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Diversity of the B Cell Repertoire to Myelin Basic Protein in Rat Strains Susceptible and Resistant to EAE

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Myelin basic protein (MBP) is a major protein of central nervous system myelin which can induce experimental autoimmune encephalomyelitis (EAE) in susceptible laboratory animals. The role of T cells in the induction of EAE has been extensively studied, but the antibody response to MBP has not been well characterized. In the present work, we immunized rats with encephalitogenic guinea-pig MBP and mapped autoreactive antibodies binding to peptides in the rat MBP sequence. We studied the responses of the Lewis rat strain, susceptible to EAE, and the responses of the Fischer and Brown–Norway (BN) rats, resistant to EAE. We found that Lewis rats immunized to guinea-pig MBP develop antibodies to a diversity of MBP epitopes with a dominance of MBP peptide p11–30 and peptides in the 71–140 region. Fischer rats showed a similar pattern of antibody specificities, but with higher titers than the Lewis rats. BN rats, in contrast, developed a very low titer of antibodies and lacked a response to p11–30. Thus, there is no clear correlation between the nature of the anti-MBP antibody response and the state of susceptibility or resistance to EAE induction in the different rat strains.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is considered a prototype T cell-mediated autoimmune disease [1]. The current concept of the pathophysiology of EAE proposes that T cells specific for an autoantigen: myelin basic protein (MBP), proteolipid protein (PLP) or other neuroantigens, are activated in the periphery, migrate to the central nervous system and cause autoimmune inflammation leading to paralysis of the experimental animal [2]. Since it was demonstrated that MBP-specific T cell clones could transfer EAE [3], most of the experimental work on EAE has focused on the role of T cells.

Immunization of experimental animals with MBP is known to lead to the production of anti-MBP antibodies. The dimensions of the B cell repertoire activated in response to MBP immunization have not been well characterized. Most papers have focused on the B cell response to encephalitogenic determinants seen by T cells [4–6] with little analysis of the antibody response to other regions of MBP. In multiple sclerosis, a recent analysis of antibodies eluted from brain tissue has pointed to a very restricted and similar range of B and T cell epitopes [7].

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Antibodies are thought by most workers not to play a pathogenic role in the induction of EAE; antibodies alone cannot transfer the disease [8, 9], and there is no direct correlation between antibody titers and disease severity [10]. However, anti-MOG antibodies are known to mediate demyelination when given with encephalitogenic T cells [11]. Thus, some authors suggest that there is a synergy between T and B cells in the induction of EAE. In support of this concept, mice and rats depleted of B cells are resistant to disease induction by MBP immunization [12, 13]. Other investigators have presented evidence for a protective role for anti-MBP antibodies; serum collected from animals recovered from EAE has been demonstrated to passively transfer resistance to EAE in naïve recipients [14, 15].

In the present work, we mapped the anti-MBP B cell repertoire in Lewis rats immunized with encephalitogenic guinea-pig MBP in CFA (MBP/CFA), using a panel of 17 synthetic overlapping peptides covering the amino acid sequence of the 18.5 kDa isoform of the rat MBP self-antigen. In addition, to uncover a possible role of the anti-MBP antibody response in the development of EAE, we compared the anti-MBP antibodies elicited using a protocol leading to EAE in Lewis rats (MBP/CFA) to a protocol that does not result in disease (MBP/IFA). The same analysis was also performed in Fischer and BN rats, which do not develop EAE in response to guinea-pig MBP/CFA. We found that the B cell repertoire to MBP is diverse

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Table 1. *Peptide sequences*

Peptide	Sequence
p1–20	ASQKRPSQRHGSKYLATAST
p11–30	GSKYLATASTMDHARHGFLP
p21–40	MDHARHGFLPRHRDTGILDS
p31–50	RHRDTGILDSIGRFFSGDRG
p41–60	IGRFFSGDRGAPKRGSGKDS
p51–70	APKRGSGKDSH—TRTTHYG
p61–80	H—TRTTHYGSLPQKSQ—
p71–90	SLPQKSQ—RSQDENPVVH
p81–100	RTQDENPVVHFFKNIVTPRT
p91–110	FFKNIVTPRTPPPSQGKGRG
p101–120	PPPSQGKGRGLSLSRFSWGA
p111–130	LSLSRFSWGAEGQKPGFGYG
p121–140	EGQKPGFGYGGRASDYKSAH
p131–150	GRASDYKSAHKGFKG-A-DA
p141–160	KGFKG-A-DAQGTLSKIFKL
p151–170	QGTLSKIFKLGGR—DSR
p161–177	GGR—DSRSGSPMARR

and peaks long after recovery from EAE. Anti-MBP antibody titers were found to be highest in the Fischer rats, followed by Lewis rats and the weakest response was detected in BN rats.

Materials and Methods

Rats

Lewis, Fischer 344 and Brown–Norway (BN) female rats were supplied by Harlan, Olac, Bicester, UK and were used at 2–3 months of age.

Proteins and peptides

Guinea-pig myelin basic protein (MBP) and rat MBP were prepared from the spinal cords of guinea-pigs or rats as described [16]. A panel of synthetic peptides covering the whole amino acid sequence of the 18.5 kDa isoform of the rat MBP molecule was synthesized with an automatic multiple peptide synthesizer (AMS 422, ABIMED, Langenfeld, Germany), using the F-MOC technique. Purity of the peptides was analysed by HLPC and amino acid composition. The sequences of the peptides are shown in Table 1.

Immunizations

EAE was induced by injecting both hind foot pads with 0.05 ml containing 25 µg guinea-pig MBP and 200 µg *Mycobacterium tuberculosis* (MT) emulsified in equal volumes of incomplete Freund's adjuvant (IFA) and phosphate buffered saline (PBS) [17]. Peptide immunization was performed by injecting the animals subcutaneously in both hind foot pads with 0.05 ml of an emulsion containing 100 µg of the test peptide emulsified in equal volumes of PBS and complete Freud's adjuvant (CFA). The CFA was prepared from the incomplete Freund's adjuvant by the addition of 4 mg/ml of *Mycobacterium tuberculosis* (MT) H37Ra. IFA and MT were purchased from Difco Laboratories Inc., Detroit, MI, USA.

Sera preparation

Immunized rats were bled under ether anesthesia from the retro-orbital plexus or by heart exsanguination. The blood was allowed to clot for 1 h at 37°C, followed by 1 h on ice. After blood clot removal, the sera were centrifuged for 7 min at 1500 rpm and were stored at −20°C.

ELISA assay

Each well of 96-well flat-bottomed microtiter plates (Maxisorp, Nunc, Denmark) was coated with 100 µl of PBS containing MBP peptides (10 µg/ml) or MBP protein (5 µg/ml), by overnight incubation at 4°C. The plates were then washed with PBS and blocked with PBS containing 1% Marvel powder milk (Premier Brands, Bourville, Birmingham, UK) for 2 h at 37°C. The plates were washed with PBS and test serum dilutions (1:10 and 1:100) in PBS 0.33%-BRIJ 35 (Sigma, Chemical Company Ltd: Israel), 1% Marvel was added to the plate (two wells/serum dilution). The plates were then incubated for 2 h at 37°C and washed in PBS. Alkaline phosphatase rabbit anti-rat IgG (Sigma) diluted 1:2,500 in PBS 0.33% BRIJ 35, 1% Marvell powder milk were added (100 µl/well) and the plates incubated for 2 h at 37°C. The plates were then washed with PBS 0.33% BRIJ 35 and with PBS; 100 µl/well of alkaline phosphatase substrate solution was added at a ratio of one tablet of phosphatase substrate (Sigma) 10 per 10 ml of 9.5% DEA, Sigma, DDW pH 9.8. The plates were read with an *anthos htll* ELISA plate reader at 405 nm (Anthos Labtec Instruments, Salzburg, Austria). The optical density (OD) background values were determined as the mean of OD values obtained from wells coated with peptides or MBP and to which no test serum was added. The background OD was subtracted from the test OD values. To compare between the ELISA assays performed at different times, OD results of each test were standardized in reference to a standard serum included in each plate. All test results were divided by the OD value of the standard serum (a single serum diluted 1:10,000 from a Lewis rat that had been immunized 75 days earlier with MBP/CFA). Antibody reactivity was considered to be positive if the OD test value was equal to, or higher than 0.1, at least 10% of the standard reference serum.

Relative antibody titer

To compare the responses of different strains, we carried out the ELISA assay with 10-fold serial dilutions of test sera, standardized in reference to the

Table 2. *The B cell epitopes of MBP in immunized Lewis rats*

Peptide	Number of positive rats (day post-immunization)				
	14	21	28	35	62
p1–20	–	–	–	–	–
p11–30	2	6	6	6	6
p21–40	–	–	–	2	2
p31–50	–	–	–	–	–
p41–60	–	–	–	–	–
p51–70	–	–	–	–	–
p61–80	–	–	–	–	3
p71–90	–	–	4	6	3
p81–100	–	–	–	–	1
p91–110	–	3	5	6	6
p101–120	–	–	–	5	5
p111–130	–	–	–	5	6
p121–140	–	3	3	6	6
p131–150	–	–	–	–	1
p141–160	–	–	–	–	–
p151–170	–	–	–	–	–
p161–177	–	–	–	–	–

Number of sera reactive with the MBP synthetic peptides, at different time points post-immunization. The sera are derived from Lewis rats immunized with MBP/CFA. The antibody reactivity was determined by ELISA, and it was scored as positive for OD values higher than 0.1.

standard serum (see above). The standard serum was arbitrarily assigned a value of 500 units/ml of anti-MBP activity.

ELISA competition assay

The ELISA competition assay was performed following a procedure similar to the one described above, except that test sera diluted 1/100 were incubated (1 h at 37°C) with MBP peptides or with rat MBP in equimolar concentrations, before addition to ELISA plates precoated with peptides p11–30 or p91–110.

Results

MBP B cell epitopes in the Lewis rats

Thirty female Lewis rats were immunized with MBP/CFA by subcutaneous hind foot pad injection, an experimental protocol that leads to paralysis. Groups of six rats were bled at different time points after immunization: days 14, 21, 28, 35 and 62. The sera were analysed for the presence of antibodies against a panel of overlapping peptides covering the amino acid sequence of rat MBP. Table 2 shows the number of rat sera, out of six, that were reactive to each of the peptides at each time point.

There was a gradual accumulation of B cell epitopes in the course of the immune response. The antibodies detected first were directed to p11–30 (at day 14), followed by p91–110 and p121–140 (day 21), p71–90 (day 28), p21–40, p101–120 and p111–130 (day 35), and p61–80, p81–100 and p131–150 (day 62 post-immunization). At day 62, which was the last time point tested, all rats were reactive to peptides p11–30, p91–110, p111–130 and p121–140 and five out of six rats were reactive to p101–120. Figure 1 shows the OD values for each of the peptides obtained with sera from days 35 and 62 post-immunization, for each of the peptides, at serum dilutions 1:10 and 1:100. The anti-MBP antibodies on day 35 (and earlier; data not shown) appeared to be directed mainly to peptide p11–30 and peptides in the 71–140 region (Figure 1A & B). At day 35, the dominant reactivity was directed to p11–30, followed by p91–110. At day 62 (Figure 1C & D), reactivity to p91–110 become dominant, followed by reactivity to p11–30. Significant antibody responses were also demonstrated to p111–130 and p121–140 (Table 2).

To test if the p11–30 and p91–110 epitopes are accessible on the intact MBP molecule, an ELISA competition assay was performed, in which a positive serum was preincubated with soluble peptides p11–30 or p91–110, or with intact MBP, in equimolar concentrations, before testing. The results show that preincubation with the intact MBP molecule led to a decrease in binding comparable to that obtained using each of the soluble peptides (Figure 2). Thus, the epitopes of p11–30 and p91–110 would appear to be accessible on intact MBP.

Detection of cryptic B cell responses to MBP

As demonstrated above, dominant B cell epitopes were found in peptides p11–30, p71–90, p91–110, p111–130 and p121–140. Since the T cell response contains a large cryptic repertoire [18], we aimed to investigate whether the antibody repertoire might also include cryptic epitopes. We immunized rats with a panel of eight peptides and tested them for the development of anti-peptide antibodies not seen when the immunogen was intact MBP. As shown in Figure 3, five of the eight peptides induced a B cell response both to the particular peptide and to intact MBP. Thus, the lack of antibodies detected to peptides such as p1–20, p21–40, or p101–120 was not due to their non-immunogenicity, but was probably the result of *in vivo* antigen processing and presentation favouring other epitopes. A similar phenomenon is seen with the T cell repertoire: immunization with MBP selects for the p71–90 response, while peptide immunization reveals additional immunogenic cryptic epitopes [18]. Unlike the T cell response, which selects a single immunodominant peptide, the B cell repertoire following MBP inoculation is diverse (Table 2).

The antibody repertoire to a non-encephalitogenic challenge of MBP in the Lewis rat

To determine whether the observed antibody specificities after MBP/CFA immunization (leading

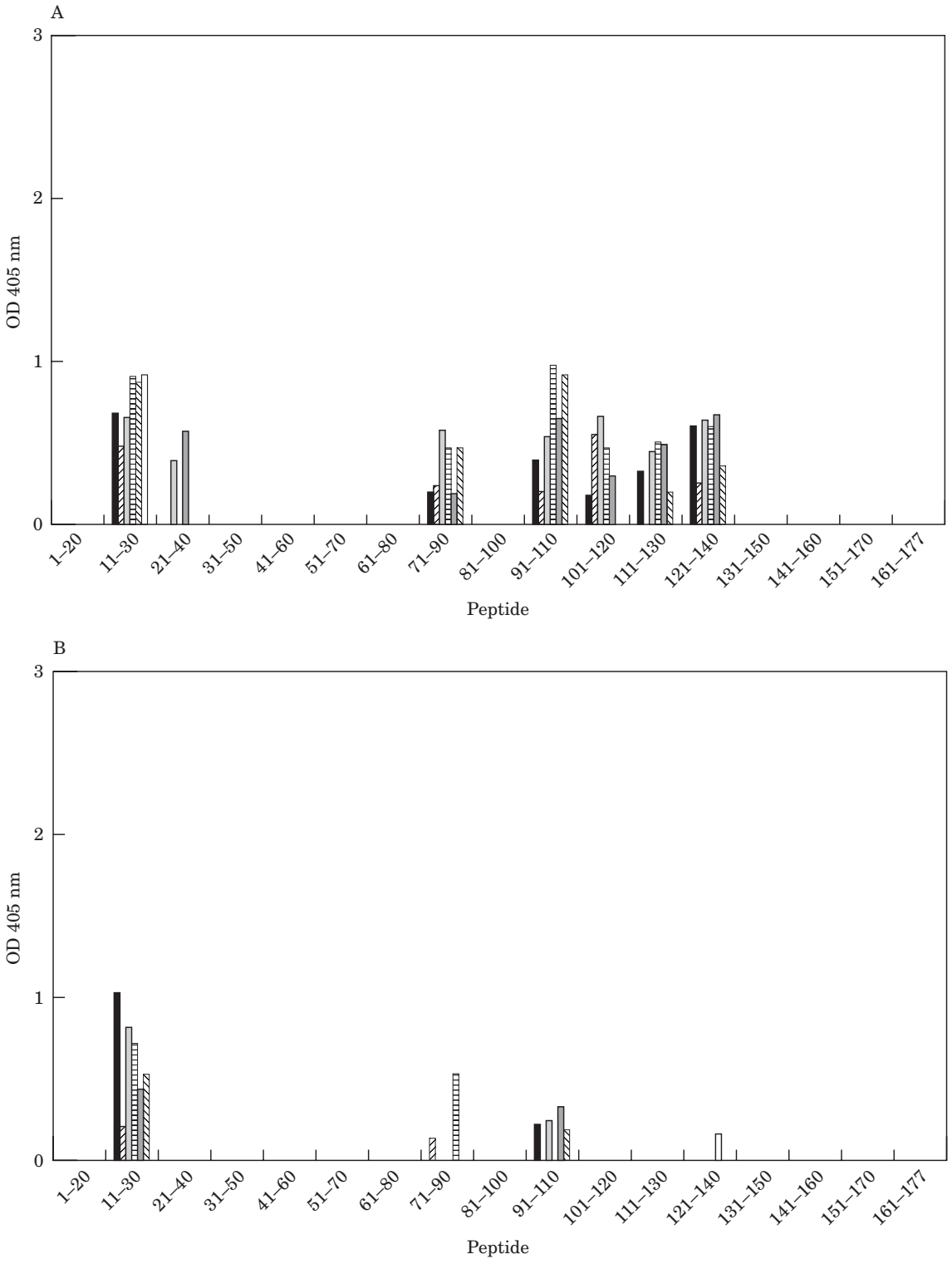

Figure 1. A & B (legend opposite)

to clinical EAE) were affected by the disease process, we immunized Lewis rats with MBP in IFA — a protocol that leads to an immune response to MBP without encephalomyelitis. The rats were bled 62 days after immunization, and the antibodies to MBP peptides were analysed. As shown in Figure 4, the overall pattern of reactivity was similar to the one seen after MBP/CFA immunization. Thus, the difference in adjuvant, CFA or IFA, did not have a significant impact on the B cell repertoire to MBP. However, the titer of anti-MBP antibodies was lower in MBP/IFA-immunized rats compared to rats immunized with MBP/CFA (Table 3).

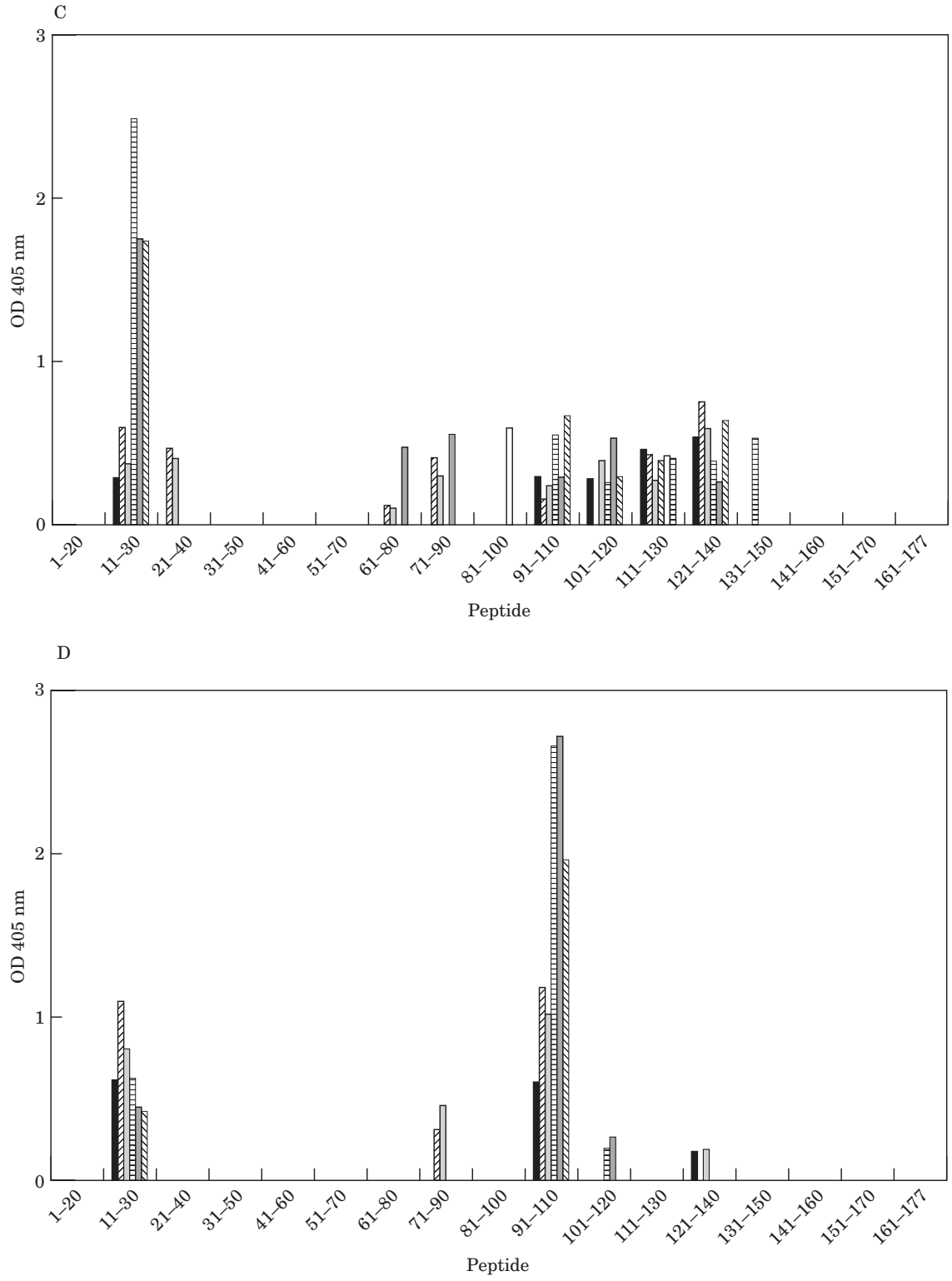

Figure 1. C & D

Figure 1. Antibodies to MBP epitopes in Lewis rats. Groups of six Lewis rats were immunized with MBP/CFA and their sera tested for antibody binding by ELISA to a panel of peptides covering the amino acid sequence of the rat MBP molecule. For each peptide, each column refers to one rat. Sera obtained on day 35 at a dilution of 1:10 (A) and a dilution of 1:100 (B), sera obtained on day 62 at a dilution of 1:10 (C) and a dilution of 1:100 (D).

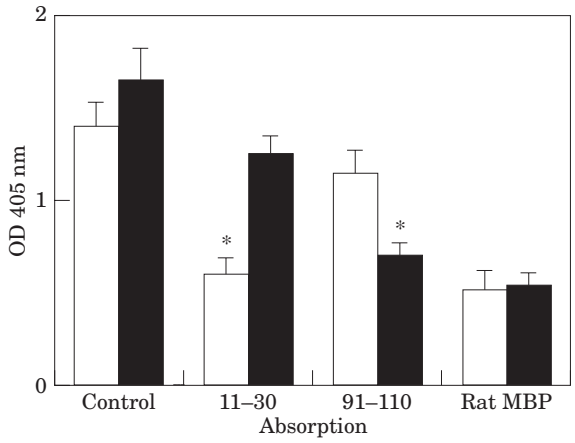

Figure 2. Absorption of peptide-specific antibodies by homologous peptide or whole MBP. Sera (1:100 dilution) were obtained from Lewis rats immunized with MBP/CFA 60 days earlier. The sera were absorbed by preincubation with p11–30, p91–110 or with intact MBP. Control sera were not preincubated. The sera were then tested for residual binding to p11–30 (open bars) or p91–110 (solid bars) in an ELISA assay. *$P<0.01$

The anti-MBP antibody response in rats resistant to EAE

The anti-MBP antibody response of Lewis MBP/CFA-immunized rats, which develop EAE, was compared to the responses of Fischer 344 and BN rats, both of which are resistant to EAE. All animals were bled at day 62 post-immunization, and their sera were analysed for antibody reactivity against the panel of peptides and for the titer of anti-MBP antibodies. The peptide antibody reactivity patterns of the three rat strains were, in general, broadly reactive. The Fischer rats, however, manifested a broader range of MBP B cell epitopes and a weaker relative dominance of their anti-p91–110 antibody reactivity (Figure 5), and a higher antibody titer compared to the Lewis rat. The antibodies developing in the BN rats showed an absence of dominant antibody reactivity towards the p11–30 peptide (Figure 6) and much lower antibody titers (Table 3). Thus, there was no correlation between the magnitude of the antibody response and the development of EAE. The two resistant strains showed either higher titers (Fischer) or lower titers (BN) than did the Lewis rat.

Prior peptide immunization affects the subsequent B cell response to whole MBP

Groups of Lewis rats were immunized with one of six MBP peptides in CFA- p11–30, p51–70, p71–90, p91–110, p131–150 or p151–170, and bled 32 days post-immunization. Three days later, the rats were immunized with MBP/CFA, and the rats were bled again 35 days after the second immunization. The first bleeding, carried out after the peptide immunization, showed that the MBP peptides p11–30, p71–90 and p91–110 induced a much stronger antibody response than did peptides p51–70, p131–150 and p151–170 (data not shown). The second bleeding, carried out after immunization with whole MBP, showed a consistent enhancement of the antibody reactivity

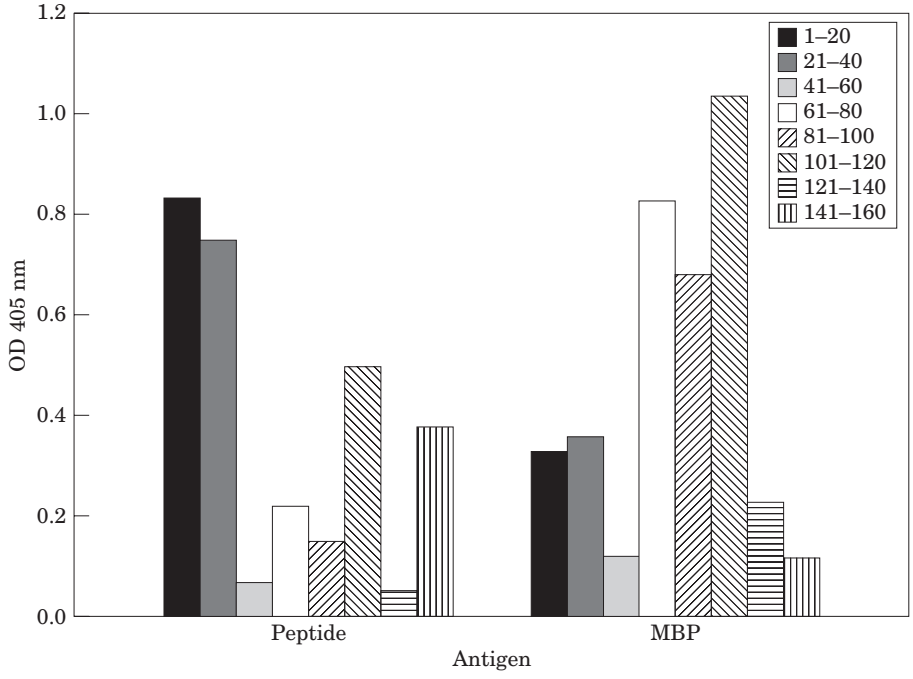

Figure 3. Immunogenic peptides induce antibodies to themselves and to intact MBP. Groups of four Lewis rats were immunized with various peptides in CFA, and, 75 days later their sera diluted 1:100 were tested for antibody binding to the homologous peptide or to intact MBP.

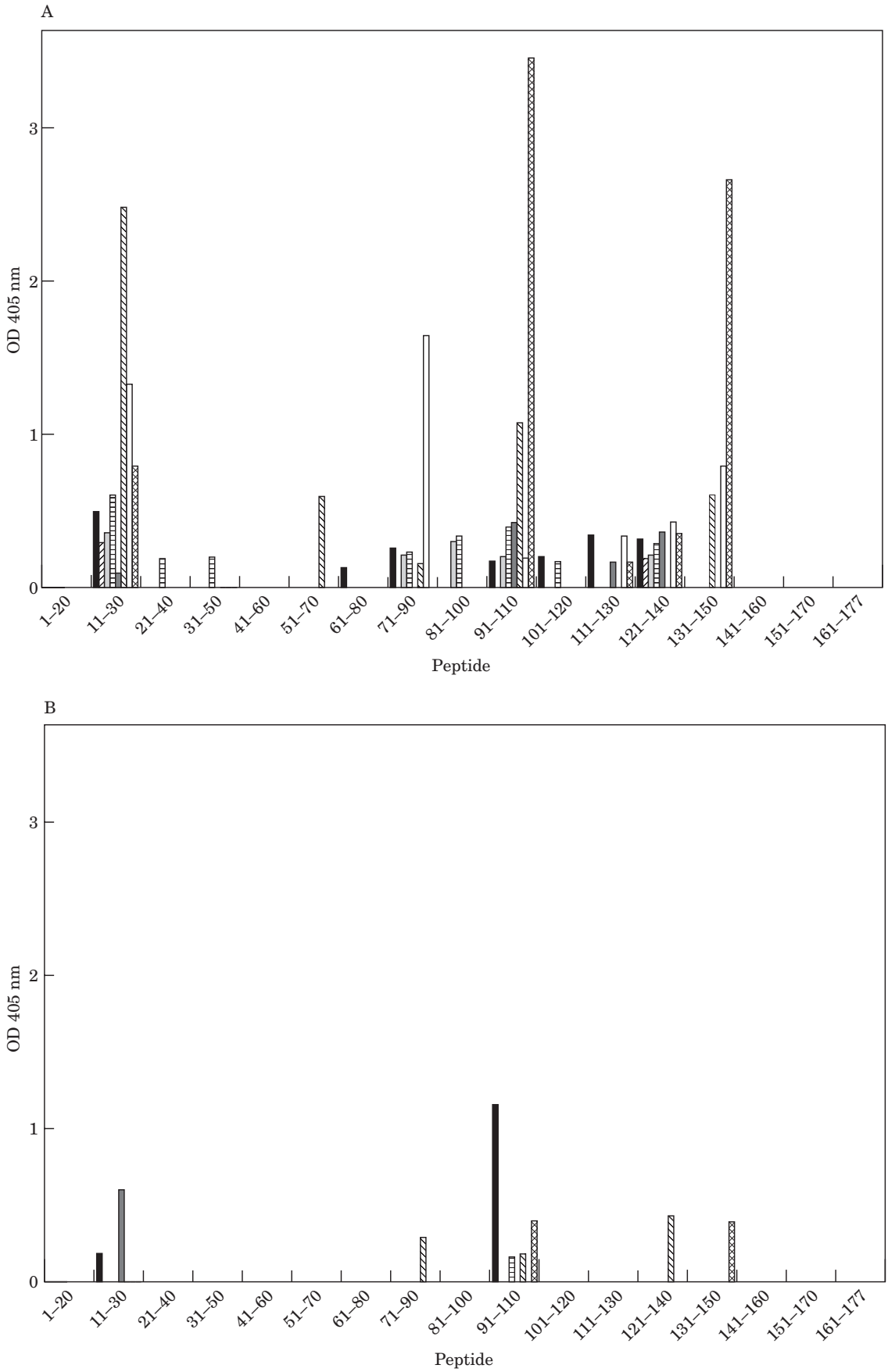

Figure 4. Induction of antibodies by immunization with MBP in IFA. The experiment was carried as described in the legend to Figure 1, except that the Lewis rats were immunized with MBP in IFA instead of CFA. Dilution of 1:10 (A) and 1:100 (B).

Table 3. *Anti MBP antibody titers in various rat strains 62 days post-immunization, determined by ELISA*

No.	Lewis GBP/CFA	Lewis GBP/IFA	BN GBP/CFA	Fischer GBP/CFA
1	574	134	13	1362
2	312	60	33	502
3	437	121	23	381
4	169	101	32	1548
5	584	113	21	623
6	239	235	13	1134
7	–	–	50	638
8	–	–	33	425
mean	386	127	27	827
SD	174	58	12	454

towards the particular peptides used on the first immunization, compared to the control group of Lewis rats first immunized with CFA without peptide. There was no effect on the antibody reactivity towards MBP peptides, not used on the first immunization. However, peptide immunization with p71–90 or p91–110 appeared to lead to partial suppression of the antibody response to the p11–30 peptide. In addition, the first immunization to p151–170 was associated with an suppression of the antibody response to whole MBP (Figure 7). Thus, prior contact with certain peptides can influence the responses to different MBP epitopes.

Discussion

We found that a broad range of antibodies to MBP epitopes appears in Lewis rats immunized with MBP/CFA. Nevertheless, dominant B cell epitopes appear to be located in the p11–30 and p91–110 sequences. The dominant T cell epitope, in contrast, is located in the p71–90 sequence [18]. The B cell epitopes in these peptides appear to be available on the intact MBP molecule; preincubation with these peptides or with the intact MBP molecule in equimolar concentrations led to an equivalent inhibition in the degree of antibody binding to the respective peptides (Figure 2).

The results also showed the progressive accumulation of antibodies to new MBP B cell epitopes during the immune response. The first antibody reactivity to MBP was detected against the p11–30 peptide (day 14) and, progressively, spread to additional peptides: p91–110 and p121–140 (day 21), p71–90 (day 28), p21–40, p101–120 and p111–130 (day 35) and p61–80, p81–100 and p131–150 (day 62). The dominant epitope was in the p11–30 peptide until day 35 post-immunization, passing to an epitope located on the p91–110 amino acid sequence by day 62 post-immunization. In contrast to this diverse B cell repertoire to MBP following MBP immunization, the T cell response to MBP is highly focused [17]. During the disease phase most T cells respond to the immunodominant peptide (P71–90) and after recovery there is also reactivity against p51–70 [17]. By immunizing with synthetic peptides, instead of whole MBP, additional epitopes can be shown to induce T cell responses (the cryptic T cell repertoire) [18].

The kinetics of antibodies to B cell epitopes developing in the course of the Lewis rat EAE does not support a pathogenic role for antibodies in the induction of EAE. At day 14 post-immunization, when the rat is paralysed, only anti-p11–30 antibodies were detected. However, immunization with this peptide in CFA does not lead to EAE, although an anti-p11–30 T cell line was shown to be encephalitogenic [2]. Moreover, the B cell response to MBP continues to evolve many weeks after clinical EAE has gone into remission by day 18–20 after immunization.

The lack of correlation between the anti-MBP antibodies and disease was confirmed by the observation that the response to MBP/CFA (which does induce EAE) did not differ from that produced in response MBP/IFA, which does not induce EAE. Moreover, Fischer and BN rats, both resistant to EAE, showed higher (Fischer) and lower (BN) titers of antibodies to MBP respectively. Thus, there seems to be no correlation between the anti-MBP antibody titer or the epitope pattern and the state of susceptibility or resistance to EAE. BN rats, in contrast to Lewis and Fischer rats, showed no significant anti-p11–30 antibodies. The mechanisms of EAE resistance in the three rat strains are probably different. Lewis rats immunized with MBP/IFA made lower titers of antibodies to rat MBP than did Lewis rats immunized with MBP/CFA (Table 3). It was reported that immunization with MBP/IFA induced weaker T cell responses [19]. Thus, IFA may be a weaker adjuvant than CFA for inducing any response to MBP. Fischer rats, identical in their MHC haplotype ($RT1.B^{1}$) to Lewis rats, also show T cell responses to the dominant p68–88 peptide [20, 21]. The fact that Fischer rats produce higher anti-MBP antibody titers than do Lewis rats suggests that their resistance to EAE might involve a deviation to an antibody response [22]. BN rats seem to be low responders to MBP in both their T and B cell populations, and their resistance to the induction of EAE may be attributed to a general lack of responsiveness to MBP.

EAE, like most autoimmune T cell mediated diseases [23, 24, 25] is considered to be a Th1 mediated disease [25, 26]. Moreover, in mice resistant to EAE, it has been shown that the immune response was of the Th2 phenotype [27]; and treatment with Th2 cytokines was associated with amelioration of diabetes in the NOD mouse [28]. More recent work modifies this view and points to a pathogenic potential of Th2 cells in EAE [28, 29] in addition to the well known pathogenic Th1 self-reactive T cells. In our work, the resistant state of Fischer rats accompanied by a strong B cell response to MBP could represent an augmented Th2 response to MBP, analogous to the situation in male SJL mice [27].

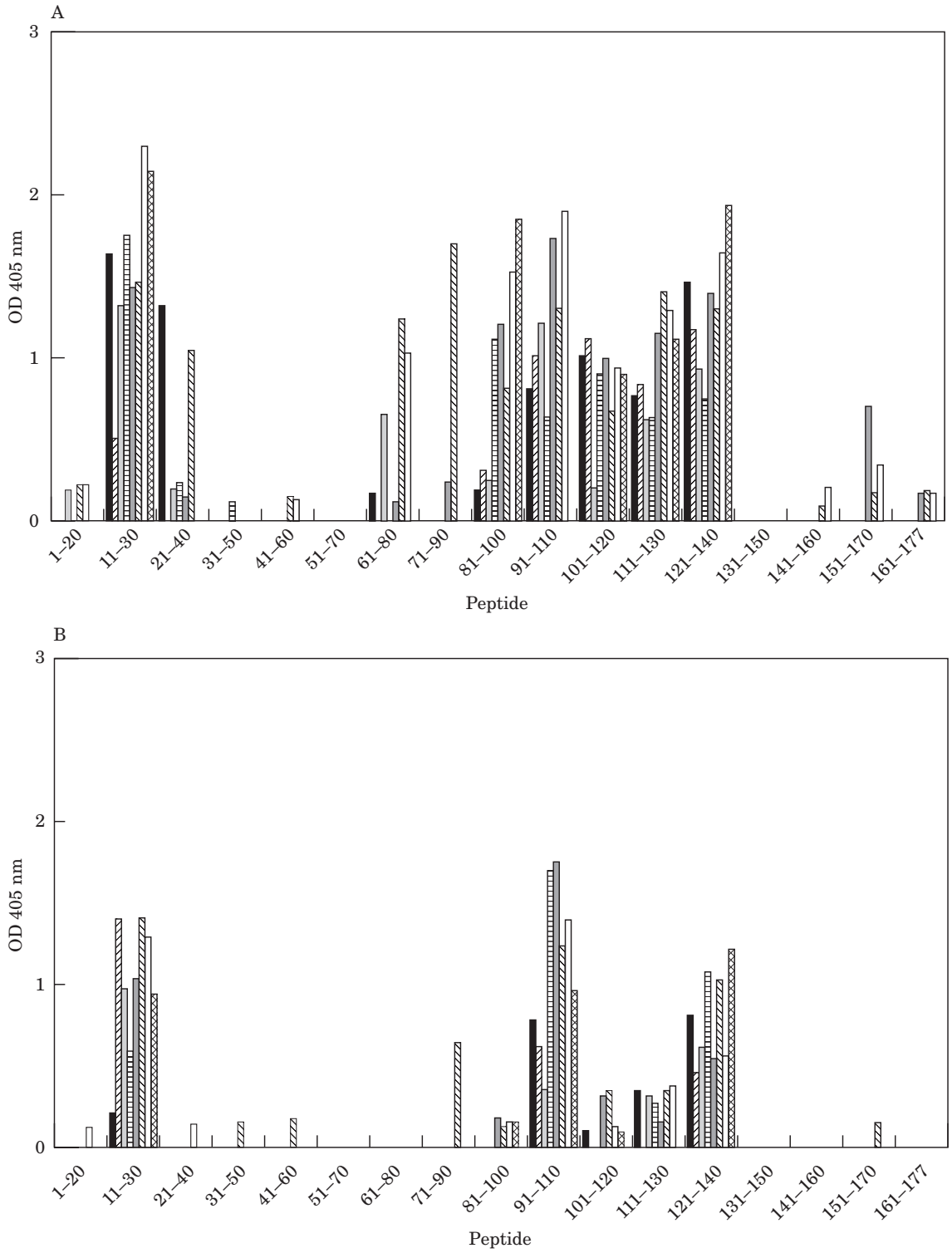

Figure 5. Antibodies to MBP peptides induced in Fischer rats in response to MBP in CFA. See legend to Figure 1. Dilution of 1:10 (A) and 1:100 (B).

To study the effect of peptide priming on the immunodominance of B cell epitopes in response to whole MBP, we first immunized Lewis rats to different peptides and then immunized them with whole MBP. The results showed a consistent enhancement of antibody reactivity towards the respective MBP peptides used on the first immunization. Moreover, the absence or weak antibody response to peptides p51–70, p131–150 and p151–170 may be due to the inherently weak immunogenicity of these stretches of the molecule. Competition among B cells with different specificities does indeed appear to play a role in the establishment of the MBP B cell immunodominant epitopes: preimmunization with p71–90 or p91–110

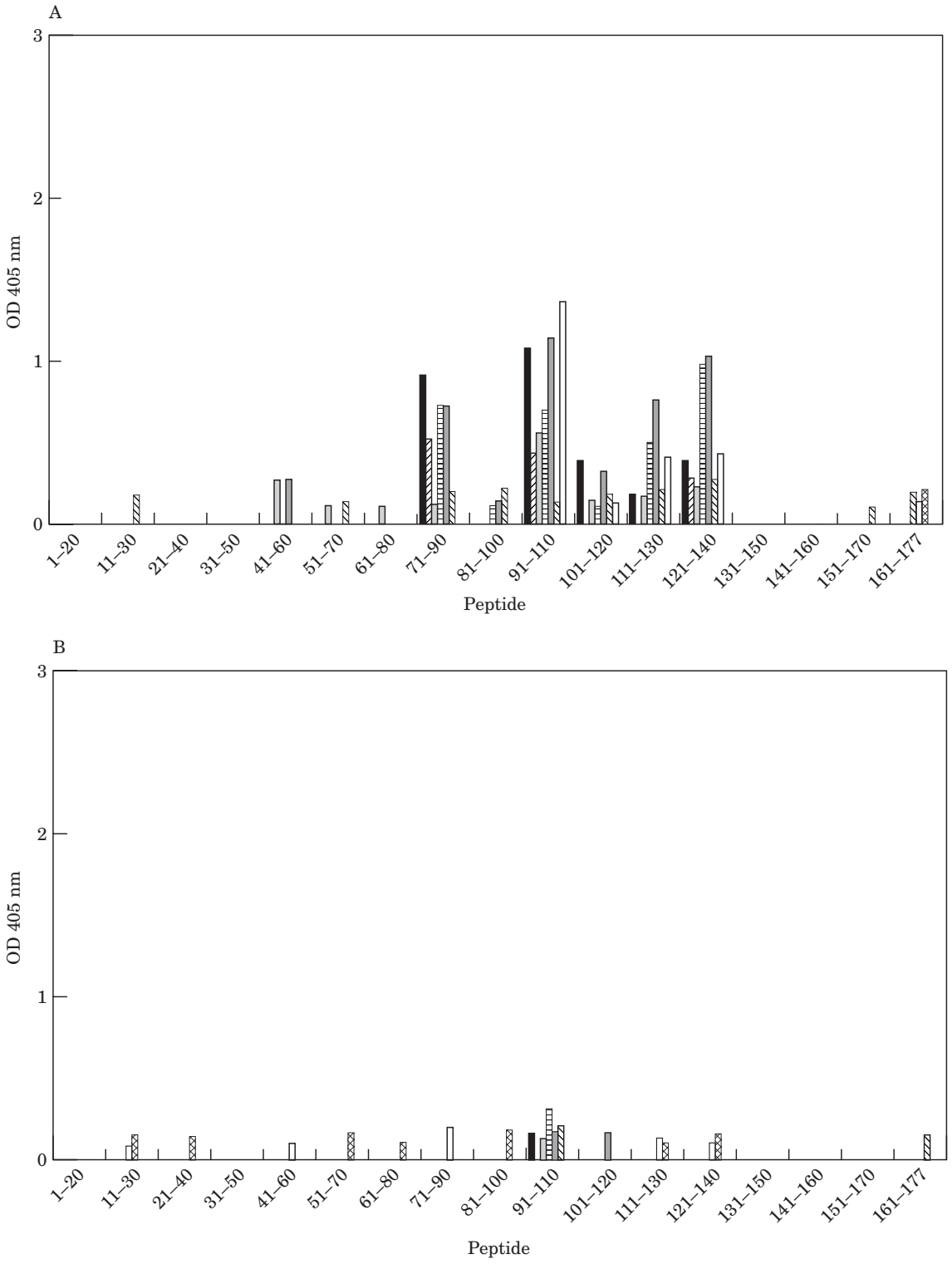

Figure 6. Antibodies to MBP peptides induced in BN rats in response to MBP in CFA. See legend to Figure 1. Dilution of 1:10 (A) and 1:100 (B).

immunodominant peptides appeared to lead to a partial suppression of the antibody reactivity towards the early MBP B cell epitope p11–30 and preimmunization with peptide p151–170 appeared to lead to an overall suppression of the B cell response to all MBP epitopes.

What can we learn about the structure of MBP from the antibody repertoire? The results presented here demonstrate that many different epitopes in the molecule induce antibody production (Table 1, Figure 1). Furthermore, when peptides were used for immunization, the antibodies always reacted with

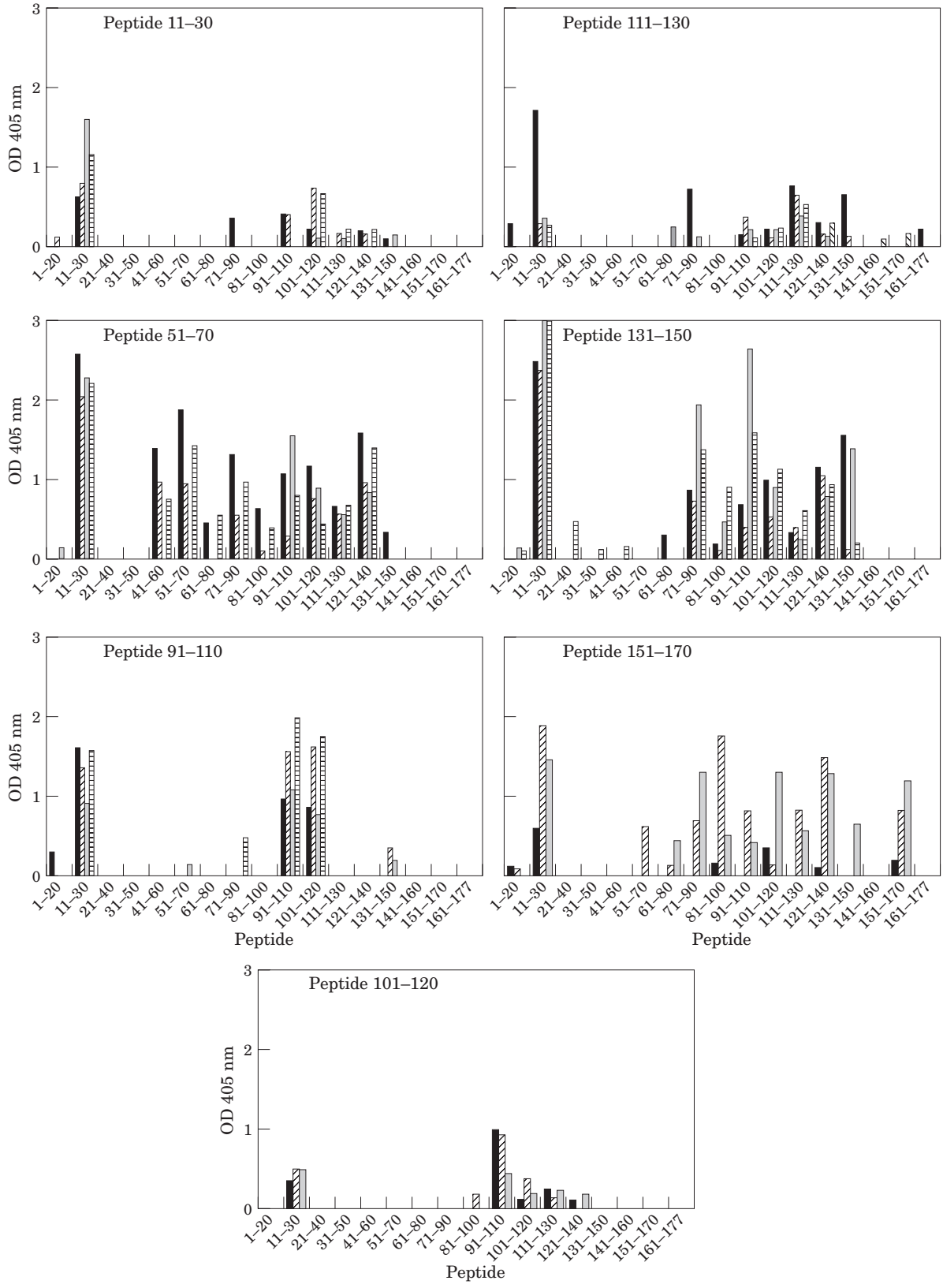

Figure 7. Antibodies to immunization with MBP in CFA following earlier immunization with specific MBP peptides. Groups of Lewis rats were preimmunized with MBP peptides in CFA, or with CFA alone. Thirty two days later the animals were immunized with intact MBP in CFA, and their sera (diluted 1:10) were tested for binding to a panel of MBP peptides.

native MBP (Figure 3). These findings provide biological confirmation for the structure of MBP in aquaous solution as a random coil [30,31]. MBP might also contain low amounts of β-structure that are increased upon phosphorylation [32]. The inability to crystalize MBP using many different protocols is considered as evidence for lack of homogeneity of tertiary structure and in favor of the random coil flexibility model of MBP [31]. Thus, many different B cell epitopes could be accessible in the intact MBP molecule.

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