Variation in Resistance of Cells from Inbred Strains of Mice to Herpes Simplex Virus Type 1

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(Accepted 7 March 1983)

SUMMARY

By several criteria, replication of herpes simplex virus type 1 (HSV-1) in primary cell cultures from inbred strains of mice resistant (R) to this virus was less than in cultures from susceptible (S) strains. The difference was not obviously related to less efficient adsorption, to more efficient production of interferon or to a larger number of lysosomes. Except at high multiplicities of infection, cells from the F1 progeny of a cross between an R and an S strain replicated virus as well as cells from the S parent; this contrasts with strain-related resistance to intraperitoneal inoculation in vivo, which is inherited as a dominant characteristic. It is suggested that diminished ability of structural cells to replicate HSV-1 may contribute to resistance in the intact animal.

INTRODUCTION

It is generally accepted that resistance to herpesvirus infections is multifactorial. Lopez (1975) showed that in inbred strains of mice, resistance to herpes simplex virus type 1 (HSV-1) is inherited as a dominant characteristic; breeding experiments suggested that at least two and possibly four or more genes might be involved. This implies that several phenotypic factors are operating, possibly differing considerably in their nature. A number of workers have attempted to identify such factors by comparing the responses to infection of strains of mice that vary in susceptibility to these agents.

We are investigating various components of resistance to HSV-1 in inbred mice. We have shown (Collier & Scott, 1982) that the response to injection of HSV-I into the ear relates to susceptibility as determined by the usual criterion of death following intraperitoneal (i.p.) inoculation. In resistant mice, e.g. C57BL/6, the response in terms of ear swelling and of replication of virus in the related dorsal root ganglia was significantly less than in a relatively susceptible strain such as DBA/2. The poor replication of virus at the inoculation site in C57BL/6 mice was apparent within the first 24 h and suggested the possibility (begging the question of any cell-mediated response by lymphocytes or macrophages) that the structural cells in which HSV-1 normally replicates might be inherently more resistant to infection than in a susceptible mouse strain. In the studies reported here we found this to be the case.

METHODS

Cell culture media. Eagle's modified minimum essential medium was prepared as described by Collier & Scott (1982) except that growth medium (GM) and maintenance medium (MM) contained respectively 10% and 1.5% foetal calf serum.

Adult mouse kidney cultures. Kidneys from male 6 to 8-week-old mice were finely minced with scissors, washed four or five times in phosphate-buffered saline solution A (PBS 'A') pH 7.2 (Dulbecco & Vogt, 1954) and suspended in 0.25% trypsin (Wellcome Reagents) in PBS 'A'; after stirring at 37 °C for 20 min, disaggregated cells were removed and kept at 4 °C. After two more trypsin treatments the cell suspensions were pooled, centrifuged at 1000 rev/min for 10 min, and resuspended in GM for seeding at a concentration of 10^5 cells/ml. After 4 days at 37 °C the GM was changed and incubation was continued for 3 to 4 days, by which time the monolayers were

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0022-1317/83/0000-5583 $02.00 © 1983 SGM
confluent. In these primary cultures a proportion of cells failed to adhere so that the final number of cells in such a monolayer was approximately the same as that originally seeded.

*Primary mouse embryo cells (MEC).* The limbs, tails and heads were removed from mouse embryos at the 14th to 16th day of gestation and discarded. The trunks were trypsinized as described above except that only two trypsin treatments were done and cultures were confluent and usable at 3 to 5 days. For secondary cultures, primary monolayers were grown in Nunc 80 cm² flasks (Gibco) for 3 days, washed three times in PBS 'A', stripped with 0.5 ml PBS 'A' containing 0-25% trypsin and 0-04% EDTA, resuspended in GM and seeded at a concentration of 5 × 10⁵ cells/ml.

**Growth curves of HSV-1 in primary MEC.** Confluent monolayers of embryo cells from several strains of mice were grown for 3 to 5 days in plastic Petri dishes (area 960 mm²). For each strain, the number of viable cells in a sample dish was counted in a haemocytometer after stripping them from the dish with EDTA-trypsin mixture and suspending them in 0-1% nigrosine. Counts ranged from 2-1 × 10⁶ to 3-4 × 10⁶ cells per dish. At zero time, GM was removed from the monolayers and 0-2 ml of virus suspension in MM was added to give multiplicities of infection (m.o.i.) of 0-1, 1 or 10. After 30 min at 37 °C, monolayers were washed twice in MM, 2 ml MM was added, and incubation was continued at 37 °C. At intervals, dishes were frozen at -70 °C and kept until the end of the observation period when they were thawed, again frozen and thawed twice, and titrated for infectivity by plaque counts in Vero cells; for each time point, the mean titre of four replicate monolayers was determined.

**Virus strains.** HSV strain SC-16 (Hill et al., 1975) was grown and titrated as described by Collier & Scott (1982) except that the adsorption period was 30 min and plaque counts are the means of those in four replicate wells. The Sendai virus was an egg-adapted strain originally isolated from mice (Fukumi et al., 1954).

*Mice.* Inbred strains were obtained as described by Collier & Scott (1982). F1 crosses from C57BL/6 and DBA/2 mice were bred at The London Hospital Medical College. For these experiments we used strains of mice whose susceptibility to HSV-1 had been characterized by determination of the LD₅₀ after i.p. inoculation (Collier & Scott, 1982). For most experiments we used the resistant (R) strain C57BL/6 (LD₅₀ > 10⁶.3 p.f.u.) and the susceptible (S) strains, A/J (LD₅₀ 10⁻³ p.f.u.) and DBA/2 (LD₅₀ 10⁻¹ p.f.u.). In addition, strains C57BL/10 (R) (LD₅₀ > 10⁻³ p.f.u.) and CBA/H (S) (LD₅₀ 10⁻⁵ p.f.u.) were employed in an immunofluorescence experiment; the latter strain was also used in the experiment on polypeptide synthesis mentioned in the Discussion.

**Immunofluorescence.** Staining with rabbit anti-HSV-1 serum by the indirect method was done as described by Collier & Scott (1982) except that the serum was used at a dilution of 1/60 and the anti-rabbit conjugate at 1/80. The wells of sterile 10-well multitest slides (Flow Laboratories) were each seeded with 0-1 ml of cell suspension in GM containing 10⁵ cells and incubated at 37 °C for 3 days in a humidified atmosphere containing 5% CO₂. The GM was removed and virus suspended in 0.05 ml MM was added at an m.o.i. of approx. 1. Incubation at 37 °C was continued and at appropriate intervals slides were removed, washed twice in PBS 'A', fixed in methanol at 4 °C, dried and stored at -20 °C until stained. Uninfected control slides were prepared similarly.

**Interferon assays.** Culture fluids were assayed for interferon-like activity by a plaque-reduction test in secondary cultures of strain A/J (S) mouse embryo fibroblasts grown in 25-well Sterilin culture plates. GM was replaced with threefold dilutions of the test fluids (five wells/dilution) or undiluted control fluids in 1 ml volumes. After overnight incubation at 37 °C, monolayers were infected with 0-1 ml of a dilution of HSV-1 calculated to form about 50 plaques/well. After adsorption at 37 °C for 60 min, 1 ml MM was added to each well and plaques were counted after 48 h at 37 °C. Appropriate controls for virus infectivity were included. Results are expressed as the dilution, obtained when necessary by interpolation, needed to reduce the plaque count to 50% of that in the control wells.

**Lysosome staining.** Semi-confluent monolayers of MEC from strains DBA/2 (S) and C57BL/6 (R) were stained by the lead nitrate method for acid phosphatase (modified from Gomori, 1950).

**RESULTs**

*Growth of HSV-1 in mouse cells*

**Adult primary kidney cells**

A suspension of HSV-1 was titrated in parallel by the 50% endpoint method in monolayers of primary kidney cells from DBA/2 (S) or C57BL/6 (R) mice. Titrations were done in 100 mm² wells of 25-well Sterilin culture plates (five wells per tenfold dilution); any well showing microplaques or more extensive cytopathic effect after 3 days at 37 °C was counted as positive. Titres were calculated in terms of 50%, tissue culture infective doses (TCD₅₀) by the method of Thompson (1947). In two similar experiments, the titre in DBA/2 (S) cells was 10⁻⁸ TCD₅₀/ml on both occasions, whereas in C57BL/6 (R) cells the 50% endpoints were respectively 10⁻⁸ and 10⁻⁹-fold lower. These experiments were hindered by the difficulty of obtaining strictly comparable monolayers from the two strains of mice: in other experiments not reported here the
Fig. 1. Growth curves of HSV-1 in primary MEC from C57BL/6 (○), DBA/2 (□) and C57BL/6 × DBA/2 (△) infected at m.o.i.s of (a) 10, (b) 1 and (c) 0·1. Vertical bars indicate ± 1 standard deviation (S.D.) of the mean yield from four monolayers.
titre was always higher in DBA/2 (S) cells, but for some reason these often did not grow as well as those from C57BL/6 (R), possibly because the increased cytopathic effect was enhanced by suboptimal growth. We accordingly tried primary MEC cultures from both strains of mice; these grew much more regularly and provided comparable monolayers suitable for one-step growth curve experiments.

Growth curves of HSV-1 in primary MEC

Fig. 1 shows an experiment in which growth were measured at m.o.i.s of 0-1, 1 and 10; at each m.o.i. there were pronounced differences between the yields of virus from R and S cells, which became apparent between 12 and 18 h after adsorption. By 24 h, R cells yielded about 10 times less virus than S cells. At m.o.i.s of 0-1 and 1, the yields from cells of the F1 cross between DBA/2 (S) and C57BL/6 (R) mice closely approximated to those from the S parent strain, but at an m.o.i. of 10 they were intermediate between those from the R and S mice. In two other experiments at an m.o.i. of 10, cells from the F1 cross behaved more like those from the R parental strain (results not shown).

Determination of TCD50 for primary MEC

The infectivity of HSV-1 was tested in parallel in embryo cells from three strains and one F1 cross. The method was similar to that used for adult kidney cell cultures, except that monolayers were prepared in flat-bottomed 96-well microtitre plates, each well being seeded with 10^5 cells. After 3 days at 37 °C, GM was removed and replaced by twofold dilutions of virus, eight wells/dilution. Fig. 2 shows that the titres in C57BL/6 (R) embryo cells were about fivefold less than in cells from DBA/2 (S) and about sixfold less than in those from A/J (S). The TCD50 for C57BL/6 × DBA/2 (F1) was similar to those of the S strains.

Adsorption of HSV-1 to primary MEC

Confluent monolayers containing 1.7 × 10^6 to 2.4 × 10^6 embryo cells were prepared in 12-well (4.5 cm²) Linbro culture plates (Flow Laboratories). After washing in MM, monolayers were infected with 0.1 ml vol. of HSV-1 suspension containing 10^3 p.f.u. (m.o.i. approx. 0.0005). Preliminary experiments showed that at 37 °C HSV-1 loses up to 75% of its infectivity in 2 h. To avoid heat inactivation, virus was allowed to adsorb at 4 °C. At intervals, the inocula were removed from four wells for each strain and retained. Each monolayer was washed twice with
Resistance of mouse cells to HSV-1

0.5 ml MM; the washings were pooled with the inocula and titrated for infectivity in Vero cells. After the second washing, 1 ml MM was added, and the monolayers were returned to the 37 °C incubator for subsequent plaque counting.

Fig. 3 (a) shows that, as judged by disappearance of infectivity from the inocula, adsorption rates were similar in the embryo cells of both the R and S strains. The titre of virus in control wells without monolayers (not shown) remained constant throughout the 2 h observation period, confirming that none of the loss of infectivity was due to thermal inactivation.

**Efficiency of plaque formation**

HSV-1 does not form plaques in primary MEC under media containing carboxymethylcellulose or anti-herpes virus serum, although small plaques do develop in the absence of these substances. Monolayers were therefore incubated under MM alone, and the plaques were counted 40 h later by low-power microscopy.
Table 1. Production of interferon by primary MEC from susceptible and resistant strains of mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 2</th>
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<tbody>
<tr>
<td>A/J (S)</td>
<td>ND*</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>DBA/2 (S)</td>
<td>325</td>
<td>1500</td>
<td>100</td>
</tr>
<tr>
<td>C57BL/6 (R)</td>
<td>162</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>C57BL/6 x DBA/2 (F1)</td>
<td>ND</td>
<td>80</td>
<td>500</td>
</tr>
</tbody>
</table>

* ND, Not done.

Fig. 3(b) shows the results of plaque counts on monolayers of primary MEC from DBA/2 (S) and C57BL/6 (R) and their F1 offspring. At zero time a small difference between cultures was already apparent, and by 90 min there were almost 2.5 times the number of plaques in the DBA/2 (S) monolayer as there were in the C57BL/6 (R) culture. The F1 cross cells behaved more like those of the susceptible strain throughout the experiment.

Immunofluorescence staining

In monolayers of CBA/H (S), C57BL/6 (R) and C57BL/10 (R) primary MEC stained at 3-h intervals after infection, specific fluorescence was first visible as a fine stippling in the cytoplasm at 6 h, by which time antigen was present in about 10% of the cells. This proportion rose to about 50% at 9 h, and by 12 h all the cells showed brilliant cytoplasmic and nuclear fluorescence. There was no obvious difference between the three types of cell in terms of rate of appearance or degree of fluorescence.

Interferon induction in MEC

We tested the possibility that the decreased susceptibility to HSV-1 of embryo cells from R mice might be explained in terms of ability to produce large amounts of interferon. Monolayers of C57BL/6 (R), A/J (S), DBA/2 (S) and C57BL/6 x DBA/2 embryo cells were grown in Nunc culture flasks (25 cm²) and treated either with 1 ml of Sendai virus with a haemagglutination titre of 1/1600 or with 10⁵ p.f.u. of HSV-1 in 1 ml for 60 min at 37 °C. To each monolayer, 7 ml MM was then added; untreated cultures served as controls. After overnight incubation at 37 °C, fluids were removed. The pH was brought to 2 with 0.1 M-HCl and fluids were kept at 4 °C for 5 days after which the pH was adjusted to 7 with 1 M-NaOH. Fluids were titrated for interferon-like activity as described in Methods. Table 1 shows that titres of interferon-like activity induced by HSV-1 or Sendai virus bore no relation to resistance.

Lysosome staining

In preparations stained by the Gomori (1950) method for acid phosphatase there was no obvious difference between the numbers of lysosomes in primary MEC from DBA/2 (S) and C57BL/6 (R) mice.

Discussion

We have shown by a number of criteria that the SC-16 strain of HSV-1 does not replicate as well in structural cells from strains of mice that are resistant (R) to i.p. inoculation of the virus as in those from comparatively susceptible (S) strains. Visual assessment of immunofluorescence-stained monolayers revealed no obvious differences in formation of virus antigen. In a preliminary experiment, however, polyacrylamide gel electrophoresis showed that, 8 h after infection, synthesis of virus-specified polypeptides was two to three times greater in DBA/2 (S) embryo cells than in C57BL/6 (R) cells; the degree of synthesis in CBA/H (S) embryo cells was intermediate, but nearer to that of DBA/2 (S) cells (L. H. Collier et al., unpublished data).

Lopez (1975) stated, without giving figures, that HSV-1 replicated to the same titre in embryo fibroblasts from S strains (A/J, BALB/c) and from an R strain (C57BL/6); he therefore
discounted this factor as an element in the resistance of certain inbred strains. Our results agree with those of Harnett & Shellam (1982) who, in a study primarily devoted to mouse cytomegalovirus, also showed that the TCD50 of HSV-1 was higher in embryo fibroblasts (4 to 8 passages) from susceptible (CBA, BALB/c) than from resistant (C57BL/6) mice. No results for F1 crosses were reported.

With regard to non-structural cells, i.e. leukocytes, Kirchner et al. (1976) reported that HSV-1 replicated in spleen cells from several S strains of mice but only after stimulation with lipopolysaccharide or poly(deoxyinosinic–deoxycytidylic) acid. Such treatments did not, however, permit replication in R (C57BL/6) cells. Lopez & Dudas (1979) reported that unstimulated peritoneal macrophages (PM) or those stimulated with proteose–peptone or thioglycollate would not support HSV-1 replication until they had been kept for 3 to 7 days in culture. Even after this treatment, however, PM from C57BL/6 mice consistently failed to replicate the virus as effectively as those from S strains.

The reason why cells from R mice are inherently more resistant to infection than those from S mice remains unclear and continues to be investigated. The studies reported here show that the deficiency in replication is not due to less efficient adsorption or to more efficient production of interferon. By contrast, Zawatzky et al. (1981) found that 8 h after i.p. injection of a large dose of HSV-1, titres of interferon were high in the sera of C57BL/6 (R) mice and at least tenfold lower in DBA/2 (S) mice; at lower doses of virus, interferon was not detectable in either strain. In vitro, spleen cells from R mice stimulated with inactivated HSV-1 produced more interferon than those from S mice.

Underwood & Greenham (1972) found that HSV (type not specified) grew better in the HEp-2 human cell line than in the L929 mouse cell line; poor replication in the latter was not related to adsorption rate or to interferon production, but may have been due to the greater number of lysosomes in the mouse cells (Underwood, 1972a) causing rapid cytolysis with consequent production of defective virions (Underwood, 1972b). Experiments with lysosome inhibitors supported this notion (Underwood, 1972c). We found no obvious difference between R and S MEC in terms of lysosome content.

Whatever the factor (or factors) diminishing the susceptibility to HSV-1 of cells from R mice, it is important to consider the question of its genetic control. We found that cells from F1 embryos of C57BL/6 (R) × DBA/2 (S) mice behaved like those from the susceptible parent, with one exception: yields of virus from MEC of the F1 cross infected at an m.o.i. of 10 were either similar to those from the C57BL/6 (R) mice or were intermediate between those from the cells of the resistant and susceptible parents. It is difficult to account for this observation without further investigation, but one explanation may be that defective interfering particles are produced by the cells of C57BL/6 (R) mice at any m.o.i., whereas the F1 cells only do so when infected at a high m.o.i.

The relative susceptibility of the F1 primary MEC to infection with HSV-1 is interesting since resistance to HSV-1 is inherited by the intact animal as a dominant characteristic (Lopez, 1975; Kirchner et al., 1976). Nevertheless, our findings with embryo fibroblasts are in accord with observations by Kirchner et al., (1976) and Lopez & Dudas (1979) in their respective experiments with spleen cells and macrophages from F1 progeny of R and S mice. These results imply that the genetic control of cell susceptibility in vitro differs in some respect from that governing resistance of the intact animal to i.p. inoculation. From these considerations, Lopez & Dudas (1979) implied that whatever determines the response to infection of cells in vitro plays no significant role in genetically determined resistance of adult mice, but this may not be true. We tend to think of resistance to HSV-1 in terms of an all-or-nothing response to an arbitrarily determined large dose, e.g. 10⁶ p.f.u. given by the i.p. route. Nevertheless, there is in inbred mouse strains a spectrum of resistance varying from very low (LD50 10 p.f.u.) to very high (LD50 > 10⁶ p.f.u.), and even in highly resistant strains some individuals succumb to very large doses. We suggest that ‘resistance’ defined in this way may be mainly due to a genetically dominant major component, possibly involving some aspect of cell-mediated immunity, and other factors that, although less decisive, still contribute to the degree of resistance or susceptibility in vitro. One such factor might be the inherent differences in cell susceptibility described in this paper; if
such susceptibility were inherited as a dominant trait in susceptible mice, it might well be detectable in vitro in the way we have described, but be to some extent obscured by the operation of more powerful components of the immune response in the intact 'resistant' animal.

We are most grateful to Dr J. Oxford (National Institute for Biological Standards and Control) for undertaking the polyacrylamide gel electrophoresis, to Miss Lynn Bonnett for her excellent technical assistance and to Dr Mary Anderson (King's College Hospital Medical School) for the Sendai virus. We also thank the Lister Institute of Preventive Medicine for the grant that supported this work. During her participation in this investigation, Alessandra Pani was in receipt of a scholarship from the Consiglio Nazionale delle Ricerche, Italy.

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Journal of Hygiene 89, 335–345.


(Received 6 January 1983)