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Interference of natural mouse hepatitis virus infection with cytokine production and susceptibility to *Trypanosoma cruzi*

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SUMMARY

Mouse hepatitis virus (MHV) infection can have a pronounced impact on several investigation areas. Reports on natural MHV outbreaks are rare and most studies have been conducted by deliberately infecting mice with MHV laboratory strains that cause moderate to severe disturbances to the immune system. We have investigated the effects of a natural acute outbreak of MHV in our otherwise specific-pathogen-free (SPF) inbred mouse colonies, and of enzootic chronic MHV infection on cytokine production and resistance to the intracellular pathogen *Trypanosoma cruzi*. We found that BALB/c and/or C57BL/6 SPF mice that had been injected with *T. cruzi* blood trypomastigotes from recently MHV-contaminated (MHV^+) mice developed significantly higher parasite blood counts, accelerated death, and showed higher IL-10 production by spleen cells than their counterparts whose *T. cruzi* inoculum was derived from MHV-negative (MHV^-) donors. Interferon-γ (IFN-γ) production by MHV^+ and MHV^- mice was not significantly different. In contrast, *T. cruzi* infection of chronically MHV-infected mice did not result in major changes in the course of infection when compared with that observed in mice from MHV^- colonies, although a trend to higher parasitaemia levels was observed in BALB/c MHV^+ mice. Nevertheless, both BALB/c and C57BL/6 *T. cruzi*-infected MHV^+ mice had diminished IFN-γ production to parasite-antigen stimulation in comparison with similarly infected MHV^- mice. Interleukin-10 (IL-10) production levels by spleen cells did not differ between chronic MHV^+ and MHV^- mice, but IFN-γ neutralization by monoclonal antibody treatment of anti-CD3-stimulated spleen cell cultures showed higher levels of IL-10 synthesis in MHV^+ BALB/c mice.

INTRODUCTION

Mouse hepatitis virus (MHV) collectively designates corona viruses of a wide range of virulence. The strains that are endemic in most mouse colonies over the world show relatively low virulence and animals from infected colonies do not present overt signs of illness. Nevertheless, although tolerated by many researchers, evidence has accumulated over the years that results from several investigation areas can be compromised by concomitant MHV infections. In particular, the study of immunological parameters that determine resistance to infections can be seriously affected by MHV infection (reviewed in refs 1,2). There are few studies on the effects of infection by natural-low-virulence enzootic MHV strains on immune responses and on their interference with experimental models of infection. Most reports deal with virulent laboratory strains, although more recently, attenuated MHV laboratory strains have been used in an attempt to mimic the prevalent strains.[2] The objective of this study was to investigate the effects of a natural acute outbreak of MHV due to accidental exposure, in our otherwise specific-pathogen-free (SPF) inbred mouse colonies, and of enzootic chronic MHV infection on cytokine production and resistance to the intracellular pathogen *Trypanosoma cruzi*.

Trypanosoma cruzi is a dygenetic protozoan that infects several kinds of mammals and is the aetiological agent of Chagas' disease in man.[3] The parasite replicates in the cytoplasm of virtually any nucleated cell type including macrophages; non-dividing forms of the parasite are found free in the blood. Efficient control of parasite load and host survival rely on T-cell-mediated immunity via T-helper cell-dependent protective antibody responses and macrophage activation for intracellular killing of the protozoan; major histocompatibility complex class I-dependent effector mechanisms also contribute to parasitism control (reviewed in refs 4,5). Among inbred mouse strains, C3H, A/J and BALB/c mice rank as susceptible to most parasite strains whereas C57BL/6 and SJL mice are

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Abbreviations: mAb, monoclonal antibody; MHV, mouse hepatitis virus; SPF, specific-pathogen-free; T-Ag, *Trypanosoma cruzi trypomastigote antigen.*

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more resistant.[6] Susceptible mice have higher parasite numbers in the blood and tissues and most animals die within 6 weeks of inoculation of as few as 50 parasites; in contrast, resistant mice have lower parasite loads and survive infection with 50 000 parasites.[7] Both *T. cruzi*-resistant and -susceptible mouse strains show elevated production of interferon-γ (IFN-γ) during infection[8] and very low interleukin-2 (IL-2) and IL-4 production.[8–11] IFN-γ and tumour necrosis factor-α (TNF-α), triggered by IL-12, are essential to control of parasitism by innate and acquired cellular immune systems in mice.[12–14] IL-10 exerts an important *in vivo* regulatory role on IFN-γ and nitric oxide production[12,15] and its absence in IL-10-gene-deprived mice[12,16] or neutralization by monoclonal antibody (mAb) treatment[17] results in lower parasitism, whereas increased parasitism occurs when mice are treated with recombinant IL-10 (rIL-10)[12] or receive IL-10- and IL-4-producing T cells.[15] IL-4, depending on the parasite strain, is also involved in negative regulation of parasitism.[18]

We found that BALB/c and C57BL/6 mice that had been injected with blood trypomastigotes from recently MHV-contaminated mice became much more susceptible to *T. cruzi* infection and produced higher IL-10 levels than their counterparts whose *T. cruzi* inoculum was derived from MHV-negative (MHV^-) donors. Comparison between mice coming from chronically MHV-infected and from MHV^- colonies, showed higher parasitaemia levels in BALB/c MHV-positive (MHV^+) mice but otherwise no major significant differences in susceptibility to *T. cruzi*. Nevertheless, quantitative differences in IFN-γ, IL-10 and nitric oxide production were found between MHV-infected and uninfected mice.

MATERIALS AND METHODS

Mice and MHV infection

C57BL/6 and BALB/c female mice (8–10-week-old), acutely or chronically infected with MHV were obtained from Biotério de Camundongos Isogênicos do Departamento de Imunologia do ICB/USP (São Paulo, SP, Brazil). Mice with negative serological tests for MHV, from both these strains, were obtained from Biotério de Camundongos Isogênicos da Universidade Estadual de Campinas (Campinas, SP, Brazil). MHV^- mice were housed and handled separate from and before those coming from MHV-contaminated animal facilities. The mice were fed autoclaved food and water, and were handled using disposable gloves. MHV infection was diagnosed by antibody testing of the sera. The MHV enzyme-linked immunosorbent assay (ELISA) diagnostic kits sold by Charles River Laboratories (Wilmington, MA) were used according to the manufacturer's instructions. When the outbreak of MHV was detected, levels of anti-MHV antibodies were very high with corrected optical density (OD) values for sera ranging from 8·5 to 23·8 (positive test values >3). These were calculated (as indicated by the manufacturer) by the following formula: [(OD obtained for the test serum diluted at 1/50 incubated with the cells containing the virus) – (OD obtained for the same serum and dilution incubated with uninfected cells)]/0·13. Fluorescence antibody testing was also performed as an additional control and ranked + + + + for serum incubated with MHV-infected cells. The colonies were serologically negative on ELISA and immunofluorescence testing for the following viruses: respiratory-enteric orphan virus (Reo 3), pneumonia virus of mice (PVM), minute virus of mice (MVM), lymphocytic chorio-meningitis virus (LCMV), Sendai virus, Ectromelia virus, mouse polio virus (GD7), and on ELISA for the bacteria *Mycoplasma pulmonis*. All procedures with the animals were in accordance with the principles of the 'Brazilian Code of Laboratory Animals Use'.

Trypanosoma cruzi *infection, parasitaemia counting and experimental design*

Infective blood trypomastigotes were obtained from Y strain *T. cruzi*-infected anaesthetized mice by drawing cardiac blood; motile blood forms were counted and the desired number of parasites was injected intraperitoneally (i.p.). Infection was maintained by weekly i.p. infection of BALB/c mice. In the experiments designated 'acute' MHV infection, parasites were maintained in mice coming from either recently infected MHV^+ or from MHV^- colonies and 500 or 5000 forms were inoculated in recipient BALB/c or C57BL/6 mice from MHV^- colonies. In later experiments, designated 'chronic' MHV infection, the *T. cruzi* strain was started anew from tissue-culture-grown trypomastigotes and maintained in MHV^- mice, whose blood was used as a source of *T. cruzi* to infect recipients derived from MHV^+ colonies that had been infected for more than 4 months or from MHV^- colonies. In these experiments the infective *T. cruzi* dose was 50, 500, or 5000 blood forms in BALB/c mice and 500, 5000 (not shown), 50 000, or 200 000 blood forms in C57BL/6 mice. As C57BL/6 mice are much more resistant to *T. cruzi* infection, the high inocula would allow comparison between MHV^- and MHV^+ C57BL/6 colonies submitted to moderate to severe *T. cruzi* infection.[7] Parasitaemia determination was performed by direct microscope ($\times 40$) counting of motile parasites in a 5 μl fresh blood sample, obtained from the lateral tail veins.

Spleen cell cultures

Spleen cell suspensions were prepared from *T. cruzi*-infected and uninfected mice, MHV^+ or MHV^-, depleted of erythrocytes by hypotonic lysis with distilled water and resuspended in RPMI-1640 (Cultilab, Campinas, SP, Brazil) complete medium containing 10% fetal calf serum (FCS; Cultilab) and supplemented with glutamine, 2-mercaptoethanol (2-ME) and antibiotics as described.[19] Spleen cell suspensions were pooled from three mice and were cultured in duplicate or triplicate in 24-well flat-bottomed plates at 10^7/ml or at 5×10^6/well and stimulated with T-Ag (5×10^6 Frozen-Thawed tissue culture trypomastigotes parasites) prepared as described.[9] Concanavalin A (Con A at 2·5 μ/ml) or plate-bound anti-CD3 [mAb 145-2C11, Americal Type Culture Collection (ATCC) CRL 1973], coated at 10 μg/ml, 500 μl/well) were also used as T-cell stimulants.[11]. Supernatants from the cultures were harvested after 20 hr from the higher cell-density cultures and after 72 hr from the lower-density cultures. The following neutralizing rat mAb anti-mouse cytokines were incorporated to the cultures in some experiments: JES5-2A5 anti-IL-10, and XMG1.2 anti-IFN-γ; both were used at final concentrations of 20 μg/ml. The anti-CD4 mAb GK1.5 was added to cultures at 10 μg/ml.

Cytokine assays

Cytokine levels in the culture supernatants were measured by two-site sandwich ELISA using the following mAb pairs of

which the second cited was biotinylated. IFN-γ, XMG1.2 and AN18; IL-10, JES-2A5 and SXC-1; IL-4, 11 B 11 and BVD6 24G2.[11] Minimum levels of detection for the assays were:. IFN-γ, 1·56 ng/ml; IL-10, 3·25 units/ml; and IL-4, 0·156 ng/ml. The rat anti-mouse cytokines producing hybridomas were a generous gift from Dr R. L. Coffman, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA. Standard curves were obtained with recombinant mouse cytokines. The supernatants were tested in serial twofold dilutions and the results were expressed as the arithmetic mean of duplicate determinations. The SD did not exceed 20% of the mean.

Statistical analysis

The significance of differences in parasitaemia between distinct experimental groups was examined by analysis of variance with repeated measurements followed by Tukey's test for multiple comparisons. Differences of 0·5 $\log_{10}$ or over were significant at least at $P<0·05$. Differences in cytokine production levels were tested by Bonferroni's multiple comparison test.

RESULTS

T. cruzi infection and cytokine production in MHV$^-$ mice inoculated with *T. cruzi* blood forms obtained from donor-mice that were serologically MHV$^+$ or MHV$^-$

Our preliminary observation, suggestive of an infection outbreak in the mouse colonies, was increased susceptibility to *T. cruzi* infection first detected in BALB/c mice. As the diagnosis of MHV infection was confirmed, we first investigated how a *T. cruzi* inoculum derived from donor mice that had concomitant MHV infection would compare with a similar inoculum originated in MHV$^-$ mice in regard to their course of infection in a *T. cruzi*-susceptible (BALB/c) and in a resistant (C57BL/6) mouse strain. As shown in Fig. 1, parasitaemia levels during the first 9 days of infection were significantly higher for both strains of mice when infected with *T. cruzi* blood forms derived from MHV$^+$ donors than when the inoculum came from MHV$^-$ donor mice. The differences in parasitaemia could be observed with inocula of 500 or 5000 blood trypomastigotes (Fig. 1a,b). Moreover, for BALB/c mice injected with MHV$^+$ inocula, mortality reached 80% by day 20 (500 *T. cruzi* forms) and 100% by day 16 (5000 *T. cruzi* forms) whereas all recipients of MHV$^-$ inocula survived to 30 days after infection. All C57BL/6 mice survived after infection whether inoculated with *T. cruzi* from MHV$^+$ or MHV$^-$ donor mice.

Serological tests for MHV became positive in *T. cruzi*-infected (from MHV$^+$ donors) C57BL/6 mice by day 28 after infection and in 30% of a group of 20 BALB/c mice that survived to 28 days after being infected with 50 *T. cruzi* forms. Recipients of inocula from MHV$^-$ mice remained serologically negative for MHV. Looking into possible causes of the observed increase in susceptibility to *T. cruzi* of mice infected with MHV$^+$-derived inocula. we investigated whether cytokine production was altered concomitant to the viral infection. Spleen cells (stimulated with Con A or T-Ag) from mice that received an MHV$^+$ inoculum of 500 *T. cruzi* produced detectable and much higher levels of IL-10 during the first 3 weeks of infection than spleen cells from mice that received an identical inoculum of *T. cruzi* derived from MHV$^-$ mice. In fact, IL-10 production in this last situation was very low and often below 3·12 units/ml (Table 1). IL-10 production, on day 11 after infection, in BALB/c (but not in C57BL/6) mice was CD4-activation dependent as treatment with GK1.5 mAb suppressed most of its synthesis (63 units/ml in untreated versus 6 units/ml in GK1.5 treated cultures in Con A-stimulated and 22 units/ml versus 6 units/ml, respectively, in T-Ag-stimulated BALB/c spleen cell cultures). In C57BL/6 mice, the values were 14 units/ml in untreated versus 10 units/ml in GK1.5-treated Con A-stimulated cultures and 13 versus 10 units/ml, respectively, after T-Ag stimulation. In spite of the higher IL-10 production by spleen cells from mice that had received the MHV$^+$ inoculum, IFN-γ levels were not significantly different from those secreted by spleen cells from MHV$^-$ *T. cruzi*

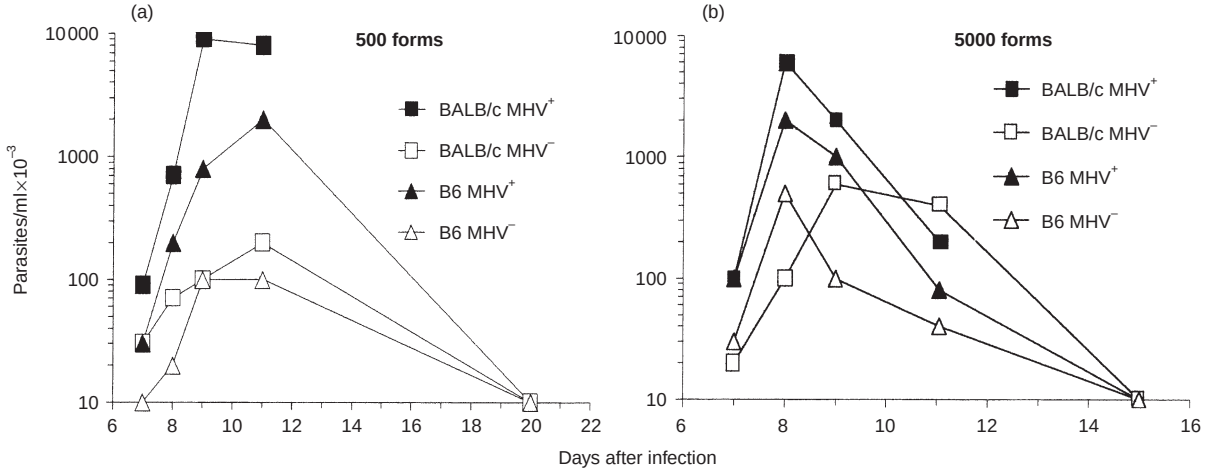

Figure 1. Blood parasitaemia of BALB/c and C57BL/6 (B6) mice, serologically negative for MHV, infected with *T. cruzi* blood trypomastigotes obtained from mice that had been recently contaminated with MHV (MHV$^+$) or from mice that were MHV$^-$. Infection with 500 forms (a) or with 5000 forms (b). Arithmetic means, $n=5$. Significant differences ($P<0·05$) between parasite counts from recipients of MHV$^+$ versus MHV$^-$ parasite donors on all infection days for mice injected with 500 forms and on days 7, 8 and 9 for mice injected with 5000 forms. Representative of two experiments.

Table 1. IL-10 production by spleen cells from MHV$^-$ mice infected with *T. cruzi* derived from MHV$^+$ or MHV$^-$ mice

Days after infection*	MHV$^+$ Con A	MHV$^+$ T-Ag	MHV$^+$ Medium	MHV$^-$ Con A	MHV$^-$ T-Ag	MHV$^-$ Medium
BALB/c mice						
11	56†	27†	10	5	<3‡	5
14/15	46†	20†	6	12	10	<3
19/20	15†	5	5	5	<3	<3
C57BL/6 mice						
11	38†	35†	19†	8	<3	<3
14/15	27†	16†	15†	6	3	<3
19/20	22†	26†	13†	<3	<3	<3

*MHV$^-$ mice were infected with 500 *T. cruzi* blood forms obtained from MHV$^+$ or MHV$^-$ donor mice. At the indicated days after infection, spleen cell cultures were stimulated with Con A or T-Ag, or maintained in culture medium and supernatants were harvested at 72 hr; †significantly different, $P<0{\cdot}01$ from values obtained for MHV$^-$ mice; ‡IL-10 concentrations are expressed in units/ml; <3 indicates less than the minimum detectable IL-10 concentration of 3·1 U/ml. Means of triplicates; representative of two experiments.

recipients and IL-4 production was below detection levels in all groups (data not shown).

Cytokine production by spleen cells from mice derived from MHV$^-$ or from chronically infected MHV$^+$ colonies

Spleen cell cultures from BALB/c and C57BL/6 mice coming from MHV$^-$ or from chronically MHV-infected (MHV$^+$) colonies produced similar amounts of lL-10 and undetectable levels of IL-4 when stimulated *in vitro* with Con A or T-Ag (data not shown). Production of IFN-γ by spleen cells from MHV$^+$ C57BL/6 and BALB/c mice cells to Con A stimulation was not significantly higher than in spleen cell cultures from MHV$^-$ animals (Fig. 2). Production of IFN-γ to Con A was under control by IL-10 both in MHV$^-$ and in MHV$^+$ mice because the addition of anti-IL-10 mAb 2A5 to cultures resulted in significant increases in IFN-γ production of the order of 40–100% (Fig. 2). We next investigated whether infecting MHV$^-$ or mice that were chronically infected MHV with *T. cruzi* would affect cytokine production and/or alter the course of infection.

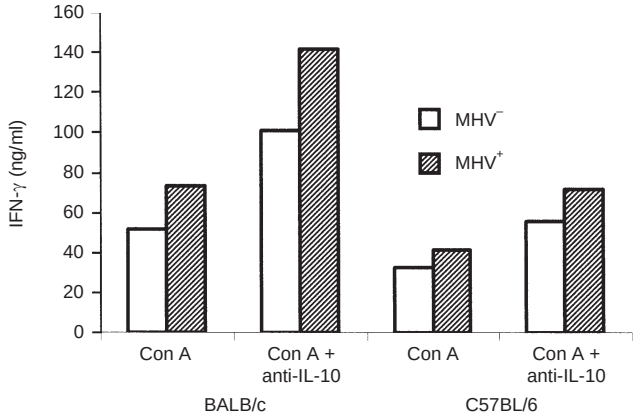

Figure 2. Interferon-γ production by Con A-stimulated spleen cell cultures from BALB/c and C57BL/6 (B6) mice coming from chronically MHV-infected or from MHV$^-$ colonies. The neutralizing anti-IL-10 mAb, 2A5, was added at the beginning of the cultures and the supernatants were harvested 72 hr later; means of triplicate cultures. Increased IFN-γ production by anti-IL-10 treatment was significant in both strains at $P<0{\cdot}05$. Other differences $P>0{\cdot}05$. Representative of two experiments with mice that were not infected with *T.cruzi*.

T. cruzi infection and cytokine production in mice from MHV$^-$ colonies or from chronically MHV-infected colonies inoculated with *T. cruzi* blood forms maintained in MHV$^-$ donor mice

In contrast with the marked increase in parasitaemia and susceptibility to *T. cruzi* determined by the situation of acute simultaneous infection with blood parasites derived from acutely infected MHV$^+$ donors as described above, no statistically significant differences of parasitaemia levels or mortality were observed between chronically infected MHV$^+$ and MHV$^-$ BALB/c or C57BL/6 mice infected with *T. cruzi*. However, peak *T. cruzi* parasitaemia levels were attained earlier and were 30–50% higher in BALB/c MHV$^+$ mice as compared to MHV-free mice (data not shown).

Differences in cytokine production between *T. cruzi*-infected MHV$^+$ and MHV-free groups of mice were only observed in the first week of infection. BALB/c mice spleen cell cultures from MHV$^+$ mice infected with *T. cruzi* produced lower IFN-γ levels to Con A and T-Ag stimulation than cultures from MHV$^-$ mice (Fig. 3). Although the potential to produce IFN-γ to polyclonal Con A stimulation was preserved in MHV+C57BL/6 mice, IFN-γ production was markedly suppressed on parasite-specific stimulation (Fig. 4). The comparison of IL-10 production levels among BALB/c and C57BL/6 mice, MHV$^-$ or MHV$^+$, infected with *T. cruzi* yielded no significant differences (data not shown). Nevertheless, when BALB/c (but not C57BL/6) spleen cell cultures stimulated with plate-bound anti-CD3 (an antigen-presenting-cell-independent polyclonal T-cell activator) were treated with neutralizing anti-IFN-γ antibody, increased production of IL-10 was observed in cultures derived from MHV$^+$ mice but not from MHV$^-$ mice (Fig. 5). Augmentation of IL-10 production was observed in cultures from *T.cruzi*-infected MHV$^+$ mice and also in cultures from *T. cruzi*-

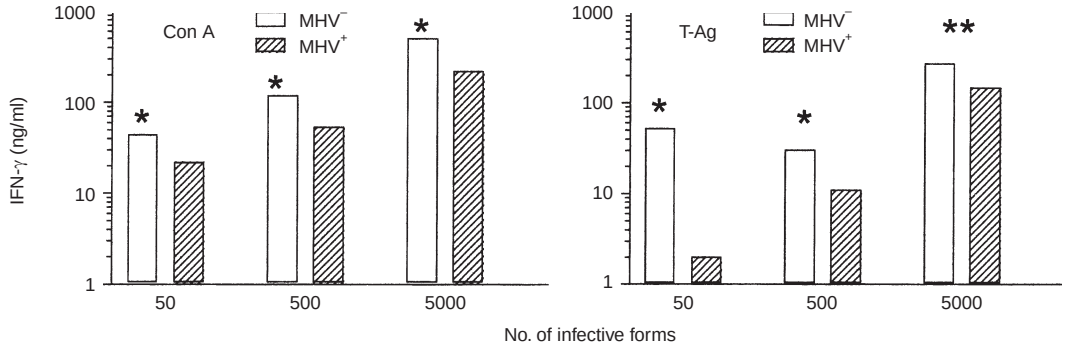

Figure 3. Interferon-γ production by Con A- and parasite-antigen-(T-Ag) stimulated spleen cell cultures from BALB/c mice coming from chronically MHV-infected or from MHV^- colonies and infected with different numbers of *T. cruzi* blood forms derived from MHV^- donors. Data from day 5 of infection, 72 hr supernatants. Means of duplicates; representative of three experiments; differences significant at $P<0{\cdot}01$ (*) or at $P<0{\cdot}05$ (**).

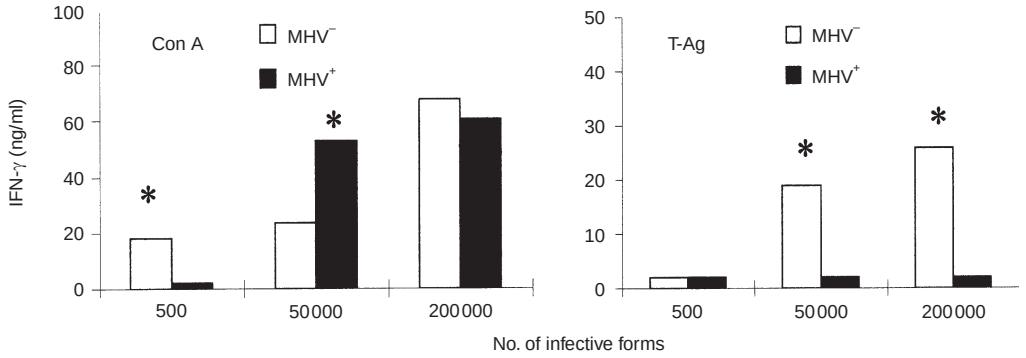

Figure 4. Interferon-γ production by Con A- and parasite-antigen-(T-Ag) stimulated spleen cell cultures from C57BL/6 (B6) mice coming from chronically MHV-infected or from MHV^- colonies and infected with different numbers of *T. cruzi* blood forms derived from MHV^- donors. Data from day 5 of infection, 72 hr supernatants. Means of duplicates; representative of three experiments; differences significant at $P<0{\cdot}01$ (*).

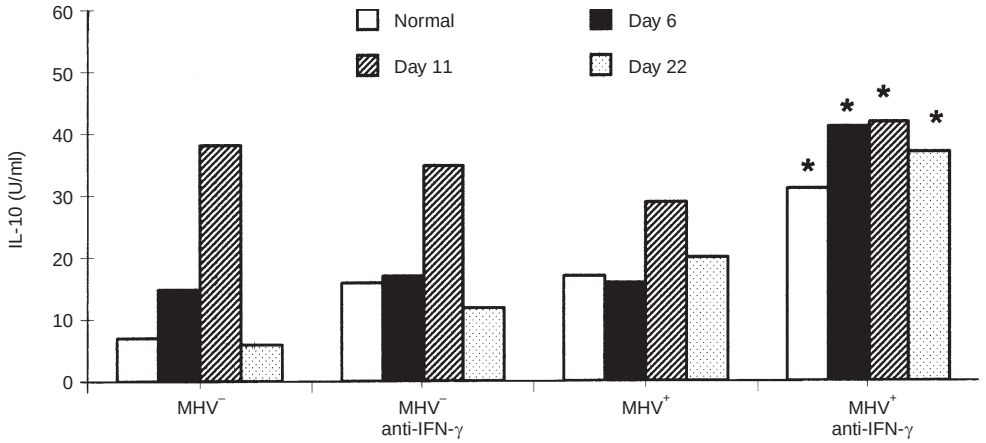

Figure 5. Interleukin-10 (units/ml) production by anti-CD3 stimulated and spleen cell cultures from BALB/c mice coming from chronically MHV-infected or from MHV^- colonies. The cultures were performed in the presence (or not) of the neutralizing anti-IFN-γ mAb XMG1.2; 72 hr supernatants. The mice were tested before *T. cruzi* infection (normal) and on days 6, 11 and 22 after infection with 500 *T. cruzi* blood forms. Means of duplicates; representative of two experiments; MHV^+ and anti-IFN-γ mAb-treated groups significantly different from untreated MHV^+ groups and from MHV^- groups at $P<0{\cdot}01$ (*).

uninfected MHV^+ mice (Fig. 5, normal group), suggesting that the potential to increased IL-10 production stimulated by MHV infection was, in these mice, maintained in check by IFN-γ. Taken together, these results indicate that in C57BL/6 mice, suppression of IFN-γ responses is limited to the antigen-specific compartment whereas MHV infection affects more severely BALB/c mice by suppression of both polyclonal and specific IFN-γ responses.

DISCUSSION

Enzootic MHV strains are of low virulence, mostly enterotropic and notoriously difficult to isolate and to grow *in vitro*.[1] When the outbreak was detected in the mouse colony, we unsuccessfully tried to isolate infective viral particles from the plasma and liver of seropositive mice. Immunosuppressing the animals with cyclophosphamide also failed to promote viral isolation. The difficulty in isolating low virulence enzootic enterotropic MHV, as opposed to polytropic laboratory MHV strains, has been frequently reported.[1,2,20] We had (as many other authors) to rely on serum antibody screening and serological conversion as a criterion to identify occurrence of MHV infection. Serological testing, although highly specific, does not distinguish between acute and chronic MHV infection and thus we will discuss the immunological alterations found in the group of mice that had recently become seropositive for MHV (recent MHV-testers) in comparison with those found in mice originating from colonies with longstanding record of MHV seropositivity. Both groups of mice were serologically negative on testing for several other mouse pathogenic viruses and for *Mycoplasma pulmonis* (see the Materials and Methods).

We have found a much more severe course of *T. cruzi* infection in MHV^- mice infected with blood forms obtained from mouse colonies that had recently become seropositive for MHV, as compared with mice inoculated with parasites derived from MHV^- donors; increased susceptibility to infection was accompanied by increased synthesis of IL-10 by spleen cell cultures. Interleukin-10 down-regulates IL-12 and IFN-γ production, besides antagonizing IFN-γ- and TNF-α-dependent macrophage activation and intracellular *T. cruzi* killing.[12,13,17,21–23] In spite of the enhanced IL-10 secretion by spleen cells from MHV^+ recipients, no decrease of IFN-γ levels, in comparison to recipients of MHV^- donors could be detected in these same cultures. However, the data on enhanced IFN-γ synthesis by anti-IL-10 mAb treatment, showed that endogenous IL-10 was down-regulating IFN-γ production in this situation of probable acute concomitant infection with the parasite and the virus. The maintenance of *in vitro* IFN-γ secretion rates in the presence of increased IL-10 concentrations has been described upon treatment of *T. cruzi*-infected mice with high doses of rIL-10 that aggravate infection.[12]

Both mouse strains, BALB/c and C57BL/6, respectively, susceptible and resistant to *T. cruzi*, showed increased parasitaemia and augmented IL-10 production, but increased mortality as a consequence of MHV infection was not observed for C57BL/6 mice and they maintained their resistant phenotype to *T. cruzi* infection. Although mouse strain resistance to MHV infection is highly dependent on the MHV strain, studies with laboratory MHV-strains have concluded that indifferently to the degree of MHV-strain virulence, replication in macrophages does always occur.[24] Viral replication inside macrophages may directly interfere with *T. cruzi* killing ability by these cells and stimulate a number of macrophage functions including IL-10 synthesis. In this regard, C57BL/6 mice are semisusceptible to most MHV strains;[1,2] as production of IL-10 by MHV-infected C57BL/6 mice (but not by BALB/c mice) was $CD4^+$-activation independent, virus-infected or virus-stimulated macrophages could be the main source of this cytokine.

Most studies on the effects of MHV infection on the immune response have been performed in situations of deliberate infection of mice with MHV laboratory strains of varying virulence that cause moderate to severe disturbances to the immune system. Mice infected with the pantropic strain of medium-virulence, JHM, show suppression of Con A-induced proliferation, decreased IL-2 and IL-4 synthesis and a delay in IFN-γ production in the first week of infection, whereas, later in infection, large amounts of IFN-γ are produced by BALB/c mice.[25–27] Macrophage function was impaired in mice infected with this strain[28] or with naturally occurring MHV strains.[29,30] Increased message levels for IFN-γ, IL-4, IL-10, TNF-α and inducible nitric-oxide synthase (iNOS) were found in the brain of mice infected with a neurotropic JHM variant strain.[31] MHV A-59 is yet another laboratory strain, but of relatively low virulence that, although subclinical, is accompanied by increased production of IFN-γ and suppression of Con A spleen responses.[32,33]

There have been few reports on immunological alterations resulting from natural MHV outbreaks. However, established 'chronic' natural infection with MHV was reported to affect mostly splenic T lymphocytes, with a 20–50% decrease in proliferative responses to Con A and resistance to the effects of nor-adrenalin or dibutyryl-cAMP.[34] We found that lymphoproliferative responses of spleen cells from BALB/c and C57BL/6 mice, MHV^+ or MHV^- recipient mice to Con A, T-Ag, or anti-CD3 stimulation were not significantly different. Suppressed lymphoproliferative responses to these stimuli, commonly seen in the course of *T. cruzi* infection,[11] were observed from day 11 of infection, with minimal levels of suppression seen on day 19 and recovery by day 27 (data not shown). Thus, in the course of *T. cruzi* infection, we did not observe, neither in recently seropositive recipient mice nor in mice from chronically MHV-infected colonies, suppression of lymphoproliferation to Con A, to parasite antigens or to anti-CD3 stimulation that could be ascribed to the viral infection. However, its occurrence could have been masked by the intense suppression characteristic of acute *T. cruzi* infection.

Our results on the aggravation of *T. cruzi* infection in BALB/c and C57BL/6 mice by using parasite inocula maintained in mice that had positive serology for MHV are in agreement with a previous report on CBA/J mice infected with *T. cruzi* derived from corona virus-positive mice.[35,36] These authors were able to demonstrate, in the plasma, infective virus particles that could be neutralized by anti-MHV antiserum. Nevertheless, the underlying mechanisms leading to increased susceptibility to the parasite were not explored.

We further investigated the influence of a longstanding (1 year) established endemic MHV infection on the course of *T. cruzi* infection and immune response. In contrast with the marked effects on the immune response observed in MHV^- hosts infected with *T. cruzi* derived from recently MHV^+ mice, the disturbances of immune response found in mice from chronically MHV-infected colonies were milder. Production of IL-10 was similar between MHV^+ and MHV^- mice, while parasite–antigen-stimulated IFN-γ production was lower in *T. cruzi*-infected BALB/c and C57BL/6 mice. However, when neutralization of IFN-γ in the cultures unmasked the full potential of IL-10 secretion, MHV^+ BALB/c mice (but not C57BL/6), showed higher IL-10 production levels. This result is consistent with the trend of enhanced *T. cruzi* susceptibility observed in MHV^+ BALB/c mice that resulted in earlier and

higher blood parasitaemia levels. Taken together, these data indicate that several alterations of the immune response occur because of chronic MHV infection. Although milder than those observed in the recent infection situation, it is worth mentioning that MHV-infected mouse colonies are subject to recurrent subacute, subclinical infections by mutant MHV strains with unpredictable consequences for the immune response.

In summary, this study describes several quantitative and qualitative modifications of cytokine production and resistance of mice to experimental infection with *T. cruzi* determined by a natural concomitant infection with enzootic MHV. It emphasizes the interference of enzootic infections with immunological parameters and with the course of infection by intracellular micro-organisms, as well as the need to use specific-pathogen-free mice when performing such studies.

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