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The autoimmune response induced by mouse hepatitis virus A59 is expanded by a hepatotoxic agent

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ABSTRACT

Mouse hepatitis virus strain A59 (MHV-A59) triggers various pathologies in several mouse strains, including hypergammaglobulinaemia, hepatitis and thymus involution. We reported previously the presence of autoantibodies (autoAb) to liver and kidney fumarylacetoacetate hydrolase (FAH) in sera from mice infected with MHV-A59.

Long-term MHV-infected mice represented a good model of non-pathogenic autoimmune response since the animals were apparently healthy in spite of the presence of autoAb. The aim of this work was to see whether a severe liver injury, which releases endogenous adjuvants, i.e. danger signals, could elicit a broader spectrum of autoAb and perhaps signs of autoimmune hepatitis. Carbon tetrachloride (CCl_4) was injected into mice 30 days after MHV infection, and serum was assayed for autoAb and total IgG 20 days later. The association of MHV infection with the toxic effects of CCl_4 resulted in hypergammaglobulinaemia and the production of autoAb to various liver and kidney proteins. Histological examination of liver samples showed tissue damages but without significant differences between the animals submitted to MHV + CCl_4 and controls, which were either infected by MHV without CCl_4, or poisoned by CCl_4 in the absence of MHV infection. Those results show that liver injury after viral infection may lead to the spreading of the immune response and to an increase of serum IgG, suggesting that the procedure used herein could simulate the onset of autoimmune hepatitis.

1. Introduction

Viruses have been long associated with autoimmune diseases [1–4]. It was proposed that these infectious agents trigger an autoimmune response by diverse mechanisms, including polyclonal B-lymphocyte activation, release of sequestered autoantigens (autoAg), antigenic mimicry, modification of self-antigen, epitope spreading of the anti-viral immune response, enhancement of major histocompatibility complex molecule expression, bystander activation or viral persistence [2,4–6].

Mouse hepatitis virus strain A59 (MHV-A59) is a corona virus that causes various mouse pathologies, including hepatitis, thymus involution, IgG2a-restricted hypergammaglobulinaemia and transient demyelination [7,8]. Its ability to infect hepatocytes and to induce hepatitis correlates with the expression of its cellular membrane receptor, CEACAM-1, previously known as Bgp1a or MHVR, on these cells [7]. We have reported the presence of autoantibodies (autoAb) in sera from various mouse strains after MHV-infection [9]. The autoAb were directed to a 40 kDa protein present in mouse liver and kidney identified as fumarylacetoacetate hydrolase (FAH), a soluble cytosolic enzyme that mediates the hydrolytic formation of fumarate and acetoacetate [9]. We later examined whether the autoimmune response to FAH induced by MHV was based on molecular mimicry and whether the epitope spreading [10–12] was involved.

Overlapping decapeptides corresponding to the entire mouse FAH sequence were prepared using the PEPSCAN method and their reactivities with sera from MHV-infected mice at different times was determined by ELISA. Results indicated that various regions of the enzyme are recognized as soon as 15 days after infection and that the autoimmune response is not restricted to peptides homologous to viral proteins [13]. As the spectrum of peptides recognized by the autoAb of a given mouse did not change significantly with time, it was likely that the MHV-elicited autoimmune response did not induce an epitope recognition spreading. It was suspected that the induction of the autoAb was not solely related to molecular or structural mimicry, but was mainly due to the tissue damages caused by the MHV infection. In other words, the danger signals [14], also called DAMPs (damage-associated molecular patterns) or alarmins, probably played a key role in the autoimmune process.

To further investigate this hypothesis, we thought that increasing the tissue damages caused by the viral infection should amplify the autoimmune response and perhaps lead to autoimmune hepatitis

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[15]. Since despite the presence of autoAb against liver and kidney FAH, MHV-infected mice remain apparently healthy [9], we associated the MHV infection with the effects of carbon tetrachloride (CCl_4), a strong hepatotoxic agent [16], injected 30 days after MHV inoculation. The sera were then assayed for autoAb and total IgG 20 days later. Briefly, this combination of viral infection and a toxic substance led to the spreading of the immune response and to an increase of serum IgG, marked signs of autoimmune hepatitis.

2. Materials and methods

2.1. Mice

The 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.

2.2. Preparation of MHV stock

The NCTC 1469 adherent cell line derived from normal mouse liver was purchased from the American Type Culture Collection. Cells growing in T-75 bottles were inoculated with MHV A59 virus at a multiplicity of 1–5 $TCID_{50}$/cell. 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.

Virus titration by endpoint method was performed by inoculating serial dilutions of the MHV stock onto cell monolayers in 96-multiwell. After 24 h wells with viral cytopathic effect were counted for each dilution and titer was expressed as 50% tissue infectious doses ($TCID_{50}$).

2.3. Viral infection and carbon tetrachloride (CCl4) treatment

Mice were inoculated intraperitoneally with 10^4 $TCID_{50}$ of MHV-A59 grown in NCTC 1469 cells [5]. Thirty days after the infection, the mice were injected intraperitoneally with 100 µl of mineral oil containing 0.16 µl of CCl_4 (dilution: 1:625). The animals were bled 24 h and 20 days after treatment. One group of animals was MHV infected and inoculated with CCl_4 ("MHV + CCl_4"), a second was only infected ("MHV alone") and the third received only CCl_4 ("CCl_4 alone"). Serum aspartate aminotransferase (AST) was determined using the GOT(AST) uni-test (Wiener lab., Rosario, Argentina).

2.4. 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/l(PBS-Tween), the plates were incubated 1 h at 37 °C with peroxidase labeled anti-mouse IgG Ab. These donkey IgG Ab (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) were used at a 1:10,000 dilution in TMS-M.

2.5. Preparation of organ lysates

Liver and kidneys 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 (PMSF). 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. [17].

2.6. Western-blot analysis

Each organ 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, non-specific Ab-binding sites were blocked 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 an Ab dilution in TBS-M-T. After several washings with TBS containing 0.1% Tween 20, bound Ab were revealed with peroxidase labeled donkey anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc, West Grove, PA, USA) diluted 1:5000 in TBS-M-T and ECL reagents (Amersham, Buckingghamshire, UK). In every experiment a negative control (pool of naïve sera from the same mice used afterward) as well as a positive control (pool sera from MHV-infected mice) were included. To avoid differences in protein expression the

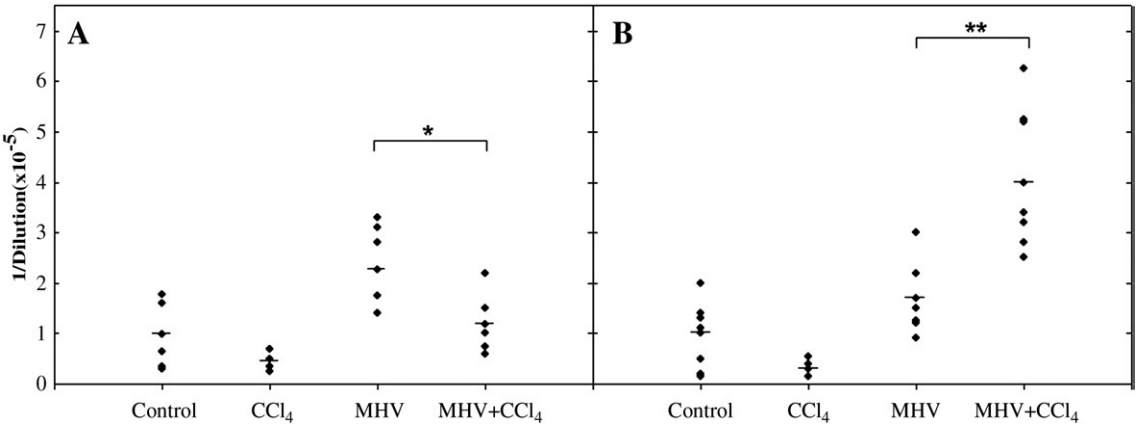

Fig. 1. IgG concentration in sera from the three groups of BALB/c mice:controls (non infected and not exposed to CCl_4), "CCl_4 alone", " MHV alone", "MHV + CCl_4". IgG concentration is expressed as the serum dilution to give an OD = 1.0 in ELISA 24 h (A) or 20 days (B) after the CCl_4 injection, i.e., 30 (A) or 50 (B) days after the MHV inoculation. Statistical analysis was performed by the Mann-Whitney *U*-test. $^*P<0.05$; $^{**}P<0.001$.