The High Prevalence of Herpes Simplex Virus Type 1 DNA in Human Trigeminal Ganglia Is Not a Function of Age or Gender

James M. Hill,1,2,3,4*, Melvyn J. Ball,5 Donna M. Neumann,1 Ann M. Azcuay,1 Partha S. Bhattacharjee,1 Saadallah Bouhanik,1 Christian Clement,1 Walter J. Lukiw,1,4 Timothy P. Foster,3 Manish Kumar,1 Herbert E. Kaufman,1,2,3,4 and Hilary W. Thompson1,4,6

Departments of Ophthalmology,1 Pharmacology,2 and Microbiology,3 Neuroscience Center of Excellence,4 Louisiana State University Health Sciences Center, New Orleans, Louisiana; Department of Pathology, Oregon Health and Science University, Portland, Oregon6; and Section of Biostatistics, School of Public Health, Louisiana State University Health Sciences Center, New Orleans, Louisiana6

Received 27 March 2008/Accepted 30 May 2008

The purpose of this study was to determine the presence and copy numbers of herpes simplex virus type 1 (HSV-1) DNA in human trigeminal ganglia (TG) with respect to age, gender, and postmortem interval (PMI). Human TG (n = 174, obtained from the Oregon Brain Bank, with data on age, gender, and PMI) were analyzed for HSV-1 DNA copies (HSV-1 DNA polymerase gene) by using real-time PCR. We found that 89.1% (131/147) of subjects and 90.1% (155/174) of TG contained HSV-1 DNA. The copy numbers of HSV-1 DNA in the positives ranged from very high (>106) to very low (5). These data confirm and strengthen our previous findings that subjects were positive for HSV-1 DNA in tears (46/50; 92%) and saliva (47/50; 94%). These TG data and tear and saliva data demonstrated considerable variability in copy numbers of HSV-1 DNA per subject. Statistical analysis showed no significant relationship between gender and copy number, age and copy number, or PMI and copy number for each pair of variables. A factorial analysis of gender, age, and PMI with respect to copy number also showed no statistical significance. This is the first study that provides statistical analysis that documents that the prevalence of HSV-1 DNA in the human TG is not a function of either gender or age.

A high percentage of the world’s population harbors herpes simplex virus type 1 (HSV-1), which can cause a variety of disorders (25, 27, 29). HSV has the ability to evade host immune defenses and seize the cellular machinery for production of progeny virus and also can shut down its own lytic replication to establish viral latency, a means for the virus to exist for the life of the host. Reactivation from latency with concomitant virus production is an efficient means of viral spreading within the population. After primary infection, latent HSV-1 can persist in neurons in sensory and autonomic ganglia in at least 90% of the adult human population (14, 18, 25, 27, 29), reactivating periodically and causing recurrent disease. Cumulatively, at least 35% of the U.S. population is affected by HSV infections (15, 25, 29).

Herpes keratitis is the leading infectious cause of blindness in the United States and is caused predominantly by HSV-1, with a reported incidence of 149 cases per 100,000 individuals and, cumulatively, up to 500,000 cases per year. Ocular herpes recurs at the rate of 10% within 1 year and 23% within 2 years (15), and an estimated 50,000 new and recurrent cases are documented each year (15). Previous findings document that 98% of subjects (49/50) spontaneously shed HSV-1 DNA in tears and saliva (12). The same PCR assay used in this study was used for analysis of the human trigeminal ganglion (TG). This study obtained demographic data on age and gender and compared these data with respect to numbers of HSV-1 DNA copies in the human TG.

This study was conducted according to the tenets of the Declaration of Helsinki, and the protocol was approved by the Oregon Health Sciences University Institutional Review Board. Samples were obtained from autopsy cases through the Oregon Brain Bank (directed by one of the authors, M. J. Ball) at Oregon Health Sciences University, Portland, OR. Each sample had protected health information removed and was identified by a “Brains for Oregon (BFO)” number. At the time of autopsy, all TG were aseptically removed and trimmed of dura and fat. The three branches of the TG were dissected away prior to immediate snap-freezing and storage at –80°C. Clinical diagnoses and evidence of disease were noted from the subjects’ medical histories. The 147 subjects were divided into groups with (i) dementia attributable to Alzheimer’s disease, (ii) neurodegenerative dementia not due to Alzheimer’s disease, and (iii) no known cognitive decline or significant pathological neurodegeneration. Each of these three groups had the same number of subjects.

Each individual TG was weighed (range, 0.26 to 1.5 g) and pulverized, under liquid nitrogen, to a fine powder. Homogeneous aliquots of the TG (~50 mg) were removed and stored at –80°C. All instruments were cleaned and sterilized between processing of each TG to avoid contamination. Rabbit TG negative for HSV-1 were used as tissue controls. TG samples were placed in cell lysis buffer (ATL buffer) and incubated for 18 h at 56°C with 150 μg of a proteinase K solution. DNA extraction was carried out using a DNA extraction kit (DNeasy tissue kit; Qiagen, Valencia, CA) per the manufacturer’s in-
The sensitivities of the primers were determined to be two to four copies and were achieved in the presence of 100 ng of human DNA, with no HSV-1 DNA endogenously present. The cycle threshold values of all unknowns were adjusted to reflect the cycle threshold value of the standard curve. The equation of the slope of the line was used to calculate the numbers of copies of unknown TG samples. All values calculated as fewer than five copies were omitted to reduce the possibility of false positives.

Statistical evaluation of the relationship between the number of HSV copies (expressed both as numbers per 100 ng of DNA or as numbers per whole TG), the age of the subject, and the postmortem interval (PMI) was conducted using linear, logarithmic, and double-logarithmic regression analyses. We assayed 100 ng of the DNA from human TG and calculated the value of 100 ng of DNA for comparison to published studies (5, 24, 28). The relationships between age and copy number, gender and copy number, and PMI and copy number were evaluated using age, PMI, or copy number as the outcome variable and gender as the class variable in t tests. The simultaneous effects of age and gender on number of copies were determined using an analysis of covariance (19), with age as the covariant and gender as a class variable. Alpha levels were set at 0.05 prior to testing, and the alpha level correction for multiple comparisons was done using the simulation method of Edwards and Berry (6). All data manipulation and analyses were performed using the Statistical Analysis System (Cary, NC).

The genders and mean ages and PMIs of the 147 subjects (174 TG) are shown in Table 1, including the means ± standard errors of the means (SEM) of results for all the subjects, with the minimum and maximum values, as well as the numbers of positives, copies per 100 ng of DNA, and copies per whole TG for all 174 TG assayed. A class distribution of copy number per 100 ng of DNA by gender and a class distribution of copy number per whole TG revealed that the majority of the TG had low to moderate copy numbers. The numbers of subjects whose TG were analyzed for HSV-1 DNA were analyzed for the primers and probe were previously reported (12).

<table>
<thead>
<tr>
<th>Gender(s)</th>
<th>No. positive/total no. (%) of:</th>
<th>Mean ± SEM (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>TG</td>
<td>100 ng</td>
</tr>
<tr>
<td>Male</td>
<td>70/78 (89.7)</td>
<td>82/91 (90.1)</td>
</tr>
<tr>
<td>Female</td>
<td>61/69 (88.4)</td>
<td>73/83 (88.0)</td>
</tr>
<tr>
<td>Both</td>
<td>131/147 (89.1)</td>
<td>155/174 (89.1)</td>
</tr>
</tbody>
</table>

* Twenty-seven subjects had both TG analyzed; all other subjects had only one TG available for analysis.

* Calculated for all 174 TG.

* One age was unavailable.

* Thirty PMIs were unavailable; none were greater than 96 h.
The relationship of gender to copy number was assessed on logarithmic transforms or log-log transforms. The relationship of gender to copy number as a covariate was, overall, not significant (for copies per 100 ng of TG DNA to age was not significant (P = 0.8470); HSV copy number from the whole TG was not related to age (P = 0.3181). The relationship between PMI and copy number was also not significant (for copies per 100 ng, P = 0.66; for copies per TG, P = 0.3861). Other regression analyses performed on logarithmic transforms or log-log transforms were not significant. The relationship of gender to copy number was not significant (for copies per 100 ng DNA, P = 0.499; for copies per TG, P = 0.6266). In addition, the results for analysis of covariates applied to copy number (100 ng of TG) as the dependent variable, gender as a class variable, and age as the covariate were, overall, not significant (P = 0.3159), with no significance related to age (P = 0.5426) or gender (P = 0.2671). We concluded that either the effects of the variables on each other were not significant or the sample size could not confer the statistical power to detect such small differences. As expected, there was a positive correlation between the number of copies per 100 ng of host DNA and the copy number in the TG, with a correlation coefficient of 0.86 (P < 0.0001).

Our results showed that 89.1% (155/174) of TG and 89.1% (131/147) of subjects were positive for HSV-1 DNA. Only 16 subjects and 19 TG were negative based on numerous assays and our stringent criteria. A number of studies have reported detection (present or absent) or quantification of HSV-1 DNA in human TG. We pulverized the whole TG to obtain a homogeneous tissue preparation prior to removing aliquots for DNA extraction; we determined the weights of both the TG and aliquots used and estimated the numbers of copies of HSV-1 DNA in the whole TG. Previous studies estimated HSV-1 DNA copy numbers in TG by using only a small fraction of tissue to extract DNA, making it very difficult to determine the percentage of tissue analyzed relative to the total size, the anatomical position of the piece of TG obtained, or the fact of whether the aliquot represented a uniform sample of the whole TG.

We have summarized the data from six reports focusing on the detection of HSV-1 DNA in Table 2 (1, 4, 7, 14, 17, 20). No copy numbers were reported, and most studies had small sample sizes (11 to 47). Table 3 is a summary of our current data and three other reports that have determined HSV-1 copy numbers plus demographic data on gender and age (7, 24, 28). While the three other reports examined only a small number (15 to 34) of TG, our study had the most extensive demographic data. Previous studies (14, 20) have suggested that age may influence the frequency of HSV-1 DNA in human TG and/or (10, 11) the frequency of HSV-1 DNA in the human brain. We did not identify age as a factor related to an increase in either positive subjects or HSV-1 copy numbers in the 147 subjects assessed.

Animal studies have reported that gender, animal strain, inoculation titer, routes of administration, and HSV-1 phenotype can be significant factors in pathogenesis and suggest that these characteristics play a role in the establishment of viral neural latency (3, 9, 21, 22, 23). Many of these variables cannot be assessed in human studies of HSV-1 in TG; however, the TG load based on gender can be assessed. In our study of 147 subjects, we found no association between gender and presence or absence of HSV-1 DNA or numbers of HSV-1 DNA copies.

Low PMI (2 to 4 h) has been shown to be critical for the accurate assessment of cellular components, such as RNA, enzymes, membrane integrity, and a number of other biological components (2, 13, 16). Viruses such as HSV and varicella-zoster virus can reactivate at the time of or shortly after death. The range of PMIs in our 147 subjects was 2 to 96 h, with a mean of 37.4 h. The majority of these cadavers were refrigerated (4°C) within 1 to 3 h after death. None of the subjects were reported to have had ocular or oral-facial lesions at the time of death, and none had received immunosuppressive therapy. The PMIs, from very short (2 h) to long (96 h), showed no relationship to the detection of HSV-1 DNA or to the HSV-1 DNA copy numbers.

### TABLE 2. Summary of six studies on the presence or absence of HSV-1 DNA in human TG

<table>
<thead>
<tr>
<th>Reference</th>
<th>No. of male subjects/total no. of subjects (%)</th>
<th>Gender identified</th>
<th>Male</th>
<th>Female</th>
<th>No. of positive subjects/total no. of subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>10/43 (23.3)</td>
<td></td>
<td></td>
<td></td>
<td>31/46 (67.4)</td>
</tr>
<tr>
<td>7</td>
<td>7/11 (63.6)</td>
<td></td>
<td></td>
<td></td>
<td>8/10 (80.0)</td>
</tr>
<tr>
<td>1</td>
<td>14/79 (72.5)</td>
<td></td>
<td></td>
<td></td>
<td>5/7 (71.4)</td>
</tr>
<tr>
<td>1b</td>
<td>1/24 (48.8)</td>
<td></td>
<td></td>
<td></td>
<td>15/21 (71.4)</td>
</tr>
<tr>
<td>20d</td>
<td>77/121 (63.6)</td>
<td></td>
<td></td>
<td></td>
<td>43/77 (55.8)</td>
</tr>
<tr>
<td>7b</td>
<td>12/20 (60.0)</td>
<td></td>
<td></td>
<td></td>
<td>11/20 (55.0)</td>
</tr>
</tbody>
</table>

`a` TG from formalin-fixed cadavers were used. Age and gender were unavailable for one subject.

`b` No fixative was used; subjects were directly frozen.

`c` TG from formalin-fixed cadavers were used. Age and gender were unavailable for one subject.

`d` TG were stored in 96% alcohol before DNA extraction.

`e` NA, not applicable.

<table>
<thead>
<tr>
<th>Reference</th>
<th>No. of positive TG/total no. of TG (%) in indicated group</th>
<th>No. of positive subjects/total no. of subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/24 (48.8)</td>
<td>15/21 (71.4)</td>
</tr>
<tr>
<td>20d</td>
<td>77/121 (63.6)</td>
<td>43/77 (55.8)</td>
</tr>
<tr>
<td>7b</td>
<td>12/20 (60.0)</td>
<td>11/20 (55.0)</td>
</tr>
</tbody>
</table>

11/13 females (85%); and for 90 years or more, 1/1 male (100%) and 6/7 females (88.2%). Age was unavailable for one of the females positive for HSV-1 in TG; the total number of females positive was 61/69 (88.4%). The total number of males positive for HSV-1 in TG was 70/78 (89.7%). Statistical analysis of the genders of subjects testing positive for HSV-1 DNA showed no significant statistical difference.

The mean copy numbers of HSV-1 DNA per 100 ng DNA (Table 1) for males and females were 130.9 ± 35.4 and 100.3 ± 26.9, respectively. Analysis of the data revealed no statistically significant difference in HSV-1 copy numbers between genders. Analysis of HSV-1 copy numbers in the total TG in males versus results for females revealed no statistically significant gender difference. Also, the majority of positive subjects (male and female) have low to moderate copy numbers.

The linear regression relationship of HSV copy numbers in 100 ng of TG DNA to age was not significant (P = 0.8470); HSV copy number from the whole TG was not related to age (P = 0.3181). The relationship between PMI and copy number was also not significant (for copies per 100 ng, P = 0.66; for copies per TG, P = 0.3861). Other regression analyses performed on logarithmic transforms or log-log transforms were not significant. The relationship of gender to copy number was not significant (for copies per 100 ng DNA, P = 0.499; for copies per TG, P = 0.6266). In addition, the results for analysis of covariates applied to copy number (100 ng of TG) as the dependent variable, gender as a class variable, and age as the covariate were, overall, not significant (P = 0.3159), with no significance related to age (P = 0.5426) or gender (P = 0.2671). We concluded that either the effects of the variables on each other were not significant or the sample size could not confer the statistical power to detect such small differences. As expected, there was a positive correlation between the number of copies per 100 ng of host DNA and the copy number in the TG, with a correlation coefficient of 0.86 (P < 0.0001).

Our results showed that 89.1% (155/174) of TG and 89.1% (131/147) of subjects were positive for HSV-1 DNA. Only 16 subjects and 19 TG were negative based on numerous assays and our stringent criteria. A number of studies have reported detection (present or absent) or quantification of HSV-1 DNA in human TG. We pulverized the whole TG to obtain a homogeneous tissue preparation prior to removing aliquots for DNA extraction; we determined the weights of both the TG and aliquots used and estimated the numbers of copies of HSV-1 DNA in the whole TG. Previous studies estimated HSV-1 DNA copy numbers in TG by using only a small fraction of tissue to extract DNA, making it very difficult to determine the percentage of tissue analyzed relative to the total size, the anatomical position of the piece of TG obtained, or the fact of whether the aliquot represented a uniform sample of the whole TG.
In this heterogeneous group of 147 subjects, the scattering of the data on HSV-1 copy numbers and the lack of a functional relationship among the variables led to the conclusion that there is considerable variability of HSV-1 copy numbers in the human TG and that this variability is unrelated to gender, age, or PMI. The data on age and copy numbers suggest these possibilities: (i) the viral load is constant once established or (ii) viral load increases are uniform and result in no change in copy number relative to age. We have shown in the rabbit eye model that HSV-1 DNA in the TG is a stable reservoir during latency (8). In this study, we show, using a very sensitive analytical method for evaluation, that a very large percentage of human subjects (89.1%) are positive for HSV-1 DNA in TG. The equal gender distribution, age distribution, and PMIs of our subjects provide an excellent representation of the population harboring HSV-1 DNA compared with previous studies which considered data primarily in older subjects (5, 17). These observations are consistent with the concept that HSV-1 infection and latency are widespread in divergent human populations. The lifelong presence of latent HSV-1, and the potential for its reactivation, could contribute to progressive, chronic, and neurological diseases, as well as recurrent ocular and oral herpetic diseases, since the vast majority of the population harbors latent HSV-1 DNA in their TG.

This research was made possible in part by National Eye Institute grants NIH AG02085 (J.M.H.), EY006311 (J.M.H.), EY002672 (H.E.K.), and F32EY016316 (D.M.N.) and was also supported in part by AG08017 (M.J.B.) and AG18031 (W.J.L.), a Research to Prevent Blindness Senior Scientific Investigator award (J.M.H.), Louisiana State University Health Sciences Center (LSUHSC) Core Grant for Vision Research NIH EY002377, and LSUHSC Translational Research Initiative grants (P.S.B. and D.V.). The Department of Ophthalmology has an unrestricted grant from Research to Prevent Blindness, New York, NY.

### REFERENCES


### TABLE 3. Summary of four studies on HSV-1 DNA copy numbers in human TG

| Source of reference | Male Positive Total Male Female Total Male Female Total Male Female Total |
|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                     | 78/147 (53.1)   | 131/147 (89.1)  | 155/174 (89.1)  | 82/91 (90.1)    | 73/83 (88.0)    | 116.3           |
|                     | 11/15 (73.3)    | 8/15 (53.3)     | 6/11 (54.5)     | 2/4 (50.0)      | 2902            |
|                     | 82/147 (56.5)   | 135/147 (92.6)  | 159/174 (91.1)  | 83/91 (92.0)    | 73/83 (88.0)    | 116.3           |
|                     | 11/15 (73.3)    | 8/15 (53.3)     | 6/11 (54.5)     | 2/4 (50.0)      | 2902            |
|                     | 113/147 (78.5)  | 121/147 (83.5)  | 150/174 (87.1)  | 81/91 (90.1)    | 72/83 (87.0)    | 116.3           |
|                     | 8/15 (53.3)     | 6/11 (54.5)     | 2/4 (50.0)      | 2902            |
|                     | 73/147 (50.7)   | 121/147 (83.5)  | 150/174 (87.1)  | 81/91 (90.1)    | 72/83 (87.0)    | 116.3           |

Note: NA not available.


