco's modified Eagle medium (Quality Biological, Inc., Gaithersburg, Md.) supplemented with 10% fetal calf serum (Sigma Chemical Co., St. Louis, Mo.) and 1% L-glutamine–aureomycin–streptomycin–penicillin (Quality Biological, Inc.) in a 5% CO2 humidified chamber at 37°C. Primary rabbit kidney cells (Biowhittaker, Walkersville, Md.) were grown in accordance with the supplier's instructions. Stocks of HSV-1 strain 17 syn+ and HSV-2 strain 333 were prepared in Vero cells and divided into cell-free aliquots, their titers were determined, and they were stored at −80°C until use.

Guinea pigs. Female Hartley guinea pigs (500 g) were housed in American Association for Laboratory Animal Care-approved facilities and studied in accordance with approved protocols. Guinea pigs were anesthetized with ketamine and xylazine and inoculated intravaginally with virus in a 25- to 100-μl volume as previously described (5). In the second experiment, 25 mg of acyclovir (Burroughs Wellcome Co., Research Triangle Park, N.C.) was given once daily by intraperitoneal injection on days 1 through 7 to animals infected with HSV-2 to reduce the high (30 to 50%) mortality rates.

Scoring of acute and latent genital lesions. Guinea pig genitalia were scored daily on a scale of 0 to 4 following inoculation as previously described (16). Recurrences were recorded from day 15 or the time of lesion resolution, whichever came later, until day 50.

Determination of the titers of vaginal swabs. Guinea pigs were swabbed vaginally with Dacron swabs during the acute infection. Swabs were immediately placed into 1 ml of Dulbecco's modified Eagle medium on ice. Dilutions were plated onto Vero cells in duplicate, and following incubation for 1 h to allow adherence, cells were washed and overlaid with medium containing 0.5% human immunoglobulin. Plaques were counted 2 days later.

Viral titers in tissues. At desired times after infection, tissue samples were obtained from each group for quantification of virus in particular anatomical sites. Sacral dorsal root ganglia and spinal cords were dissected free of surrounding tissue and placed into 1 ml of Dulbecco's modified Eagle medium on ice. Tissues were homogenized by using a Tissumizer (Tekmar, Cincinnati, Ohio) and
frozen and thawed once. Homogenized tissues were spun briefly in a microcentrifuge, and dilutions of the supernatant were plated onto primary rabbit kidney cell monolayers in duplicate. Following incubation for 1 h to allow adherence, cells were washed and overlaid with medium containing 0.5% human immunoglobulin. Plaques were counted 2 days later.

Supernatants from some samples (200 μl) were also extracted for quantitative competitive DNA PCR assays.

**Extraction of nucleic acids from ganglia.** Guinea pigs were sacrificed by carbon dioxide inhalation, and their sacral dorsal root ganglia were removed with sterile instruments. Ganglia were placed into 300 μl of cell lysis solution (0.001% sodium dodecyl sulfate–0.0001% Triton X-100 in buffer containing Tris-HCl at 10 mM and EDTA at 1 mM) containing 0.6 mg/ml proteinase K (Sigma Chemical Co., St. Louis, Mo.) and incubated overnight at 50°C. DNA was extracted by using the RNAsigkine kit (Gentra Systems, Minneapolis, Minn.) in accordance with the manufacturer's instructions. RNA was extracted by using the RNeasy kit (Promega, Madison, Wis.) in accordance with the manufacturer's instructions following mechanical dispersion of the ganglia with 10 to 25-gauge needles and syringes in succession. DNA was stored in buffer containing Tris-HCl at 10 mM and EDTA at 1 mM at 4°C. Ganglion RNA was stored in diethylpyrocarbonate-treated water.

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guinea pigs infected with HSV-1 had lesions, while 15 of 16 infected with HSV-2 had lesions ($P < 0.001$; Fig. 5C).

Important differences were also seen in virion genome and LAT copy numbers in latently infected ganglia from these animals, as quantitated by competitive DNA and RNA PCR assays (an example is shown in Fig. 3B). The geometric mean number of latent HSV-2 DNA copies per 200 ng of ganglion DNA was over three times that of latent HSV-1 DNA ($P < 0.11$; Table 1). Dorsal root ganglia from guinea pigs experiencing recurrent outbreaks contained greater numbers of latent HSV DNA than did those from animals without recurrences, regardless of the viral type (geometric mean latent viral DNA copy numbers per 200 ng of ganglion DNA of $8 \times 10^{3}$ for animals without recurrences and $4.9 \times 10^{4}$ for animals with recurrences, $P < 0.02$). Ganglia latently infected with HSV-2 also contained 15-fold more copies of HSV-2 than HSV-1 LATs ($P < 0.01$; Table 1). These results suggested that the burden of latent viral DNA and the levels of LAT expression may be important determinants of recurrence frequency. To further test this hypothesis, we analyzed recurrence rates in animals bearing a different ratio of HSV-1 to HSV-2 DNA and LAT copy numbers.

Latent HSV infection and recurrent genital herpes in guinea pigs infected with a 10-fold higher inoculum of HSV-1 and HSV-2. It was postulated that HSV-1 infection would recur as frequently as HSV-2 infection if the input inoculum of HSV-1 was sufficiently increased to achieve levels of latent HSV-1 DNA equivalent to or higher than those of latent HSV-2 DNA. Guinea pigs were infected intravaginally with either $10^6$ PFU of HSV-1 (five times the amount used in the first experiment) or only $10^5$ PFU of HSV-2 (half of the amount used in the first experiment). In this experiment, suboptimal acyclovir therapy was given to the HSV-2-infected animals for the first 7 days...
with the goal of reducing somewhat the mortality that results from the primary infection. Prior studies showed that this extent of therapy does not alter genital recurrence frequency (15). Daily observations revealed that the disease recurred in similar proportions of animals infected with HSV-1 and HSV-2 (P > 0.5; Fig. 5D). Although the median number of recurrences per animal with HSV-1 was three times greater than that of animals with HSV-2, three (range, zero to six) and one (range, zero to four), respectively, the distributions of recurrences were not statistically significantly different (P = 0.22; Fig. 5B). The median numbers of days with lesions, 5.5 days (range, 0 to 33) for HSV-1 and 9.5 days (range, 0 to 23) for HSV-2, were also not statistically significantly different (P > 0.5), nor were the numbers of days until the first recurrence (P > 0.5). In comparing experiment 1 (Fig. 5A and C) with experiment 2 (Fig. 5B and D), it was noted that the 10-fold elevation in the HSV-1 inoculum led to a significantly enhanced likelihood of disease recurrence (P < 0.01). The likelihood of HSV-2 recurrence was unchanged (P = 0.34) by decreasing its inoculum by half.

In accord with the increased likelihood of HSV-1 recurrence with a greater inoculum, quantitation of DNA and LAT contents in latently infected ganglia demonstrated corresponding increases in the latent HSV-1 genome and LAT contents to levels that were higher than those found in HSV-2-infected ganglia (P = 0.01 for LAT copies; Table 1).

DISCUSSION

Genital herpes recurrence rates are influenced by the quantity of latent virus in the ganglia. We found that the number of copies of latent viral DNA and LATs were higher, often significantly so, in the groups of animals experiencing higher rates of genital outbreaks. When the titer of virus with which the animals were infected was increased, the levels of latent viral DNA and RNA increased and there were corresponding increases in the likelihood and duration of recurrences. We believe that the quantity of LATs merely reflects the level of latent viral DNA and is not, by itself, an efficient determinant of reactivation rates. In fact, our recent analyses of a series of HSV-2 mutants that produce high, intermediate, or very low levels of LATs in guinea pig ganglia showed that only very profound (>5-log) reductions in LAT expression but unchanged levels of latent viral DNA result in modest (50 to 90%) reductions in the rates of disease recurrence (21). Although HSV mutants deficient in LAT expression showed reduced rates of reactivation, these mutants have not always been rigorously assessed for the levels of latent DNA that they achieve in sensory ganglia (2, 4, 9, 13). The present data also do not negate the recent findings that the type specificity of the LATs influences the rate of reactivation, since latent DNA levels were not quantitated precisely in those studies (23). A recent study by Maggioncalda et al. verified decreased numbers of latently infected mouse neurons and rates of induced reactivation by explant cultivation with selected LAT region mutants of HSV-1 (14).

The present results have implications regarding antiviral therapy and vaccine development for HSV infections. Were one able to reduce the quantity of virus that can establish recurrent genital lesions in independent experiments in which animals were infected with equivalent titers of HSV-1 or HSV-2 (experiment 1 in A and C) or with 10-fold higher inocula of HSV-1 (experiment 2 in B and D). Following resolution of the acute infections, the presence of lesions was noted daily from day 15 through day 50. The data are displayed at the top as the percentages of animals with no lesions, with lesions for 1 to 5 days, and with lesions for more than 5 days in the study interval (A and B). Below (C and D), the data indicate the cumulative percentage of animals experiencing a first genital recurrence in the study interval (Kaplan-Meier curves).

FIG. 5. Recurrent genital lesions in independent experiments in which animals were infected with equivalent titers of HSV-1 or HSV-2 (experiment 1 in A and C) or with 10-fold higher inocula of HSV-1 (experiment 2 in B and D). Following resolution of the acute infections, the presence of lesions was noted daily from day 15 through day 50. The data are displayed at the top as the percentages of animals with no lesions, with lesions for 1 to 5 days, and with lesions for more than 5 days in the study interval (A and B). Below (C and D), the data indicate the cumulative percentage of animals experiencing a first genital recurrence in the study interval (Kaplan-Meier curves).

TABLE 1. Geometric mean numbers of latent HSV-1 and HSV-2 DNA copies and LAT copies in lumbosacral gangliaa

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<thead>
<tr>
<th>Expt and virus</th>
<th>DNA copy no.</th>
<th>LAT copy no.</th>
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<td>DNA copy no.</td>
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<td>1, Equal-titer inocula</td>
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<tr>
<td>HSV-1 (n = 6)</td>
<td>1.5 x 10^6 (6.6 x 10^-5)</td>
<td>7.4 x 10^5 (3.2 x 10^-5)</td>
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<tr>
<td>HSV-2 (n = 6)</td>
<td>4.9 x 10^6 (1.6 x 10^-5)</td>
<td>1.7 x 10^6 (3.4 x 10^-5)</td>
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<tr>
<td>2, 10-fold higher HSV-1 inoculum</td>
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<tr>
<td>HSV-1 (n = 4)</td>
<td>2.2 x 10^6 (1.4 x 10^-5)</td>
<td>1.1 x 10^7 (8.7 x 10^-5)</td>
</tr>
<tr>
<td>HSV-2 (n = 5)</td>
<td>1.7 x 10^6 (4.2 x 10^-5)</td>
<td>1.1 x 10^6 (2.9 x 10^-5)</td>
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a Number of copies per 200 ng of ganglion DNA or RNA are shown. Geometric means are shown with 95% confidence intervals in parentheses.
latency, the likelihood and rate of disease reactivation should decrease. However, multiple trials have proven that acyclovir is not initiated sufficiently early in the course of first episodes of genital herpes to alter subsequent-recurrence rates (10), and vaccines have failed to induce protective immunity in humans (3), but more potent antiviral drugs and more immunogenic vaccines may prove effective.

More immediately, the present data may explain the disproportionate rates at which HSV-1 and -2 cause recurrent genital herpes outbreaks in humans (11). Although there might be tissue-specific or immunologic obstacles to virus reactivation at a particular anatomic site, the lower rate at which HSV-1 genital infections recur could simply reflect a lower burden of latent virus in the lumbosacral ganglia. The present data establish that the quantity of latent virus correlates with the rate at which HSV infections recur and suggest that it is one of its major determinants.

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REFERENCES