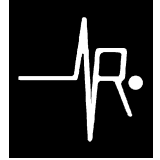Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Original Contribution

Association between nitric oxide synthesis and vaccination-acquired resistance to murine hepatitis virus by *spf* mice

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Abstract

Murine hepatitis virus strain 3 (MHV-3) produces a strain-dependent pattern of disease, with A/J and BALB/c mice being considered models of resistance and susceptibility, respectively. A role for nitric oxide in controlling infection remains debatable; thus, we monitored nitric oxide levels in blood and liver of immunized and nonimmunized *spf* mice during infection by electron paramagnetic resonance. In parallel, liver histology, virus titers, and plasma alanine aminotransferase (ALT) activity were monitored. Nitric oxide synthesis was barely detectable in BALB/c mice, which showed a progressive increase in virus titers and ALT activity. These animals died with a shorter survival time than A/J mice. The latter displayed a less severe infection and presented detectable levels of nitric oxide as nitrosyl complexes in blood and liver at 72 hpi. Immunized mice from both strains became resistant to MHV-3 and showed comparable levels of nitrosyl complexes in blood and liver at an early time (24 hpi). Thereafter, nitric oxide levels decreased but remained detectable in blood up to 96 hpi. Immunized mice were capable of clearing the virus and clearance was inhibited by administration of a nitric oxide synthase inhibitor. Overall, the results support a role for nitric oxide in controlling MHV-3 infection.
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Keywords: MHV-3; Nitric oxide; Immunized and nonimmunized *spf* mice; Fulminant murine hepatitis; Resistance/susceptibility; Free radicals

Murine hepatitis virus strain 3 (MHV-3) is a single-stranded, positive-sense RNA virus belonging to the *Coronaviridae* family, which produces a strain-dependent pattern of disease in inbred laboratory mice, depending on the virus strain, infection route, age, genetic background, and immune status of the host [1,2]. MHV-3 was isolated in 1956 [3] and has been used as a model for the study of host resistance/susceptibility to human hepatitis virus. Indeed, several studies using inbred lines of mice have reported that A/J mice show an innate resistance and BALB/c mice an innate susceptibility to experimental MHV-3 infection [4–6]. Even though the resistance of A/J mice to MHV-3 has been considered innate, there are data suggesting that this resistance is also dependent on the antibodies naturally acquired or induced by immunization [7,8]. Also, it has been shown that in coronavirus-free colonies, A/J mice can rapidly acquire resistance through natural or experimental infection with other MHV strains, whereas BALB/c mice cannot [1].

Several mechanisms have been proposed to explain susceptibility/resistance to MHV-3 [4,9–15], including those that emphasize oxidative macrophage mechanisms and nitric oxide synthesis. The conclusions about the role of nitric oxide in MHV-3 infection, however, have been contradictory. For instance, it has been demonstrated that peritoneal macrophages isolated from resistant A/J mice produced a fivefold-higher level of nitric oxide and higher levels of mRNA transcripts of inducible nitric oxide synthase (iNOS) in response to IFN-γ than macrophages isolated from susceptible BALB/c mice [16]. In contrast, it has been proposed that IFN-γ acts as an antiviral effector [1,10,17] by a mechanism that is independent of nitric oxide [18] but dependent on down-regulation of the main virus receptor Bgp1[a] [19].

Abbreviations: MHV-3, murine hepatitis virus strain 3; iNOS, inducible nitric oxide synthase; PFU, plaque-forming units; *spf*, specific-pathogen-free; ALT, alanine aminotransferase; IFN-γ, interferon-γ.
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Nitric oxide is a radical with several physiological and pathophysiological roles [20], which has been proposed to act as a nonspecific antiviral effector [16,21–29]. However, the role that nitric oxide plays in many diseases, including those of viral origin, remains controversial [16,21,26,30–32]. Depending on the experimental model and conditions, opposing effects are often attributed to nitric oxide, creating much confusion in the literature [33]. A possible explanation for the many discrepancies is the extensive use of nonspecific NOS inhibitors [32] as well as the intrinsic difficulties in detecting nitric oxide, a short-lived free radical, under physiological conditions.

Studies of the role of nitric oxide in MHV-3 infection have been performed mostly in vitro or with inbred mice [16,18]. Because the conclusions have been controversial, further studies are warranted. Here, we monitored nitric oxide formation in vivo by examining the blood and liver of immunized and nonimmunized *spf* mice during MHV-3 infection by direct electron paramagnetic resonance (EPR) spectroscopy. In parallel, we monitored several infection parameters and administered a relatively selective iNOS inhibitor, 1400 W. Our results indicate that an early and sustained synthesis of nitric oxide is important for virus and disease control.

Materials and methods

Mice, virus, and infection

MHV-3 and MHV-A59 viruses (kindly provided by Dr. J.P. Martin, Laboratoire de Virologie, Strasbourg, France) were propagated and plaque assayed on L929 cells cultured in modified Eagle's medium (MEM) containing 10% fetal bovine serum at 37°C, as previously described [10]. Aliquots containing 4×10^5 PFU ml^{-1} were stored at −80°C and used in all experiments. *Spf*-grade female A/J and BALB/c mice (6 to 8 weeks of age) were purchased from the Instituto de Ciências Biomédicas, Universidade de São Paulo, and maintained in a laminar-flow rack in microisolator cages. Mice were infected ip with 0.2 ml of phosphate-buffered saline (PBS) containing 10^3 PFU of MHV-3; control animals were injected with 0.2 ml of sterile PBS alone. Mice were immunized ip with 10^3 PFU of MHV-A59 [34] in a volume of 0.2 ml and challenged with 10^3 PFU of MHV-3, 2 weeks later. The determination of serum antibodies to MHV-3 was performed by serially diluting mice sera with 2 vol of MEM containing 10% fetal bovine serum. A 0.2-ml sample of each dilution was mixed with the same volume of 200 PFU of MHV-3 in MEM containing 10% fetal bovine serum and incubated at 37°C for 45 min. Then, neutralizing antibodies were determined and expressed as the reciprocal of the highest serum dilution producing 100% inhibition of the cytopathic effect induced by MHV-3 on L929 cells [35].

Tissue collection and EPR measurements

At designated periods, anesthetized mice were bled from the orbital plexus into heparinized tubes. The plasma was separated by centrifugation; red blood cells were drawn into a plastic syringe and immediately frozen in liquid nitrogen. The liver was perfused with 10 ml cold phosphate-buffered saline, extruded into a plastic syringe, and immediately frozen in liquid nitrogen. EPR spectra were obtained with a Bruker ER 200-SRC spectrometer using a fingertip liquid nitrogen dewar (77 K). Spectrometer conditions are specified in the figure legends. The data were fed into an IBM/AT computer with which baseline subtraction and double integration were performed as previously described [36,37]. The concentration of nitrosyl complexes in blood was estimated by double integration of the EPR spectra and comparison with those of standards containing known concentrations of hemoglobin–nitrosyl complexes [36].

Viral titration

Animals were periodically sacrificed, and the liver tissue samples were ground and resuspended in MEM containing 10% fetal bovine serum plus gentamicin (50 μg ml^{-1}). Liver tissue (1 mg ml^{-1}) was serially diluted and plaque assayed on L929 cells as described previously [10]. Virus titers were expressed as PFU mg^{-1} liver.

Histopathology

Liver samples were fixed in a solution containing 60% methanol, 30% chloroform, and 10% glacial acetic acid. Samples were embedded in paraffin, cut, stained with hematoxylin and eosin, and observed under a light microscope.

Plasma alanine aminotransferase (ALT) activity

Plasma ALT activity was spectrophotometrically determined by a standard enzymatic method using a commercial kit from LabTest Diagnóstica (Minas Gerais, Brazil).

Treatment of animals with the selective iNOS inhibitor 1400 W

Female immunized mice (A/Ji, $n=8$, and BALB/ci, $n=6$) 6–8 weeks of age, approximately 20 g, were experimentally infected with 10^3 PFU of MHV-3 15 days after immunization and then treated with 1400 W (*N*-(3-(aminomethyl) benzyl) acetamidine dihydrochloride; Cayman Chemical Co.). A stock solution was made by dissolving 1400 W in ethanol and stored at −20°C. The stock solution was diluted into sterilized PBS (pH 7.2) and mice were administered 15 mg 1400 W kg^{-1} ip [38] 1 h before the injection of MHV-3 and then daily, for 5 days. Control animals (6 A/Ji and 13 BALB/ci) received an ip injection of the vehicle. Nitric oxide production in blood from treated and untreated animals was assayed at 24 hours postinfection (hpi) by EPR as described above. Viral titers from liver tissue samples from both groups were monitored at 96 hpi. The animals were observed for 30 days and the mortality was recorded.

Results

Infection course and nitric oxide synthesis

Resistance to MHV-3 has been associated with mouse genetic factors and immune status [1,4–8] and therefore we monitored several infection parameters in immunized and nonimmunized *spf* mice (Figs. 1–7).

BALB/c and A/J mice were immunized with MHV-A59, an attenuated strain of MHV [34]. The titers of neutralizing antibodies against MHV-3 in the sera of the animals were determined as previously described [35], and no neutralizing activity was detected in the sera of nonimmunized mice. In contrast, sera from immunized mice of both strains (BALB/ci and A/Ji) were found to contain neutralizing antibodies at a titer of 1/512 2 weeks postvaccination (MHV-A59 inoculation). This titer of neutralizing antibodies was enough to inhibit virus multiplication in the liver of immunized mice of both strains (Fig. 4) that became fully resistant to the infection and displayed no mortality up to 30 days after (Fig. 1A). These animals were also resistant to a second challenge with 10^3 PFU of MHV-3 10 days after the first experimental infection. In this case, the survival rate was 100 and 90% for A/Ji ($n=10$) and BALB/ci ($n=10$), respectively. The animals were monitored for 35 days after the second challenge and showed no clinical sign of disease up to 7–10 days after the second challenge (Fig. 1B). In contrast, the survival time of nonimmunized mice (BALB/c and A/J) varied with strain, i.e., 100% of BALB/c mice died at 72 to 96 hpi (mean survival time: 84 hpi), whereas A/J mice died between 96 and 144 hpi (mean survival time: 120 hpi) (Fig. 1A). The different survival rates of the strains of nonimmunized mice and the resistance developed after immunization by both mouse strains confirmed that genetic and immune factors play a role in the development of resistance against MHV-3 infection.

To examine the potential role of nitric oxide in MHV-3 infection, its synthesis was monitored by low-temperature EPR of blood and liver samples of infected mice during the course of the disease. In nonimmunized mice, production of nitric oxide was detectable by the characteristic EPR spectra
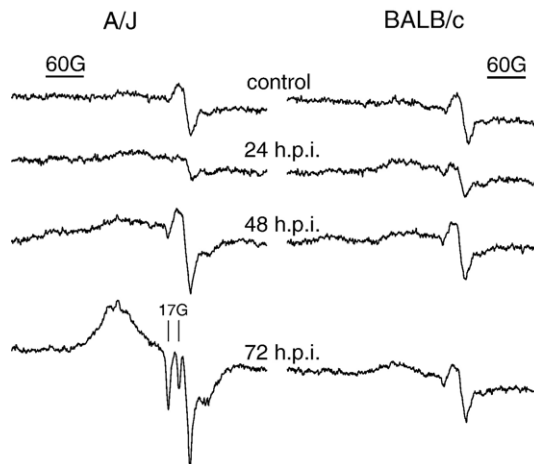

Fig. 2. Representative low-temperature EPR spectra obtained from blood of nonimmunized mice (A/J and BALB/c) after various times of infection with 10^3 PFU of MHV-3. The marked hyperfine splitting constant value of 17 G characterizes the hemoglobin–nitrosyl pentacoordinated complex [40]. Spectrometer conditions: microwave power, 20 mW; modulation amplitude, 5 G; time constant, 163 ms; scan rate, 3.58 G/s.

of heme–nitrosyl complexes in blood (Fig. 2) and liver (Fig. 3) of A/J mice at 72 hpi [36,37,39,40]. The EPR signal detected in blood is due to hemoglobin–nitrosyl complexes (at around 10 ± 2 μM levels), whereas that in liver is probably due to cytochrome P450–nitrosyl complexes [40] because the organ was extensively perfused to minimize blood contamination. At other infection times or in samples from BALB/c mice, heme–nitrosyl complexes were barely detectable (Figs. 2 and 3).

Nitric oxide synthesis, virus titers, liver damage, and death

Relationships between nitric oxide synthesis, virus titers, liver damage, and animal death were established. Thus, in the case of BALB/c mice, virus titers in the liver increased gradually to a peak of 3.2×10^3 PFU ml^{-1} at 72 hpi (Fig. 4), when most mice died of acute hepatitis (Fig. 1A). In the case of A/J mice, virus titers were lower, a maximum of 1.1×10^3 PFU ml^{-1} at 72 hpi. Thereafter, virus titers decreased substantially
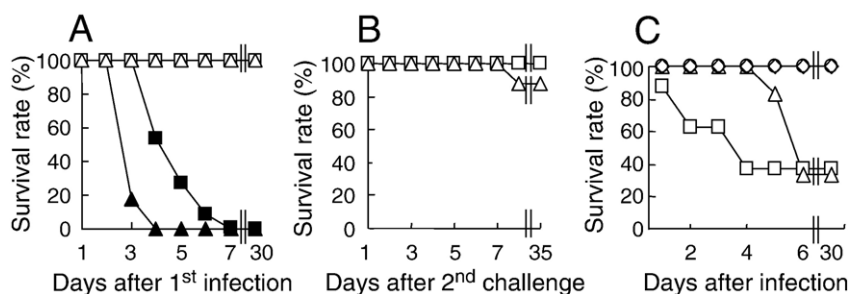

Fig. 1. Mortality of immunized (BALB/ci (△) and A/Ji (□)) and nonimmunized (BALB/c (▲) and A/J (■)) mice (10 of each strain) after ip MHV-3 infection (10^3 PFU). (A) Mice were immunized with a single dose of 10^3 PFU of MHV-A59, then infected (ip) with 10^3 PFU MHV-3, and monitored for 30 days. (B) Immunized mice were reinfected 10 days after the first infection and monitored for 35 days. (C) Resistance-immunized mice (6 BALB/ci (△) and 8 A/Ji (□)) were challenged with 10^3 PFU of MHV-3 ip and treated 1 h before the infection and daily with 1400 W (15 mg kg^{-1} ip) for 5 days. Control mice (6 A/Ji (○) and 13 BALB/ci (◇)) were injected with vehicle. Mice were monitored for 30 days.
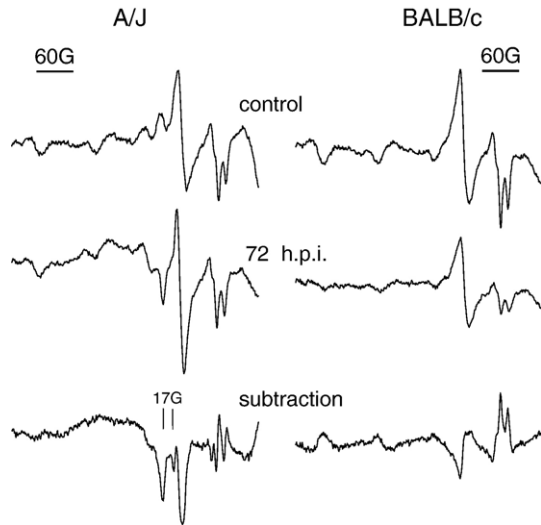
Fig. 3. Representative low-temperature EPR spectra obtained from livers of nonimmunized mice (A/J and BALB/c) 72 h post-infection with 10^3 PFU of MHV-3. The last spectrum (top to bottom) is the computer subtraction of the second (infected mice) from the first spectrum (control). Spectrometer conditions: microwave power, 20 mW; modulation amplitude, 5 G; time constant, 163 ms; scan rate, 3.58 G/s.

(88 PFU ml^{-1} at 96 hpi) (Fig. 4) but, even so, the animals started dying (Fig. 1A). Virus titer variation during the course of the disease paralleled liver damage as assessed by histology (Fig. 5) and by measurements of plasma ALT activity (Fig. 6). No abnormalities were found in livers from uninfected A/J (Fig. 5A) and BALB/c mice (Fig. 5B) but the two strains developed a different pattern of lesions when infected. At 24 hpi, A/J mouse livers appeared unaltered (Fig. 5D), whereas BALB/c livers presented a discrete degeneration of hepatocyte cytoplasm (Fig. 5C). At 48 hpi, discrete cytoplasm degeneration occurred in A/J mice but numerous foci of inflammatory cells and necrotic lesions were evident in the liver of BALB/c mice (Fig. 5F). At 72 hpi, cytoplasm degeneration was observed for both strains (Figs. 5G and 5H) but it was strikingly more intense in the liver of BALB/c animals, which showed extensive necrosis and high amounts of polymorphonuclear cell infiltrates (Fig. 5H). These observations were consistent with those reported by other investigators for inbred laboratory mice [41,42].

Progression of liver damage was also consistent with biochemical findings, showing a time- and strain-dependent increase in plasma ALT activity (Fig. 6). Thus, BALB/c mice did not synthesize nitric oxide upon MHV-3 infection and fast succumbed to hepatitis. A/J mice presented a milder evolution of the disease and were able to synthesize nitric oxide and to decrease virus titers but also died, although with an average longer survival time.

Vaccination leads to nitric oxide synthesis

In contrast to nonimmunized mice, the immunized A/Ji and BALB/ci mice became resistant to the disease (Fig. 1) and synthesized comparable levels of nitric oxide early in the infection (24 hpi) as attested by EPR analysis of blood (Fig. 7)

and liver samples (data not shown). Nitric oxide levels in blood remained considerably high up to 96 hpi (Fig. 7B). In parallel, a complete virus clearance was observed for both mouse strains (Fig. 4). As could be anticipated, liver damage was also controlled in these animals because plasma ALT levels showed a small increase up to 48 hpi but decreased to control levels at 72 hpi and thereafter (Fig. 6). The control of the disease by the immunized mice (A/Ji and BALB/ci) was also attested by liver histology that showed marginal abnormalities except for some localized foci of inflammatory cell infiltrates that decreased with time (data not shown).

Most relevant, treatment of resistant immunized mice with the selective iNOS inhibitor 1400 W significantly increased mortality after MHV-3 infection (Fig. 1C). Indeed, administration of 1400 W to immunized A/Ji and BALB/ci mice returned mortality 4 to 6 days after infection to levels similar to those of nonimmunized mice (Fig. 1A), that is, 63 and 67% for A/Ji ($n=8$) and BALB/ci ($n=6$), respectively (Fig. 1C). That 1400 W treatment inhibited early nitric oxide synthesis was confirmed by EPR analysis of blood. Treatment with 1400 W decreased hemoglobin–nitrosyl complex levels at 24 hpi by 40–50% in both A/Ji and BALB/ci mouse strains (Fig. 7). Moreover, 1400 W treatment permitted viral replication in both animals. Indeed, the viral titers found in the livers of treated A/Ji and BALB/ci mice at 96 hpi were 30 and 145 PFU mg^{-1} of liver, respectively, values that are similar to those detected at 24 hpi in the nonimmunized mice (Fig. 4).

Discussion

Our results demonstrate that nitric oxide synthesis occurs in vivo during fulminant murine hepatitis virus infection (Figs. 2, 3, and 7) and that an early nitric oxide production plays a role in protecting the immunized–challenged mice against the virus (Figs. 1 and 4). Partial inhibition (40–50%) of the early nitric oxide synthesis by a specific iNOS inhibitor was shown to increase virus replication (30 and 145 PFU mg^{-1} of liver in A/Ji and BALB/ci mice, respectively) and animal mortality (Fig.
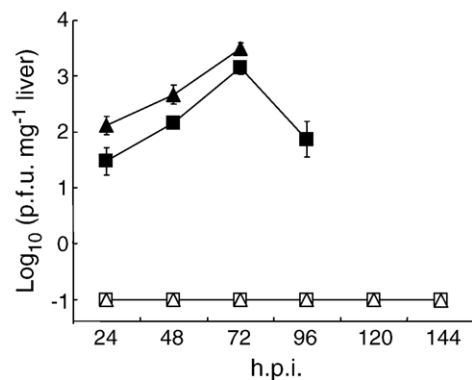

Fig. 4. Viral growth in the liver of nonimmunized (A/J (■) and BALB/c (▲)) and immunized (A/Ji (□) and BALB/ci (△)) mice after ip infection with 10^3 PFU of MHV-3. Samples were collected at the times indicated and the values are the means±SD of three different determinations.
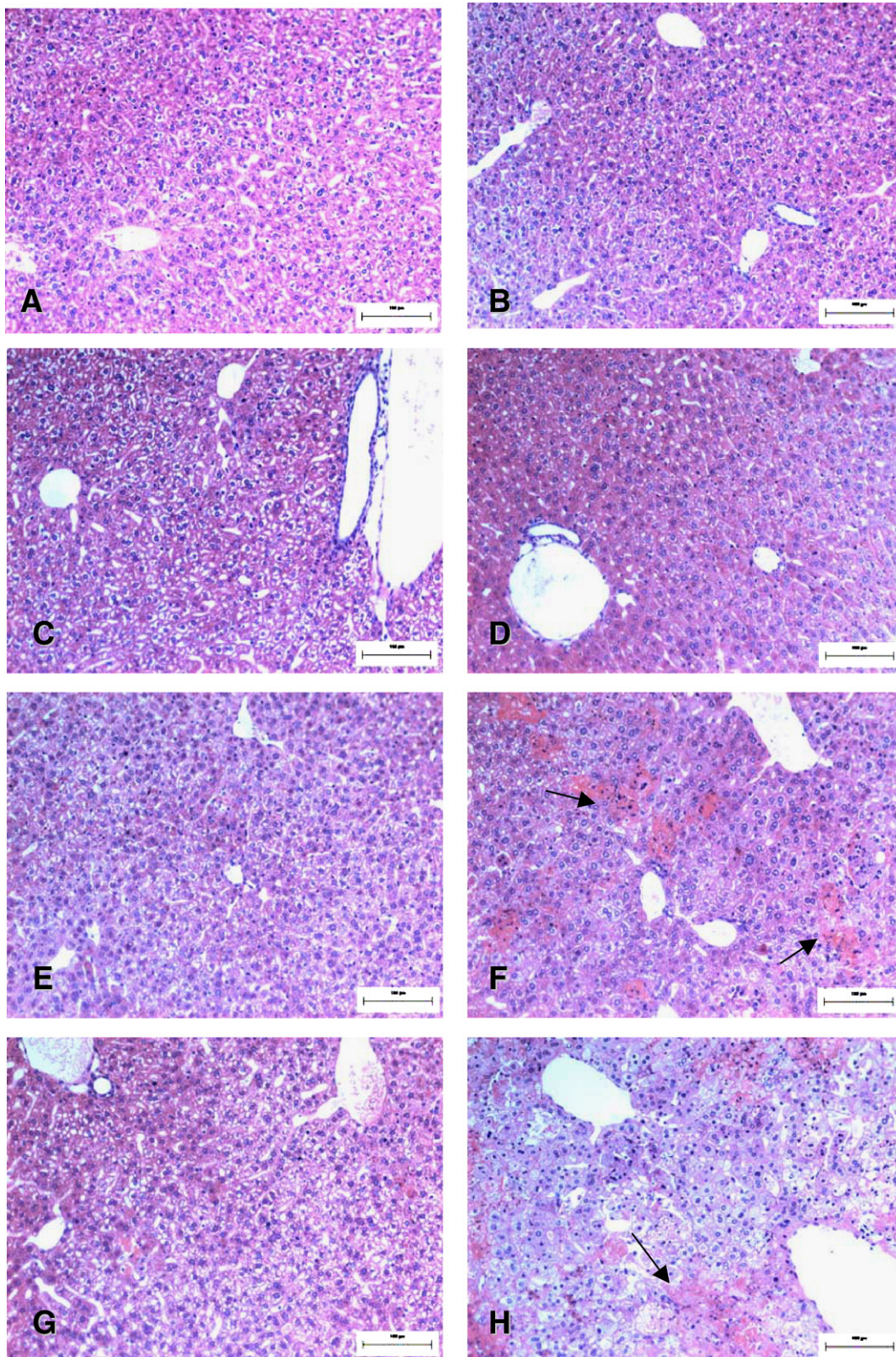
Fig. 5. Histopathological changes in the livers of nonimmunized mice after ip infection with 10^3 PFU of MHV-3. A/J mice: (A) uninfected, (C) 24 hpi, (E) 48 hpi, and (G) 72 h. BALB/c mice: (B) uninfected, (D) 24 hpi, (F) 48 hpi, and (H) 72 hpi. Note the areas of hepatic necrosis (arrows). Bars, 100 μm. Samples were stained with hematoxylin and eosin.
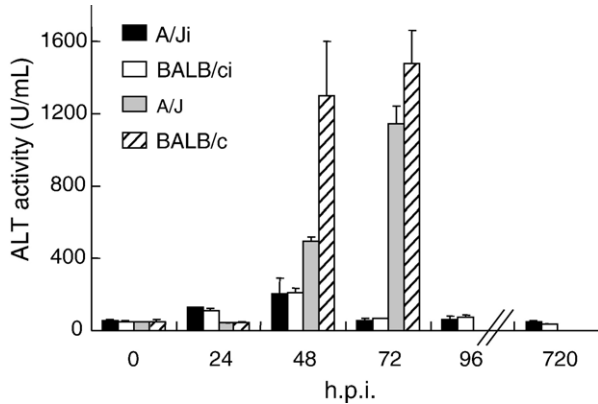
Fig. 6. Plasma ALT activity in nonimmunized (A/J and BALB/c) and immunized (A/Ji and BALB/ci) mice after ip infection with 10^3 PFU of MHV-3. Samples were collected at the times indicated. Data are expressed as the means±SD of three different experiments. Levels of plasma ALT activity in uninfected mice were <60 U ml^{-1}.

1C). Taken together, these results indicate a casual relationship between nitric oxide synthesis and control of MHV-3 infection.

The results obtained with *spf* nonimmunized mice were less clear because even the mouse strain that synthesized nitric oxide, A/J (Figs. 2 and 3), succumbed to the disease (Figs. 1 and 4). However, nitric oxide synthesis occurred at a late time and although not sufficient to fully protect the host, it decreased viral load (Figs. 2–4) and extended liver integrity (Figs. 5 and 6) and survival (Fig. 1) of the A/J strain. These results suggest that, to be efficient, nitric oxide synthesis should occur before viral load becomes too high. In agreement, it was previously reported that an early nitric oxide synthesis is required for the control of murine infection by the protozoon *Leishmania amazonensis* [36,37]. Also, previous studies have associated an early and strong iNOS expression with genetic and acquired resistance after vaccination against Marek disease herpesvirus in chicken [28]. Moreover, our results confirm previous suggestions that a specific humoral immune response, albeit important, is not enough to neutralize virus infections [43–45]. Because it has been shown that development of resistance to MHV-3 infection in mice is not exclusively dependent on antibodies or T cells [9], our results suggest that nitric oxide may be the additional protective factor against MHV-3 infection. In agreement, treatment of the resistance-immunized mice with a specific iNOS inhibitor resulted in loss of the resistance of immunized–challenged mice (Fig. 1C). Thus, an early and sustained nitric oxide synthesis resulting from immunization and infection is essential for the vaccination-acquired resistance to murine hepatitis virus by *spf* mice.

Although our studies confirm and extend previous suggestions about the roles of nitric oxide in protecting against virus infections [16,21–29], they do not address the mechanisms by which nitric oxide levels affect MHV-3 load and disease development (Figs. 1–7). Nitric oxide can function as an anti-

or proapoptotic signal, depending on the biological milieu [46–48]. Therefore, one possibility to consider is that extended nitric oxide synthesis triggers apoptosis, limiting virus replication and liver injury. In agreement, previous studies have demonstrated that induction of apoptosis in resistant mouse macrophages limited virus replication and hepatic injury during MHV-3 infection [13]. These authors suggested that programmed cell death may represent a mechanism for eliminating cells expressing a host gene that is potentially harmful to host survival, such as the *fgl2* prothrombinase gene, which is responsible for fibrin deposition and hepatic necrosis [14]. Alternatively, nitric oxide may be acting through an antiapoptotic mechanism by preserving hepatocytes [49] while affecting virus replication through the inhibition of enzymes essential for its survival. Among them, ribonucleotide reductase [21], ornithine decarboxylase [50], *S*-adenosylmethionine decarboxylase [51], and cysteine proteases [13,52] have been shown to be inhibited by nitric oxide. Enzyme inhibition is frequently attributed to nitrosation of crucial cysteine residues, a process that requires nitric oxide metabolites such as nitrogen dioxide and dinitrogen trioxide [53–56]. Further studies will be required to clarify the mechanisms by which nitric oxide limits virus replication and liver injury.

In conclusion, our data strongly support a role for nitric oxide in the development of resistance against MHV-3 infection, suggesting that nitric oxide donors may be a useful adjuvant therapy for controlling some viral infections.
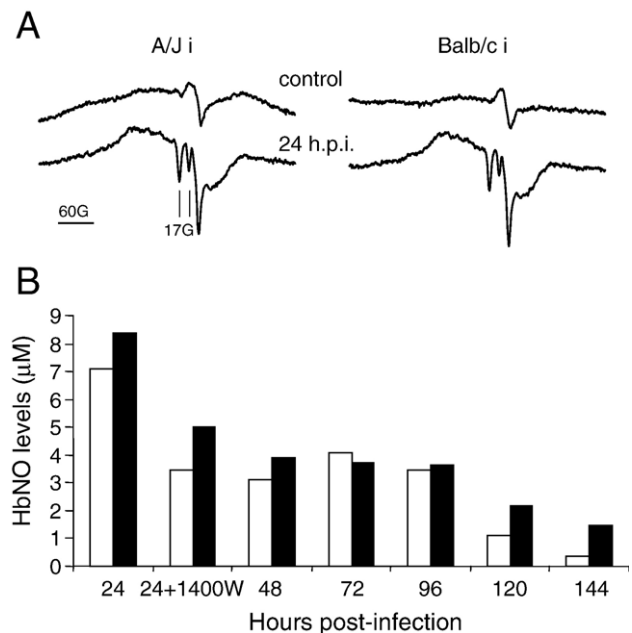

Fig. 7. (A) Representative low-temperature EPR spectra at 24 hpi and (B) hemoglobin–nitrosyl complex levels obtained from blood of immunized mice (A/Ji and BALB/ci) after infection with 10^3 PFU of MHV-3. In (B), the hemoglobin–nitrosyl complex levels at various infection times for A/Ji and BALB/ci mice are shown by open and filled bars, respectively; the hemoglobin–nitrosyl complex levels 24 hpi in animals treated with 1400 W are also shown. Spectrometer conditions: microwave power, 20 mW; modulation amplitude, 5 G; time constant, 163 ms; scan rate, 3.58 G/s.

Acknowledgments

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