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Correlation of Persistent Mouse Hepatitis Virus (MHV-3) Infection with Its Effect on Mouse Macrophage Cultures

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With 3 Figures

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Summary

MHV 3 has three distinct effects in different strains of mice: strain A mice are completely resistant, most strains (including C57BL, DBA/2, BALB/c and NZB strains) die of acute hepatitis whereas in certain strains (eg. C3H and A2G) the virus produces a persistent infection with neurological symptoms. In cultures of peritoneal macrophages from susceptible strains, MHV-3 replicated freely, with giant cell formation. No replication was observed in macrophages from strain A mice. In contrast to this full susceptibility or resistance, macrophage cultures from strains of mice in which persistent infections occur showed an intermediate susceptibility, as judged by the intensity of the cytopathic effect, the presence of viral antigens in the cytoplasm and levels of viral replication. Possible ways in which the intermediate susceptibility of macrophages and persistent infections might be related are discussed.

Introduction

The susceptibility of animals to viruses is often genetically determined (1). Bang and his colleagues have shown that the inherited capacity of the mouse macrophage to restrict virus growth is the basis of the resistance of certain strains of mice to mouse hepatitis virus type 2 (2). Mouse hepatitis virus type 3 (MHV-3), a closely related coronavirus (6), has the ability to produce a very characteristic and easily recognizable cytopathic effect (CPE) in mouse macrophages by fusing them into multinucleated giant cells (5). We have previously reported that the disease induced by intraperitoneal (IP) infection with MHV-3 is different in various strains of mice (3, 4, 7). Mice from the A strain are fully resistant to MHV-3. They develop a mild disease and 100 per cent survive. Mice from the C57 Bl, BALB/c, NZB and DBA/2 strain are fully susceptible to this virus. All infected mice of these strains develop a fulminating hepatitis and die in 4 to 8 days after infection.

Animals from the A2G strain, and mice of C3H strain aged more than 3 months, have an intermediate susceptibility to MHV-3: most of them survive the acute stage of the infection but become chronic virus carriers and develop a progressive disease which lasts for weeks or months. They show signs of neurological involvement especially incoordination and paralysis of one or more limbs. The neuropathological lesions in A2G mice are dominated by a chronic choroido-ependymitis and meningitis leading to hydrocephalus and hydromyelia, whereas an immunopathological vasculitis appears to be responsible for the central nervous system lesions in mice of the C3H strain (8).

Persistent infections with MHV-3 are of interest from several points of view: their development is determined by the genotype of the virus and of the host. Moreover, they represent models of chronic infection involving the central nervous system and some at least seem to have an immunopathological basis. Indeed the infection of C3H mice represents a good model of vasculitis associated with the presence of immune complexes containing virus-specific antigens. As part of an investigation of the pathogenesis of these persistent infections, we have examined the capacity of macrophages from different mouse strains to support replication of MHV-3.

We now report the finding that the *in vitro* susceptibility to MHV-3 of peritoneal macrophages is closely correlated with the severity of the disease induced by the virus *in vivo*.

Materials and Methods

Mice

Five inbred strains of mice were used: A/J mice were supplied by the National Institute for Medical Research, Mill Hill. A2G/Lac mice were supplied by the Laboratory Animal Centre, Carshalton. C3H/He-mg, C57BL/10 Crc and DBA/2 Crc were obtained from the specific-pathogenfree unit of the breeding centre in the Clinical Research Centre. All mice were used as young adults (5 to 10 weeks old), except C3H/He mice which were used at 3 or more months of age, since semi-susceptibility in this strain has been shown to be age-dependent, and is maximal in aged animals (4, 7). Macrophages from the outbred VSBS/NIMR strain (T0 mice) were used for virus titrations *in vitro*.

Virus

MHV-3 virus (Mill Hill strain) was prepared from livers of C57BL mice injected intraperitoneally 4 days previously with 0.1 ml of a mouse liver suspension containing 10^4 LD_{50} per ml. A 10 per cent mouse liver suspension, containing $10^{4.6}$ LD_{50} per ml as judged by its capacity to kill C57BL mice, was stored at —70° C in 0.2 ml aliquots. This stock suspension titrated 10^5 macrophages—infecting doses (MID) when tested in cultures of macrophages from T0 mice.

In vivo *Infection*

Groups of 3 to 6 mice of the fully resistant or fully susceptible strains, and groups of 25 mice of the strains showing intermediate susceptibility were injected IP with 0.1 ml of our stock MHV-3 suspension diluted 10 times in phosphate-buffered saline (equivalent to $10^{2.6}$ LD_{50} for susceptible animals). The mortality and clinical symptoms were recorded during several weeks following infection.

Macrophage Cultures

Mice from batches tested for their *in vivo* susceptibility to MHV-3 as described above were killed with anesthetic ether and their peritoneal cavity was washed with

5 ml of medium 199 containing 3 per cent bicarbonate, 100 units of heparin, penicillin and streptomycin per ml. Glass tubes (1.4 cm diameter) containing half of a cover slip each and closed with a loose metallic cap were placed in a tube holder inclined at 15° C. One ml of a suspension containing 5×10^5 peritoneal cells was incubated (for 1 hour at 37° C in a CO_2 incubator) per tube. The cover-slips were removed with sterile forceps, washed thoroughly in 3 consecutive bechers containing L 15 medium to remove non-adherent cells, and placed in another tube containing culture medium (medium 199 with 3 per cent bicarbonate, 10 per cent fetal calf serum, penicillin and streptomycin).

In vitro *Infection of Macrophages*

Cultures of adherent peritoneal cells were infected, 24 to 72 hours after seeding, by removing the medium and replacing it with 1 ml of a virus suspension diluted in culture medium. The appearance of giant cells was checked examining unfixed cultures with an inverted microscope. When a permanent quantitative record of the intensity of the CPE was needed, coverslips were stained with Giemsa.

Infectivity Assay in Macrophage Supernatants

Peritoneal macrophages from outbred, susceptible, unstimulated VSBS/NIMR mice were seeded in plastic plates (Linbro Scientific, New Haven, Connecticut) containing 24 flat-bottomed, 1.5 cm diameter wells (8×10^5 peritoneal cell per well). After 2 hours incubation at 37° C the cultures were washed 3 times in L 15 medium and incubated for 24 hours in culture medium. Duplicate cultures were then incubated with 1 ml of graded ten fold dilutions of a pool of 3 supernatants of infected macrophages from various mouse strains. Cultures were checked 72 and 96 hours after infection with an inverted microscope. The number of macrophages infecting doses (MID) was the reciprocal of the last dilution giving a clear CPE.

Immunofluorescence

A mouse anti-MHV-3 serum was obtained from resistant mice of the A strain and conjugated with fluorescein isothiocyanate (FITC) as described previously (8). A FITC conjugated goat anti-mouse immunoglobulin antiserum was purchased from Nordic Pharmaceuticals. All antisera were absorbed with mouse liver powder and passed through a Sephadex G 50 column just before use. For direct immunofluorescence, infected and control cultures on half coverslips were washed inPBS, fixed for 15 minutes in acetone at —20° C and incubated for 30 minutes in a humid chamber with a $^1/_{10}$ dilution of FITC conjugated mouse anti-MHV-3 serum. For the indirect technique, coverslips were successively incubated with a $^1/_{15}$ dilution of anti-MHV-3 serum and a $^1/_{20}$ dilution of FITC conjugated goat anti-mouse antiserum. The coverslips were thoroughly washed in phosphate-buffered saline for 1 hour and mounted on glass slides with 50 per cent glycerol-phosphate.

Results

CPE in Infected Macrophages from Different Strains

Monolayers of macrophages from mice of different strains with a comparable density were infected simultaneously and checked for the appearance of multinucleated giant cells at different times after infection with graded dilutions of MHV-3. In repeated experiments cultures of macrophages from mice of the A strain consistently failed to produce any giant cells, except in cultures infected with very heavy inocula (10^4 MID). Table 1 shows that, after infection with 10^2 MID, cultures of macrophages from the A strain did not show any CPE whereas in C57BL macrophages there was an early and rapidly progressive CPE. Macrophages from the C3H and the A2G strains showed a CPE of intermediate intensity. Giant cells appeared later and the fusion process was slower. Moreover, this process was incomplete, since cultures from the A2G and C3H strains checked

6 days after infection showed about 50 per cent of apparently healthy individual macrophages, while cultures from C57BL or DBA/2 mice were completely destroyed at that time, due to death and detachment of giant cells. Only a few individual cells (less than 1 per cent) survived after a week, possibly because of contamination of peritoneal macrophages by other adherent cell types.

Table 1. *Cytopathic effect induced by MHV-3 in mouse macrophages from different strains. Results of a representative experiment. The number of crosses represent the percentage of giant cells in triplicate tubes 0 = No CPE, + ⩽25, ++ ⩽50, +++ ⩽75, ++++ ⩽100 per cent. The cultures were infected with a dilution of a mouse liver suspension containing 100 infecting units of C 57 Bl macrophages*

Time after infection	A	C3H	A2G	C57Bl
5 hours	0	0	0	0
24 hours	0	0	+	++
48 hours	0	+	++	+++
72 hours	0	++	++	++++

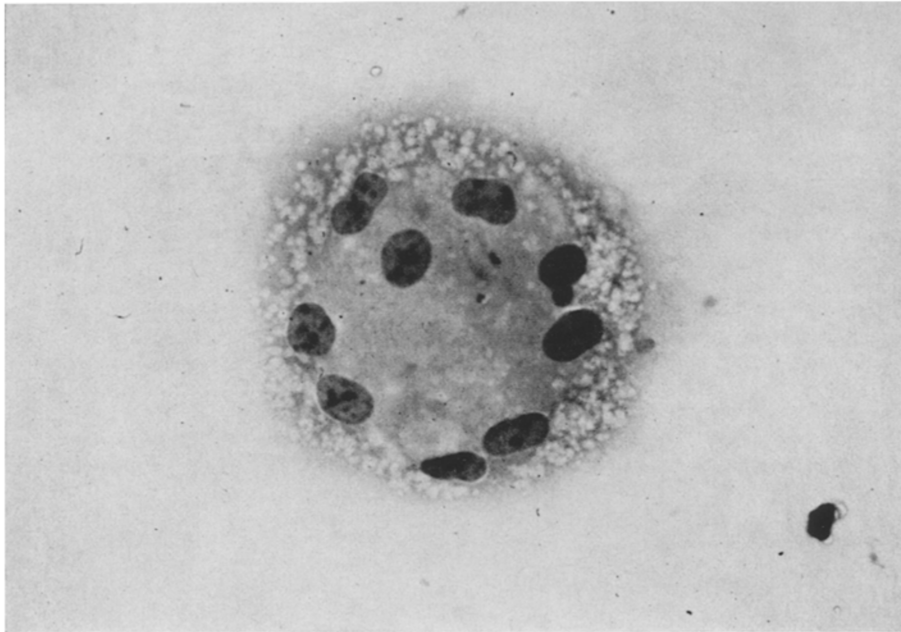

Fig. 1. Multinucleated giant cell in a culture of C57BL macrophages 24 hours after infection. Giemsa stain. (× 900)

Immunofluorescence

In cultures of C57BL macrophages examined 24 hours after infection with the direct and indirect immunofluorescence technique, bright cytoplasmic fluorescence was found in all giant cells and in many surrounding individual macrophages. The early fusion of infected macrophages from C57Bl mice led to the creation of a denuded area around most giant cells, as shown in Figure 1. As shown in Figure 2, cultures from A2G mice had also brightly fluorescent giant cells, but

these were closely surrounded by individual macrophages which were not actually fusing with the giant cells and lacked virus-specific antigen. Uninfected macrophages from all strains, and infected macrophages from the A strain, were consistently negative when examined by immunofluorescence.

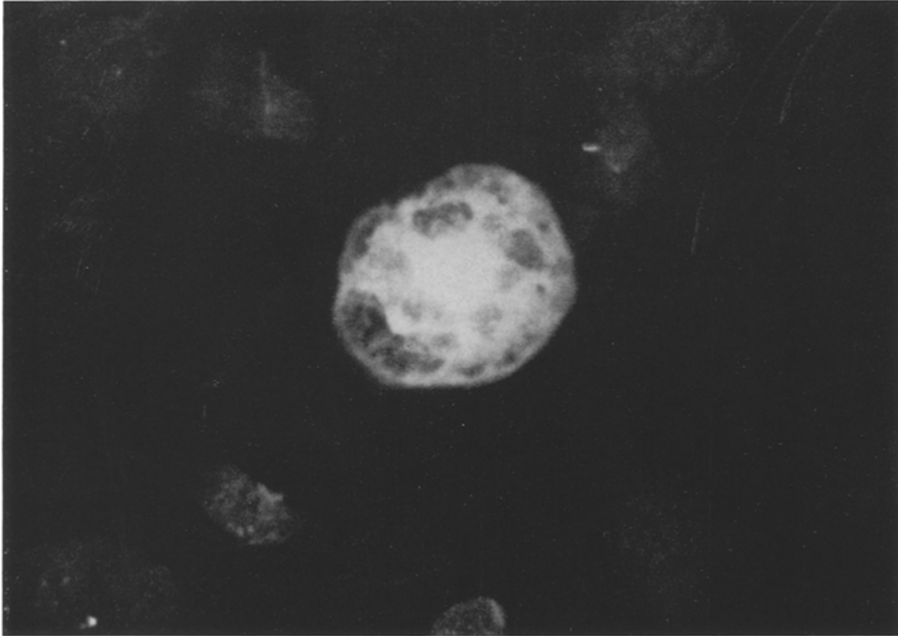

Fig. 2. Multinucleated giant cell in a culture of A2G macrophages 24 hours after infection. Direct immunofluorescence. Note the presence of virus-specific antigen in the giant cell but its absence in the surrounding individual cells. (×750)

Virus Replication in Macrophages

Macrophage cultures from A, C3H and C57BL mice were infected with 100 MID of MHV-3. Pools of triplicate culture supernatants were collected 5, 24 and 48 hours after infection and tested for infectivity on susceptible VSBS/NIMR mice macrophages. Figure 3 shows that cultures from A mice did not produce any detectable virus whereas C57BL macrophages produced large amounts of virus. Cultures from C3H animals produced 10 times less virus than cultures from C57BL mice, thus showing an intermediate level of replication.

In vivo *Infection*

Since mice from different batches of the same strain may have a variable susceptibility to a virus, groups of mice of the same batch used for macrophage harvest were infected with MHV-3. Table 2 shows that, while mice of the A strain always survived the infection, mice of the C57BL and DBA/2 strains always developed a fulminant hepatitis and died within a few days. A2G and C3H animals became virus carriers, appeared healthy for 2 to 12 weeks, and 50 to 100 per cent of them developed signs of a progressive neurological disease as described previously (7, 8).

Table 2. *Correlation between* in vitro *and* in vivo *susceptibility to MHV-3 of different mouse strains*

Mouse strain	*In vitro* susceptibility of peritoneal macrophages	Course of the disease after *in vivo* infection
A	Not susceptible	Short (<7 days) viral disease. 100 per cent survival
A2G (young adults) C3H (>3 months)	Intermediate susceptibility	Persistence of virus, chronic encephalitis. 50—100 per cent late deaths
C57Bl DBA/2	Full susceptibility	Fulminant hepatitis. 100 per cent acute deaths

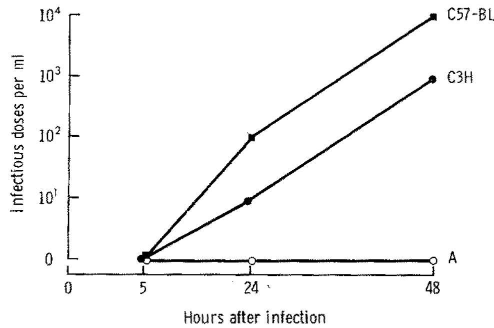

Fig. 3. Infectivity assay on supernatants of macrophage cultures from 3 different mouse strains infected with 100 MID. Supernatants were collected 5, 24 and 48 hours after infection and tested in macrophage cultures from susceptible VSBS/NIMR mice. ○ A strain, ● C3H strain, ■ C57BL strain

Discussion

Our results confirm and extend those of BANG and his colleagues (2) showing that the susceptibility of mouse macrophages to murine hepatitis viruses parallels the susceptibility of adult animals of different strains. The cellular basis of the resistance of A strain macrophages is still unknown. Our finding that this resistance can be overcome by increasing the infecting dose suggest that such macrophages do not entirely lack receptors for MHV-3. However, when infected with moderate doses of virus, these macrophages failed to produce infective virus and no viral antigens were found in their cytoplasm, indicating a complete restriction of virus growth.

The correlation between *in vitro* and *in vivo* susceptibility to MHV-3 was shown to be remarkably precise, since intermediate suceptibility of macrophages was associated with viral persistence *in vivo*. Indeed, even within the C3H strain, we have found a correlation between the virus titers in the liver or serum 4 days after infection and the evolution of the disease in individual animals: the higher the titer, the earlier the death (4). The intermediate susceptibility of macrophages appeared to have some predictive value. Indeed, while our previous *in vivo* finding in the C3H strain led us to investigate MHV-3 replication in C3H macrophages, it was our discovery of an intermediate susceptibility in A2G macrophages which led us to find persistent infection in this strain.

The mechanism by which intermediate susceptibility of macrophages leads to *in vivo* virus persistence is likewise unknown. The virus appears not to persist

in vivo in macrophages, since viral antigen is found exclusively in vascular structures (C3H strain) and in ependymal and meningeal cells (A2G) (8). Macrophages appear to be prime early targets after infection. We suggest that in strains of intermediate susceptibility there is enough replication in macrophages for the virus to have access to its target organs, but the replication in macrophages is slow enough to permit the appearance of an immune response. This may limit further virus replication protecting the host from acute hepatitis and death. Indeed, when C3H mice were immunosuppressed with one injection of 200 mg/kg of cyclophosphamide 24 hours before infection, they developed acute hepatitis and died (Virelizier, unpublished observation). Since macrophages are thought to be relatively resistant to immunosuppressive chemicals, it is likely that cyclophosphamide-susceptible lymphocytes contribute to host defence against MHV-3. That resistance to mouse hepatitis virus is unlikely to be due exclusively to an inborn resistance of macrophages has already beeen suggested by Allison (1). Thus macrophages from a MHV-1 resistant strain are susceptible when collected from neonatally thymectomised adult donors. Furthermore, preliminary data in our laboratory have shown that *in vitro* pre-incubation with either fibroblastic or lymphocytic interferon can induce resistance to MHV-3 in macrophages from susceptible strains (Virelizier, De Maeyer and Allison, in preparation). These observations suggest that the immune response can modulate the susceptibility of macrophages to MHV-3. Further work is needed to establish whether a "physiological cooperation" between lymphocyte and macrophages, acting through soluble products of activated lymphocytes, are operative in host defence against MHV-3.

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