Influence of Type and Concentration of Sera in vitro on Susceptibility of Genetically Resistant Cells to Mouse Hepatitis Virus

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SUMMARY

The type and concentration of sera used to support macrophages was found to influence the in vitro destruction of macrophages from genetically resistant C3H mice by the PRINCETON strain of mouse hepatitis virus, and the accompanying conversion of mouse hepatitis virus (PRINCETON) to the variant virus, mouse hepatitis virus (C3H). Cells incubated in 20% horse serum were more susceptible to destruction than those in 90% horse serum. Susceptibility was greatest in the presence of 10% foetal calf serum, while cells in 10% mouse sera were the most resistant. Differences in susceptibility were as great as 10,000 TCD 50. The sera had no direct effect upon either mouse hepatitis virus (PRINCETON) inoculum virus or the released variant virus, but appeared to influence intracellular adaptation of virus. In plaque titrations using reduced concentration of horse sera, mouse hepatitis virus (PRINCETON) produced small plaques on C3H cells. The variant virus, mouse hepatitis virus (C3H), produced both large plaques characteristic of mouse hepatitis virus (C3H) and small plaques characteristic of mouse hepatitis virus (PRINCETON). The results suggest that a fraction of mouse hepatitis virus (PRINCETON) virus multiplies in resistant C3H cells, and is then converted to mouse hepatitis virus (C3H), and that mouse hepatitis virus (PRINCETON) continues to be carried in stocks of mouse hepatitis virus (C3H) during passage in C3H cells. The outcome of infection of genetically resistant C3H macrophages with mouse hepatitis virus (PRINCETON) was greatly influenced by the type and concentration of sera used to support macrophages.

INTRODUCTION

The PRINCETON strain of mouse hepatitis virus (MHV(Pri)) grows to high infectivity in, and is destructive for, cultures of macrophages obtained from susceptible Princeton (Pri) strain mice. Similar cultures obtained from resistant strain C3H mice are highly resistant to the virus (Bang & Warwick, 1960). Hybrids of the resistant and susceptible strains of mice have shown that susceptibility is dominant, and further studies demonstrated the Mendelian inheritance of susceptibility (Kantoch et al. 1963). However, C3H cells were occasionally destroyed after infection with undiluted MHV(Pri) virus, but this was delayed for several

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days. Virus recovered from such cultures had an increased pathogenicity for C3H mice and cultured C3H cells, and is referred to as variant virus MHV(C3H); the change in the virus population from MHV(PRI) to MHV(C3H) is called conversion (Shif & Bang, 1970b). Virus MHV(C3H) formed plaques on monolayers of both C3H and Pri cells, while the original MHV(PRI) formed plaques on Pri cells only.

We report here the experiments on the effect of type and concentration of sera on the susceptibility of C3H cells to MHV(PRI).

**METHODS**

**Cells.** Cultures of mouse peritoneal macrophages were prepared by the method of Kantoch et al. (1963). Princeton (Pri) and C3H mice, 4 weeks old, were injected with 2 ml. of sterile thioglycollate broth. Three days later, 5 ml. of Hanks's balanced salt solution (BSS) containing 100 units/ml. of penicillin, 100 µg. of streptomycin, and 5 units/ml. of heparin were injected into the peritoneum and withdrawn after 1 or 2 min. The exudate was centrifuged at 1000 rev./min. for 10 min., cells were resuspended in appropriate media, and dispensed into 10 x 100 mm. Wasserman tubes or 35 x 10 mm. Falcon plastic tissue culture dishes. One ml. containing 1 x 10⁶ cells was put into tubes, incubated in a stationary position overnight and then transferred to a roller drum at 37°C for the duration of the experiment. Culture dishes were seeded with 2-5 x 10⁶ cells in 2 ml., and maintained at 37°C in a humidified atmosphere of 5 % CO₂ in air.

**Sera and media.** Sera, yielding the following final concentrations, were added to the base medium of 1-65 % lactalbumin hydrolysate in Earle's BSS (GIBCO), 2 % beef embryo extract, 100 units/ml. penicillin, and 100 µg/ml. streptomycin: horse serum, 90, 30 and 20 %; Pri mouse serum, 10 %; C3H mouse serum, 10 %; foetal calf serum, 10 %; rat serum, 10 %. Horse blood was obtained locally and sera were processed and filtered (Seitz) in this laboratory. Such sera were free of inhibiting factors for mouse hepatitis virus (PRI) when tested on susceptible Pri cells. C3H and Pri mouse sera were obtained from 5- to 6-week-old animals and filtered (0.45 nm. Millipore) before use. Foetal calf and rat sera were obtained commercially (GIBCO). All sera were heat-inactivated (56°C, 30 min.). Overlay media consisted of 20 % horse serum, NCTC-109 medium, 1 % agar, penicillin and streptomycin.

**Viruses.** The Princeton strain of mouse hepatitis virus (MHV(PRI)) (Nelson, 1952), twice plaque-purified on Pri cells, was obtained from Dr I. Shif of this laboratory. A stock of virus was prepared as a 10 % liver homogenate from a single moribund Pri mouse 2 days after inoculation of a 10⁻⁶ dilution of virus. MHV(C3H) was obtained from C3H cell cultures undergoing destruction 3 days after inoculation of undiluted MHV(PRI). Cell lysates were passed at 10⁻⁶ dilution to fresh C3H cell cultures. After 48 hr, when destruction was nearly complete, virus was harvested from several cultures, pooled, and used as stock MHV (C3H).

**Infectivity assays.** MHV(PRI) and MHV(C3H) viruses were titrated on 2- to 3-day-old Pri and C3H cell cultures as follows: 0.05 ml. of serial tenfold dilutions of virus were inoculated into tube cultures having 1 ml. of media. Usually 3 or 4 cultures were used for each dilution in Hanks's BSS with 1 % horse serum. Cultures were observed daily for cellular destruction and final readings were made at 7 or 8 days after inoculation; no cellular destruction was found beyond that time. Infectivities were calculated according to the method of Reed & Muench (1938) and expressed as TCD₅₀/ml. of inoculum.

**Plaque assays.** Monolayers of Pri and C3H cells, 2 to 3 days old in tissue culture dishes, were inoculated with 0.3 ml. of tenfold dilutions of virus and incubated at 37°C in 5 % CO₂ in air for 2 hr. During this time, cultures were agitated gently every 15 to 20 min. to redis-
Altering susceptibility to MHV in vitro

perse the inoculum. The inoculum was then aspirated from the cultures and 2 ml. of overlay medium was added and allowed to solidify at room temperature before cultures were returned to the incubator. After 2 or 3 days, depending upon the cell-virus system, neutral red was added to a final concentration of 1/9000, and plaques were counted 1 hr later. When MHV (Pri) and MHV (C3H) viruses were titrated on Pri cells, plaques were enumerated on the second day after inoculation; plaques of these viruses on C3H cells were read on the third day.

RESULTS

Influence of type and concentration of sera in media on titrations of MHV(PRI) and MHV (C3H) viruses in resistant C3H and susceptible Pri macrophage cultures

C3H and Pri cells were harvested and seeded in tubes with media containing 20 % horse serum. The following day, fluids were removed and replaced with media containing the following types and concentrations of sera: horse serum, 90 and 20 %; C3H mouse serum, 10 %; Pri mouse serum, 10 %; rat serum, 10 %; foetal calf serum 10 %; and a mixture of 20 % horse and 10 % C3H mouse sera. After incubation for 24 hr cultures were inoculated with 0.05 ml. of tenfold serial dilutions of stock MHV(PRI) virus. Three or four cultures were used for each dilution. The destructive end-point (TCD50) was determined 8 days after inoculation and the results of three experiments are given in Table 1. C3H cells incubated in 90 % horse serum were destroyed only by high concentrations of virus. The effect was observed regularly with undiluted virus and at 1/10 and occasionally at 1/100 for virus preparations of infectivity 10^2 to 10^5 TCD50/0.05 ml. for Pri cells. However, when the horse serum was reduced to 20 % the destructive effect was observed at 5 dilution of virus in some cases. In each of several cultures tested, destruction was accompanied by virus multiplication as indicated by a high infectivity of released virus for fresh Pri cells. When C3H cells were incubated with either 90 or 20 % horse serum destruction was observed at 4 to 6 days after inoculation. The effect is commonly referred to as ‘delayed destruction’ since susceptible Pri cultures usually were destroyed within 48 hr. In Pri (genetically susceptible) cell cultures, there was no significant difference between cells incubated with 90 or 20 % horse serum. This was expected since maximum infectivity of MHV(PRI) virus has been obtained consistently in cultures with 90 % horse serum.

Since C3H cells were more susceptible to MHV(PRI) virus in reduced horse serum concentration, it was of interest to observe the effect of mouse and other sera. As shown in Table 1, C3H cells incubated with C3H or Pri mouse sera were highly resistant to MHV(PRI)

Table 1. Influence of type and concentration of serum on titration of MHV(PRI) virus in cultures of C3H and Pri macrophages

<table>
<thead>
<tr>
<th>% and type of sera in medium</th>
<th>Infectivity of virus (log TCD50/0.05 ml.)*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>On C3H cells</td>
</tr>
<tr>
<td>90, horse</td>
<td>1.3, 2.5, &lt; 1.0</td>
</tr>
<tr>
<td>20, horse</td>
<td>3.8, 4.8, 4.5</td>
</tr>
<tr>
<td>10, C3H mouse</td>
<td>&lt; 1.0, &lt; 1.0</td>
</tr>
<tr>
<td>20, horse + C3H mouse</td>
<td>&lt; 1.0, &lt; 1.0</td>
</tr>
<tr>
<td>10, Pri mouse</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>10, foetal calf</td>
<td>5.0</td>
</tr>
<tr>
<td>10, rat</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Results of 3 different experiments.
virus and destruction occurred only with undiluted virus. Cells incubated with foetal calf serum were highly susceptible, since destruction was found at dilutions of $10^{-5}$. Cells incubated in rat serum had intermediate susceptibility. However, when susceptible Pri cells were incubated with C3H or Pri mouse sera the infectivities obtained were maximal and cellular destruction occurred within 72 hr. This finding was important since it demonstrated that inoculated virus was not inactivated by serum, and suggested that the observed resistance was due to an effect of serum upon the cells. Interestingly, cells incubated in serum from susceptible Pri mice were as resistant as cells incubated in serum from resistant C3H mice. Finally, an incubation mixture of 20% horse serum and 10% C3H mouse serum resulted in high resistance, indicating that C3H serum inhibits susceptibility even in the presence of low concentrations of horse serum.

### Table 2. Titration of MHV(C3H) virus in C3H macrophage cultures incubated with different types and concentrations of sera

<table>
<thead>
<tr>
<th>% and type of sera in medium</th>
<th>Infectivity (log TCD50/0.05 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90, horse</td>
<td>5.3</td>
</tr>
<tr>
<td>20, horse</td>
<td>4.5</td>
</tr>
<tr>
<td>10, C3H mouse</td>
<td>4.5, 5.3</td>
</tr>
<tr>
<td>10, rat</td>
<td>5.0</td>
</tr>
<tr>
<td>10, foetal calf</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Several C3H cultures which showed delayed destruction following infection with MHV (Pri) virus were tested for virus (MHV(C3H)) now capable of destroying C3H cells at high infectivity. Of 8 cultures tested, 6 were found to cause rapid (48 hr) destruction of fresh C3H cell cultures, while 2 still caused delayed destruction between the 3rd and 4th days. This indicated that MHV(C3H) virus was produced and suggested that some of the inhibitory sera might inactivate released MHV(C3H) virus, but not MHV(Pri) virus. C3H cultures were therefore incubated with aliquots of sera and treated as in previous experiments on MHV (Pri) virus and C3H cells. They were then inoculated with tenfold dilution of a stock of MHV(C3H) virus derived from MHV(Pri) as described in Methods. The infectivity obtained in each serum fell within the range of the maxima for that stock of MHV(C3H) virus (Table 2). This indicated that the sera did not inactivate preformed MHV(C3H) virus, and supported the hypothesis that certain sera, notably C3H and Pri mouse sera, and high concentrations of horse serum, suppressed intracellular conversion, or adaptation, of MHV(Pri) virus to the variant virus MHV(C3H).

The choice of serum concentrations in the above experiments was largely determined by the finding that mouse macrophages did not survive well at serum concentrations below 10% or, for most sera, above 15 to 20%. Survival of mouse macrophages in high concentrations of horse serum was exceptional.

### Plaque formation by MHV(Pri) and MHV(C3H) viruses on Pri and C3H cell monolayers

Since MHV(Pri) virus fails to produce plaques on C3H cells when these are grown in 90% horse serum, and since reduced concentrations of horse serum increase the susceptibility of C3H cells to MHV(Pri) virus, the capacity of MHV(Pri) virus to produce plaques on C3H cells in 20% horse serum was tested. Monolayers of CH3 and Pri cells were prepared using media containing this concentration of horse serum. When C3H cell monolayers were inoculated with MHV(Pri) virus, small plaques 1 mm. in diameter appeared after about 72 hr; these failed to increase in size during the next 24 hr. In contrast, MHV(Pri) and
MHV(C3H) viruses on susceptible Pri cells produced large plaques of 2 to 3 mm. in diameter within 48 hr, increasing to 3 to 4 mm. by 72 hr. Both large and small plaques appeared on C3H monolayers inoculated with MHV(C3H) virus (Table 3), and the number of plaques produced was proportional to the concentration of virus applied. Infectivities indicated by plaque-forming units (p.f.u./ml.) were in close agreement with TCD50/ml. values for each virus of the given cell type. The occurrence of MHV(PRI) virus as a small plaque on C3H cells suggested multiplication of virus in these cells. The finding that MHV(C3H) virus produced on C3H cells both large plaques characteristic of MHV(C3H) virus and small plaques characteristic of MHV(PRI) virus suggested that a fraction of MHV(PRI) virus type was carried in MHV(C3H) virus stock. No tests were made for the type of virus which emerged as plaques on the plates.

Table 3. Plaque titrations of MHV(PRI) and MHV(C3H) viruses on monolayers of Pri and C3H macrophages

<table>
<thead>
<tr>
<th>Titration no.</th>
<th>Pri cells</th>
<th>C3H cells</th>
<th>Pri cells</th>
<th>C3H cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5·6 × 10⁸ (L)</td>
<td>1·3 × 10⁷ (S)</td>
<td>---</td>
<td>4·0 × 10⁷ (L), 2·6 × 10⁷ (S)</td>
</tr>
<tr>
<td>2</td>
<td>3·3 × 10⁶ (L)</td>
<td>7·8 × 10⁵ (S)</td>
<td>8·2 × 10⁵ (L)</td>
<td>---</td>
</tr>
</tbody>
</table>

* Large (L) and small (S) plaques are described in the text. Plaques on C3H monolayers were counted 3 days after inoculation; plaques on Pri monolayers 2 days after inoculation.
† Both large and small plaques appeared on individual monolayers.

DISCUSSION

The effect of the type and concentration of sera in the media on the susceptibility of C3H cells to MHV(PRI) virus is believed to be at least partly due to an inhibition by some sera of conversion of MHV(PRI) to MHV(C3H) virus which was found, in the presence of destruction, in all of 8 cases. It appears that mouse sera possess a conversion-inhibiting activity, that a similar activity of horse sera is manifest only at a high serum concentration, and that these sera were acting upon the cells, and not upon the virus.

Whereas the susceptibility of C3H macrophage cultures to large concentrations of MHV(PRI) virus was observed earlier (Gallily, Warwick & Bang, 1967), multiplication of MHV(PRI) virus in such cultures has not been conclusively demonstrated. On the contrary, the results of Shif & Bang (1970b) indicated that destruction of C3H cultures was always accompanied by appearance of the variant virus. Conversely, in C3H cultures given non-destructive doses of MHV(PRI) virus, no multiplication was observed. The question remained, however, as to whether there was any multiplication of MHV(PRI) virus when MHV(C3H) emerged in and destroyed C3H cells. Multiplication of MHV(PRI) virus might not be detected because the emergent variant, MHV(C3H) virus, readily destroys both Pri and C3H cells. Thus, the observation that small plaques appeared on C3H monolayers infected with MHV(PRI) virus when cells were incubated and overlaid with media containing reduced concentration of horse serum, suggests that MHV(PRI) virus may multiply to a limited degree in conditions which favour the concomitant conversion to MHV(C3H) virus. Furthermore, MHV(C3H) virus grown on C3H cells produced both large plaques characteristic of MHV(C3H) and small plaques characteristic of MHV(PRI). This may be a manifestation of
intermediate stages in the conversion of MHV(PR) to MHV(C3H) as suggested by Gallily et al. (1967).

Of particular interest are the findings (Shif & Bang, 1970a) that C3H cells inoculated with non-destructive doses of MHV(PR) virus adsorbed virus at a similar rate and to the same extent as did susceptible Pri cells. Furthermore, following adsorption of virus, resistant C3H cells retained infectious virus intracellularly without multiplication for several days before virus finally disappeared. It is conceivable that intracellular processes governing the eventual disappearance or multiplication of virus are influenced by environmental factors related to the serum composition of the medium. In this connexion Cohn & Benson (1965) have shown that changes in the concentration of sera in media have a dramatic influence on the formation of lysome-like granules and the yield of hydrolytic enzymes in cultures of mouse macrophages. Such changes could influence the intracellular fate of virus.

REFERENCES


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