Role of macrophages, interferon gamma and procoagulant activity in the resistance of genetic heterogeneous mouse populations to mouse hepatitis virus infection

R. C. Vassão, I. G. C. Mello, and C. A. Pereira

Laboratorio de Imunologia Viral, Instituto Butantan, Sao Paulo, Brasil

Summary. Genetic heterogeneous mouse populations selected for high (H_{III}) and low (L_{III}) antibody response were used to study some aspects of mouse hepatitis virus 3 (MHV3) infection, such as the resistance pattern, virus replication in the liver and peritoneal exudate or in cultured peritoneal macrophages, the interferon (IFN) synthesis in the serum and peritoneal exudate and the procoagulant activity (PCA) of the peritoneal exudate (PEC) and spleen cells (SC). The H_{III} mice, when compared to their L_{III} mice counterparts, were susceptible to MHV3 infection showing higher virus titres in the liver and peritoneal exudate, comparable IFN alpha/beta or IFN gamma titres in the peritoneal exudate or in the serum, and higher levels of PCA of PEC and SC. A higher virus titre was detected in the supernatants of H_{III} mouse macrophages infected with MHV3. The activation of H_{III} mouse macrophages with LPS, IFN alpha/beta or IFN gamma, in contrast to that of L_{III} mouse macrophages, did not induce an antiviral effect with partial restriction of the MHV3 replication. The LPS antiviral activity was shown to be partially exerted by IFN alpha/ beta synthesis. The IFN gamma was shown to be more effective in inducing an antiviral state in L_{III} macrophages, when compared to IFN alpha/beta. The data obtained are consistent with the notion that the resistance mechanisms to the MHV3 infection involve the PCA and the sensitivity of macrophages to IFN.

Introduction

The mouse hepatitis virus (MHV) group of coronaviruses is widespread, occurring endemically in mouse colonies, many of which have antibodies against MHV [5, 10, 17, 21, 25]. The MHV3 was isolated by Dick et al. [5] and has been used as a model of viral infection in which resistance is dependent on the genetic background of the mouse strain [1, 6, 8, 13–15, 24]. Genetic homogeneous A/J mice have been reported to be resistant to MHV3 infection because

they develop only a mild disease which disappears 4 to 6 days later. Several other genetic homogeneous mouse strains, such as the BALB/c, have been reported to be susceptible because they develop an acute hepatitis after MHV3 infection and die 3 to 4 days later.

The expression of a monokine with procoagulant activity (PCA), the antiviral state induced by interferon (IFN) and the virus replication in target cells have been implicated in the resistance/susceptibility of these genetic homogeneous mouse strains to the MHV3 infection [1, 7, 12, 18, 24]. We have shown that resistance to MHV3 infection in these genetic homogeneous mouse populations can be a consequence of a T-cell-dependent mechanism, in which the production of IFN gamma and the sensitivity of macrophages to IFN gamma play an essential role [13–15].

The use of genetic heterogeneous lines of mice, which can be more representative of a natural population since inbreeding was avoided during the selective process, permits a further approach to the study of resistance mechanisms involved in the MHV3 infection. Mice selected for high (H) and low (L) responsiveness, were obtained by bidirectional selective breeding of high or low antibody responder lines of mice and the effect of the polygenic regulation of responsiveness to selection antigens is essentially non-specific. The same group of genes regulates the antibody response to many complex immunogens unrelated with those used during the selective breeding, the antibody response being, as far it has been studied, a consequence of a diversity of immune functions, mainly the regulatory role of the macrophage [2, 3, 11, 22, 23, 26].

H and L mice of the selection III (H_{III} and L_{III}) obtained by several selective breeding experiments based on responsiveness to the flagellar antigen of *Salmonellae* [22] were used in this study with the aim to investigate aspects of the MHV3 infection that could further elucidate the mechanisms involved in the resistance/susceptibility to this virus infection.

Materials and methods

Mice

High and low antibody responder mice, 8 to 12-weeks old, from the selection III (H_{III} and L_{III}), obtained by selective breeding based on responsiveness to the flagellar antigen of *Salmonellae* [22], from the Laboratorio de Imunogenetica, Instituto Butantan, were used in the experiments. Certain animals were periodically sacrificed, and the peritoneal exudate and liver tissue 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 presence of MHV in L929 cells. The peritoneal exudates were collected by peritoneal lavage with 5 ml of medium, centrifuged at 750 g for 10 min and the presence of MHV in the supernatants tested on L929 cells. No animal was found to have MHV in the liver or peritoneal exudate. Nevertheless, all the animals tested were found to have antibodies against MHV3 in comparable titres, as measured by ELISA of blood from retroorbital sinus.

Virus

MHV3, originally obtained from Dr. J. L. Virelizier, Institut Pasteur, Paris, France, was cloned by limiting dilution. One plaque was selected and amplified on L929 cells to serve as the inoculum for future stocks [16] to limit spontaneous mutations. The stocks were always titrated by plaque assay on L929 cells as previously described [19]. Aliquots containing 2×10^5 plaque forming units per milliliter (PFU/ml) were stored at $-80\,°C$ and used in the experiments. The MHV3 titres in the peritoneal exudate or liver of infected animals or in supernatants of macrophage cultures were expressed as PFU/ml of peritoneal exudate or cell supernatant and as PFU/g of liver.

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, U.S.A.) at a concentration of 2×10^5 cells per well. The cells were incubated for 2 h at $37\,°C$ in 5% CO_2 and washed three times with medium after vigorous shaking to remove nonadherent cells. Ninety per cent of the cells were macrophages as determined by their ability to take up zymosan particles.

Virus replication assay

Peritoneal macrophages were treated with 10 µg/ml of lipopolyssacharide (LPS) from *Escherichia coli* O111:B4 (Difco Laboratories, Detroit, U.S.A.) or 100 U/ml of murine IFN alpha/beta (laboratory standard purified to 10^7 units of protein per mg from supernatants of L929 cells infected with Sendai virus devoid of IFN gamma activity) or of murine recombinant IFN gamma (Holland Biotechnology, Leiden, Holland). Twenty-four hours later, activated or nonactivated cultures were infected with MHV3 at a multiplicity of infection (moi) of 0.01, 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 titre by plaque assay [19].

Interferon assay

A cytopathic effect reduction test technique, described in detail elsewhere [19, 20] was used for the IFN titre determinations. Briefly, monolayers of L929 cells in microtitre plates were incubated for 18 h with different dilutions of samples. Supernatants were removed, and the monolayers were infected with 200 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. Microtitre plates were incubated for 24 h, and the IFN titre, in units/ml (U/ml) was expressed as the reciprocal dilution of the supernatant able to inhibit 50% of virus replication. For characterization of IFN alpha/beta or IFN gamma in the samples, polyclonal antibodies to mouse IFN alpha/beta and monoclonal antibodies to mouse recombinant IFN gamma (Holland Biotechnology, Leiden, Holland) with activity of 2×10^3 and 2×10^5 neutralizing units per mg, respectively, were always used. The antibodies showed no cross-reactivity, and controls for IFN characterization included internal and well-known preparations of IFN alpha/beta, IFN gamma and a mixture of both to assure the assay specificity.

Procoagulant activity (PCA)

The PCA was determined by a technique already described [9]. Briefly, phosphate buffer saline (PBS), pH 7.2-suspended peritoneal exudate (PEC) and spleen cells (SC) from mice noninfected or infected with 10^3 PFU of MHV3, obtained 2 days after infection, were freeze-thawed, sonicated and assayed for the capacity to shorten the clotting time of rabbit citrated platelet-poor plasma. For the determination of standard milliunits of PCA (mU PCA), a rabbit brain thromboplastin standard (Roche Laboratories, Rio de Janeiro, Brasil) was used to construct a standard curve of PCA, where 100% of thromboplastin activity corresponded to 10 000 mU of PCA/10^6 cells. The data were linear with rabbit citrated platelet-poor plasma and expressed as the mean ± standard deviation of assays from 5 mice in 3 different experiments.

Results

Susceptibility of H_{III} and L_{III} mice to MHV3 infection

As shown in Tables 1 and 2, the H_{III} mice were fully susceptible and the L_{III} mice were fully resistant to the MHV3 infection, regardless the dose of virus used to the experimental infection and whether they were inoculated intraperitoneally, intravenously or subcutaneously. The mean survival time (mst) of H_{III} mice inoculated ip with 10^1 to 10^3 PFU of MHV3 was of 4.0 ± 0.3 to 4.2 ± 0.4, with no correlation with the MHV3 dose used for the experimental infection (Table 1). The mst of H_{III} mice inoculated sc with 10^3 PFU of MHV3 was longer (7.0 ± 1.6) than that observed for the H_{III} mice inoculated ip or iv (4.0 ± 0.4 and 4.2 ± 0.5, respectively) with the same dose of virus (Table 2).

Virus replication in the liver and peritoneum of MHV3 infected H_{III} and L_{III} mice

Table 3 shows that the virus replication in the liver and peritoneum of ip MHV3 infected H_{III} and L_{III} mice correlated with their susceptibility. In both tissues,

Table 1. Susceptibility of H_{III} and L_{III} mice to the MHV3 infection[a]

Mice	Virus (PFU)	Mortality		
		n	%	mst ± sd
H_{III}	10^1	13/13	100	4.0 ± 0.3
	10^2	25/25	100	4.2 ± 0.4
	10^3	40/40	100	4.0 ± 0.6
L_{III}	10^1	0/13	0	–
	10^2	0/25	0	–
	10^3	0/40	0	–

[a] 8 to 12-weeeks-old mice were ip inoculated with MHV3, observed for 30 days and the mortality and mean survival time (*mst*) ± standard deviation (*sd*) recorded

Table 2. Susceptibility of H_{III} and L_{III} mice to MHV3 infection. Route of inoculation dependence[a]

Mice	Route of inoculation	Mortality		
		n	%	mst ± sd
H_{III}	ip	50/50	100	4.0 ± 0.4
	iv	15/15	100	4.2 ± 0.5
	sc	15/15	100	7.0 ± 1.6
L_{III}	ip	0/50	0	–
	iv	0/15	0	–
	sc	0/15	0	–

[a] 8 to 12-weeks-old mice were intraperitoneally (*ip*), intravenously (*iv*) or subcutaneously (*sc*) inoculated with 10^3 PFU of MHV3, observed for 30 days and the mortality and the mean survival time (*mst*) ± standard deviation (*sd*) recorded

Table 3. Virus replication in tissues of MHV3 infected H_{III} and L_{III} mice[a]

Mice	Tissue	Time after infection	MHV3 titre
H_{III}	liver	48 h	$8 ± 0.3 × 10^4$
		96 h	$2 ± 0.1 × 10^6$
	PE	48 h	$6 ± 0.3 × 10^4$
		96 h	$1.4 ± 0.2 × 10^5$
L_{III}	liver	48 h	$1.1 ± 0.2 × 10^5$
		96 h	0
	PE	48 h	$3.5 ± 0.4 × 10^4$
		96 h	0

[a] 8 to 12-weeks-old mice were ip inoculated with 10^3 PFU of MHV3 and 24 and 48 h after, groups of 5 mice were killed, the liver and peritoneal exudate (PE) obtained and the virus titrated. The results expressed in PFU/ml of peritoneal exudate or PFU/g of liver are the average of 5 experiments ± standard deviation

the virus titres increased during the first 48 h of infection, when comparable titres were found in both mouse lines. After this time, the virus titres continued to increase in H_{III} mice reaching a high titre at 96 h of infection, when the animals died. On the other hand, after 48 h of infection the virus titres decreased in L_{III} mice, no virus being found in these animals at 96 h of infection.
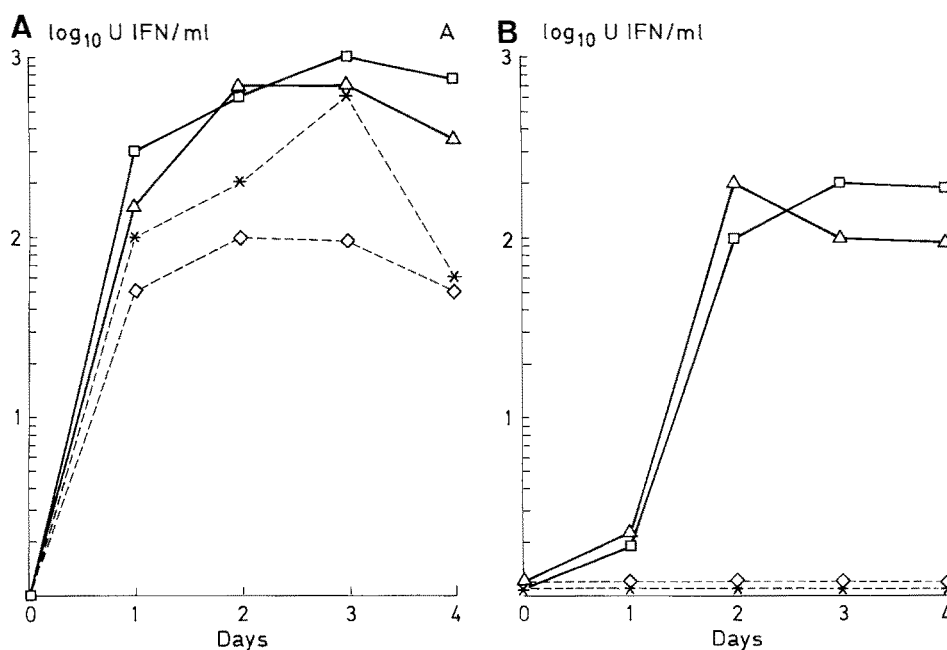
IFN alpha/beta and IFN gamma synthesis in serum and peritoneal exudate of MHV3 infected H_{III} and L_{III} mice

Figure 1 shows the kinetics of IFN alpha/beta and IFN gamma synthesis in serum and peritoneal exudate of mice infected ip with MHV3. In both mouse lines, comparable levels of IFN alpha/beta or IFN gamma synthesis were found in peritoneal exudate or in serum. No IFN alpha/beta was found in their sera. Except in this case, a peak of IFN alpha/beta or IFN gamma in both mouse lines was observed 2 to 3 days after the MHV3 infection, and higher levels of IFN gamma synthesis were always detected in both H_{III} and L_{III} mice.
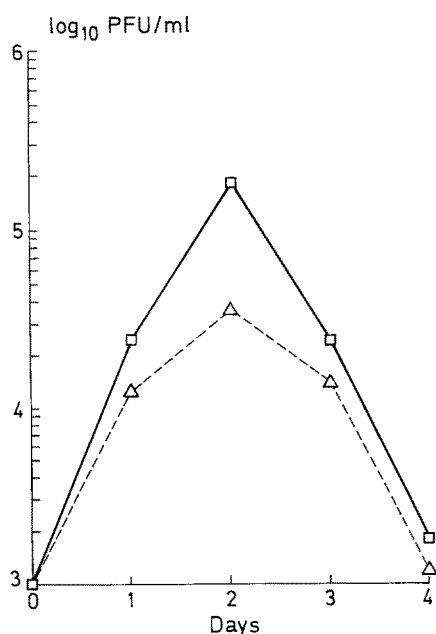
MHV3 replication in macrophages from H_{III} and L_{III} mice

As shown in Fig. 2, the maximal MHV3 titers in macrophage cultures of H_{III} or L_{III} mice were observed 2 days after infection, with a higher virus titre in cell cultures from the susceptible H_{III} mice. Typical syncytia of MHV3 replication could be found in both cell cultures (data not shown).

The results shown in Table 4 indicate that the macrophages from resistant L_{III} mice were sensitive to the induction of an anti-MHV3 state by LPS, IFN alpha/beta or IFN gamma. However, at the same conditions, no anti-MHV3 effect could be induced in macrophages from H_{III} mice. Macrophages from L_{III} mice were shown to be more sensitive to IFN gamma than to IFN alpha/beta.

Fig. 1. Kinetics of IFN alpha/beta (– – – –) and IFN gamma synthesis (————) in peritoneal exudate (**A**) and serum (**B**) of MHV3 infected H_{III} (□, *) and L_{III} (△, ◇) mice. Animals were inoculated ip with 10^3 PFU of MHV3 and different times after, groups of 5 mice were killed, tissues were obtained and IFN alpha/beta and IFN gamma were titrated. The results expressed in Log_{10} U IFN/ml are the average of 5 different experiments

Fig. 2. Kinetics of MHV3 replication in cultured peritoneal macrophages from H_{III} ($\square$) and L_{III} ($\triangle$) mice. Cells were infected with 0.01 moi of MHV3 and, different times after, the supernatants were collected and the virus titrated. The MHV3 titres, reported as Log_{10} PFU/ml, are the average of 5 different experiments

Table 4. Induction of anti-MHV3 state by LPS, IFN alpha/beta and IFN gamma in H_{III} and L_{III} mouse macrophages[a]

Macrophages	Treatment	MHV3 titre (PFU/ml)
H_{III}	none	$3.5 \pm 0.5 \times 10^4$
	LPS	$1.3 \pm 0.2 \times 10^4$
	IFN alpha/beta	$1.2 \pm 0.3 \times 10^4$
	IFN gamma	$1.4 \pm 0.2 \times 10^4$
L_{III}	none	$1.2 \pm 0.4 \times 10^4$
	LPS	$4.5 \pm 0.3 \times 10^2$
	IFN alpha/beta	$6.5 \pm 0.3 \times 10^3$
	IFN gamma	$6.7 \pm 0.1 \times 10^2$

[a] H_{III} and L_{III} mouse macrophages were treated with 10 µg/ml of LPS or 100 U/ml of IFN alpha/beta or IFN gamma, 18 h prior to infection with 0.01 moi of MHV3. The MHV3 titres determined in the supernatants obtained 24 h after infection, are the average of 5 experiments ± standard deviation

The H_{III} and L_{III} mouse macrophage treatment with LPS, led to the synthesis of comparable titres of IFN alpha/beta (data not shown), which was shown to be partially responsible for the antiviral state induced by LPS in L_{III} mouse macrophages (Table 5).

Table 5. Effect of antibodies against IFN alpha/beta on the LPS activation of L_{III} mouse macrophages[a]

Cell treatment	MHV3 titre (PFU/ml)
None	$1.3 \pm 0.3 \times 10^4$
LPS	$3.7 \pm 0.6 \times 10^2$
LPS + ab IFN alpha/beta	$2.1 \pm 0.6 \times 10^3$

[a] L_{III} mouse macrophages were treated with 10 µg/ml of LPS alone or together with antibodies against IFN alpha/beta, 18 h prior to infection with 0.01 moi of MHV3. The MHV3 titres determined in the supernatants obtained 24 h after infection, are the average of 5 experiments $\pm$ standard deviation

Table 6. Procoagulant activity (PCA) of PEC and SC of MHV3 infected H_{III} and L_{III} mice[a]

Mice	Virus	PCA (mU)	
		PEC $\pm$ sd	SC $\pm$ sd
H_{III}	–	60 ± 3	108 ± 7
	MHV3	383 ± 9	720 ± 15
L_{III}	–	55 ± 2	112 ± 4
	MHV3	63 ± 2	187 ± 8

[a] 8 to 12-weeks-old mice were ip inoculated with medium (–) or 10^3 PFU of MHV3 and 48 h later they were sacrificed, the peritoneal exudate cells (*PEC*) or spleen cells (*SC*) were obtained and the PCA determined. The results expressed in milliunits (*mU*) $\pm$ standard deviation (*sd*) are the average of 3 experiments

The "in vitro" treatment with LPS, IFN alpha/beta or with IFN gamma, which induce an anti-MHV3 effect only in L_{III} mouse macrophages, correlated with the "in vivo" resistance observed in L_{III} mice after MHV3 infection.

Procoagulant activity (PCA) of peritoneal exudate and spleen cells from MHV3 infected H_{III} and L_{III} mice

The data obtained of PCA of PEC and SC from MHV3 infected or noninfected H_{III} and L_{III} mice (Table 6) show that 48 h after the infection, an elevation of 6.3 to 6.6 fold in the PCA, could be observed only for cells originated from H_{III} mice (from 60 ± 3 to 383 ± 9 mU and from 108 ± 7 to 720 ± 15 mU for the PEC and SC respectively), whereas no elevation of PCA was found in those

originated from L_{III} mice (from 55 ± 2 to 63 ± 2 mU and from 112 ± 4 to 187 ± 8 mU for the PEC and SC respectively). Noninfected mice from both lines showed comparable levels of PCA in PEC and SC. The elevation of PCA observed in cells from MHV3-infected susceptible H_{III} mice, correlated with their susceptibility pattern to MHV3 shown in Tables 1 and 2.

Discussion

The mouse hepatitis virus strains of coronavirus are viruses responsible for well known epizootics of enteritis which occurs endemically in most mouse colonies. Most of the animals in these colonies were found to have antibodies against different types of MHV, such as MHV3, which have been isolated from a variety of mouse strains under diverse conditions [1, 5, 10, 15, 21, 25].

This study was undertaken in an attempt to investigate the resistance or susceptibility of genetic heterogeneous mouse populations to our strain of MHV3. The H_{III} and L_{III} lines of mice obtained by several selective breeding based on antibody responsiveness to an unrelated antigen [22], are representative of a natural state of a mouse population concerning genetical and immunological aspects, since inbreeding was avoided during the entire selective process. Furthermore, we have observed a reconstitution of the normal distribution of the original F0 genetically heterogeneous population among an F2 segregant population [2, 3, 11]. In addition, these mice are very useful for the studies of resistance/susceptibility to infectious agents due to their characteristic macrophage activity [4].

Both lines of mice, although showing no MHV in their tissues, had in their sera comparable levels of antibodies against MHV3. These antibodies have been shown to exert an "in vitro" neutralizing activity, but their involvement in resistance mechanisms against MHV3 have not been clearly demonstrated. H_{III} mice were shown to be fully susceptible and L_{III} mice fully resistant to the experimental infection with MHV3, regardless the route of inoculation or the dose of virus used for the infection (Tables 1 and 2). The MHV3 replication in their tissues (Table 3) correlated with the resistance/susceptibility pattern (Tables 1 and 2) and the high levels of IFN gamma detected in sera and mainly in the peritoneal exudates (Fig. 1), can be explained by the immunological primed state of the animals. In this case, the experimental virus infection provided a secondary stimulus for the immune system to synthesize the early and high levels of IFN gamma.

As it has already been shown in genetic homogeneous mouse populations [7, 13], the two proposed mechanisms of resistance to MHV3 infection, or, the activation of macrophages by IFN gamma and the PCA, were shown to be correlated with resistance/susceptibility in these genetic heterogeneous mouse populations (Tables 4 and 6). Only macrophages originated from resistant L_{III} mice were able to be activated by IFN gamma in order to restrict the MHV3 replication (Table 4) and only PEC and SC from susceptible H_{III} mice were able to show an elevation of the PCA (Table 6).

During the experimental infection with MHV3, the high levels of IFN gamma synthesis that are observed in already MHV-primed mice are able to efficiently restrict the MHV3 replication only in resistant mice, which have IFN gamma-sensitive macrophages, allowing time for the local and systemic responses to clear the infective particles. On the other hand, and acting synergistically, the higher PCA in susceptible mice, which demonstrates prothrombin cleaving activity [6], was shown to induce abnormalities of the microcirculation, such as granular blood flow and sinusoidal microthrombi, that may disturb the virus clearance and precedes the higher viral replication in susceptible animals [6, 7], which are unable to restrict the virus replication by an impaired macrophage activity. As a consequence, focal avascular lesions appear and progress to confluent necrosis (data not shown) that results in mortality.

The IFN alpha/beta seems therefore to play a secondary role in the resistance of L_{III} mice to MHV3, since it is synthesized in lower amounts during the MHV3 infection (Fig. 1), and because L_{III} mouse macrophages are less sensitive to IFN alpha/beta than to IFN gamma (Table 4). The same reasoning could be applied to the LPS, which exert its function partially through the IFN alpha/beta synthesis (Table 5).

In conclusion, these data from the study of MHV3 infection using genetic heterogeneous mouse populations, which better represents a natural population, confirm those obtained in studies with genetic homogeneous mouse strains [7, 13], representing individuals in a given population in the sense that the two main mechanisms involved in the resistance/susceptibility of mice to MHV3 infection are: 1. The synthesis of IFN gamma and the sensitivity of macrophages to IFN gamma, and 2. The PCA levels exhibited by peritoneal exudate and spleen cells.

Acknowledgements

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Authors' address: Dr. C. A. Pereira, Laboratorio de Imunologia Viral, Instituto Butantan, Av. Vital Brasil 1500, 05503–900 São Paulo SP, Brasil.