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A Major Role of Macrophage Activation by Interferon-Gamma during Mouse Hepatitis Virus Type 3 Infection. I. Genetically Dependent Resistance

MARIA A. LUCCHIARI\textsuperscript{1,2} and CARLOS A. PEREIRA\textsuperscript{1}

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Abstract

Resistance of mice to mouse hepatitis virus type 3 (MHV3) infection is genetically determined. Normal adult A/J mice are resistant, and BALB/c mice are susceptible. Higher titers of virus and interferon (IFN) \textit{in vivo} were found in MHV3-infected BALB/c mice compared with A/J mice. \textit{In vitro} activation of macrophages (Mφ) by lipopolysaccharide (LPS) delayed MHV3 replication only in cells that originated from A/J mice, although cell populations from both A/J and BALB/c mice were able to synthesize comparable amounts of IFN-α/β. Using specific antibodies, we have shown that the delayed MHV3 replication in LPS-activated A/J Mφ was due, in part, to IFN-α/β. A/J Mφ were found to be more sensitive to IFN-γ than to IFN-α/β, and BALB/c Mφ did not develop an antiviral state to either IFN. Cultured spleen cells from A/J mice synthesized more IFN-γ than BALB/c spleen cells after specific or non-specific stimulation. The results indicate that IFN-activated Mφ may play a crucial role in the resistance to MHV3 infection. Since IFN-γ is produced in large amounts by A/J spleen cells after specific stimulation with MHV3 and is efficient in activating the A/J Mφ, a T cell-dependent mechanism is likely to be involved.

Introduction

Mouse hepatitis virus type 3 (MHV3) constitutes a model of viral infection in which resistance is dependent on the genetic background of the animal (1–5). Adult A/J mice have been reported to be resistant following MHV3 infection, since they develop a mild disease which disappears 4 to 6 days later. BALB/c mice are susceptible, and after the virus infection, they develop an acute hepatitis and die 3 to 5 days later (2, 4). It has been

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Abbreviations: MHV3 = mouse hepatitis virus type 3; IFN = interferon; LPS = lipopolysaccharide; Mφ = macrophage(s); Con A = concanavalin A; FCS = fetal calf serum; PFU = plaque-forming units; \textit{i.p.} = intraperitoneally.
speculated that the development of the immune response, the expression of a monokine that demonstrates procoagulant activity, the antiviral state induced by interferon (IFN) and the virus replication in target cells are crucial in determining the resistance or susceptibility (3–5). Since the resistant mice are able to clear the virus 4 to 6 days after infection, it is reasonable to consider that non immune or immune mechanisms generated early in infection play an essential role in the resistance against MHV3.

We have recently described that a nutritionally induced impairment of the Kupffer cell functions, such as the sensitivity to IFN, was pertinent to the loss of resistance as shown by hypercholesterolemic A/J mice during the MHV3 infection (6). The genetically determined capacity of macrophages to restrict MHV3 multiplication has been considered to be one of the most important factors in determining in vivo susceptibility or resistance (1, 4, 7). However, the treatment of resistant mice with anti-lymphocytic sera or X-irradiation induces susceptibility (8), indicating that MΦ alone probably do not account for all instances of resistance.

Dupuy et al. proposed that two complementary mechanisms are required to confer resistance against MHV3: Firstly, the capacity of MΦ to restrict viral multiplication and secondly, via cells capable of mounting an efficient immune response (9).

Our aim was to clearly define the ability of MΦ to restrict the MHV3 growth and to investigate possible links with the generation of the immune response. The findings of this study are consistent with the notion that the resistance against MHV3 infection is a consequence of a T cell-dependent mechanism, in which the production of IFN-γ and the sensitivity of MΦ to IFN-γ play an essential role.

Materials and Methods

Mice

Six-week-old mice of the inbred A/J and BALB/c strain obtained from the Institut Pasteur, Paris, France, were bred in our mouse colony. Before use, mice were bled from the retroorbital sinus, and the sera were tested for antibodies to MHV by complement fixation and neutralization tests. Certain animals were periodically sacrificed, and the peritoneal exudate, liver and lung tissue were obtained. These were ground, resuspended in 2 ml of RPMI 1640 medium containing 10% fetal calf serum (FCS) (Gibco Ltd., Paisley, Scotland), penicillin (100 U/ml) plus streptomycin (100 μg/ml) and tested for the presence of MHV in L929 cells. The peritoneal exudates were collected by peritoneal lavage with 6 ml of medium, centrifuged at 750 × g for 10 min and the virus in the supernatants titrated on L929 cells. No animal was found to have either pre-existing antibody in serum or virus in the tissues.

Virus

MHV3 was cultivated and titrated by plaque assay on L929 cells at 37°C as previously described (10). Aliquots containing $2 \times 10^5$ plaque-forming units per ml (PFU/ml) were stored at −80°C and used in all experiments. The MHV3 titers in the peritoneal exudates or tissues of infected animals, obtained as described above, were expressed as PFU per milliliter of peritoneal exudate (PFU/ml), or PFU per gram of tissue (PFU/g).
Macrophage cultures

Peritoneal exudate cells were collected by peritoneal lavage with RPMI 1640 containing 10% of FCS and cultured on 96-well plates (Limbro Chemical, Hamden, CN, U.S.A.) at a concentration of $2 \times 10^5$ cells per well. The cells were incubated for 2 h at 37°C in 5% CO$_2$ and were washed three times with medium after vigorous shaking to remove nonadherent cells. Ninety per cent of the cells were MΦ as determined by their ability to take up zymosan particles.

Virus replication assay

Peritoneal MΦ were treated with LPS from *Escherichia coli* 0111:B4 (Difco Laboratories, Detroit, MI, U.S.A.), IFN-α/β (laboratory standard purified to $10^7$ units of protein per mg from supernatants of L929 cells infected with Sendai virus devoid of IFN-γ activity) or with recombinant IFN-γ (11), kindly provided by Prof. BILLIAU, Leuven, Belgium. Twenty-four hours later, activated or non activated cultures were infected with MHV3 at a multiplicity of infection (MOI) of 0.1 in order to study the inhibition of MHV3 replication. The supernatants of cell cultures, collected at various intervals after infection, were tested for the virus titer by plaque assay (10). Controls included non treated MΦ and MΦ cultivated in the presence of 800 neutralizing units of antibodies to mouse IFN-α/β, produced in rabbits, or with monoclonal antibodies to recombinant mouse IFN-γ (obtained from Prof. BILLIAU, Leuven, Belgium), with activity of $2 \times 10^3$ and $2 \times 10^5$ neutralizing units per mg, respectively. One unit was defined as the amount of antibodies sufficient for neutralizing one unit of IFN-γ.

Interferon assay

A cytopathic effect reduction test technique, described in detail elsewhere (10, 12), was used for the IFN titer determinations. Briefly, monolayers of L929 cells in microtiter plates were incubated for 18 h with different dilutions of samples. Supernatants were then removed, and monolayers were infected with 100 tissue culture infective doses 50% (TCID$_{50}$) of encephalomyocarditis virus. Unadsorbed virus was removed 2 h later by washing the monolayers, and fresh minimum essential medium (MEM) supplemented with 10% FCS was added. Microtiter plates were incubated for 72 h, and the IFN titer was expressed as the reciprocal dilution of the supernatant able to inhibit 50% of virus replication. Each titration contained an internal reference: IFN-α/β or recombinant IFN-γ. For characterization of IFN-α/β or IFN-γ in the samples, antibodies to mouse IFN-α/β and monoclonal antibodies to recombinant mouse IFN-γ were always used, showing no cross-reactivity. IFN titrations were determined in sera, peritoneal exudates and supernatants of peritoneal macrophages after LPS treatment or, alternatively, spleen cells cultured in RPMI 1640 medium were supplemented with 10% FCS, and stimulated with concanavalin A (10 µg/ml) (Con A, Pharmacia Fine Chemicals AB, Uppsala, Sweden) or 10^3 PFU of MHV3 for 2 h, washed twice, and finally incubated for 48 h in fresh medium.

Statistical analysis

The Student’s t-test was used to evaluate the data shown in Figure 2. Standard deviations are shown in Tables 1 and 2.

Results

In vivo virus and IFN titers in MHV3-infected mice

It can be clearly seen in Figure 1 that after MHV3 infection, higher virus titers were observed in the liver, lung and peritoneal exudate of BALB/c mice than in A/J mice. In BALB/c mice, the virus titers increased gradually
Figure 1. Virus titers detected in the liver (▲, △), lung (■, □) and peritoneal exudate (●, ○) of MHV3-infected A/J (-----) and BALB/c (---) mice. Animals were i.p. inoculated with 10³ PFU of MHV3. At subsequent intervals, groups of 5 mice were sacrificed, the tissues obtained and the virus titrated. The MHV3 titers, reported as log 10 PFU/g of tissue (for the livers and lungs) or log 10 PFU/ml (for the peritoneal exudates), are the average of 5 different determinations.

Figure 2a

Figure 2b

Figure 2. IFN-α/β (-----) and IFN-γ (---) detected in sera (○) and peritoneal exudates (●) of MHV3-infected A/J (a) and BALB/c (b) mice. Animals were i.p. inoculated with 10³ PFU of MHV3. At subsequent intervals, groups of 5 mice were sacrificed, the sera and peritoneal exudates obtained and the IFN titrated. IFN-α/β or IFN-γ titers, reported as U IFN/ml × 100, are the average of 5 different determinations. Significant difference between IFN-γ and IFN-α/β titer 24 or 48 h after infection: P < 0.01.
to a peak of $10^5$ PFU/g or ml after 4 days of infection, when the mice died of acute hepatitis. In A/J mice, the virus titers increased during the first 2 or 3 days of infection and then decreased, with no virus being found after 5 days of infection. All the A/J mice survived the infection. The kinetics of IFN-α/β and -γ synthesis in the serum and peritoneal exudate of A/J and BALB/c mice infected with MHV3 is shown in Figure 2. The higher levels of IFN-α/β and principally IFN-γ found in BALB/c mice correlated with the virus titers found in the tissues of these animals after MHV3 infection.

**Effect of LPS MΦ activation on the MHV3 growth**

The activation of macrophage cultures from A/J mice with LPS led to a partial restriction of the MHV3 growth in contrast to the BALB/c macrophage activation, where the kinetics of the virus growth was comparable to that in non activated cultures (Fig. 3).

Although, the final MHV3 yield was similar in LPS-activated and non activated A/J MΦ cultures, maximal titers were always observed 24 h later in activated cells. A 2 log restriction of virus replication occurred always 24 to 48 h after infection (Fig. 3a).

Syncytia and virus-containing cytoplasmic vacuoles, typical of MHV3 replication, could be found in all cell cultures. However, these events were observed with a delay of approximately 24 h in the LPS-activated A/J MΦ cultures (data not shown). In both cell cultures from BALB/c mice or in non activated cultures from A/J mice, cell fusion spread rapidly over the entire monolayer, whereas in the activated cultures of the A/J mice, reduced cell numbers and delayed spreading were observed.

![Figure 3a](image1)  ![Figure 3b](image2)

**Figure 3.** Effect of LPS treatment on the kinetics of MHV3 multiplication in cultured peritoneal MΦ from A/J (a) and BALB/c (b) mice. Cells were treated with 10 μg/ml of LPS for 24 h before infection with 0.1 MOI of MHV3. Supernatants were collected after infection and the virus titrated on L929 cells. The MHV3 titer, reported as log 10 PFU/ml, is the average of 5 different experiments. (●) control MΦ; (○) LPS-treated MΦ. Arrow indicates 60-fold difference from control MΦ.
Table 1. Effect of antibodies to IFN-α/β on LPS activation of cultured peritoneal macrophages from A/J mice

| Macrophage treatment          | MHV3 titer (PFU/ml) |
|------------------------------|----------------------|
| -                            | 5.8 ± 0.9 × 10⁴     |
| LPS                          | 1.5 ± 0.4 × 10⁵     |
| LPS + Ab anti-IFN            | 1.6 ± 0.7 × 10⁵     |

* MΦ were treated with LPS (10 μg/ml) alone or together with antibodies to IFN-α/β (Ab anti-IFN) prior to infection with MHV3 (0.1 MOI). Virus titers were determined in the supernatants collected 24 h after the infection. Results are the mean values of 3 separate experiments ± S.D.

The in vitro ability of A/J activated MΦ to restrict the MHV3 growth correlated with the in vivo resistance of these mice after MHV3 infection. The LPS-activated MΦ cultures from both mouse strains were able to synthesize comparable amounts of IFN-α/β (400 ± 50 U/ml in BALB/c and 450 ± 50 U/ml in A/J cells).

Table 1 shows the effect of LPS activation of A/J MΦ cultured in medium containing antibodies IFN-α/β. The antibodies in the supernatant solution neutralized the IFN synthesized (no IFN was found in the supernatants of these cells), preventing the exogenous antiviral action of IFN. Even though the IFN released was neutralized by the antibodies, some inhibition of virus replication was still observed, raising the possibility of an antiviral effect induced by intracellular IFN or by an IFN-dependent mechanism.

Figure 4. Dose-dependent sensitivity of cultured peritoneal MΦ from A/J (a) and BALB/c (b) mice to IFN-α/β and IFN-γ. Cells were treated with the indicated units of IFN/ml (U IFN/ml) 24 h before infection with 0.1 MOI of MHV3. Supernatants were collected 24 h after infection and the virus titrated on L929 cells. The MHV3 titer, reported as log 10 PFU/ml, is the average of 3 different experiments. (○) IFN-γ-treated MΦ; (●) IFN-α/β-treated MΦ. Arrows indicate 10- to 20-fold difference from IFN-γ-treated MΦ.
Table 2. Synthesis of IFN-γ in cultured spleen cells from A/J and BALB/c mice

| mouse strain | immunization | stimulation | IFN-γ titer (U/ml) |
|--------------|--------------|-------------|--------------------|
|              |              |             | 5 ± 4.1            |
| A/J          |              | Con A       | 1250 ± 120.5       |
|              |              | MHV3        | 20 ± 5.3           |
|              | MHV3 uv      |             | 2200 ± 286.4       |
|              | MHV3 uv      | Con A       | 4200 ± 348.6       |
|              | MHV3 uv      | MHV3        | 5 ± 4.1            |
|              |              | Con A       | 300 ± 40.8         |
|              |              | MHV3        | 10 ± 3.6           |
| Balb/c       | MHV3 uv      |             | 5 ± 4.1            |
|              | MHV3 uv      | Con A       | 500 ± 81.6         |
|              | MHV3 uv      | MHV3        | 1500 ± 163.3       |

The mice were i.p. immunized with 10³ PFU of ultraviolet (uv)-inactivated MHV3. Ten days later, the mice were sacrificed and the spleen cells cultured. They were stimulated with Con A (10 µg/ml) or MHV3 (10³ PFU), and the IFN-γ titer was determined in supernatants. The results are the mean values of 3 separate experiments ± S.D.

Effect of IFN MΦ activation on the MHV3 growth

The results shown in Figure 4a indicate that A/J MΦ were sensitive to the induction of an anti-MHV3 state by IFN-α/β or -γ. However, no anti-MHV3 effect could be induced in BALB/c MΦ cultures (Fig. 4b). The results of the IFN-α/β or -γ dose dependence shown in Figure 4a demonstrate that A/J MΦ were more sensitive to IFN-γ.

The in vitro IFN induction of an anti-MHV3 effect in MΦ correlated with the in vivo resistance seen in A/J mice after MHV3 infection, and indicates a major role of IFN-γ.

Synthesis of IFN-γ by specific or non-specific stimulation of cultured spleen cells

As shown in Table 2, spleen cells from A/J mice were capable of producing higher amounts of IFN-γ than cells from BALB/c mice after a non-specific stimulation with Con A. Also, spleen cells from MHV3-immunized A/J mice synthesized higher amounts of IFN-γ after MHV3 stimulation. In cells from both mouse strains, higher amounts of IFN-γ were always found after the specific stimulation when compared with non-specific stimulation.

Discussion

Coronavirus induce in their respective hosts acute, subacute or chronic diseases which are of a complex nature. Viral and host factors play a
pathogenetic role; therefore, for some diseases, the availability of susceptible and resistant inbred animal strains permit the study of the resistance mechanisms involved. There is a wide variance in virulence and organ tropism among established strains of MHV. MHV2 and MHV3 are of high virulence for mice, multiplying not only in the liver but also in the nervous system. MHV4, a highly neurotropic strain, is extremely virulent for mice and capable of causing encephalomyelitis with demyelination when intracerebrally injected (13, 14).

Previous work on MHV3 postulated that both resistance gene(s) controlling the degree of viral replication in target cells and an intact immune response are required for resistance (9, 15). It has also been shown that the susceptibility is linked to the MΦ procoagulant activity and controlled by two non-H-2-linked genes (16). We have shown that a nutritionally induced hypercholesterolemia in resistant A/J mice caused susceptibility to the MHV3 infection, and the inhibition of the host resistance was a consequence of an impairment of Kupffer cell functions. Although the Kupffer cells from these mice were capable of synthesizing normal levels of IFN-α/β, they showed a markedly decreased sensitivity to the induction of an anti-MHV3 state by this type of IFN (6).

We further explored previously published data (2, 15) showing that endogenous IFN is important in the resistance of MHV3, since injection of anti-IFN-α/β globulin accelerated the appearance of disease in MHV3-infected mice. We studied several aspects of MΦ activation by IFN, which has been recognized as a crucial step during several bacterial, parasitic and other viral infections, in resistant and susceptible mouse strains.

During the MHV3 infection, the BALB/c mice, when compared with A/J mice, showed higher titers of virus (Fig. 1), IFN-α/β and -γ (Fig. 2) in the tissues. This correlation can be explained by a direct virus stimulation in the case of IFN-α/β synthesis and by an indirect stimulation via the immune system in the case of IFN-γ. Surprisingly, significant amounts of IFN-γ were systematically detected very early in sera and principally in peritoneal exudates of infected mice. Although under investigation, it can be speculated that the ability of cultured spleen cells from these animals, of synthesizing high amounts of IFN-γ after a specific stimulation (Table 2), and the rapid virus growth after infection can account for this early in vivo IFN-γ synthesis that was observed. The titers of IFN-γ were higher in BALB/c mice than in A/J mice, but both mouse strains 48 h after infection showed comparable levels of lymphocytes present in the peritoneal cavity.

The kinetics of the MHV3 multiplication in LPS-activated peritoneal MΦ isolated from A/J and BALB/c mice showed that although both cell populations were capable of producing IFN-α/β, only those originating from the resistant A/J mice partially restricted the virus growth (Fig. 3). The reduction in virus titer temporarily resulted in a delay in the exponential rise in virus titer rather than an absolute inhibition of virus growth. This is probably due to the fact that the MΦ release IFN-α/β which was shown
to be much less efficient in inducing an anti-MHV3 state when compared to IFN-γ (Fig. 4a). Nevertheless, more than one mechanism seems to be involved in this partial restriction that was observed, because when these cells were cultivated in the presence of antibodies to IFN-α/β (Table 1), LPS treatment was still able to induce some inhibition of MHV3 growth. In this situation, all the released IFN was neutralized by the antibodies present in the supernatants. Although the presence of intracellularly active IFN cannot be repudiated, a role of an IFN-independent mechanism developed during the LPS activation must be considered. This notion is consistent with a previous study where it was shown that mice can be protected from MHV3 infection by an IFN-independent mechanism of MΦ activation by Corynebacterium parvum (17).

In further support of the role of MΦ sensitivity to IFN, the data presented in Figure 4 show clearly that peritoneal MΦ that originated from resistant A/J mice were very sensitive to IFN-α/β and IFN-γ, as evidenced by a rapidly acquired anti-MHV3 state. In contrast, peritoneal MΦ that originated from susceptible BALB/c mice were not able to develop an anti-MHV3 state (Fig. 4b). These data correlated, respectively, with the in vivo resistance or susceptibility shown by these mouse strains after MHV3 infection.

It has been shown that an efficient immune response is required to confer resistance against MHV3 infection (3, 8), and we demonstrated here that A/J MΦ show a higher sensitivity to IFN-γ when compared to that induced by IFN-α/β in a dose-dependent experiment (Fig. 4a). Thus, it was of interest to study the ability of immune spleen cells from both mouse strains to synthesize IFN-γ (or immune IFN) after a specific or non-specific stimulation. The data presented in Table 2 show that A/J and BALB/c spleen cells were able to synthesize IFN-γ, but those originating from the resistant A/J mouse strain were capable of producing higher amounts after stimulation with ConA or MHV3. The greater ability of immune spleen cells after specific stimulation to produce IFN-γ can be linked to the very early IFN-γ synthesis in vivo after MHV3 infection. The observation that infected BALB/c mice synthesize more IFN-γ than infected A/J mice does not correlate with the levels of the in vitro IFN-γ synthesized by their spleen cells, and can be explained by the different level of stimulation occurring in vivo, with much virus in BALB/c mice stimulating the immune system, and in vitro, with the same amount of virus for cell stimulation.

Our results show that there is a link between both mechanisms that have been proposed for the resistance to MHV3. We believe the capacity of MΦ from mice to restrict viral multiplication depends partially on their sensitivity to IFN, which is synthesized very early in the immune response. Therefore, this may explain why the resistant mice can rapidly clear the virus and the susceptible mice die shortly after infection. Resistant mice, which have MΦ that are very sensitive to IFN-γ in order to develop an anti-MHV3 state, are also capable of producing reasonable amounts of IFN-γ in
the course of the immune response against MHV3 (Fig. 2a and Table 2). Thus, soon after initiation of infection, the virus particles could be neutralized and the infection cleared in a few days. Alternatively, we speculate that susceptible mice have MΦ that are not sensitive to IFN in order to develop an anti-MHV3 state (Fig. 4b), and despite high concentrations of IFN-γ in the serum and peritoneal fluid during the first days of infection (Fig. 2b), the MΦ cannot display a restriction of virus multiplication. Accordingly, these animals die 5 to 6 days after the infection with high concentrations of IFN-γ, reflecting the virus replication and stimulation of the immune system. Nevertheless, the participation of other mechanisms in the susceptibility, such as the expression of procoagulant activity (5, 16), is not excluded and may be involved in the course of the disease.

In conclusion, our findings are in agreement with previously proposed mechanisms and may explain the relationship between the MΦ restriction of virus multiplication and the dependence on the immune response.

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Dr. CARLOS A. PEREIRA, Instituto Butantan, Laboratorio de Imunologia Viral, Av. Dr. Vital Brasil 1500, CP 65, 05504 São Paulo, Brazil