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Uric acid and HMGB1 are involved in the induction of autoantibodies elicited in mice infected with mouse hepatitis virus A59

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Abstract
We have shown that mice infected with mouse hepatitis virus A59 develop autoantibodies (autoAb) to liver and kidney fumarylacetoacetate hydrolase (FAH). Because it has been proposed that the immune system is stimulated by alarm signals called damage-associated molecular patterns or alarmins, we investigated the participation of uric acid and high-mobility group box protein 1 (HMGB1) in the autoimmune response elicited by mouse hepatitis virus (MHV). Mice subjected to MHV infection had increased plasmatic uric acid concentration that significantly decreased after 20 days of daily treatment with allopurinol and, simultaneously, autoAb to FAH were undetected. Furthermore, this autoAb disappeared after 30 days of treatment with ethyl pyruvate, along with a substantial reduction in serum HMGB1 concentration. Both results indicated a remarkable relationship between the autoimmune process induced by the virus and uric acid and HMGB1 liberation. Unexpectedly, it was found that allopurinol and ethyl pyruvate inhibited the release of both uric acid and HMGB1. Because HMGB1 is activated through binding to interleukin 1β, and that this cytokine is produced by the NLRP3 inflammasome that could be stimulated by uric acid, we propose that both alarmins could be acting in concert with the induction of the autoAb to FAH in MHV-infected mice.

Keywords: *Autoantibodies, autoimmunity, mouse hepatitis virus, uric acid, HMGB1*

Introduction

Mouse hepatitis virus strain A59 (MHV-A59) is a coronavirus that triggers various pathologies in susceptible mice, such as hepatitis and thymus involution, IgG2a-restricted hypergammaglobulinemia and transient demyelination [1,2]. We have reported the presence of autoantibodies (autoAb) to liver and kidney fumarylacetoacetate hydrolase (FAH) in sera from various mouse strains after mouse hepatitis virus (MHV) infection [3]. It was demonstrated that the autoAb could recognize either cryptic or native FAH epitopes and that they bound mainly to N- and C-terminal FAH regions, the most reactive sequences being 1–50 and 390–420, respectively [4,5]. Surprisingly, although FAH sequence 1–50 shares a high degree of homology with various MHV proteins, the C-terminal portion does not. Moreover, although the autoAb reacted with homologous peptides surrounding residues 70, 160, and 360, non-similar sequences around residues 130, 210, 240, 250, and 300 were also recognized, indicating that autoAb were not restricted to epitopes with sequence homologies [4,5]. Besides, three minimal linear epitopes corresponding to fragments 9–15, 36–42, and 396–404 were detected, and mutational analysis permitted the identification of key residues energetically important for the Ab binding [6].

As reviewed by Routsias et al. [7], in basic research focusing on autoimmune processes, the most intriguing question is why a particular autoAg, among the myriads of molecules expressed in the organism, is selected as a target for the immune system. We have demonstrated that anti-FAH Ab produced after experimental immunization with purified rat liver FAH recognized essentially the same mouse FAH regions than the autoAb from MHV-infected mice, suggesting that the immune system was "mislead" and recognized the FAH protein as a foreign antigen [5].

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Thus, the possible involvement of endogenous adjuvants [8,9] on the autoimmune response induced by MHV was analyzed in this paper, focusing on two alarm signals: uric acid, a product of purine metabolism [10], and high-mobility group box protein 1 (HMGB1), a non-histone DNA-binding protein that operates as a proinflammatory cytokine once in the extracellular milieu [11,12].

Our results indicated an intimate association between the reactivity of autoAb to FAH and the presence of high levels of both uric acid and HGMB1 in mouse serum, suggesting that the adjuvant effect of both alarmins, together with the fact that the FAH molecule shares some similarity with MHV proteins [4–6], was implicated in the autoimmune response elicited by the viral infection.

Methods

Mice

Specific-pathogen-free (SPF) female BALB/c mice from the University of La Plata, Argentina, were used at the age of 8–10 weeks. All animals were maintained in isolators, on standard laboratory chow, under SPF conditions until the end of the experiments, and received care in compliance with international legal requirements. Animals were grouped randomly and assigned to a specific experiment.

Preparation of MHV stock

The NCTC 1469 adherent cell line derived from a normal mouse liver was purchased from the American Type Culture Collection. Virus titration by the endpoint method was performed by serial dilutions of the MHV stock onto cell monolayers in 96-multiwell. After 24 h, wells with the viral cytopathic effect were counted for each dilution and titer was expressed as 50% tissue infectious doses (TCID50) [5]. After an adsorption period of 1 h at 37°C, 15 ml of NCTC 135 medium with 10% fetal calf serum was added to each bottle and incubated at 37°C. Several cycles of freezing and thawing were used to release the virus 24 h after inoculation. The harvested virus was centrifuged at 400*g* for 10 min to removed debris and the supernatant was frozen at −70°C for storage.

Viral infection

Mice were inoculated intraperitoneally with 10^4 TCID50 of MHV-A59 grown in NCTC 1469 cells [5] and bled at different times.

Immunoglobulin assays

For total IgG determination in mouse serum, microplates (Nunc Maxi-Sorb) were coated with 100 μl of phosphate buffer saline (PBS) containing a 1:500 diluted rabbit antiserum directed against mouse Ig. The plates were blocked 1 h at 37°C with 0.01 M Tris, 0.13 M NaCl, pH 7.4 (TMS) containing 5% of non-fat milk (TMS-M) and were incubated with serial dilutions of mouse serum in the same medium. After 2 h at 37°C and washing with PBS containing 0.125 ml of Tween 20 per liter (PBS-Tween), the plates were incubated 1 h at 37°C with peroxidase-labeled anti-mouse IgG Ab. These donkey's IgG Ab (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) were used at a 1:10,000 dilution in TMS-M.

Preparation of liver lysates

The livers from non-infected BALB/c mice were removed, soaked in chilled PBS and ground in a Dounce homogenizer at 4°C with 5 volumes of PBS containing 10^{-3} M phenylmethyl-sulfonyl fluoride. The homogenates were centrifuged for 15 min at 400*g* and the clarified extracts kept at −20°C until used. A sample of each suspension was solubilized by heating for 30 min at 100°C in 1 M NaOH and protein concentration was determined by the method of Lowry et al. [13].

Preparation of nuclear and cytoplasmic liver fractions

Frozen liver tissues were suspended in a buffer that contained 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% Triton X-100 and homogenized. Nuclei were recovered by microcentrifugation at 7500 rpm for 5 min. The supernatant that contained cytoplasmic and membrane proteins was collected and stored at −80°C for Western blot analysis. Nuclear proteins were extracted at 4°C by gentle resuspension of the nucleus pellet in a buffer that contained 20 mM Tris, pH 7.5, 20% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, and 0.1% Triton X-100, followed by 1 h incubation at 4°C with occasional vortexing. After microcentrifugation at 13,000 revolutions/min for 15 min at 4°C, the supernatant that contained nuclear protein was collected. Protein concentration was determined as described by Bradford [14].

Western-blot analysis

Determination of autoAb anti-FAH. Essentially, reactivity of autoAb anti-FAH was determined as indicated previously [3]. Briefly, total liver extract (100 μg of protein) was subjected to 10% SDS-PAGE and then transferred onto nitrocellulose sheets (Amersham, Buckingghamshire, UK). After reversible staining with Ponceau S to check satisfactory transfer, we blocked non-specific Ab-binding sites by incubating the sheets with 5% non-fat milk in 30 mM Tris, 0.14 M

NaCl, 0.1% (v/v) Tween 20, pH 8.0 (TBS-M-T) for 1 h at room temperature with shaking. The strips were then incubated overnight at 4°C with the indicated serum dilution from control or treated mice diluted in TBS-M-T. After several washings with TBS containing 0.1% Tween 20, bound Ab were revealed with peroxidase-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:10,000 in TBS-M-T and ECL plus reagents (Amersham). The apparent molecular mass (kDa) of the detected bands was determined using a wide range protein standard (BDH Laboratory Supplies Poole BH15 1TD, UK). Identification of FAH position (the unique liver protein recognized by serum from MHV-infected BALB/c mice) was done using as a positive control, a pooled serum from MHV-infected mice.

Determination of various proteins by Western-blot. The same technique described above was used, except that the gel was loaded with the appropriate organ extract of protein, as indicated in the legend of each Figure. Rabbit polyclonal Ab to subunit p65 of NFkB (lot #6312), P38 (lot #F0605) and phosphorylated P38 (lot #K1004) were from Santa Cruz Biotechnology, whereas polyclonal Ab to β-actin were provided by Sigma-Aldrich, Inc. (Illinois, MO, USA). HMGB1 was revealed with MAb anti-HMGB1 HAP46.5 (Santa Cruz Biotechnology).

In some experiments, Western blot bands were quantified by densitometry and submitted to the image analysis software, using either endogenous Ig heavy chain or β-actin as loading controls (indicated in the Figure legends).

Determination of HMGB1 in serum

Mouse sera were filtered with Centricon YM-100 (Millipore Corp., Billerica, MA, USA) to clear the samples from macromolecular complexes and then concentrated 15-fold with Centricon YM-30 and separated on 12% SDS-polyacrylamide gels. Western blot analysis was carried out as described above, and HMGB1 was revealed with MAb anti-HMGB1 HAP46.5 (Santa Cruz Biotechnology) diluted 1:1000 and peroxidase-labeled goat anti-mouse IgG. Recombinant human HMGB1 (HMGBiotech SRL, Milano, Italy) was used as a positive control.

Preparation of monosodium urate crystals

Monosodium urate crystals (MSU) were prepared by dissolving uric acid at a concentration of 5 mg/ml in 0.1 M sodium borate buffer, pH 8.5–9.0. The solution was then warmed to 55°C and, after filtering, the supernatant was left to sit for more than 72 h, whereupon monosodium crystals formed [15].

Measurement of uric acid concentration

Uric acid concentration was determined enzymatically using the assay kit Uricostat (Wiener Lab, Rosario, Argentina) in 1:50 diluted mouse sera as indicated by the manufacturer.

Treatments

i) Mice were infected with MHV as indicated above, inoculated simultaneously i.p. with 100 μl of MSU 5 mg/ml in saline (dose: 20 mg/kg), and bled at days 0, 15, and 20.
ii) Mice were infected with MHV as indicated above and inoculated subcutaneously daily with 100 μl of a solution of allopurinol 10 mg/ml in saline (dose: 40 mg/k) through 30 days [16]. Animals were bled at days 0, 15, 20, 30, 40, 50, and 60, and in some cases killed to take off their livers.
iii) Mice were infected with MHV as indicated above, inoculated simultaneously i.p. with 200 μl of ethyl pyruvate 0.6 mg/ml in Ringer's solution [17] (dose: 4 mg/kg) and bled at days 0, 15, 20, 30, 40, 50, and 60.
iv) Mice were infected with MHV as indicated above, and inoculated i.p. with 200 μl of ethyl pyruvate 0.6 mg/ml in Ringer's solution [17] (dose: 4 mg/kg) at the time of infection (day 0) and continued three times a week throughout 4 weeks [18]. The animals were bled at days 0, 15, 20, 30, and 45, and in some cases killed for liver analysis.

In every treatment, infected controls received an equivalent amount of sterile 0.9% NaCl solution or Ringer's solution as appropriate.

Statistical analysis

Statistical significance between experimental values was calculated using the Student's *t*-test.

Results

Uric acid administration failed to increase autoAb reactivity to liver FAH

The possibility of enhancing the autoAb reactivity to FAH by uric acid administration was explored. Thus, we infected seven BALB/c mice with MHV and simultaneously inoculated them with MSU at a dose of 20 mg/kg. The animals were bled 15 and 20 days later and autoAb to liver FAH were measured by Western blot. Results indicated that MSU administration did not increase the Ab reactivity to FAH, because the extent of autoAb binding to the enzyme was similar in MHV-infected mice and in mice

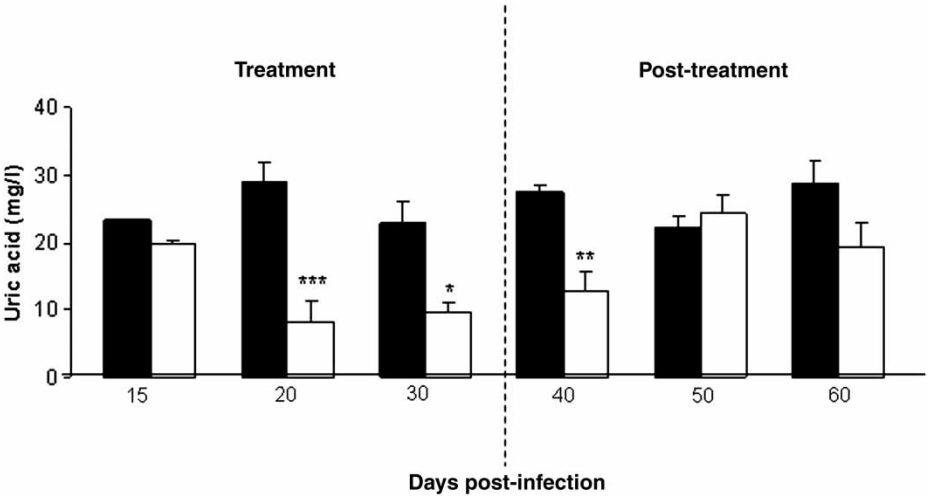

Figure 1. Daily treatment with allopurinol decreased uric acid levels in sera from MHV-infected mice. The animals were infected with MHV as indicated in Materials and Methods and inoculated daily with 100 μl of a solution of allopurinol 10 mg/ml in saline (white bars) or saline (black bars) (Treatment II, Materials and Methods). At the indicated times, the mice were bled and uric acid determined with a commercial kit. Results are mean ± SE of three independent determinations performed with pooled serum from seven BALB/c mice. A significant inhibition of uric acid concentration in sera from treated mice in comparison to non-treated but infected animals is shown as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Dotted line indicates the end of the treatment with allopurinol. Values of uric acid in sera from non-treated control mice were 2.1 ± 0.6 and 6.9 ± 0.9 mg/l in sera from normal mice inoculated with allopurinol.

infected and treated with MSU (data not shown). On the other hand, the treatment with MSU slightly affected the hypergammaglobulinemia induced by the virus and did not increase the IgG levels in its absence (data not shown).

Effect of allopurinol treatment on the levels of serum uric acid and autoAb reactivity to liver FAH

Mice infected with MHV showed an increase in plasmatic uric acid concentration of more than 10 times the control values through 15 to 60 days post

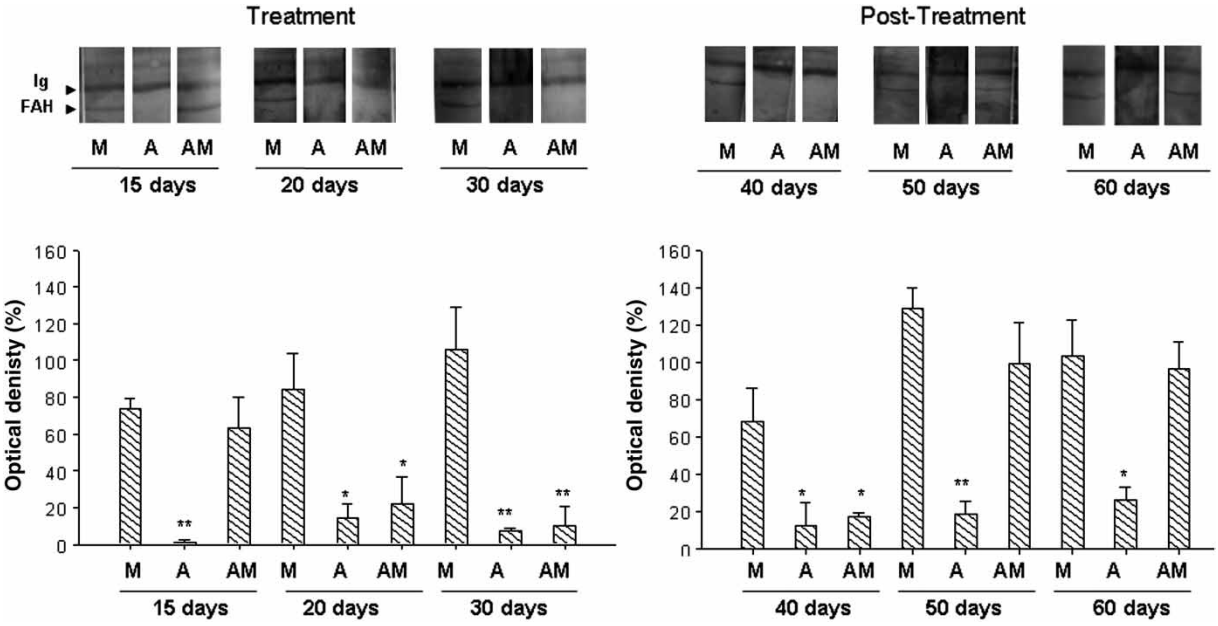

Figure 2. AutoAb to FAH lost reactivity after allopurinol treatment of MHV-infected mice. Detection of Ab to mouse liver FAH during treatment and post treatment were determined by Western blot assays as described in Materials and Methods. Results were obtained at the post-treatment indicated times with the same pooled sera used in Figure 1 diluted 1:100. A, allopurinol (no infection); M, MHV; AM, allopurinol + MHV. The position of the bands corresponding to FAH and endogenous Ig heavy chain are indicated on the left side of each panel. Proteins in each lane were quantified by densitometry and FAH concentration was expressed as percent of loading control, i.e. endogenous Ig heavy chain. Results are indicated as mean ± SE of three independent determinations. Statistical significance of optical density (OD) values obtained with sera from mice under treatment with A and/or AM in comparison with those shown by sera from MHV animals is indicated by $* P < 0.05$, and $**P < 0.01$.

infection (Figure 1). As expected, uric acid levels significantly decreased following daily injection of allopurinol (1,5-dihydro-4Hpyrazolo[3,4]pyrimidin-4-one), a widely used reagent to treat gout [19], and raised to initial levels 20 days after the end of the treatment (Figure 1).

Western blot analysis of autoAb to liver FAH performed with the same sera used in Figure 1 showed the lack of Ab reactivity to the enzyme at 20 and 30 days after allopurinol treatment (Figure 2), whereas the autoAb to FAH reappeared once the reagent inoculation was halted for at least 20 days (Figure 2). Comparison of data from Figures 1 and 2 indicates a strong correlation between the decrease in plasmatic uric acid concentration and autoAb reactivity, suggesting that the induction of anti-FAH Ab was related to the uric acid liberation produced by the virus infection. Additionally, as allopurinol could decrease the level of serum Ig under some experimental conditions [20] and thus impair the autoAb detection, we tested whether MHV-infected mice treated with the drug maintained the hypergammaglobulinemia normally induced by the virus. Results from ELISA assays showed that Ig levels were similar in serum from MHV-infected and infected and treated animals at least until 30 days post infection (data not shown).

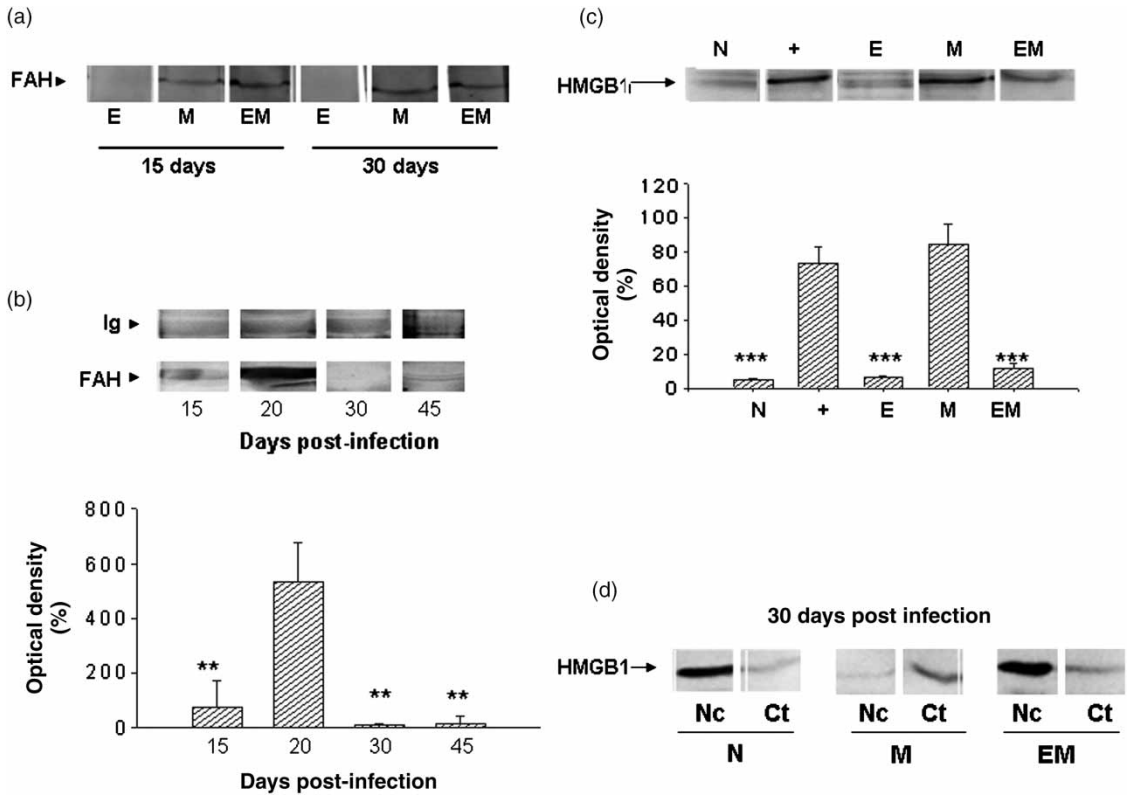

Figure 3. Reduction in anti-FAH autoAb reactivity and HMGB1 serum levels following ethyl pyruvate treatment of MHV-infected mice. Detection of mouse liver FAH or HMGB1 were determined at the indicated times by Western blot assays as described in Materials and Methods. (a) BALB/c mice were infected with MHV and inoculated with 200 μl of ethyl pyruvate 0.6 mg/ml in Ringer's solution at the time of infection.(Treatment III, Materials and Methods). The animals were bled at various times and results for 15 and 30 days post treatment are shown. The position of the bands corresponding to FAH is indicated on the left side of each panel. Results were obtained with pooled serum from seven mice. E, ethyl pyruvate (no infection); M, mice infected with MHV as indicated in Materials and Methods; EM, mice infected with MHV and treated with ethyl pyruvate as described previously. (b) Results were obtained with a pool of sera from seven MHV-infected BALB/c mice inoculated with 200 μl of ethyl pyruvate 0.6 mg/ml in Ringer's solution at the time of infection (day 0) and continued three times a week throughout four weeks (Treatment IV, Materials and Methods). The position of the bands corresponding to FAH and endogenous Ig heavy chain are indicated on the left side of each panel. Proteins were quantified by densitometry and FAH concentration was expressed as percent of loading control, i.e. endogenous Ig heavy chain. Results are indicated as mean ± SE of three independent determinations. Statistical significance of OD values obtained with sera from mice under treatment during 15, 30, and 45 days in comparison with those shown at day 20 is indicated by $^{**}P < 0.01$. (c) Determination of HMGB1 in the same pool of mouse sera used in B, after 30 days of treatment with ethyl pyruvate. N, normal, non-treated animals; E, ethyl pyruvate (no infection); M, MHV; EM, ethyl pyruvate + MHV. The position of the bands corresponding to HMGB1 is shown on the left side of the panel. Proteins were quantified by densitometry and expressed as percent of positive control (+), i.e. 2.5 μg of human recombinant HMGB1. Results are indicated as mean ± SE of three independent determinations. Statistical significance of OD values obtained with sera from mice subjected to the different treatments in comparison with positive control is indicated by $^{***}P < 0.001$. (d) Determination of HMGB1 in nuclear (Nc) and cytoplasmic (Ct) liver fractions from control mice (N), MHV-infected (M) and animals infected and treated with ethyl pyruvate as indicated in B (EM). Results were obtained 30 days post treatment or infection, using a pool of material from two mice in each case.

Effect of ethyl pyruvate treatment on autoAb reactivity to liver FAH and levels of plasmatic HMGB1

Because it has recently been reported that ethyl pyruvate therapy attenuates experimental acute pancreatitis via reduced serum HMGB1 levels in rats [21], we treated MHV-infected mice with this drug to evaluate its influence in both HMGB1 serum levels and anti-FAH Ab reactivity. Results obtained after one inoculation with ethyl pyruvate (Treatment III, Material and Methods) did not show any modification in the autoAb reactivity to FAH measured by Western blot (Figure 3(a)). By contrast, animals inoculated three times a week with the drug (Treatment IV, Material and Methods) showed undetectable levels of anti-FAH autoAb and a significant decrease in plasmatic HMGB1 levels after 30 days of treatment (Figure 3(b) and (c)). Moreover, once the ethyl pyruvate inoculation was stopped, the autoAb to FAH were still absent for 15 days more (Figure 3(b)). Besides, HMGB1 was detected in nuclear liver extracts from control mice; whereas in MHV-infected animals, the protein was found in the cytosolic fraction (Figure 3(d)). This situation was reversed after 30 days of ethyl pyruvate treatment, because Western blot experiments demonstrated the location of HMGB1 in the nuclear fraction of hepatocytes (Figure 3(d)).

Effect of ethyl pyruvate treatment on the levels of P38 MAPK and NFκB signaling pathways

The inhibition of the HMGB1 release produced by ethyl pyruvate could be due to its action on several intracellular pathways, including P38 MAPK and NFκB activation [22,23]. Therefore, the levels of total (P38) and phosphorylated P38 (pP38), the activated form of the enzyme, were analyzed by Western blot in liver lysates from mice subjected to MHV-infection and ethyl pyruvate treatment. The amount of pP38 was very much lower than total P38 in normal animals as well as in those treated with the drug (Figure 4(a)). However, the proportion of pP38 augmented to about 100% of P38 at 15 and 30 days post MHV-infection, whereas decreased to normal figures following treatment for 30 days with ethyl pyruvate (Figure 4(a)). These results suggest that P38 MAPK pathway was activated by the viral infection

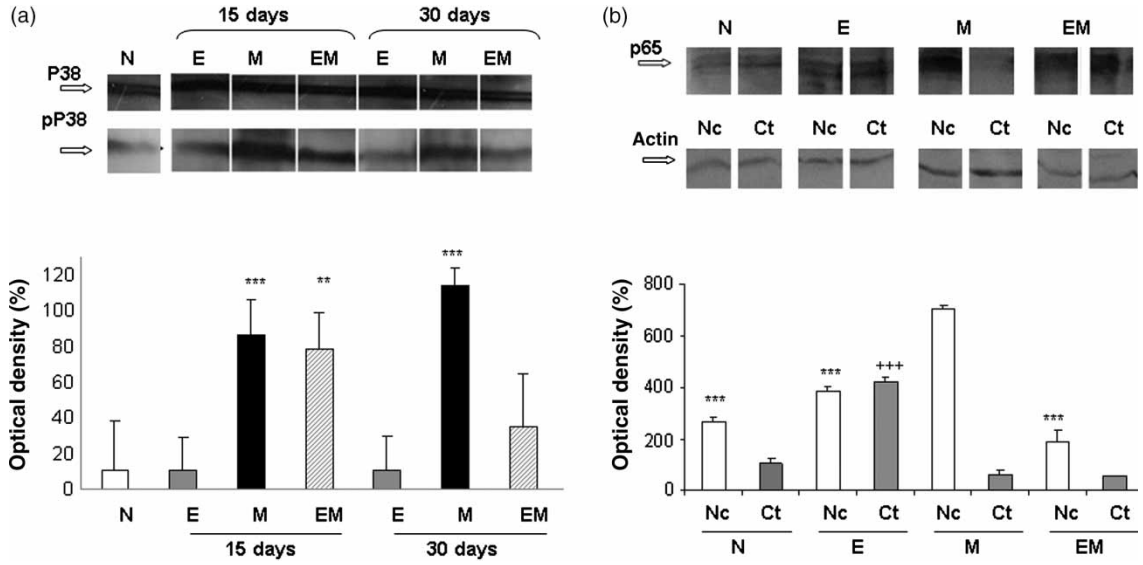

Figure 4. (a) Levels of total liver phosphorylated P38 (pP38) decreased after 30 days of ethyl pyruvate treatment of MHV-infected mice and (b) activation of NFkB was inhibited by the drug. (a) Detection of P38 and pP38 were determined in liver extracts by Western blot assays as described in Materials and Methods. Ab to P38 were diluted 1:500 and Ab to pP38 1:2000. The positions of the bands corresponding to both proteins are shown on the left side of each panel. N, normal, non-treated animals; E, ethyl pyruvate (no infection); M, MHV; EM, ethyl pyruvate + MHV. Results were obtained with the same pool of sera from seven BALB/c mice used in Figure 3, bled at the indicated times. The position of the bands corresponding to P38 and pP38 are indicated on the left side of each panel. Proteins in each lane were quantified by densitometry and pP38 concentration was expressed as percent of P38 in each experimental condition. Results are expressed as mean ± SE of three independent determinations. Statistical significance of OD values obtained with sera from mice subjected to the different treatments in comparison with normal control is indicated by $^{**}P < 0.01$ and $^{***}P < 0.001$. (b) Identification of p65 was performed by Western blot assays in nuclear (Nc) and cytosolic (Ct) liver fractions prepared as described in Materials and Methods using Ab to p65 diluted 1:500. Results were obtained with the same pool of sera from seven BALB/c mice used in A, after 30 days of ethyl pyruvate treatment and/or MHV-infection. The position of the bands corresponding to p65 and β-actin are indicated on the left side of each panel. Proteins were quantified by densitometry and p65 concentration was expressed as percent of loading control, i.e. β-actin. Results are expressed as mean ± SE of three determinations. Statistical significance of OD values obtained with each liver fraction (Nc and Ct) from mice subjected to the different treatments were calculated in comparison with positive control (M). Thus, $^{***}P < 0.001$ indicates a decrease in p65 in nuclear extract (Nc) in N, E, and EM animals versus infected mice (M), whereas $+++P < 0.001$ shows an increase in values for Ct (E) compared with Ct (M).

and that ethyl pyruvate treatment hampered at least partially this process.

Because ethyl pyruvate could affect the activation of the transcription factor NFκB [24], the degree of p65 subunit (also called relA) in the nuclear and cytosolic fractions of liver from infected and treated mice was evaluated. At 30 days post infection, the p65 subunit was found mainly in the nuclei of hepatocytes from MHV-infected animals, suggesting that the viral infection did activate NFκB. However, after ethyl pyruvate treatment for 30 days, the p65 protein was located in the nucleus as well as in liver cytosol, in the same way as results found in control and non-infected treated animals (Figure 4(b)).

Dual effects of allopurinol and ethyl pyruvate

As both allopurinol and ethyl pyruvate treatments abolished the autoAb reactivity to FAH almost totally, we asked whether the two drugs had double effects, i.e. whether ethyl pyruvate could affect plasmatic uric acid concentration and allopurinol decreased HGMB1 serum levels. As shown in Figure 5(a), ethyl pyruvate inoculation during 15 and 30 days lowered plasmatic uric acid concentration in MHV-infected mice. In the same way, allopurinol treatment did decrease significantly HGMB1 serum levels after 20 and 30 days post infection (Figure 5(b)).

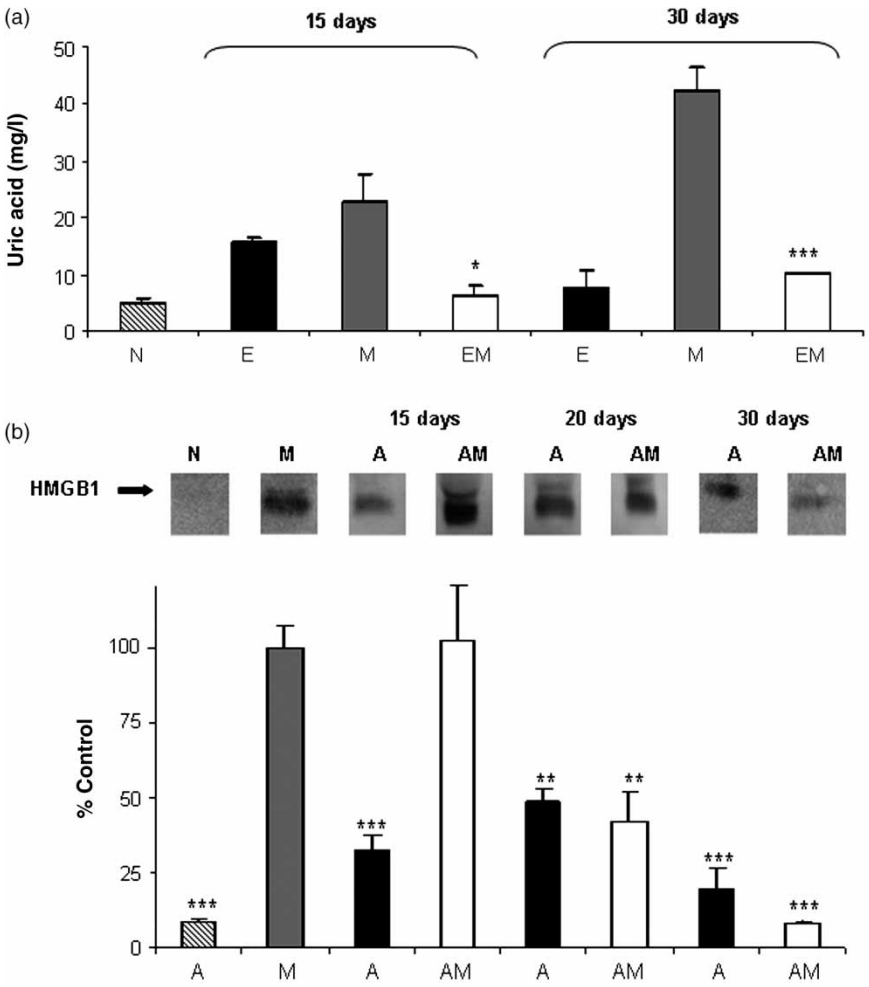

Figure 5. Allopurinol decreases HGMB1 concentration (a) and ethyl pyruvate affects the levels of uric acid (b) in serum from MHV-infected mice. (a) Seven MHV-infected BALB/c mice were inoculated with 200 μl of ethyl pyruvate 0.6 mg/ml in Ringer's solution at the time of infection (day 0) and continued three times a week throughout 4 weeks (Treatment IV, Materials and Methods). At the indicated times, the mice were bled and uric acid determined with a commercial kit. Results are means ± SE of three determinations. N, normal, non-treated animals; E, ethyl pyruvate (no infection); M, MHV; EM, ethyl pyruvate + MHV. A significant inhibition of uric acid concentration in comparison to non-treated but infected mice is shown by $*P < 0.05$ and $***P < 0.001$. (b) BALB/c mice were infected with MHV and inoculated daily with 100 μl of a solution of allopurinol 10 mg/ml in saline (Treatment II, Materials and Methods). Serum HMGB1 was identified by Western blot assays as described in Materials and Methods. Proteins in each lane were quantified by densitometry at the indicated post-infection times. Results were calculated as percent of positive control (MHV = 100%) and expressed as mean ± SE of three independent determinations ($**P < 0.01$; $***P < 0.001$). N, normal, non-treated animals; A, allopurinol (no infection); M, MHV; AM, allopurinol + MHV.

Discussion

It has been reported that signals that originate from dying cells, the damage-associated molecular patterns (DAMPs), determine apoptotic cell engulfment, dendritic cell (DC) activation, antigen processing, DC maturation, and T-cell activation [9,25]. Thus, as it was suggested that structural features of autoAg as well as their locations, catabolism during cell death and translocation to cells that present antigens to the immune system could contribute to selection of the autoimmune repertoire [26], we asked whether some DAMPs, i.e. uric acid and HMGB1, could be involved in the autoimmune response elicited by MHV.

It has been shown that MSU activated DC and increased the cytotoxic T-cell response in animals that were previously faced with Ag [15]. Herein, we reported that MHV infection strongly enhanced uric acid concentration in mouse sera, and that elevated levels of the metabolite were found as long as 60 days after viral inoculation. These results were rather unexpected, because we had found that transaminases as well as hypergammaglobulinemia decreased to normal levels after 30 days of MHV infection, indicating the resolution of the hepatitis elicited by the virus [3,27]. However, autoAb to liver FAH as well as Ab to MHV proteins were still detected 90 days post infection [3,5] and, very recently, focuses on cell necrosis were observed in liver from mice after 50 days of MHV-infection [27], suggesting a long but not evident persistence of the pathogen in the host. At the light of those results, it is easy to explain why the administration of uric acid to MHV-infected mice did not enhance autoAb reactivity: the metabolite was already released by the virus necrotic action and a further increase in its concentration was unable to produce any supplementary effect.

Once the MHV-infected mice were inoculated daily with allopurinol, which is known to inhibit urico-poieisis via xanthine oxidase suppression [19,20], plasmatic uric acid levels significantly decreased after 20 days of treatment. These values were low even 10 days after concluding the drug administration. Remarkably, autoAb to FAH were not detected when plasmatic uric acid was depleted, indicating a close relationship between the alarmin liberation and the autoimmune process induced by the virus.

On the other hand, HMGB1 is a non-histone nuclear protein that has a dual function. Inside the cell, HMGB1 binds DNA, regulating transcription and determining chromosomal architecture. Outside the cell, HMGB1 activates the innate system, interacts with receptors that include toll-like receptor-2 (TLR-2) and TLR-4, and mediates a wide range of physiological and pathological responses [11,12]. Our results proved that the autoAb to FAH induced by MHV were undetected after 30 days of treatment with ethyl pyruvate, concomitantly with a considerable decrease in serum HMGB1 concentration, suggesting that the release of HMGB1 and Ab to FAH were intimately related.

Signaling pathways that are mainly affected by ethyl pyruvate are P38 MAPK and NFκB [16,28,29], both associated with the release of various proinflammatory cytokines [28]. We found that the levels of pP38 augmented after MHV infection, but decreased to normal values when the animals were treated with ethyl pyruvate for 30 days, indicating that the partial inhibition of this process could be a consequence of the anti-inflammatory action of the drug [30].

NFκB is the generic name of a family of transcription factors composed of hetero-and homo-dimerization of Rel family proteins [24]. We showed that MHV infection induced the translocation of p65, a component of the NFκB dimer form, from cytoplasm to the cell nucleus and that ethyl pyruvate treatment partially reestablished the p65 location, indicating that the drug could decrease the expression of several inflammatory proteins.

It was striking to find that the slow down of the release of each alarmin, in an independent way, was able to totally abolish the autoimmune response elicited by the virus. To clarify this point, we checked whether allopurinol, in spite of decreasing serum uric acid, was able to reduce HMGB1 concentration and, at the same time, whether ethyl pyruvate induced a reduction in uric acid liberation. Unexpectedly, results showed that the two treatments affected the simultaneous release of both alarmins, suggesting that this was the reason for their similar and whole effects on the decrease in autoAb to FAH.

Unlike apoptosis, where the cell content remains packed in the apoptotic bodies that are removed by macrophages, and thereby inflammation does not occur, during necrosis, the cell membrane breaks and the cytosolic constituents, including HMGB1 and uric acid, are released into the extracellular space [31,32]. Lim et al. [29] showed that ethyl pyruvate prevented the HMGB1 release by inducing the switch from necrosis to apoptosis through prevention of reactive oxygen species (ROS) production. In the same way, it was reported that allopurinol is an inhibitor of xanthine oxidase, whose activity increases in a variety of pathological conditions, and the ROS generated during these enzymatic reactions take part in the oxidative tissue damage [16].

In fact, although allopurinol and ethyl pyruvate are well-known anti-inflammatory drugs, it was astonishing to detect their suppressive effect on the simultaneous release of uric acid and HMGB1. We can speculate that these drugs suppressed the necrosis induced by the virus and/or that both alarmins act by a cyclic pathway: uric acid inducing the release of HMGB1, and HMGB1 inducing the release of uric acid.

Recent reports demonstrated that active, mature interleukin 1β (IL-1β) is produced by cleavage of the

inactive pro-IL-1β precursor by caspase-1, which is activated in a large multiprotein complex called the inflammasome [33]. The NLRP3 inflammasome, containing the Nod-like receptor protein NLRP3 (also called cryopyrin) and caspase-1, is vital for the production of mature IL-1β and is stimulated by a variety of stress-associated danger signals, including uric acid [33,34]. On the other hand, it was found that HMGB1 induces only minimal cytokine production by macrophages and murine neutrophils unless bound to proinflammatory mediators, such as IL-1β, when it acquires markedly enhanced cell-activating properties [35]. Thus, we propose that uric acid and HMGB1 are acting in concert, because the release of the former could trigger the NLRP3 inflammasome to produce the mature IL-1β that HMGB1 needs to become activated. This situation may explain a certain delay in the decrease in both alarmins and the administration of allopurinol or ethyl pyruvate.

In any case, to our knowledge, this is the first report showing the double effect of allopurinol and ethyl pyruvate on uric acid and HGMB1's simultaneous release. These results could also explain the beneficial effects observed by the administration of both drugs in animal models of different pathological situations, in autoimmune disorders or in human diseases [12,17,22,23,36,37]. In fact, as shown in studies on patients, HMGB1 can play an important role in the pathogenesis of rheumatoid arthritis, systemic lupus erythematosus, polymyositis, dermatomyositis and Sjögren's syndrome (reviewed in reference [12]). On the other hand, an abnormally high uric acid level has been correlated with gout, hypertension, cardiovascular, and renal disease, whereas a reduced uric acid concentration has been linked to multiple sclerosis, Parkinson's disease, Alzheimer's disease, and optic neuritis (reviewed in reference [38]).

Finally, because the decrease in uric acid and HGMB1 in serum from MHV-infected mice were coincident with the loss of reactivity of autoAb to liver FAH, we propose that the autoimmune reaction elicited by the virus was related to the liberation of these endogenous adjuvants that induced the compromised DC to "confound" the liver enzyme with a foreign Ag because its similitude with some MHV proteins.

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