Host Genetic Control of Mouse Hepatitis Virus Type 4 (JHM Strain) Replication. I. Restriction of Virus Amplification and Spread in Macrophages from Resistant Mice

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SUMMARY

Peritoneal macrophages were used to analyse host genetic control of mouse hepatitis virus type 4 (MHV-4) infection. Both infectious centre and immunofluorescence assays indicated that only a subset of macrophages from either susceptible (BALB, C3H or C57BL/6J) or resistant (SJL/J) mice were initially infected with MHV-4 during the first cycle of infection. However, compared to macrophages from susceptible mice, three- to sixfold fewer SJL/J macrophages were infected, and there was no amplification of virus replication by involvement of adjacent cells during the second cycle of infection. Treatment of macrophages from susceptible mice with interferon beta could not duplicate the aborted second cycle of infection that occurred in macrophages from resistant mice.

INTRODUCTION

Resistance to viral infection may be regulated by host defence mechanisms at the level of virus–cell interaction or the immune system. The resistance of mice to infection by a particular virus may be a manifestation of lack of a receptor for virus attachment, inappropriate intracellular processing and replication, or failure of virus assembly or release, and may reflect genetically controlled responses (for reviews, see Brinton & Nathanson, 1981; Brinton et al., 1983). Furthermore, lymphokines, such as interferon, influence the replication of numerous viruses (De Maeyer & De Maeyer-Guignard, 1979; Haller et al., 1981), including a strain of murine coronavirus (Virelizier, 1981).

Mouse hepatitis virus type 4 (MHV-4, JHM strain), a member of the coronavirus family, is attracting increasing interest as an agent of both virus-induced encephalomyelitis and virus-induced demyelination (Bailey et al., 1949; Waksman & Adams, 1962; Lampert et al., 1973; Weiner, 1973; ter Meulen et al., 1981; Knobler et al., 1981, 1982). Resistance to MHV-4-induced fatal central nervous system disease is inherited as a single autosomal recessive trait, independent of the major histocompatibility complex, expressed at the level of the neuronal cell (Knobler et al., 1981). Resistance is mirrored by peritoneal macrophages elicited from SJL/J mice (Stohlman & Frelinger, 1981; Knobler et al., 1981).

Since macrophages are readily obtained, handled and manipulated in culture, we analysed MHV-4 replication in these cells as a guide towards understanding their response to infection. We learned that only a subset of macrophages from both susceptible and resistant mice are infected during the first cycle of replication, and that there is restriction of virus amplification limiting the spread of infection in macrophages from resistant mice, which cannot be duplicated by interferon treatment of macrophages from susceptible mice.

METHODS

Virus. A stock of MHV-4 initially provided by Dr L. Weiner (Departments of Neurology and Microbiology, University of Southern California, Los Angeles, Ca., U.S.A.) was plaque-purified three times and grown as a
stock, containing $5 \times 10^3$ p.f.u./ml, in L-24-1 cells. The handling of virus, culture and plaque assay conditions have been reported elsewhere (Haspel et al., 1978; Knobler et al., 1981). Vesicular stomatitis virus (VSV), Indiana strain, was titrated by plaque assay on L-929 cells (Casali et al., 1981).

Mice. Inbred and F1 mice strains were obtained from the breeding facility of the Scripps Clinic and Research Foundation vivarium (BALB/cSt, BALB/Wehi, C3H/St, C57BL/6J, SJL/J, BALB/Wehi × SJL/J, and SJL/J × BALB/Wehi). Male mice, 4 to 6 weeks of age, were used as the source of macrophages in these experiments.

Resident macrophages. These cells were obtained from the peritoneal cavities of mice by lavage while the animal was under ether anaesthesia. These animals had not previously been injected intraperitoneally with any reagents. The peritoneal cavity was lavaged with 5 to 6 ml of sterile Eagle's MEM which contained 20 units/ml heparin, 1% glutamine, 100 μg/ml penicillin, and streptomycin. Cells were plated to provide $1 \times 10^6$ adherent cells/dish as reported previously (Brautigam et al., 1979; Knobler et al., 1981), and identified as macrophages by their ability to ingest zymosan particles or sheep erythrocytes previously reacted with antibody and C5-deficient mouse serum, and by their morphology (van Furth et al., 1978). Homogeneity was usually > 95% with a range of 95 to 99%.

Elicited peritoneal cells. Macrophages were also obtained from the peritoneal cavities of mice inoculated intraperitoneally with 2 ml of 38% thioglycollate broth (Brewer's modified thioglycollate; Becton, Dickinson & Co., Cockeysville, Md., U.S.A.). Peritoneal exudate cells were harvested from animals anaesthetized with ether 5 days later by peritoneal lavage and identified as macrophages as described above.

Cultured cells. The tissue culture MC57 line was used, and is a transformed macrophage line derived from C57BL/6J mice. The L-24-1 line has been used for growing and assaying MHV-4 (Haspel et al., 1978), and the L-929 line was used for assaying interferon activity.

Infectious centre assay. Peritoneal exudate cells ($1 \times 10^6$) were incubated in suspension with MHV-4 (m.o.i. of 2.5) on a tube rotator (Becton, Dickinson & Co.), at 4°C for 60 min. The cells were centrifuge-washed five times with medium. Tenfold dilutions of the cells, in 200 μl aliquots, were added to 60 mm plastic tissue culture plates (Falcon), and subsequently handled as reported by Brautigam et al. (1979).

Immunofluorescent labelling of MHV antigens. Cultured cells on glass coverslips were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M-phosphate buffer pH 7.3, washed in phosphate-buffered saline (PBS), and incubated with either polyclonal mouse antibody to mouse hepatitis virus (provided by the Resource Branch of the National Cancer Institute, Bethesda, Md., U.S.A.) or monoclonal antibodies to MHV-4 (Collins et al., 1981) for 30 min. The coverslips were washed three times in PBS and incubated with the F(ab)$_2$ fragment of rabbit antibody to mouse IgG, conjugated with fluorescein isothiocyanate or peroxidase (Cappel Laboratories, Cochranville, Pa., U.S.A.) for 30 min, as reported by Knobler et al. (1981). All reactions took place at room temperature. The monoclonal and polyclonal reagents gave equivalent results. Labelled cells were observed through a Zeiss photomicroscope with epifluorescence.

Interferon determinations. Interferon was assayed by a VSV plaque-reduction assay on L-929 cells, with serial twofold dilutions of each sample assayed in duplicate. Samples were centrifuged at 100000 g for 1 h to remove viral particles. For these experiments, a dilution of virus providing 40 to 50 plaques was used. Control plates were preincubated with a known dilution of an interferon standard (G002-904-511; kindly provided by the National Institute of Allergy and Infectious Diseases, Bethesda) that reduced the number of VSV plaques by 50%, i.e. 1 International Unit (IU) of interferon. The sensitivity of this assay is 1 IU/ml. Poly(I):poly(C), an interferon inducer, was purchased from P-L Biochemicals, and purified mouse fibroblast (β) interferon was purchased from Lee Biomolecular, La Jolla, Ca., U.S.A.

RESULTS

MHV-4 replication in macrophages correlates with host susceptibility

MHV-4 replication was similar in thioglycollate-stimulated or -unstimulated (resident) macrophages obtained from mice susceptible to MHV-4 infection in vivo (BALB/cSt, C3H/St, C57BL/6J). Thioglycollate-elicited macrophages were used in subsequent experiments. After MHV-4 infection at an m.o.i. of 0.1, virus was first detected within 10 to 12 h ($10^3$ to $10^4$ p.f.u./ml of culture fluid), peaked in 24 to 36 h ($10^5$ to $10^6$ p.f.u./ml of culture fluid), and then rapidly declined. Infected cells underwent cytopathic effects by fusing to form syncytiain which MHV antigens could be demonstrated. In contrast, macrophages derived from SJL/J mice released less than 10 p.f.u./ml of virus into culture fluids after infection at an m.o.i. of 0.1; they contained less than 10 p.f.u./ml of cell-associated MHV-4, and did not develop cytopathic effects.

Macrophages from F1 hybrid mice between susceptible BALB/Wehi and resistant SJL/J mice were productively infected with MHV-4, consistent with inheritance of resistance as an autosomal recessive trait (Knobler et al., 1981). F1 macrophages usually yielded 1 to 2 log$_{10}$ units more virus than the permissive parent.
Host control of MHV-4 replication

Table 1. Macrophage infectious centres after infection with MHV-4*

<table>
<thead>
<tr>
<th>No. of cells plated</th>
<th>BALB/cSt</th>
<th>SJL/J</th>
<th>L-24-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \times 10^5$</td>
<td>TNTC†</td>
<td>TNTC</td>
<td>TNTC</td>
</tr>
<tr>
<td>$2 \times 10^4$</td>
<td>TNTC</td>
<td>36</td>
<td>106</td>
</tr>
<tr>
<td>$2 \times 10^3$</td>
<td>45</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>$2 \times 10^2$</td>
<td>6</td>
<td>Nil</td>
<td>3</td>
</tr>
</tbody>
</table>

* Macrophages ($1 \times 10^6$) were infected with MHV-4, m.o.i. 2.5, in a 5 ml suspension. Cells were centrifuge-washed with medium five times, and resuspended in 1 ml of medium, from which tenfold dilutions were made. Aliquots of 200 μl were plated; the cells were allowed to adhere, then overlaid with L-24-1 cells and agarose. Duplicate experiments showed similar results. Control BALB/cSt, SJL/J and L-24-1 cells were infected and then frozen and thawed twice before being overlaid with L-24-1 cells and agarose; these showed no infectious centres. 
† TNTC, Too numerous to count; approximately 100 infectious centres can be reliably counted.

Table 2. Number of antigen-positive macrophages after infection with MHV-4*

<table>
<thead>
<tr>
<th>Strain</th>
<th>6 h</th>
<th>18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/cSt</td>
<td>6 (1.2%)</td>
<td>364 (72.8%)</td>
</tr>
<tr>
<td>C3H/St</td>
<td>9 (1.8%)</td>
<td>400 (80%)</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>6 (1.2%)</td>
<td>214 (42.8%)</td>
</tr>
<tr>
<td>SJL/J</td>
<td>2 (0.4%)</td>
<td>5 (1%)</td>
</tr>
</tbody>
</table>

* Macrophages ($1 \times 10^6$) were infected with MHV-4, m.o.i. 1. Numbers represent cells containing MHV-4 antigen out of 500 cells counted at 6 h and at 18 h after infection, with percentages in parentheses. Data were similar in triplicate experiments.

Only a subset of macrophages are infected with MHV-4 during the first cycle of infection

The SJL/J macrophage cultures, infected at an m.o.i. of 1, yielded less than 10 p.f.u./ml (the limit of detection) of MHV-4 by standard plaque assay at 24 h post-infection. In a more sensitive bioassay, 0.03 ml of this fluid, but not tenfold or greater dilutions, contained sufficient virus to kill all of ten 2-week-old suckling BALB/cSt mice when injected intracerebrally. This did not result from carry-over of the virus inoculum, since 0.03 ml aliquots of the third wash from macrophages after virus adsorption did not kill any of ten suckling BALB/cSt mice. Thus, SJL/J macrophages support limited MHV-4 replication, and yield low levels of infectious virus.

To determine the number of macrophages infected during the first cycle of infection, an infectious centre assay was performed. Fewer than 3% of the cells from either strain were productively infected during the first cycle, with sixfold fewer SJL/J macrophages than BALB/cSt macrophages productively infected (Table 1). Similar findings were obtained by counting virus antigen-positive cells. In this assay, fewer than 2% of the cells from either strain contained viral antigen during the first cycle of infection (6 h post-infection), and threefold fewer SJL/J macrophages than BALB/cSt macrophages expressed viral antigens (Table 2). During the second cycle of virus replication (18 h post-infection), the number of antigen-positive SJL/J macrophages remained low, while the number of BALB/cSt macrophages expressing viral antigens increased to more than 70% (Table 2). These results demonstrate that only a small subset of macrophages from both susceptible and resistant mice are initially infected during the first cycle of infection, and in resistant SJL/J macrophages both virus amplification and spread of infection is limited in the second cycle of infection.

Restriction of MHV-4 amplification and spread in SJL macrophages is neither associated with detectable interferon nor duplicated by interferon administration

Since interferon can play a role in regulating the spread of virus, the role of interferon in regulating MHV-4 infection was evaluated. Culture fluids, collected 24 h after MHV-4 infection of BALB/cSt or SJL/J macrophages at an m.o.i. of 0.1, were assayed for interferon, which was not detectable at a 1:2 dilution of these culture fluids. However, uninfected BALB/cSt and SJL/J macrophages yielded measurable levels of interferon, when incubated with 150μg
### Table 3. Virus released from macrophages at 24 h post-infection with VSV Indiana or MHV-4 from interferon- or poly(I)-poly(C)-treated cells*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Macrophage source</th>
<th>Treatment</th>
<th>C.p.e.</th>
<th>P.f.u./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV+</td>
<td>BALB/cSt</td>
<td>No IFN</td>
<td>++</td>
<td>3-5 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 IU IFN</td>
<td>+</td>
<td>1 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 IU IFN</td>
<td>±</td>
<td>1 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 IU IFN</td>
<td>±</td>
<td>1 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000 IU IFN</td>
<td>±</td>
<td>2 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(I)-poly(C)</td>
<td>±</td>
<td>4 x 10^4</td>
</tr>
<tr>
<td>VSV†</td>
<td>SJL/J</td>
<td>No IFN</td>
<td>++</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 IU IFN</td>
<td>+</td>
<td>2.5 x 10^1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 IU IFN</td>
<td>±</td>
<td>2.5 x 10^1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 IU IFN</td>
<td>±</td>
<td>1 x 10^1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000 IU IFN</td>
<td>±</td>
<td>1 x 10^1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(I)-poly(C)</td>
<td>±</td>
<td>2.5 x 10^1</td>
</tr>
<tr>
<td>MHV-4†</td>
<td>BALB/cSt</td>
<td>No IFN</td>
<td>++++</td>
<td>3.0 x 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 IU IFN</td>
<td>++++</td>
<td>9.75 x 10^5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 IU IFN</td>
<td>++++</td>
<td>2.2 x 10^5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 IU IFN</td>
<td>++++</td>
<td>1.6 x 10^5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000 IU IFN</td>
<td>++++</td>
<td>3.6 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(I)-poly(C)</td>
<td>++++</td>
<td>9.5 x 10^4</td>
</tr>
</tbody>
</table>

* Macrophages (1 x 10^6) were incubated for 18 h with several concentrations of purified mouse fibroblast (β) interferon (IFN) or 150 μg poly(I)-poly(C), then infected with either VSV Indiana or MHV-4, m.o.i. = 0.1. C.p.e., cytopathic effect.
† These are the results of one of three experiments which showed similar results.

poly(I)-poly(C), a known inducer of interferon (Merigan & Finkelstein, 1968) for 18 h, and reduced the number of VSV plaques by 50%. This was not due to the transfer of inducer with the culture fluids tested, because the poly(I)-poly(C) was inactivated by exposure to RNase before testing. Thus, macrophage culture fluids do not contain detectable interferon following infection with MHV-4, although interferon is inducible in uninfected macrophages upon exposure to poly(I)-poly(C).

BALB/cSt or SJL/J macrophages show cytolytic effects of infection with VSV Indiana (m.o.i. 0.1), and produce 4 to 5 log_{10} units of virus in a 24 h period. Either mouse fibroblast (β) interferon or prior incubation with 150 μg of poly(I)-poly(C) reduced the yield of VSV by 3 to 4 log_{10} units (Table 3) and protects these cells from the spread of the cytolytic infection. In contrast, despite incubation with up to 2000 IU/ml of interferon or 150 μg of poly(I)-poly(C), BALB/cSt macrophages produced significant yields of MHV-4 (10^4 p.f.u./ml) (Table 3), and formed syncytia. Thus, interferon has little effect on limiting the replication of MHV-4.

**DISCUSSION**

In this report, we provide several new findings regarding host genetic control of MHV-4 susceptibility. First, only a small subset within the macrophage population from both susceptible and resistant mice is initially permissive to MHV-4 replication (Table 1). Thus, MHV-4 infection defines a unique small subset of macrophages, which have only recently been appreciated as a heterogeneous population with regard to a variety of parameters (Booss, 1980; Lee, 1980; Miller et al., 1982). Second, MHV-4 replication in resistant SJL/J macrophages is restricted by limitation of virus amplification and lack of involvement of adjacent cells in the formation of syncytia in the second cycle of infection (Table 2). Third, the limitation of virus amplification and spread in SJL/J macrophages cannot be duplicated by interferon treatment of susceptible BALB/c macrophages (Table 3).

The molecular basis of susceptibility or resistance to MHV-4 is presently not understood. However, three- to sixfold fewer resistant SJL/J macrophages are infected during the first cycle of infection compared to permissive BALB/cSt macrophages (Tables 1, 2). Both reduced virus replication and limited syncytium formation had been found in SJL/J primary glial cell cultures.
infected with MHV-4 (Collins et al., 1983). Cell–cell fusion and spread of infection is a function of the interaction of cell surface and MHV-4 E-2 glycoprotein (Collins et al., 1982). The limitation of infection during the first cycle and subsequent restriction of virus amplification in the second cycle of infection in SJL/J macrophages may reflect a genetically controlled processing defect of the E-2 glycoprotein by resistant SJL/J cells. Lack of an active proteolytic enzyme, necessary for cleavage of the E-2 glycoprotein and fusion (Sturman & Holmes, 1984), or another processing molecule are potential mechanisms of genetic resistance to virus infection (Brinton & Nathanson, 1981; Brinton et al., 1983).

Resistance to murine coronavirus MHV-2, MHV-3 and MHV-4 is determined by a single host gene inherited as an autosomal recessive trait. C3H mice are resistant to MHV-2 (Bang & Warwick, 1960), A/J mice are resistant to MHV-3 (Virelizier & Allison, 1976) and SJL/J mice are resistant to MHV-4 (Stohlman & Frelinger, 1978; Knobler et al., 1981). Interferon has been implicated in the limitation of MHV-2 replication (Bang, 1981) and protects in vivo and in vitro against MHV-3 infection (Virelizier et al., 1977; Virelizier, 1981; Arnheiter & Haller, 1981; Schindler et al., 1982; Sorensen et al., 1982). Unlike MHV-2 and MHV-3, MHV-4 neither induces interferon (Stohlman et al., 1978) nor can purified exogenous mouse interferon protect macrophages from MHV-4 infection (Table 3), although similarly treated macrophages were protected from VSV. The present study indicates that resistance to MHV-4 in SJL macrophages operates through restriction of virus amplification and spread by a presently unidentified mechanism. Current studies using newly available hybrid inbred strains such as the BALB/c × SJL/J (CXJ) strains and SWR/J × SJL/J (SWXJ) strains, should provide definitive linkage information for localizing the gene determining resistance to MHV-4 and the possibility of identifying the unique gene product regulating MHV-4 infection.

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