Comparative Replication of HSV-1 in BALB/c and C57BL/6 Mouse Embryo Fibroblasts In Vitro

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Herpes simplex virus type 1 (HSV)-host cell interactions were studied in fibroblasts from inbred mice by measuring virus replication, virus adsorption, infectious center formation, and single-cell virus production. BALB/c mouse embryo fibroblasts (MEF) produced more intracellular and extracellular virus than C57BL/6 MEF, with differences in virus production first appearing at 16 hr after inoculation. Virus yield in C57BL/6 cells peaked earlier (16 hr) and at a lower level than in BALB/c cells (20 hr). These results were explained by a difference in single-cell virus replication, rather than less efficient adsorption or the presence of cells that could not be infected. Host-related variation in the ability of infected cells to support HSV replication may account, in part, for differences in the severity of HSV ocular disease. Invest Ophthalmol Vis Sci 27:909-914, 1986

Materials and Methods

Cell culture medium: Growth medium (GM) was Eagle’s minimal essential medium (MEM) containing 5% fetal calf serum, 5% newborn calf serum and antibiotics as described previously. Maintenance medium (MM) was MEM containing 1% fetal calf serum, 1% newborn calf serum and antibiotics.

Continuous cell lines: Vero and HEp-2 cell lines were grown and maintained as previously described.

Primary mouse kidney cells: Kidneys from 6- to 8-wk-old BALB/c and C57BL/6 mice were removed, washed, and minced. Animals were handled in compliance with the ARVO Resolution on the Use of Animals in Research. Tissue fragments were rinsed in calcium and magnesium-free phosphate-buffered saline (0.01 M, pH 7.6) and suspended in 0.25% trypsin in Hanks’ salt solution. After slow stirring at 4°C overnight, cell suspensions were filtered through sterile mesh cloth, resuspended in GM (1.0 X 10^6 cells/ml) and placed in 75 cm^2 tissue culture flasks. Medium was changed every 3 to 4 days. Confluence was reached 3 to 4 days after seeding.

Primary mouse embryo fibroblasts (MEF): MEF were cultured as previously described and used at the second to fifth passage.

Mouse keratocytes: Keratocytes were cultured as previously described and used at the third passage.

Virus: Isolate 4, the herpes simplex virus type 1 used in these studies, was obtained from a patient with herpetic keratitis and produces stromal keratitis in mice. Virus stocks were prepared in HEp-2 cells and stored at −70°C. The titer was determined in vero cells. For use in experiments, stock virus was diluted in PBS.
(0.01 M, pH 7.3), containing 1% fetal calf serum and 0.1% glucose (PBS-A).

**Kinetics of HSV replication in vitro:** Confluent monolayers (0.7-1.0 × 10^6 cells/25 cm² flask) were infected with HSV at a multiplicity of five plaque-forming units (PFU)/cell. Virus was allowed to adsorb to the cells for one hour at 4°C. The inocula were then aspirated, the cell monolayers were washed twice with PBS, MM (3.0 ml) was added to each flask, and the flasks were incubated at 37°C. At intervals, extracellular and intracellular infectious virus were separated by the following procedure. To recover extracellular virus, 1.0 ml of culture supernatant fluid was removed from each flask, centrifuged (500 × g, 10 min), and the supernatant fluid was frozen at -70°C. To recover intracellular virus, the infected cells were scraped from the surface of the flask, added to the cells remaining in the centrifuge tube, and again centrifuged. The supernatant fluid was discarded. The cells were washed twice in PBS, resuspended in MM (3.0 ml), freeze-thawed, sonicated, centrifuged (500 × g, 10 min), and supernatant fluid was frozen. All samples from each experiment were thawed and assayed on vero cells at the same time.

**Relative sensitivity of cells to HSV infection:** Confluent cell monolayers were inoculated with the same stock solution of HSV and plaques were counted. The diameter of 20 individual plaques was determined microscopically using a stage micrometer slide graduated in 0.1-mm increments.

**Adsorption assay:** Confluent monolayers of mouse embryo fibroblasts were trypsinized and suspended in GM. Cell suspensions were centrifuged (500 × g, 10 min) and the cell pellets were washed twice with PBS. Cells were then counted and adjusted to a concentration of 1.0 × 10⁶/ml. Cell suspensions (1.0 ml) were transferred to small tubes, centrifuged (500 × g, 10 min), and the supernatant fluids were discarded. Cell pellets were resuspended in 1.0 ml of PBS-A containing HSV (1.0 × 10⁶ PFU/ml) and incubated at 4°C with occasional agitation. Virus incubated in tubes without cells was used to determine zero adsorption. After 1 hr, the tubes were centrifuged (500 × g, 10 min), and the amount of virus present in the supernatant fluids was determined on vero cells by plaque assay. The percent of virus adsorbed was calculated according to the following formula:

\[
\text{Adsorption (\%)} = \frac{\text{Titer of virus in tubes without cells} - \text{Titer of virus in tubes with cells}}{\text{Titer of virus in tubes without cells}} \times 100.
\]

**Infectious center assay:** MEF monolayers were infected with HSV at a multiplicity of five. After incubation (37°C, 16 hr), cells were scraped into the medium and a single cell suspension was obtained by repeated pipetting. One hundred cells in 1.0 ml were inoculated onto a vero cell monolayer and incubated at room temperature for 5 hr. One ml of MM containing 0.2% human gamma globulin (Cutter Biological, Inc.; Berkley, CA) was then added to each well and the incubation was continued for 48-72 hr (37°C, 5% CO₂). The number of infectious centers per 100 cells was determined after monolayers were stained with crystal violet (0.5% in 20% ethanol).

**Single cell virus production:** Confluent MEF monolayers were infected with HSV as above. Infected monolayers were incubated for 7 hr (37°C, 5% CO₂), then the cells were scraped into the medium. After centrifugation (500 × g, 10 min), the cells were rinsed twice with PBS and resuspended in MM at a concentration of 2 cells/ml. Aliquots of 0.2 ml were transferred to micro test tubes so that single cells would be expected to be found in two out of five tubes. Tubes were incubated for 16 hr (37°C, 5% CO₂). The virus present in each tube was quantified by plaque assay using inocula of 0.2 ml.

**Results**

**Replication of HSV in MEF monolayers:** Intracellular and extracellular virus production in BALB/c, C57BL/6, and HEp-2 cell monolayers was measured at 24 hr post-inoculation (PI). The titer of intracellular virus produced in BALB/c MEF varied between 8.7 and 19 × 10⁶ PFU/10⁶ cells; the titer of intracellular virus produced in C57BL/6 MEF was 9- to 12-fold less (0.98 to 1.6 × 10⁶ PFU/10⁶ cells) (Table 1). Quantification of extracellular virus gave similar results, with 8- to 13-fold more virus produced in BALB/c MEF than in C57BL/6 MEF. In all experiments, both intracellular and extracellular virus replication were more efficient in HEp-2 cells.

**Kinetics of replication of HSV in MEF cells:** Differences in total intracellular virus present after 24 hr of incubation were not due solely to differences in the kinetics of virus replication, since virus production was greater in BALB/c than C57BL/6 monolayers at all times after eclipse (Fig. 1). Peak intracellular virus production was more than tenfold greater in BALB/c MEF monolayers than in C57BL/6 MEF monolayers, although peak production occurred at different times (20 hr for BALB/c vs 16 hr for C57BL/6). Similar results were obtained for extracellular virus, with differences in virus production becoming evident sooner and peak production occurring at 24 hr for BALB/c and 16 hr for C57BL/6 (Fig. 2). Both intracellular and extracellular virus production in MEF monolayers was less than that in the continuous human cell line HEp-2 (Figs. 1 and 2).
Table 1. Production of herpes simplex virus by infected mouse embryo fibroblast monolayers at 24 hr postinoculation

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HEp-2</th>
<th>BALB/c</th>
<th>C57BL/6</th>
<th>P-value*</th>
<th>HEp-2</th>
<th>BALB/c</th>
<th>C57BL/6</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.20 × 10⁷†</td>
<td>8.70 × 10⁶</td>
<td>9.83 × 10⁶</td>
<td>&lt;0.01</td>
<td>9.2 × 10⁵</td>
<td>2.77 × 10⁵</td>
<td>2.13 × 10⁶</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2</td>
<td>9.00 × 10⁷</td>
<td>1.30 × 10⁷</td>
<td>1.31 × 10⁷</td>
<td>&lt;0.05</td>
<td>1.14 × 10⁷</td>
<td>9.90 × 10⁷</td>
<td>1.29 × 10⁷</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>3</td>
<td>1.04 × 10⁹</td>
<td>1.91 × 10⁷</td>
<td>1.57 × 10⁸</td>
<td>&lt;0.05</td>
<td>3.19 × 10⁷</td>
<td>2.69 × 10⁸</td>
<td>2.13 × 10⁷</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* BALB/c vs C57BL/6 (Student’s t-test). For all three experiments combined, BALB/c monolayers produced more virus than C57BL/6 monolayers (P < 0.001).

Sensitivity of cells to HSV infection: Keratocytes, kidney cells, and MEF from BALB/c and C57BL/6 mice were inoculated with the same stock virus suspension. Eight- to nine-fold more plaques were formed in BALB/c monolayers, compared to C57BL/6 monolayers for all cell types examined (Fig. 3). The average plaque diameters in BALB/c and C57BL/6 MEF were not statistically different (0.82 mm and 0.85 mm respectively; P > 0.8, Student’s t-test).

Adsorption of HSV by MEF: Host-related differences in HSV replication may be due to differences in virus adsorption by MEF.

Fig. 1. Quantification of intracellular HSV production in cultured inbred mouse cells. BALB/c and C57BL/6 MEF and HEp-2 monolayers were infected with HSV at a multiplicity of five. Intracellular virus production was measured at 0–28 hr post-inoculation. Differences in intracellular virus production by BALB/c and C57BL/6 MEF were statistically significant (Wilcoxon matched pairs signed rank test; P < 0.01).

Fig. 2. Quantification of extracellular HSV production in cultured inbred mouse cells. BALB/c and C57BL/6 MEF and HEp-2 monolayers were infected with HSV at a multiplicity of five. Extracellular virus production was measured at 0–28 hr post-inoculation. Differences in extracellular virus production by BALB/c and C57BL/6 MEF were statistically significant (Wilcoxon matched pairs signed rank test; P < 0.05).
Plaque Formation by HSV in Cultured Inbred Mouse Cells

![Plaque Formation by HSV in Cultured Inbred Mouse Cells](image)

**Fig. 3.** Plaque formation by HSV in cultured inbred mouse cells. Confluent monolayers of HEp-2 cells, BALB/c MEF, and C57BL/6 MEF were infected with HSV and plaques enumerated. More plaques were produced in BALB/c cells than in C57BL/6 cells (Student's t-test; \( P < 0.001 \) for all tissues).

adsorption or differences in post-adsorption events. No significant differences in the ability of BALB/c and C57BL/6 MEF to adsorb virus were found (Table 2).

**Infectious center assay:** Since the MEF were used during early passages (two through five), these cultures were likely to contain diverse cell types. Increased virus production by BALB/c MEF compared to C57BL/6 MEF could therefore have resulted from infection of only a fraction of the cells present, with a higher percentage of infectable cells in the BALB/c MEF monolayers than in the C57BL/6 MEF monolayers. To examine this possibility, infectious center assays were performed. Three separate experiments showed no difference in the percentage of BALB/c and C57BL/6 cells that were infected at a multiplicity of five (Table 3).

**Replication in isolated cells:** Cultures of BALB/c MEF produce more HSV after infection than do cultures of C57BL/6 MEF (Table 1). Since the cell cultures have a similar ability to adsorb virus (Table 2), and essentially all cells in a culture can be infected (Table 3), then each BALB/c cell should produce more virus than each C57BL/6 cell. This hypothesis is confirmed by the data presented in Table 4, which show that single infected BALB/c MEF produce 4- to 6-fold more virus than single infected C57BL/6 MEF.

### Discussion

The severity of herpetic ocular disease in inbred mice is dependent on both the host and virus strain. Following topical ocular inoculation with HSV, more BALB/c mice develop stromal keratitis than do similarly inoculated C57BL/6 mice.\(^1\)\(^2\) More infectious virus can be recovered from the inoculated eye, the ipsilateral trigeminal nerve, and the ipsilateral trigeminal ganglion of susceptible BALB/c mice than from corresponding tissues of resistant C57BL/6 mice.\(^3\)

Lopez reported that replication in vitro of HSV in embryo fibroblasts from susceptible (A/J and BALB/c) strains of mice was not different from replication in the resistant (C57BL/6) strain studied.\(^9\) Cook and Stevens found no significant difference in the ability of these strains to support HSV replication.

### Table 2. Adsorption of herpes simplex virus by BALB/c and C57BL/6 mouse embryo fibroblasts

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Unadsorbed</th>
<th>Adsorbed</th>
<th>Percent adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.00*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>None</td>
<td>4.22</td>
<td>6.78</td>
<td>62</td>
</tr>
<tr>
<td>HEp-2</td>
<td>3.00</td>
<td>8.00</td>
<td>73</td>
</tr>
<tr>
<td>BALB/c</td>
<td>3.37</td>
<td>7.63</td>
<td>69</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>5.20</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>BALB/c</td>
<td>1.28</td>
<td>3.90</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1.70</td>
<td>50</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>1.60</td>
<td>69</td>
</tr>
</tbody>
</table>

* PFU \( \times 10^2 \). There is no significant difference in adsorption of HSV by BALB/c and C57BL/6 fibroblasts \( P > 0.10 \); (Student's t-test).

### Table 3. Infectious center assay for BALB/c and C57BL/6 mouse embryo fibroblasts

<table>
<thead>
<tr>
<th>Experiment</th>
<th>BALB/c</th>
<th>C57BL/6</th>
<th>HEp-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>93</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>2</td>
<td>102</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>3</td>
<td>96</td>
<td>94</td>
<td>98</td>
</tr>
</tbody>
</table>

* Results are not significantly different for BALB/c and C57BL/6 (Student's t-test; \( P > 0.80 \)).

### Table 4. Herpes simplex virus production by single isolated cells infected in vitro

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mouse strain</th>
<th>P-value (BALB/c vs C57BL/6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEp-2</td>
<td>8.8* (12/16)‡</td>
<td>6.8 (5/17) 1.2 (5/16) 0.004‡</td>
</tr>
<tr>
<td>BALB/c</td>
<td>4.6 (13/24)‡</td>
<td>6.7 (13/24) 1.5 (13/24) 0.002‡</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>5.2 (6/15)‡</td>
<td>4.7 (6/15) 1.2 (6/15) 0.001‡</td>
</tr>
</tbody>
</table>

* Mean number of plaques per positive culture.
† Positive cultures per number of cultures seeded.
‡ Mann–Whitney U test.
ity of spinal ganglia from susceptible and resistant inbred mice to support HSV replication in vitro. These authors concluded that the intrinsic ability of structural cells to support virus replication plays no role in the development of disease. In contrast, we and others have shown that embryo fibroblasts and keratocytes from susceptible strains replicate more virus during a 24-hr period than do fibroblasts and keratocytes from resistant strains. The data presented (Table 1) corroborate these results.

Further experiments were performed to determine if this difference in virus produced during 24 hr was due solely to differences in the kinetics of virus replication with peak production occurring at different times and the total amount of virus recovered being influenced by virus inactivation during the incubation period. The time of peak virus production was indeed different for the two strains (Figs. 1 and 2), but the amount of virus recovered from BALB/c MEF was greater than that recovered from C57BL/6 MEF at all times. These findings confirm genetically determined differences in the ability of host cells isolated from the influence of the immune system to support HSV replication. The data are in agreement with the results of Collier et al, who found host strain-related differences in HSV replication using DBA/2 and C57BL/6 embryo fibroblasts.

The difference in the in vivo susceptibility of different mouse strains to HSV parallels the in vitro replication of HSV in structural cells from these same strains. This finding suggests that variation in the ability of structural cells to restrict HSV replication may explain, at least in part, differences in host susceptibility to HSV ocular disease. Our in vitro assays were designed to elucidate further the mechanism by which cells from resistant mouse strains interact with HSV in vitro.

Eight to nine times more plaques are produced by HSV in BALB/c MEF than in C57BL/6 MEF (Fig. 3). Not only do infected monolayers from susceptible BALB/c mice produce more virus, but also more foci of infection are established under similar conditions of inoculation. Thus, quantitative differences in virus recovery following topical ocular inoculation in vivo may be due not only to differences in the ability of cells to support virus replication, but also to differences in the number of foci of infection that are established.

Adsorption assays revealed that HSV adsorbs to C57BL/6 MEF as efficiently as to BALB/c MEF. This result suggests that restricted HSV replication in C57BL/6 MEF is not related to cell surface antigen heterogeneity or to variation in the ability of virus to bind to cell receptors.

Since early passage monolayers were used in these experiments, the cell populations in each monolayer could be heterogenous, with some cells being incapable of becoming infected with HSV. Therefore, differences in virus yield could be related to variation in the number of infectible cells present in the monolayers. The infectious center assay (Table 3) showed that virtually all cells of both strains can be infected at a multiplicity of five, indicating that the presence of uninfected cells does not account for host strain-related differences in virus production.

Since the amount of HSV adsorbed by BALB/c and C57BL/6 MEF is the same and noninfectible cells are not present, isolated BALB/c MEF should support replication of HSV better than isolated C57BL/6 MEF. Experiments measuring HSV replication in isolated individual cells (Table 4) confirm this to be the case; however, the strain-related differences seen with single cells were not as great as differences seen in MEF monolayers. Virus yield on a per cell basis was also higher in monolayers (Table 1) than in single cells (Table 4). These observations are consistent with those of other investigators.

The data indicate that differences in virus production by MEF monolayers from inbred mice are due to differences in post-adsorption events, rather than differences in the ability of the cells to adsorb virus or to differences in the proportion of infectible cells present. Therefore, host-determined restriction of virus production must involve virus penetration, uncoating, transcription, translation, and/or assembly.

Differences in the level of interferon produced by inbred mouse strains infected by Newcastle disease virus and HSV are known to exist. Although cells cultured in vitro are isolated from cellular and humoral mediators of the immune response, the cultured cells might produce interferon after infection of HSV. Interferon, which is produced by virus-infected cells, acts by the induction of antiviral protein in uninfected cells. Thus, host-determined differences in virus production by single isolated cells (Table 4) are unlikely to be due to the action of interferon.

In humans, HSV may produce a relatively benign, self-limited corneal epithelial infection or a more severe stromal keratitis. It has been hypothesized that differences in the host immune response or genetically determined virus factors account for these individual differences in the severity of ocular disease produced by HSV. Our results suggest that genetically determined differences in the ability of host cells to support HSV replication may also be important in determining the subsequent course of infection.

Key words: herpes simplex, virus, inbred mice, replication, fibroblasts, keratitis, BALB/c, C57BL/6
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References