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Journal of Autoimmunity 27 (2006) 203–209

journal of autoimmunity

www.elsevier.com/locate/issn/08968411

The peptide specificities of the autoantibodies elicited by mouse hepatitis virus A59

Maite Duhalde-Vega, María E. Loureiro, Patricia A. Mathieu, Lilia A. Retegui*

Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Junín 956, 1113 Buenos Aires, Argentina

Received 15 June 2006; revised 24 August 2006; accepted 19 September 2006

Abstract

Synthetic decapeptides ($N = 206$) covering the entire sequence of mouse liver fumarylacetoacetate hydrolase (FAH) were used to analyze the specificities of the autoantibodies (autoAb) elicited towards this enzyme in mice infected with mouse hepatitis virus (MHV). These autoAb bound mainly to N- and C-terminal FAH peptides, the most reactive sequences being 1–50 and 390–420, respectively. Surprisingly, although FAH sequence 1–50 shares a high degree of homology with various MHV proteins, the C-terminal portion does not. Moreover, whereas 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. There was also a lack of correlation between the amount of anti-MHV or anti-FAH antibodies produced and the reactivity towards the peptides. Moreover, the spectrum of peptides recognized by the autoAb of a given mouse did not change significantly with time, which suggests that the MHV-elicited autoimmune response does not induce an epitope recognition spreading. Finally, anti-FAH Ab produced after immunization with rat liver FAH recognized essentially the same mouse FAH regions than autoAb from MHV-infected mice. Results indicated that the induction of the autoAb is not only related to molecular or structural mimicry, but rather supports the Danger model, in which any aggression, in this case the MHV infection, is susceptible to trigger the production of autoAb.

Keywords: Mouse hepatitis virus; Autoantibody; Autoimmune response; Molecular mimicry; Danger model; Fumarylacetoacetate hydrolase

1. Introduction

Mouse hepatitis virus strain A59 (MHV-A59) is a coronavirus that triggers various pathologies in susceptible mice, including hepatitis and thymus involution, IgG2a-restricted hypergammaglobulinaemia and transient demyelination [1,2]. In a previous paper we reported the presence of autoantibodies (autoAb) in sera from various mouse strains after MHV infection [3]. The autoAb were directed to a 40-kDa protein present in mouse liver and kidney, later identified as fumarylacetoacetate hydrolase (FAH), a soluble cytosolic enzyme that mediates the hydrolytic formation of fumarate and acetoacetate [3].

Since molecular mimicry of viral antigens with self determinants could be the mechanism involved in the MHV induction of autoAb to liver FAH, the putative cross-reaction between the enzyme and MHV proteins was afterward examined [4]. ELISA and Western blot competition assays indicated that the autoAb could recognize either cryptic or native FAH epitopes, the response being different between individuals [4]. Furthermore, to analyze the Ab repertoire to sequential FAH epitopes in MHV-infected mice, a set of 24 decapeptides displaying at least 20% of identity between the sequence of mouse FAH and viral proteins E2, nucleocapside, E1 and RNA polymerase was used. The results suggested that the FAH sequence 1–20 was one of the epitopes recognized by the MHV-elicited autoAb [4].

Herein we examined whether the MHV-elicited autoimmune response was based on molecular mimicry and whether the epitope spreading, an immune diversification originated from only a single autoreactive determinant frequently associated with autoimmune disorders [5–7], occurs in the present

* Corresponding author. Tel.: +54 11 4964 8289; fax: +54 11 4962 5457.
E-mail address: lretegui@mail.retina.ar (L.A. Retegui).

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doi:10.1016/j.jaut.2006.09.003

anti-FAH autoimmune response. 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, including sequence 1–20, are recognized as soon as 15 days after infection and that the autoimmune response is not restricted to peptides homologous to viral proteins. Besides, the determinant spreading phenomenon was discarded because individual mouse sera did not display the corresponding pattern of response.

2. Materials and methods

2.1. Mice

Female pathogen free BALB/c mice from the University of La Plata, Argentina, were bred in isolators and used for experiments at the age of 8–10 weeks.

2.2. Viral infection

Several mice were inoculated intraperitoneally with 10^4 50% tissue culture infectious doses ($TCID_{50}$) of MHV A59, grown in NCTC 1469 cells [2] and bled at different times.

2.3. 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 remove 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 ($TCID_{50}$).

Before using in ELISA assays the virus was inactivated by incubating the MHV stock 1 h at 56 °C [8]. Protein concentration in both MHV and NCTC stocks was determined by Lowry et al. [9].

2.4. Determination of anti-MHV and anti-FAH Ab by ELISA

To test anti-MHV Ab, ELISA plates were coated with 100 μl of UV-inactivated MHV-A59, 2×10^7 PFU/well, diluted in 0.02 M glycine, 0.03 M NaCl, pH 9.2. After overnight incubation at room temperature and washing with phosphate buffer saline containing 0.125 ml of Tween 20 per liter (PBS–Tween), the plates were blocked 2 h at 37 °C with 0.01 M Tris, 0.13 M NaCl, pH 7.4, containing 5% of fetal calf serum (TMS-FCS), which minimizes non-specific binding. The plates were then incubated 2 h at room temperature with mouse serum diluted in TMS-FCS and after washing with PBS–Tween, the bound Ab were revealed with peroxidase labeled donkey IgG anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) diluted 1:10,000 in TMS-FCS. As a substrate, *ortho*-phenylene-diamine-dihydrochloride (OPD, Sigma Chemical Co, St. Louis, MO) with freshly added H_2O_2 was used. The reaction was stopped after 10 min by addition of 1 M H_2SO_4. The absorption was measured by ELISA reader (Metertech Inc., Taipei, Taiwan) at 490 nm. Non-specific values of optical density were obtained in the absence of mouse serum.

Essentially the same procedure was used to test anti-FAH Ab, except that ELISA microplates were coated with 100 μl of 0.1 M $NaHCO_3$, pH 8.9, containing 10 μg of rat liver FAH prepared as indicated before [10].

2.5. Immunization of mice with rat liver FAH

As reported previously [4], 10-week-old BALB/c mice were immunized subcutaneously on day 0 with 20 μg of purified rat FAH in 50 μl of saline, emulsified in an equal volume of complete Freund's adjuvant (DIFCO Laboratories, USA). The animals were boosted on day 15 with the same amount of FAH in incomplete Freund's adjuvant (DIFCO Laboratories, USA) and bled 15 days after the last inoculation.

2.6. Alignment of peptide sequences

LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html) using two different algorithms or matrices (pam120.mat, blosum80.mat) was utilized to locate multiple matching sub-segments in two protein sequences. Sequences of MHV A59 surface glycoprotein (E2), membrane glycoprotein (E1), nucleocapside (N), RNA-direct RNA polymerase (RNA), hemagglutinin-esterase and 30 kDa non-structural protein were aligned with the mouse liver FAH amino acid sequence. Our minimum criterion for homology was the existence of at least 30% of sequence identity between FAH and each viral protein.

2.7. Peptide synthesis and sera reactivity

A set of 206 overlapping peptides (10 mers) representing the entire sequence of mouse liver FAH were synthesized according to the method of Geysen et al. [11] onto activated polyethylene pins, in a standard 96-well microtiter plate format (Mimotopes, San Diego, CA). Each consecutive peptide was offset by two residues from the preceding one (i.e. 1–10, 3–12,…, 410–420).

Serum reactivity with synthetic peptides was determined by ELISA as follows: immobilized pins were blocked for 1 h at room temperature with PBS, pH 7.2, containing 2% BSA and 0.1% Tween 20. After washing with PBS, pH 7.2, for 10 min at room temperature, pins were incubated overnight at 4 °C in 150 μl of each serum, diluted 1:300 in the above-described

blocking buffer. Pins were then washed four times with PBS, pH 7.2, and incubated for 1 h at room temperature with peroxidase labeled donkey IgG anti mouse IgG diluted 1:1500 in PBS, pH 7.2, containing 1% FCS and 0.1% Tween 20. After several washes, the bound Ab were detected by incubating the pins for 45 min at room temperature in 200 μl of 0.5 mg/ml 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) (ABTS) dissolved in 0.1 M Na_2HPO_4, 0.1 M citric acid, pH 4.0, containing 0.01% H_2O_2. The absorption was measured by ELISA reader at 405 nm and control values obtained with non-immune serum were subtracted in each experiment.

3. Results

3.1. Kinetics of Ab production against MHV and FAH by MHV-infected mice

To follow the production of Ab to viral proteins and liver FAH, both kinds of Ab were assayed by ELISA in individual mice at different times after viral infection. Representative results obtained with four infected mice showed that low but significant titers of Ab to MHV were present 15 and 30 days after viral infection, the amount of Ab sharply increasing after 45 days post-infection and persisting at least 90 days in mice #1, #2 and #4 (Fig. 1). By contrast, serum from mouse #3 contained significant Ab to viral proteins only 60 days after infection (Fig. 1).

Since mouse FAH was not available, rat liver FAH was used to assay by ELISA the amount of autoAb produced by MHV-infected mice. Using rat FAH rather than mouse FAH should not change the results markedly since rat and mouse FAH share 97% of sequence identity [3].

The titers of anti-FAH Ab were significantly lower than those of anti-MHV Ab and the kinetics of the production of these two Ab were quite different (Fig. 1). Large variations were also observed between each mouse. AutoAb were detectable only at 45 and 60 days post-infection in mouse #1, after 30 and 45 days in mouse #3 whereas, in mice #2 and #4, anti-FAH Ab appeared at various times after MHV inoculation (Fig. 1).

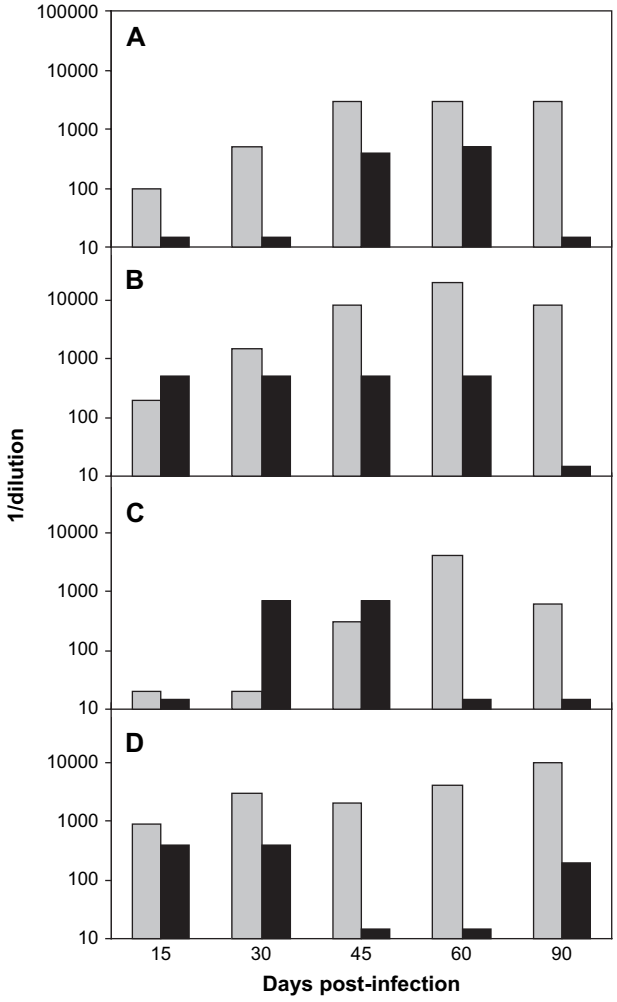

Fig. 1. Time course of Ab to MHV and rat liver FAH in serum from MHV-infected mice. ELISA microplates were coated with either UV-inactivated MHV-A59 (2×10^7 PFU/well) or 10 μg of rat liver FAH, and incubated with diluted serum (1:50 to 1:500,000) from MHV-infected BALB/c mice bled at the indicated days after infection. Bound Ab were detected with peroxidase labeled donkey anti-mouse IgG. Results are expressed as the mean serum dilution to reach an optical density value of 0.5 (anti-MHV Ab, grey bars) or 0.3 (anti-FAH Ab, black bars). Representative results for mice #1 (A), #2 (B), #3 (C) and #4 (D) are shown.

3.2. Reactivity of Ab from MHV-infected mice with synthetic peptides covering the entire mouse liver FAH sequence

Overlapping decapeptides ($N = 206$) corresponding to the mouse liver FAH sequence were prepared using the PEPSCAN method, and their reactions with sera from various MHV-infected mice were tested by ELISA. Sera were collected at 15, 30, 45, 60, and 90 days after viral infection. Representative results showed that no correlation was found between the binding of the Ab to the peptides and to the viral proteins (Figs. 1 and 2). Sera from mice #1 and #2 (Fig. 2A and B) reacted more strongly with peptides than did sera from mice #3 and #4 (Fig. 2C and D), whereas sera from mice #1, #2 and #4 displayed the highest anti-MHV titers (Fig. 1). Similar discrepancies were found when the bindings of Ab to peptides were compared to the amount of autoAb detected by ELISA (Figs. 1 and 2). Sera from mouse #2 displayed more autoAb than sera #1, #3 and #4, whereas Ab from mouse #1 displayed the most potent binding to peptides (Figs. 1 and 2).

Ab titers to viral proteins were low 15 and 30 days post-infection, compared with values for 45, 60, and 90 days in mice #1, #2 and #4 (Fig. 1). In contrast, the sera collected later did not react with peptides more strongly than the sera obtained earlier (Fig. 2A, B and D). Similar observation could be ascribed to serum from mouse #3, since peptide reactivity did not raise 60 days post-infection, when the highest amount of anti-MHV Ab was detected (Figs. 1 and 2C).

Data obtained with mouse serum #1 and #2 showed that Ab would recognize essentially the same FAH regions. In fact, both sera reacted mainly with N-terminal (residues 1–50) and C-terminal (residues 390–420) portions of the enzyme, whereas sequences around residues 70, 130, 210, 250, 300,

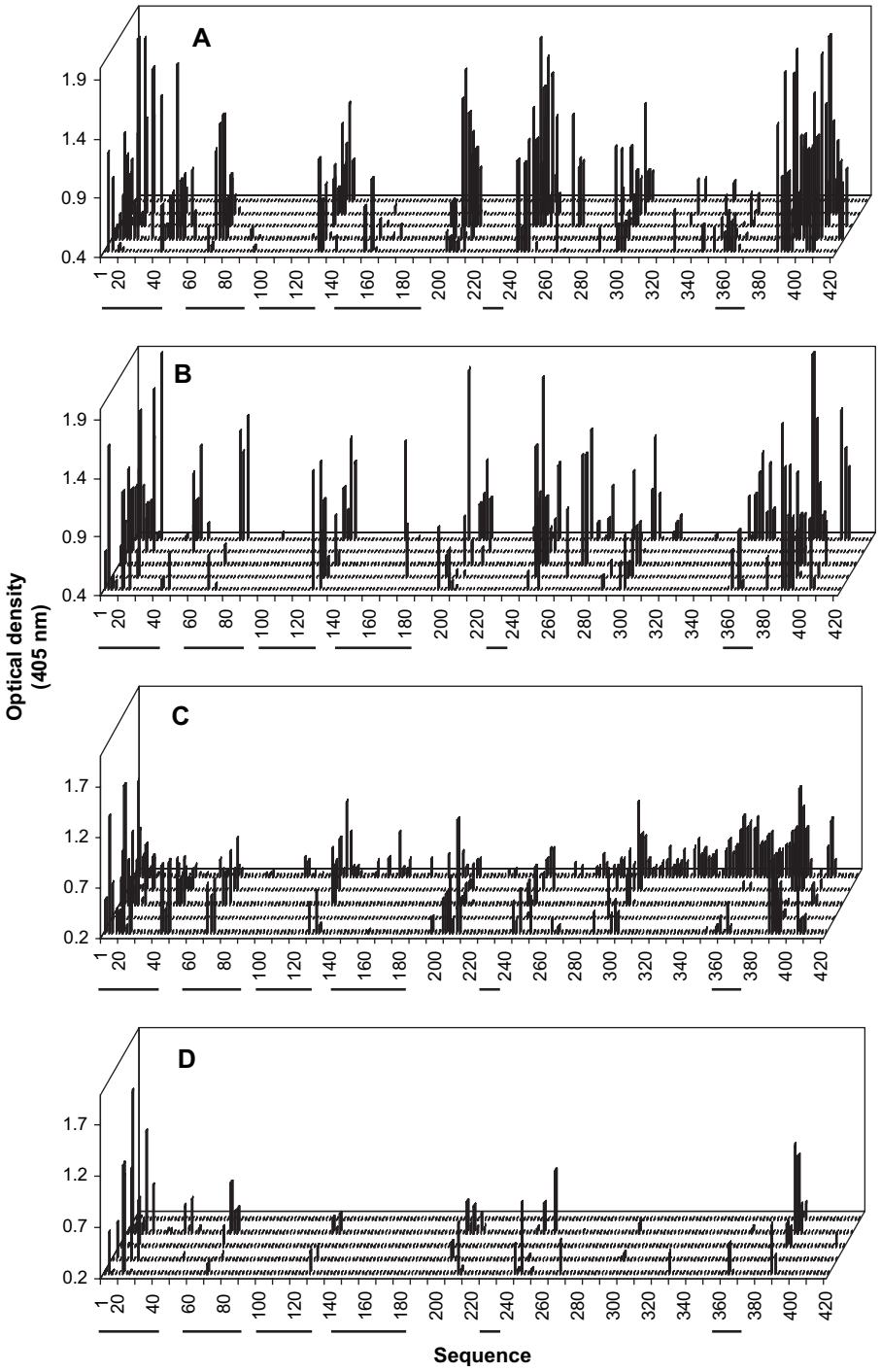

Fig. 2. Reactivity of sera from MHV-infected mice with synthetic peptides. Ab binding to the overlapping decapeptides covering the entire mouse liver FAH was determined by ELISA as indicated in Section 2. Results are expressed as specific optical density values for BALB/c mouse #1 (A), #2 (B), #3 (C) and #4 (D) after 15, 30, 45, 60 and 90 days of MHV infection (lines from front to rear). Horizontal bars indicate sequence homology (30% or more) between mouse liver FAH and MHV proteins according to data presented in Fig. 3. Thus, homologous sequence 1–46 correspond to overlapping FAH peptides 1, 2, 3, 4, 10, 11, 14, and 19, sequence 60–92 to peptides 5, 6, 7, and 20, sequence 100–127 to peptides 15, 21 and 22, sequence 140–187 to peptides 12, 16, 17, 18, and 23, sequence 223–232 to peptide 8 and sequence 359–378 to peptides 9 and 13 (see Fig. 3).

and 360 were recognized at diverse extents (Fig. 2A and B). Moreover, although Ab from mouse serum #3 displayed lower reactivity than the former, essentially the same FAH regions were recognized (Fig. 2C), and the scarce reaction of mouse serum #4 with peptides was limited to N- and C-terminal FAH sequences (Fig. 2D).

Various mouse FAH portions exhibiting 30–70% of identity with peptides from different MHV proteins (Fig. 3) were placed as solid bars below the mouse FAH sequence (Fig. 2). It was observed that although homologous sequences 1–46, 60–92, and 359–378 did react with Ab, others did not, i.e., sequences 100–127, 140–187, and 223–232 (Fig. 2A, B and C).

Peptide number	Sequence	Identity (%)
1	FAH (13-22) PI**QN**L**PYG**VF E2 (1102-1111) LV**QN**A**PYG**LY	50
2	FAH (23-32) S**TQ**SN**PK**PRI E2 (491-500) T**TQ**TK**PK**SAF	40
3	FAH (34-43) V**AIGD**QILD**L** E2 (501-510) V**NVGD**HCEG**L**	40
4	FAH (37-46) **GD**QILD**LSVI** E2 (504-513) **GD**HCEG**LGVL**	40
5	FAH (62-71) FDETT**LNN**FM E2 (1004-1013) ANAEA**LNN**LL	30
6	FAH (74-83) **GQ**AAW**KE**AR**A** E2 (590-599) **GQ**GVF**KE**VK**A**	50
7	FAH (83-92) **ASLQ**NLLSAS E2 (1025-1034) **ASLQ**EILTRL	50
8	FAH (223-232) EH**IF**GMV**LMN** E2 (547-556) CQ**IF**ANI**LLN**	40
9	FAH (369-378) G**TK**AID**VGQG** E2 (38-47) S**TE**TVE**VSQG**	40
10	FAH (1-10) **MSFIPVAE**DS N (1-10) **MSFVPGQE**NA	50
11	FAH (9-18) **DSDFPIQNLP** N (308-317) **DPQFPI**LAEL	40
12	FAH (143-152) **GKENALLPNW** N (117-126) **GQQKQLLPRW**	50
13	FAH (359-368) **FGSMLELSWK** N (326-335) **FGSKLELVKK**	70

Peptide number	Sequence	Identity (%)
14	FAH (9-18) DSD**FPIQNLP** E1 (163-172) GTG**FSLSDLP**	30
15	FAH (115-124) MHLPATI**GDY** E1 (134-143) VYVRPI**IEDY**	30
16	FAH (155-164) LPV**GY**HG**RAS** E1 (51-60) L**QFGYTSRSM**	40
17	FAH (159-168) **YH**GRASS**IVV** E1 (142-151) **YH**TLTAT**IIR**	30
18	FAH (178-187) **GQMRPDNSKP** E1 (209-218) **GNYRLPSNKP**	40
19	FAH (11-20) **DFPIQNLPYG** RNA (2317-326) **DYAFEHVVYG**	30
20	FAH (60-69) H**VFDETTLNN** RNA (1137-1146) Y**VFDKSELTN**	50
21	FAH (100-109) K**EL**RQ**RAFTS** RNA (728-737) R**EL**QK**RLYSN**	30
22	FAH (118-127) **PA**TIGD**YTDF** RNA (746-755) **PA**FVSE**YYEF**	40
23	FAH (140-149) M**FRGKENALL** RNA (2176-2185) L**FDGRDNGAL**	40

Fig. 3. Homologous synthetic peptides from mouse liver FAH and various MHV A59 proteins. Alignment of mouse liver FAH amino acid sequence with MHV A59 proteins was realized with LALIGN program. Left, upper panel: surface glycoprotein E2; left, lower panel: nucleocapside (N); right, upper panel: membrane glycoprotein E1; right, lower panel: RNA-direct RNA polymerase (RNA). Identical residues are shown in bold.

Surprisingly, the highly reactive C-terminal mouse FAH sequence (residues 390–420) does not display significant homology with any viral protein (Fig. 2A, B and C). Besides, results obtained with serum #4, even if very low, followed basically the same pattern of reactivity than sera #1–3 (Fig. 2).

Same results as those described in the last two paragraphs could be distinguish when the sum of optical density values at all time points for each peptide was collected for the four representative mice shown in Fig. 2 plus the addition of values displayed by sera from other three animals (Fig. 4A). Furthermore, optical density values exhibited by different pooled serum samples obtained after 15 and 30 days of MHV infection also indicated that autoAb reacted with the FAH sequence portions listed before, even though residues around position 240 as well as the homologous sequence 140–187 were also reactive (Fig. 4B and C).

Finally, sera reacted similarly with peptides from the autoAg at the various times after MHV infection, i.e., there was no evidence of a major sequence being the first target of the autoimmune response, suggesting the lack of spreading of the immune response (Fig. 2).

3.3. Reactivity of Ab from mice immunized with purified rat FAH

Various mice were immunized with purified rat liver FAH [3] and the Ab tested for their binding to the mouse FAH synthetic peptides. Results indicated that anti-rat FAH Ab reacted like the autoAb from MHV-infected mice. N- and C-terminal peptides were recognized, as well as regions surrounded residues 80, 130, 210, 250, 300, and 360 (Fig. 4D). The only remarkable difference was that anti-rat FAH Ab also bound to peptides corresponding to mouse FAH sequence 180–200, whereas sera from MHV-infected mice did not (Fig. 4).

4. Discussion

We have reported that mice infected with MHV produced autoAb to mouse liver and kidney FAH [3]. Competition assays indicated that the autoAb detected both conformational and cryptic FAH epitopes in ELISA but only cryptic determinants in Western blot assays, whereas anti-MHV Ab were directed to native epitopes of the viral proteins [4]. Such results suggested that MHV infection could trigger a cross-reaction of either sequential or conformational epitopes between the viral proteins and the autoAg and that MHV-infected mice produce at least three different Ab populations: Ab specifically directed either to viral proteins or to the autoAg, and cross-reacting Ab [4].

Molecular mimicry between viral proteins and self-Ag is one of the most probable mechanisms that explain autoimmune responses induced by viral infections [12, 13]. Murine adenovirus, Semliki forest virus, lactate dehydrogenase-elevating virus, herpes simplex virus type-1, hepatitis B virus, encephalomyocarditis virus, Theiler's murine encephalomyelitis virus,

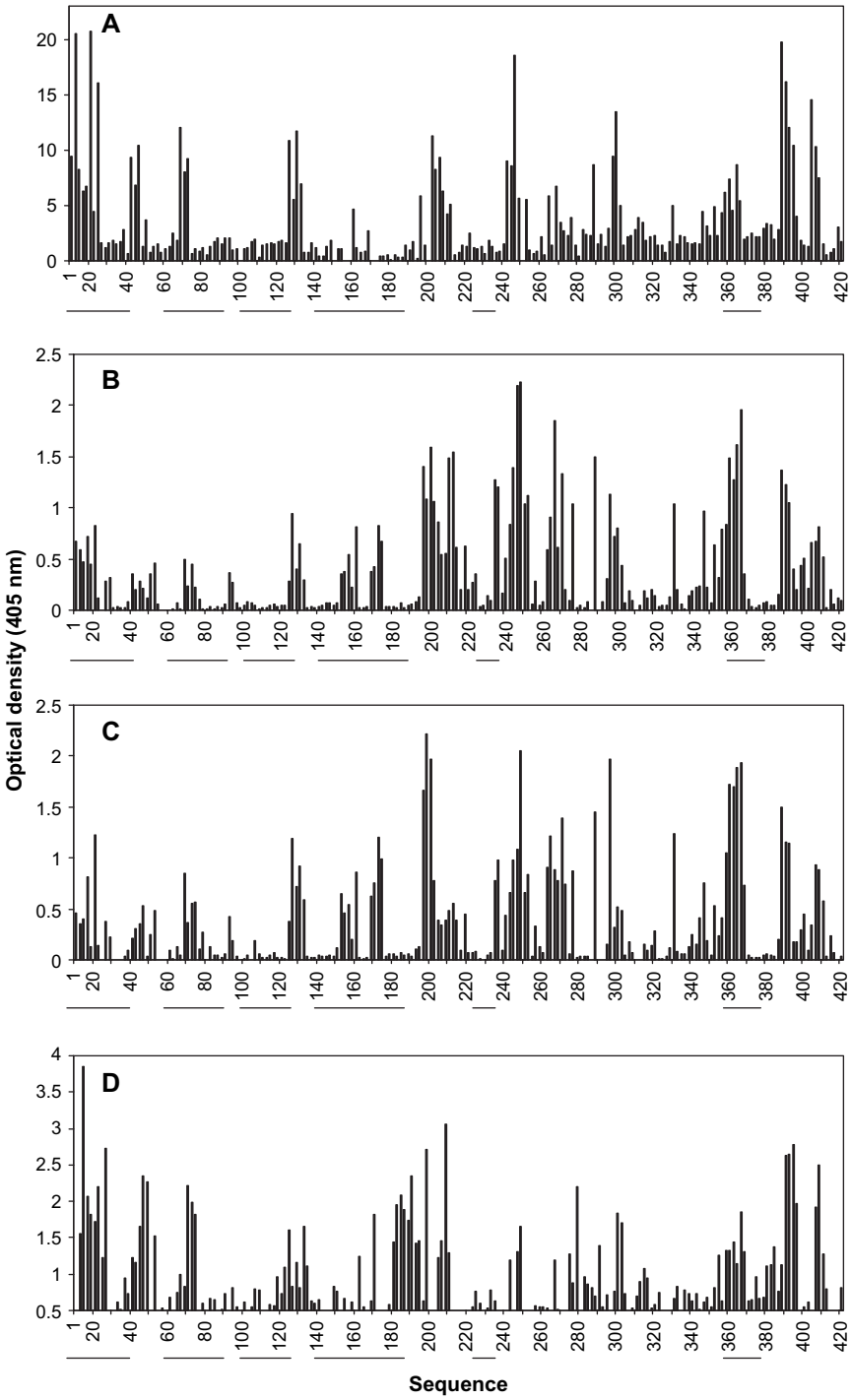

Fig. 4. Reactivity of synthetic peptides with sera from mice infected with MHV or immunized with rat liver FAH. Ab binding to the overlapping decapeptides covering the entire mouse liver FAH was determined by ELISA as indicated in Section 2. Results are expressed as specific optical density values (OD). Each panel shows results obtained with different animal groups. Horizontal bars indicate sequence homology (30% or more) between mouse liver FAH and MHV proteins (see Fig. 3 and legend of Fig. 2). (A) Results are presented as the sum of OD values at all time points for each peptide displayed in Fig. 2 for four individual mice, plus results obtained in a similar way with other three mice. (B) Results are the mean of two determinations performed with pooled serum from five BALB/c mice obtained after 15 days of MHV infection. (C) Results are the mean of two determinations performed with pooled serum from four BALB/c mice obtained after 30 days of MHV infection. (D) Results are presented as the sum of OD values for each peptide obtained with individual serum from three BALB/c mice immunized with rat liver FAH as indicated in Section 2.

Coxsackie virus and cytomegalovirus have been found to mimic physiologically important host proteins [12].

We previously observed that most autoAb from MHV-infected mice reacted with the FAH N-terminal sequence (residues 1–20) [4]. However, it could be expected that other sequences were also recognized. Thus, in the present work, we extended the study of the autoAb specificities using peptides spanning the entire sequence of mouse liver FAH. Moreover,

to examine the possible occurrence of determinant spreading [5–7], we tested the sera from individual animals at various times post-infection.

Representative results from four different mice showed that, in spite of individual disparity, the autoAb recognized mainly N- and C-terminal peptides, the most reactive sequences being 1–50 and 390–420, respectively. Strikingly, whereas the N-terminal portion of FAH shares a high degree of homology with various MHV proteins, sequence 390–420 does not display any similarity with viral proteins. Furthermore, the autoAb reacted with homologous peptides surrounding residues 70, 160 and 360, but non-homologous sequences around residues 130, 210, 240, 250 and 300 were also recognized. Thus, autoAb were not restricted to similar sequences, suggesting that structural patterns other than linear epitopes were involved in the autoimmune response. Accordingly, it has been proposed that viral regions that are able to initiate autoimmune responses do not need to have sequences analogous to the autoantigen [14] and, since the only requirement should be structural similarity, some authors propose that "molecular mimicry" should be changed to "structural mimicry" [15].

The present data also show a lack of correlation between the amount of autoAb and Ab to MHV proteins determined by ELISA, and Ab binding to the peptides. These facts could be explained by the production of three kinds of Ab, as mentioned above: virus-specific Ab, FAH-specific autoAb, and cross-reacting Ab, together with previous observation indicating than mainly conformational epitopes are recognized by ELISA assays [4,10]. Moreover, no immune diversification originated from a single autoreactive determinant was observed, indicating that the autoimmune response induced by MHV is not associated with determinant spreading as described in other autoimmune processes [5–7].

When the specificities of the MHV-elicited autoAb were compared with those of Ab induced by injections of rat liver FAH, no significant differences were observed. The same mouse FAH regions were recognized, with the exception of sequence 180–200, recognized only by the anti-rat FAH Ab.

The Danger model proposes that the immune system is more concerned with damage than with foreignness, and is called into action by alarm signals from injured tissues rather than by the recognition of non-self [16]. Thus, it was suggested that structural features of autoantigens, their locations, and catabolism during cell death and their translocation to cells that can present antigens to the immune system could contribute to selection of the autoimmune repertoire [16,17]. MHV is known to be lymphotropic and to induce diverse alterations of immune responses that depend on the mouse genetic background [1–4,18,19], but why liver FAH was chosen as autoantigen among the large variety of liver proteins? As said by P. H Plotz [17]: "The repertoire of target autoantigens is a wunderkammer – a collection of curiosities – of molecules with no obvious linking principle". In the present model of MHV-induced anti-FAH autoAb, it seems that antigen mimicry – FAH N-terminal sequence is about 50% homologous with a MHV protein – together with the alarm signals released by the MHV-injured tissues leads to the autoimmune response.

Acknowledgements

The authors are indebted to Drs. Pierre L. Masson (ICP, Brussels, Belgium) and Leonor P. Roguin (IQUIFIB, Buenos Aires, Argentina) for helpful discussions and critical revision of the manuscript. This work was supported by grants from CONICET, FONCYT and Universidad de Buenos Aires, Argentina.

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