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¹ Instituto Butantan, Laboratório de Imunologia Viral, São Paulo, Brasil, and ² Max Planck Institute of Immunobiology, Freiburg, Germany

***In vivo* Depletion of Interferon-Gamma Leads to Susceptibility of A/J Mice to Mouse Hepatitis Virus 3 Infection**

MARIA A. LUCCHIARI^{1,2}, MANUEL MODOLELL², KLAUS EICHMANN², and CARLOS A. PEREIRA¹

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Abstract

The possible role of interferon-gamma (IFN- γ) in the resistance of A/J mice to MHV3 infection was investigated. Monoclonal antibodies specific for IFN- γ , CD4 and CD8 molecules were administered *in vivo* to deplete selectively the IFN- γ synthesized or the appropriate subset of T cells. The animals were then infected with MHV3 and the course of infection was followed by studying different parameters, such as, the mortality, the virus growth in the tissues and the IFN- γ synthesis in sera and peritoneal exudates. After MHV3 infection, a full resistance of control A/J mice was observed, in contrast to the high mortality rate observed among the depleted animals, where higher virus titers were found in different tissues. The IFN- γ synthesis in sera and peritoneal exudates of depleted mice, after MHV3 infection, drastically decreased when compared to that detected in control mice. The data presented are consistent with the hypothesis that IFN- γ plays an essential role in the resistance of A/J mice to MHV3 infection.

Introduction

The 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 (1, 2). The mechanisms suggested to be involved in the resistance include the antiviral state induced by IFN, the virus replication in target cells and the expression of a monokine that demonstrates procoagulant activity (3–8). We have recently shown that the resistance of A/J mice

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Abbreviations: MHV3 = mouse hepatitis virus 3; IFN = interferon; FCS = fetal calf serum; PFU = plaque-forming units; *ip* = intraperitoneally; *Ab* = antibodies; *PBS* = phosphate buffer solution

against our strain of MHV3 is acquired after immunization and the mechanism involved is dependent on the IFN- γ synthesis and the macrophage sensitivity to IFN- γ (9).

CD4 and CD8 T lymphocytes are essential for the development of protective immunity to several infections (10–12). One of the functions of these cells is the production of IFN- γ , a major T cell lymphokine, which can also be produced by NK cells. IFN- γ activates macrophages, as assessed by the modulation of antiviral state (6), the increased expression of class II major histocompatibility complex gene products (13), the enhancement of phagocytosis (14), the induction of production of reactive oxygen intermediates (15) and tumor necrosis factor (16).

In the experiments described here, with the aim to investigate the *in vivo* participation of IFN- γ in the development of resistance to MHV3 infection, mice have been depleted *in vivo* of the IFN- γ synthesized after infection or the CD4 and CD8 subsets of T cells, using monoclonal antibodies.

The data show a direct evidence that the *in vivo* neutralization of synthesized IFN- γ during the infection led to susceptibility to MHV3 and that mice depleted of CD4 or CD8 T lymphocytes were unable to synthesize the high levels of IFN- γ that were found in the controls after MHV3 infection, and died of acute hepatitis. These results are further support to the already proposed crucial role of IFN- γ in the expression of resistance of A/J mice to MHV3 (6, 7, 9).

Materials and Methods

Mice

Six-week-old mice of the inbred A/J strain obtained from the Institut Pasteur, Paris, France were bred in our mouse colony. The animals were periodically tested for the preexisting coronaviruses or antibodies against MHV3 following procedures already described (9).

Virus

MHV3 was cultivated and titrated by plaque assay on L929 cells at 37°C as previously described (17). Aliquots containing 2×10^5 plaque-forming units per ml (PFU/ml) were stored at -80°C and used in all experiments. For the determination of MHV3 titer in tissues, the animals were sacrificed, and the peritoneal exudates and livers were obtained. These were ground, resuspended in 2 ml of RPMI 1640 medium with 10% fetal calf serum (FCS) (Gibco Ltd, Paisley, Scotland), penicillin (100 U/ml) plus streptomycin (100 μ g/ml) and the virus titrated on L929 cells. The peritoneal exudates were collected by peritoneal lavage with 6 ml of medium, centrifuged at 750 \times g for 10 min and the virus in the supernatants titrated on L929 cells. The MHV3 titer obtained were expressed as PFU per milliliter of peritoneal exudate (PFU/ml), or PFU per gram of liver (PFU/g).

Antibodies

Cells producing rat monoclonal antibodies against mouse IFN- γ (R4-6A2) was kindly donated by G. L. SPITALNY, Trudeau Institute, NY, USA (18) and cells producing rat monoclonal antibodies specific for CD4 and CD8 determinants (YTS 191.1 and YTS 169.4.2

respectively) were a gift of S. COBBOLD and H. WALDMANN, Cambridge University, Cambridge, U.K. (19). The antibodies were purified from tissue culture supernatants by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden). The immunoglobulin concentration was determined by spectrophotometric measurement at 280 nm. They were concentrated by dialysis and sterilized by filtration. The activity of the antibodies against IFN- γ or CD4 and CD8 was tested, respectively, by neutralization of cytopathic effect or inhibition of T cell proliferation and cell binding by cytofluorometric assay. Sera of 5 normal rats in a pool were used as a source of normal rat antibodies.

Depletion of IFN-gamma and CD4 and CD8 T lymphocytes

Groups of A/J mice were immunized once by intraperitoneal (ip) injection of 10^3 PFU of MHV3 inactivated by ultraviolet (uv) radiation. After a period of 30 days they were injected ip with normal rat antibodies (controls) or purified antibodies against IFN- γ , CD4 or CD8 in PBS. Groups of mice were injected ip with 200 μ g of purified antibodies at day -5, -1, 0, 1, 2, 3, 4 and 500 PFU of MHV3 at day 0. The mice were maintained under sterile conditions for the duration of the experiment and the T cell depletion, mortality, virus growth in the tissues and IFN synthesis determined.

Interferon assay

A cytopathic effect reduction test technique using monolayers of L929 cells and encephalomyocarditis virus, described in detail in previous papers (17, 20), was used as an IFN assay. For characterization of IFN- α/β and IFN- γ antibodies to mouse IFN- α/β , produced in rabbits, and monoclonal antibodies to recombinant mouse IFN- γ (Holland Biotechnology, Leiden, Holland) with activity of 2×10^3 and 2×10^5 neutralizing units per mg, respectively, were always used. One unit was defined as the amount of antibodies sufficient for neutralizing one unit of IFN. These antibodies showed no cross-reactivity.

Results

Resistance of mice to MHV3 infection

As shown in Table 1, the treatment of A/J mice with antibodies against IFN- γ or CD4 or CD8 T lymphocytes led to a high susceptibility to MHV3 infection. Control A/J mice were fully resistant to the infection and the animals showed only a mild disease that disappeared 4 days later. In

Table 1. Resistance of A/J mice after *in vivo* depletion of IFN- γ or CD4, CD8 T lymphocytes^a

treatment	mortality		period of disease
	n	%	days
normal rat Ab	0/15	0	2 to 4
Ab anti-IFN- γ	13/15	86.6	1 to 8
Ab anti-CD4	11/15	73.3	1 to 8
Ab anti-CD8	10/15	66.6	1 to 6
Ab anti-CD4/CD8	14/15	93.3	1 to 7

^a A/J mice, previously immunized 30 days before with MHV3 uv, were injected ip with 200 μ g of antibodies at days -5, -2, -1, 0, 1, 2, 3 and 4. 500 PFU of MHV3 were injected ip at day 0. They were observed during 30 days and the mortality recorded.

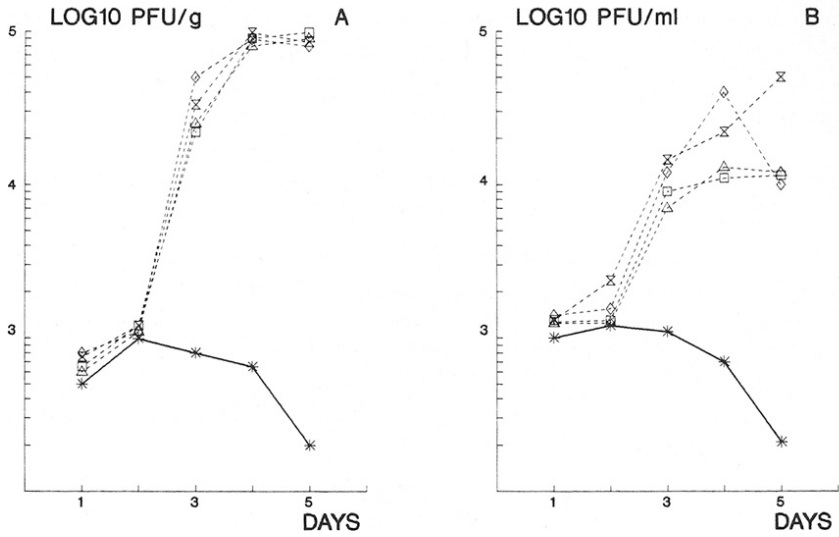


Figure 1. Kinetics of virus growth in the peritoneal exudate (A) and liver (B) of normal rat antibodies (*), anti-IFN- γ (□), anti-CD4 (◇), anti-CD8 (Δ) and anti-CD4 and CD8 (\times) treated mice. The MHV3 titers, reported as Log_{10} PFU/ml are the average of 5 different determinations. The maximal variation was of $9.5 \pm 1.8 \times 10^4$ PFU/ml in the peritoneal exudate of anti-CD8 treated mice at day 4.

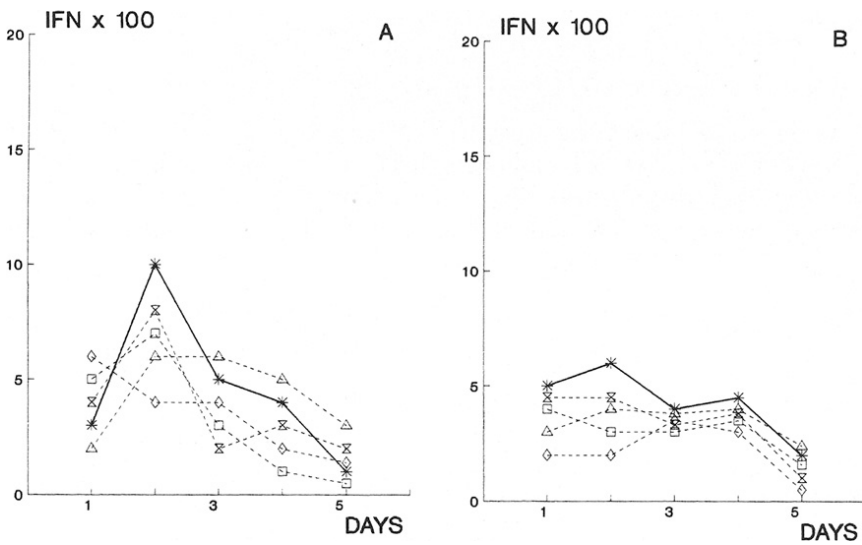


Figure 2. Kinetics of IFN- α/β synthesis in peritoneal exudate (A) and sera (B) of normal rat antibodies (*), anti-IFN- γ (□), anti-CD4 (◇), anti-CD8 (Δ) and anti-CD4 and CD8 (\times) treated mice. The titers, reported as $\text{IFN} \times 100/\text{ml}$, are the average of 5 different determinations. The maximal variation observed was of 550 ± 50 U IFN/ml in the peritoneal exudate of normal rat antibodies and anti-IFN- γ treated mice at day 2.

contrast, the treated animals had a period of disease characterized by ruffled fur, hunching, loss of weight, diarrhea and general lassitude, that started earlier and remained for a longer period. Most of the animals died of acute hepatitis 7 to 8 days after the infection.

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 peritoneum and liver of mice treated with specific antibodies than those observed in the controls (injected with normal rat antibodies). The virus titers increased gradually to a peak of 10^4 to 10^5 PFU/ml or g after 4 to 6 days of infection, when the mice died of acute hepatitis. In control mice, the virus titers increased during the first 2 days of infection and then decreased, with no virus being found after 5 days of infection.

The kinetics of IFN- α/β and IFN- γ synthesis in the serum and peritoneal exudate of control or specific antibody treated A/J mice infected with MHV3 are shown in Figure 2 and 3. It can be seen that the treatment with antibodies against IFN- γ , CD4 and CD8 cells was able to neutralize or inhibit the synthesis of IFN- γ that was observed in control A/J mice during the MHV3 infection. On the other hand, no significant inhibition of IFN- α/β synthesis during the infection was observed in treated mice.

The neutralization/inhibition of IFN- γ synthesis observed in the treated A/J mice during the infection, correlated with the lack of resistance showed by these animals to MHV3 infection.

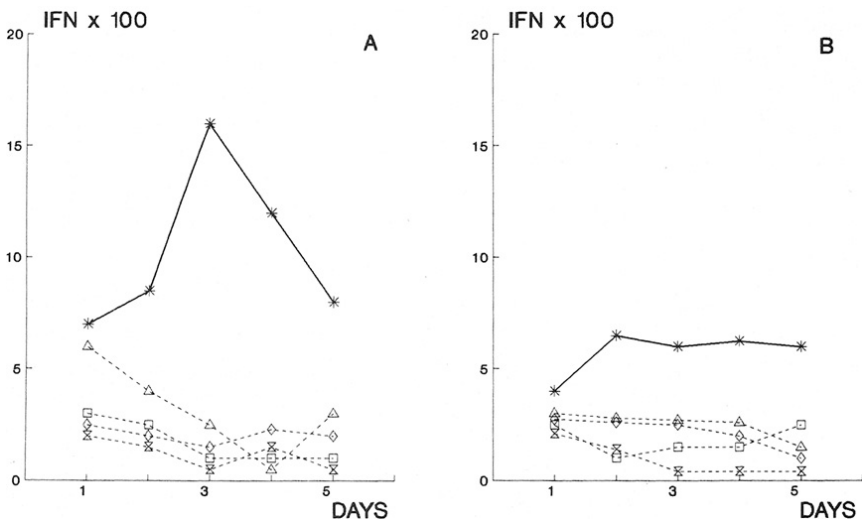


Figure 3. Kinetics of IFN- γ synthesis in peritoneal exudate (A) and sera (B) of normal rat antibodies (*), anti-IFN- γ (□), anti-CD4 (◇), anti-CD8 (△) and anti-CD4 and CD8 (X) treated mice. The titers, reported as IFN \times 100/ml, are the average of 5 different determinations. The maximal variation observed was of 1600 ± 100 U IFN/ml in the sera of control treated mice at day 3.

Discussion

Previous work on MHV3 infection postulated that both resistance gene(s) controlling the degree of viral replication in target cells and the intact immune response are required for resistance (8, 21). We have shown that a nutritionally induced hypercholesterolemia in resistant A/J mice caused susceptibility to MHV3 infection, and that the inhibition of the host resistance was a consequence of an impairment of Kupffer cell functions, such as the sensitivity to the induction of an anti-MHV3 state by IFN (22).

Recently, we have proposed that the mechanism involved in the A/J mice resistance to MHV3 is dependent on the T cells activity and rely on the IFN- γ production and the macrophage sensitivity to IFN- γ (6, 7, 9). These mice have macrophages that are very sensitive to IFN- γ in order to develop an anti-MHV3 state and are capable of producing reasonable amounts of IFN- γ in the course of the immune response against MHV3. Thus, soon after initiation of infection, the virus particles could be neutralized and the infection cleared in a few days. Alternatively, susceptible BALB/c mice have macrophages that are not sensitive to IFN- γ in order to develop an anti-MHV3 state, and despite high concentrations of IFN- γ produced during the first days of infection, the macrophages cannot display a restriction of virus multiplication and these animals die 5 to 6 days after infection (6).

In order to investigate the *in vivo* role of IFN- γ during the MHV3 infection, we followed a direct approach by treating the A/J mice, previously immunized 30 days before with uv-inactivated MHV3, since the resistance is acquired after immunization (9), with monoclonal antibodies against IFN- γ , which has been shown to neutralize different activities of the IFN- γ (18). Since one of the features of CD4 and CD8 T lymphocytes is to produce and release IFN- γ , it was of interest to investigate whether A/J mice treated with monoclonal antibodies against CD4 or CD8 molecules, in such a way that a depletion of CD4 or CD8 T lymphocytes occurred, could be rendered susceptible by an inhibition of IFN- γ synthesized during the course of the infection by MHV3.

The data obtained showed that A/J mice treated with anti-IFN- γ , anti-CD4 or anti-CD8 antibodies became susceptible to MHV3, showing a longer period of disease (Table 1), and that the treatment induced a neutralization or inhibition of the IFN- γ synthesis (Fig. 3) that normally occurred in control mice during the MHV3 infection. The effect was due to the treatment with specific antibodies against IFN- γ , CD4 or CD8, since mice treated with normal rat antibodies behave like those only infected with MHV3, showing that nonspecific effects of control antibodies were not involved. The finding that depletion of either CD4 or CD8 T cell subsets showed the same effect on the inhibition of IFN- γ synthesis, mainly in the sera, after MHV3 infection is rather surprising and has been one of the subjects of our present investigations.

Although, in view of the crucial regulatory function of CD4 cells and the important role of CD8 cells as cytotoxic effector cells during a virus-specific immune response, the results cannot unequivocally exclude the interpretation that susceptibility of the immunosuppressed animals may be due to the absence of proper immune effector mechanisms, the lack of clearance of the virus in the peritoneum and liver of A/J treated and infected mice (Figure 1), which leads to high rates of mortality (Table 1), may be attributed to the lack of IFN- γ in the serum or peritoneal exudate of them (Fig. 3), which has been shown *in vitro* to be effective to restrict the MHV3 growth in target cells such as macrophages (6, 7, 9). Furthermore, our previous observation that the lack of Kupffer cells sensitivity to IFN correlated with the susceptibility of hypercholesterolemic A/J mice to MHV3 (22), is in further support to the idea that IFN- γ plays a crucial role in the resistance to MHV3.

Beside the direct evidence that IFN- γ depleted mice became susceptible to MHV3 infection, our findings show the crucial intervention of CD4 and CD8 lymphocytes by one of their products, the IFN- γ , in the development of A/J mice resistance to MHV3 infection. Although the participation of other factors linked to the generation of the immune response can not be excluded, these results are in further support to the involvement of IFN- γ in the mechanism of resistance against MHV3 infection.

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Dr. C. A. PEREIRA, Instituto Butantan, Laboratório de Imunologia Viral, AV. Dr. Vital, Brasil 1500, CP 65, 05504 São Paulo, Brasil