Endogenous Myelin Basic Protein Inactivates the High Avidity T Cell Repertoire

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Summary

To study the contribution of endogenous myelin basic protein (MBP) to the positive and/or negative selection of the MBP-specific T cell repertoire, we studied the T cell response to MBP in MBP-deficient shiverer and MBP-expressing congenic C3H mice. Immunization with MBP induced a vigorous T cell response in shiverer mice directed against a single I-A^k–restricted immunodominant determinant, the core of which is peptide MBP:79-87 (DENPV-VHFF). Injection of this peptide induced a high avidity T cell repertoire in shiverer mice that primarily consisted of clones capable of recognizing the native MBP protein in addition to the peptide itself. These data show that endogenous MBP is not required for the positive selection of an MBP-specific T cell repertoire. C3H mice, in contrast, were selectively unresponsive to the MBP protein and injection of MBP:79-87 peptide induced a low avidity repertoire that could be stimulated only by the peptide, not by the protein. Therefore, endogenous MBP induced profound inactivation of high avidity clones specific for the immunodominant determinant making that determinant appear cryptic.

Key words: cryptic determinant • T cell tolerance • MBP-specific repertoire • T cell affinity • determinant hierarchy

There have been three mechanisms implicated in the development of self tolerance: clonal deletion, anergy, and ignorance. At present, it is unclear which of these mechanisms underlies the naturally developing self tolerance to myelin basic protein (MBP)[1], an abundant central nervous system protein and the best-studied autoantigen. For decades it was thought that MBP is sequestered behind the blood–brain barrier, but more recently MBP transcripts have been detected in the thymus and in other lymphoid organs (1–3), and evidence that MBP is presented in the thymus has emerged (4). However, the extent to which endogenous MBP contributes to the positive and/or negative selection of the MBP-reactive T cell repertoire remained unclear. Studies using three independently generated TCR transgenic mice showed that MBP:Ac1-11–specific T cells are positively selected in the thymi of MBP-expressing *H-2*^{u} mice and, in the absence of cross-reactive environmental stimulation, persist as naive T cells in their secondary lymphoid tissues (5–7). Although they do not yield conclusions about the role of endogenous MBP in positive selection, these results seemed to imply that the dominant determinant of MBP does not cause clonal deletion in *H-2*^{u} mice and that ignorance is the primary tolerance mechanism to MBP. Only when activated by immunization or environmental antigens would these MBP-reactive T cells induce the inflammatory central nervous system (CNS) disease, experimental allergic encephalomyelitis (EAE), an animal model of human multiple sclerosis (8). In support of this data, MBP-reactive T cells are present in unmanipulated, healthy mice, but these T cells do not cause EAE without immunization with MBP and treatment with pertussis toxin to open up the blood brain barrier (9). Do such T cells in MBP-expressing hosts represent the full MBP-reactive repertoire or are they low avidity remnants that slipped through the negative selection process? How fully do the existing TCR-transgenic models reflect the mechanisms of self tolerance to MBP?

We studied the contribution of endogenous MBP to positive and negative selection by comparing the MBP-specific T cell repertoires in MBP-deficient Shiverer mice (*H-2*^{k}) and in the congenic, MBP-expressing C3H strain. Shiverer mice have a deletion in the last five exons of the MBP gene; the first two MBP exons are present but their transcripts are unstable (10, 11). We report here, first, that, shiverer mice can generate a vigorous high avidity T cell response to MBP and its immunodominant determinant suggesting that endogenous MBP is not required for the positive selection of MBP-reactive T cells. Second, we re-

[1]*Abbreviations used in this paper:* CNS, central nervous system; EAE, experimental allergic encephalomyelitis; HEL, hen eggwhite lysozyme; MBP, myelin basic protein; gpMBP, guinea pig MBP; mMBP, mouse MBP.

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port that the MBP-expressing C3H mice are selectively unresponsive to this protein and its immunodominant peptide, but retain low avidity MBP-reactive T cells. Therefore, endogenous MBP induces profound clonal inactivation of the high avidity MBP-reactive T cell repertoire.

When *H-2*^{u} mice (which express MBP) are immunized with MBP, Ac1-11 is the peptide that elicits the strongest immune response and, hence, has been defined as the immunodominant determinant for this haplotype (12, 13). When the inflammatory CNS lesions develop, additional MBP peptides are targeted by T cells; these include the peptide 121–141, which has been designated as cryptic (14, 15). This assignment of determinant hierarchy has defined the immunotherapeutic approach to the EAE model. It assumes that determinant hierarchy within the T cell response to MBP follows the rules of T cell responses to foreign antigens, where the most abundantly presented peptide is likely to be the immunodominant one (16, 17). However, if endogenous MBP induces tolerance, its dominant determinant(s) might be the one that inactivates T cells most efficiently, as was observed after tolerogenic injection of hen egg lysozyme protein (18) and the transgenic expression of that protein (19). If the MBP-specific T cell repertoire were shaped in a similar fashion by endogenous MBP, one might expect the determinant that is immunodominant in the MBP-deficient shiverer mouse to be a cryptic determinant in the MBP-expressing congenic strain. Here we report evidence for this apparent reversal of determinant hierarchy.

Materials and Methods

Mice, Antigens, and Immunizations. Shiverer C3HeB/FeJ-*Mbp*$^{shi/+}$ mice, congenic C3HeB/FeJ mice, and B10.A(2R) and B10.A(4R) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and bred at CWRU facilities under specific pathogen-free conditions. Hen eggwhite lysozyme (HEL) and ovalbumin (OVA) were purchased from Sigma Chemical Co. (St. Louis, MO). Guinea pig MBP (gpMBP) and mouse MBP (mMBP) were prepared as described (20). MBP:79-87 peptide (DENPVVHFF) was purchased from Princeton Biomolecules (Columbus, OH). IFA was purchased from GIBCO BRL (Gaithersburg, MD) and CFA was made by adding *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI) at 2 mg/ml to IFA. Individually, antigens or peptides were mixed with the adjuvant to yield the emulsions, 50 μl of which were administered in a single injection. The antigen/peptide dose deposited within the injection is specified in the text and figure legends.

Cytokine ELISA Spot Assays. Plates (ImmunoSpot®; Autoimmun Diagnostika (US) Inc., Beltsville, MD) were coated overnight at 4°C with the cytokine-specific capture antibodies specified below. The plates were then blocked with 1% BSA in PBS for 1 h at room temperature and washed 4× with PBS. Subsequently, the antigens, peptides, and freshly isolated single cell suspensions of spleen or LN cells were added under the conditions specified for proliferation assays. After 24–48 h of cell culture in the incubator, the cells were removed by washing 3× with PBS and 4× with PBS containing 0.05% Tween (PBST), then the detection antibodies were added and incubated at 4°C overnight (the detection antibodies were either HRP-labeled or biotinylated). The plates were washed 3× with PBST. If biotinylated detection mAbs were used, streptavidin-HRP conjugate (Dako Corp., Carpenteria, CA) was added at 1:3,000 dilution, incubated for 2 h at room temperature, and removed by washing twice with PBST and twice with PBS. The spots were visualized by adding HRP substrate AEC (3amino-9-ethylcarbozole) (Pierce Chemical Co., Rockford, IL). We used the following combinations of capture and detection mAbs for IL-2, IL-4, IL-5, and IFN-γ assays, respectively: JES6-1A12 (5 μg/ml) and JES6-5H4-biotin (2 μg/ml), BVD4-1D11 (2 μg/ml) and BVD4-24G2-biotin (2.5 μg/ml), TRFK5 (5 μg/ml) and TRFK4-HRP (2 μg/ml), and R46A2 (5 μg/ml) and XMG1.2-HRP (2 μg/ml). Image analysis of ELISA spot assays was performed on a Series 1 ImmunoSpot® Image Analyzer (Autoimmun Diagnostika (US) Inc.) customized for analyzing ELISA spots to meet objective criteria for size, chromatic density, shape and color.

Proliferation Assays. Spleen cell proliferation assays were performed as previously described (14). In brief, single cell suspensions were prepared and 1×10^6 spleen cells per well were plated in flat-bottom 96-well microtiter plates in serum-free HL-1 medium (BioWhittaker, Inc., Walkersville, MD) supplemented with L-glutamine at 1 mM. Antigens and peptides were added at the final concentrations specified in the text and legends. During the last 18 h of a 4-d culture, [^{3}H]thymidine was added (1 μCi/well); incorporation of label was measured by liquid scintillation counting. Assays of LN followed the same protocol except that 5×10^5 cells were plated per well. CTLL proliferation assays for measuring IL-2 production by antigen-activated T cell hybridomas were performed according to standard protocols. In brief, 5×10^4 T cell hybridomas and 5×10^5 irradiated spleen cells were plated per well with or without antigen. After 24 h in cell culture, 50 μl of supernatant was collected and frozen at −85°C until tested for their ability to induce CTLL cells (1×10^4/well) to proliferate for 24 h. [^{3}H]Thymidine (1 μCi/well) was added for the last 16 h of the assay; incorporation of label was measured by liquid scintillation counting.

Determinant Mapping with Overlapping Peptides. A series of nonamer peptides covering the entire murine MBP sequence in steps of single amino acids (Chiron Mimotopes, Raleigh, NC) were dissolved at 560 μM in pH-optimized buffer (either 0.2 M Hepes or 0.2 M acetic acid) and aliquoted into 96-well plates and stored frozen at −85°C. Freshly thawed peptides were tested at 7 μM in the ELISA spot and proliferation assays, as described.

Generation of T Cell Hybridomas. T cell hybridomas from shiverer and C3H mice were generated using a standard protocol (21). In brief, 9 d after immunization with 50 μg of MBP peptide 79–87 in CFA, subcutaneously, draining LN cells were activated in vitro with 50 μM MBP:79-87 as specified for proliferation assays. After 78 h, 10 ng/ml of recombinant human IL-2 (a gift from Sandoz Ltd., Basel, Switzerland) was added overnight and the cells were fused with BW1100 T lymphoma cells (provided by Dr. C. Harding, Case Western Reserve University, Cleveland, OH). The cells were plated under limiting dilution conditions. The resulting hybrids were tested for MBP:79-87 specificity in CTLL assays and positive hybridomas were recloned under conditions of limiting dilution. Restriction of the hybridomas was mapped using recombinant mouse strains and the *I-A*^{k}–transfected cell line C3F6 (22), the *H-2* allele expression of which is shown in Fig. 1 *B*.

Results

MBP$^{-/-}$ *Mice Respond to MBP, but MBP*$^{+/+}$ *Mice Do Not.* We immunized C3H (*MBP*$^{+/+}$) and congenic shiverer (*MBP*$^{-/-}$) mice with two control "foreign" antigens,

OVA and HEL, and tested the antigen-specific recall response in draining LN. We performed cytokine ELISA spot assays to measure the frequency of antigen-specific T cells in freshly primed cell populations. The magnitude of the IFN-γ recall response to these control antigens was comparable in C3H and shiverer mice (Table 1). The immunizations with OVA and HEL also induced similar frequencies of memory cells that produced IL-2 but did not induce detectable production of IL-4 or IL-5 (data not shown). These data show that C3H and shiverer mice mount type-1 responses of comparable magnitudes to two prototypic "foreign antigens"; despite their neurologic defect, shiverer mice are fully immunocompetent.

When immunized with either mMBP or gpMBP (the two proteins differ at 15 residues scattered over the 168–amino acid sequence), shiverer mice developed a vigorous response that could be recalled with either protein (Table 1). As with the aforementioned control antigens, IL-2 was detected in addition to IFN-γ, a result confirmed in proliferation assays, whereas neither IL-4 or IL-5 was detected (data not shown). In two experiments performed using Shiverer mice (six mice per group), the frequency of the MBP-specific IFN-γ–producing memory cells was three to eight times higher in MBP-immunized shiverer mice than the frequency of memory T cells specific for HEL or OVA in mice immunized with the respective antigen (Table 1). Because C3H mice are classic high responders for HEL and OVA, these data show that mice of the C3H (*H-2*^{k}) background are high-responders to MBP when MBP is a "foreign" antigen.

Unlike the case in shiverer mice, mMBP did not induce an IFN-γ recall response in C3H mice (Table 1), nor was MBP-specific proliferation or the production of IL-2, IL-4, or IL-5 detected in immunized mice (data not shown). Immunization with gpMBP resulted in a response that was not recalled with mMBP (Table 1). Despite the extensive sequence similarities between the two proteins, the anti-gpMBP response did not cross-react with mMBP but seemed to be directed against the species-foreign sequences of the protein. In light of the fact that *MBP*$^{-/-}$ mice respond strongly to mMBP, the complete absence of spontaneous mMBP-reactivity in the unimmunized *MBP*$^{+/+}$ mice suggests that endogenous MBP did not prime a type-2 immune response, that is, the spontaneous development of tolerance to MBP did not involve immune deviation. Instead, the failure of C3H mice to respond to mMBP even after immunization with gpMBP suggests that their endogenous MBP had inactivated the majority of MBP-reactive precursor T cells.

IFN-γ responses to MBP (Table 1) were not detected in C3H or shiverer mice immunized with control antigens, nor was production of IL-2, IL-4, or IL-5 or a proliferative response seen (data not shown). Exposure to potentially cross-reactive environmental antigens did not, therefore, result in significant spontaneous priming of MBP-reactive T cells.

MBP Peptide 79–87 Is the Core Immunodominant Determinant in Shiverer Mice. We immunized shiverer mice with mMBP and mapped the fine specificity of the response using an overlapping nonamer peptide series. Fig. 1 *A* shows that, from the entire series, only three peptides induced a recall-response, 78–86, 79–87, and 80–88, with the response to peptide 79–87 by far the strongest. No reactions were detected to other peptides, including peptide Ac1-11, which has been defined as an I-A^k–restricted determinant (23). This result identifies peptide 79–87 on the MBP molecule as the core immunodominant determinant in shiverer mice. Further characterization showed that this MBP:79-87–specific recall response could be blocked with anti-CD4 mAb (data not shown) and that this peptide is I-A^k–restricted (Fig. 1 *B*).

Immunization of C3H mice with mMBP (or with gpMBP) did not result in a recall response to any of the mMBP peptides (Fig. 1 *A*); therefore, the MBP:79-87 peptide that was immunodominant in the *MBP*$^{-/-}$ mouse was non-stimulatory in the congenic, *MBP*$^{+/+}$ strain after the injection of the MBP protein.

The Functional MBP:79-87–specific T Cell Repertoire Is of High Avidity in MBP$^{-/-}$ *Mice and of Low Avidity in MBP* $^{+/+}$ *Mice*. Negative selection was shown to primarily affect T cell clones with high avidity and spare those with low avidity (24). To address this possibility, we tested the dose-response characteristics of the MBP:79-87 peptide-specific functional T cell repertoires in shiverer and C3H mice. Groups of mice from each strain were immunized with 50, 5, or 0.5 μg of the MBP:79-87 peptide, and their draining LN were tested in cytokine ELISA spot assays titrating the peptide's concentration in the recall culture (Fig. 2 *A*). Immunizing shiverer mice with 50 μg of the peptide primed a vigorous IFN-γ recall response that had a dose response curve that plateaued at 10^{-4} M of MBP:79-87 and induced IFN-γ production in 365 cells of the one million LN cells tested (cells producing IL-4 and IL-5 were not detected,

Table 1. *Immunogenicity of MBP in Shiverer vs. C3H Mice*

Immunization*		Antigen-Induced‡ IFN-γ spots§				
Mice	Antigen	Medium	mMBP	gpMBP	OVA	HEL
Shiverer	mMBP	<3	81 ± 7	181 ± 40	<3	<3
(*MBP*$^{-/-}$)	gpMBP	<3	83 ± 20	227 ± 95	<3	<3
	OVA	<3	<3	<3	31 ± 13	<3
	HEL	<3	<3	<3	<3	12 ± 1
C3H	mMBP	<3	<3	<3	<3	<3
(*MBP*$^{+/+}$)	gpMBP	<3	<3	23 ± 5	<3	<3
	OVA	<3	<3	<3	21 ± 4	<3
	HEL	<3	<3	<3	<3	11 ± 4

*50 μg/mouse in CFA, subcutaneously.
‡50 μg/ml
§ Number of spots/million draining lymph node cells tested on day 9 by IFN-γ ELISA spot (mean ± SE for three mice per group tested in two experiments). SD for triplicate wells for individual mice was <10%.

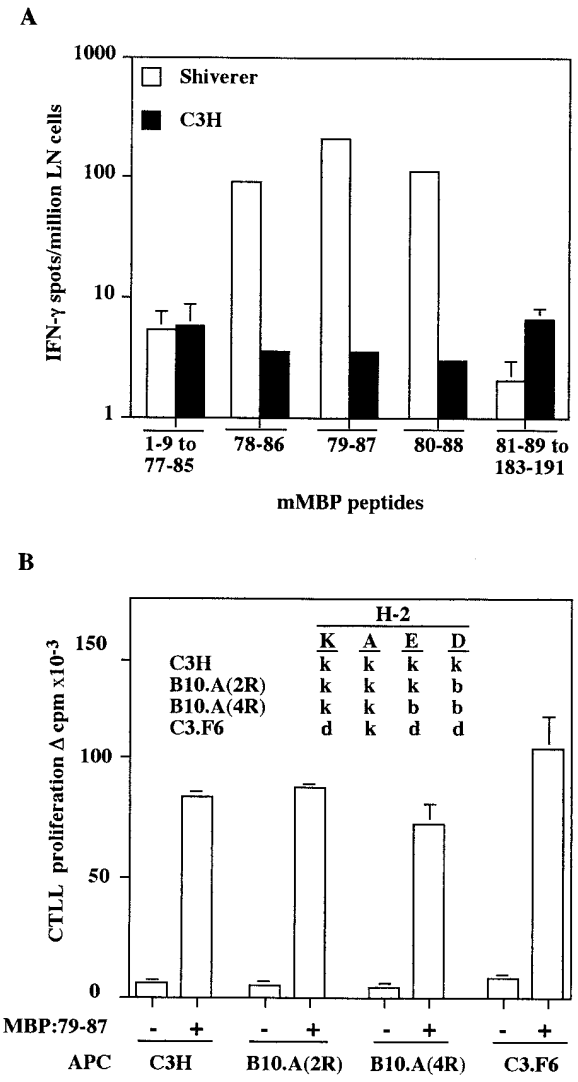

Figure 1. Mapping of the MBP response in shiverer mice. (*A*) Fine specificity of the response. Shiverer mice (*open bars*) and C3H mice (*filled bars*) were immunized with 50 μg mMBP in CFA, subcutaneously. 10 d later, the draining LN and spleen cells of three mice in each group were pooled and tested by IFN-γ ELISA spot assay using a nonamer peptide series that covered the entire murine MBP sequence. The results were read with an automated ELISA spot image analyzer. The medium background in this experiment was 6 ± 3 spots. Because no significant spot formation (SI < 2) was seen with peptides 1–9 to 69–77 or 89–97 to 183–191, these results are represented in a single bar. The results shown are from one experiment, which was reproduced four times. (*B*) MHC restriction of peptide 79–87. A shiverer mouse–derived 79-87–specific T cell hybridoma 2G9.4 was tested in the presence of the indicated MHC-disparate APC. The inserted table shows the MHC alleles expressed by these APC, which were either spleen cells from *H-2* recombinant mice or the *I-A*^{k} transfected cell line, C3F6. IL-2 production was measured by the CTLL assay.

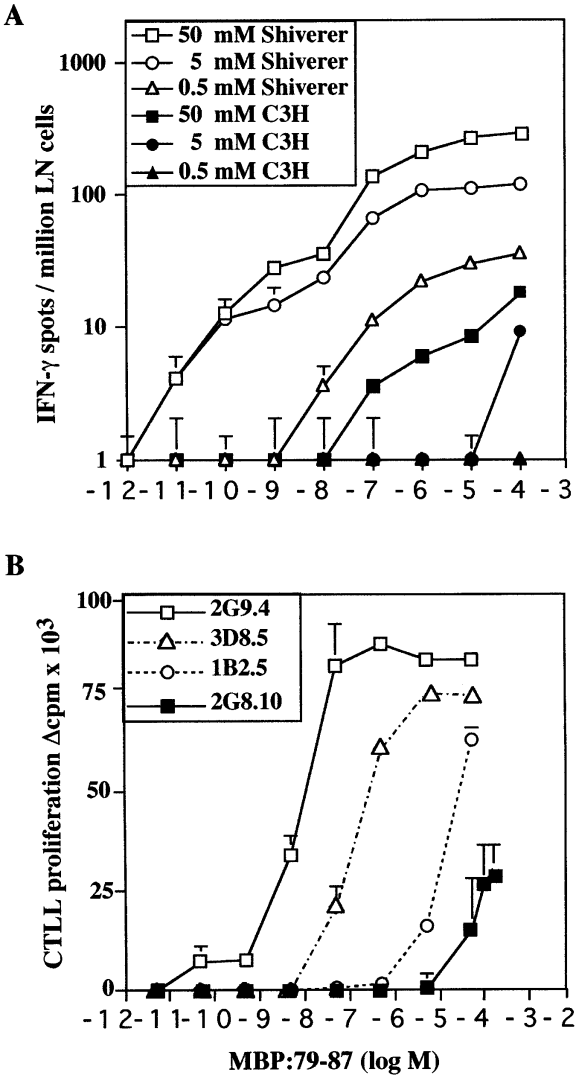

Figure 2. Functional avidity of the MBP:79-87–specific T cell repertoire induced by immunization with this peptide. (*A*) Dose–response curves of peptide-induced IFN-γ production in freshly isolated draining LN cells as measured by ELISA spot. Shiverer mice (*open symbols*) and C3H mice (*filled symbols*) were immunized with the dose of MBP:79-87 peptide indicated (in CFA, subcutaneously) and tested on day 9 for the frequency of IFN-γ–producing cells at various concentrations of the peptide. Data shown are mean ± SD of triplicate wells from individual mice. Where not visible, the SD where smaller than the size of the symbols. Unimmunized or HEL-immunized mice did not respond to this peptide by cytokine production or proliferation (data not shown). These data are fully representative of 16 individual mice of both strains tested in four independent experiments. (*B*) Response characteristics of MBP:79-87–specific T cell hybridomas from shiverer (*open symbols*) and C3H mice (*filled symbols*). Mice were immunized with MBP:79-87 as in *A*; day 9 LN cells were fused and cloned as described in Materials and Methods. Individual T cell hybrid clones with characteristic response patterns are shown as measured in CTLL proliferation assays using C3H spleen cells as APC.

data not shown). In C3H mice, on the contrary, the 50 μg immunization dose primed 19 IFN-γ–producing cells per million LN cells when tested at the same 10^{-4} M MBP:79-87 recall concentration, the highest that was not toxic to the cells. No cells producing IL-4 or IL-5 were detected. The dose–response curve in C3H mice did not approximate a plateau and, compared with that of shiverer mice, was shifted 4 Log units to the right. Immunizations with the 0.5 μg MBP:79-87 peptide dose still primed a vigorous response in shiverer mice but failed to induce a significant response in the C3H strain; the 5 μg immunizing dose gave intermediate results.

Conclusions about the functional avidity of the MBP:79-87 peptide-specific repertoires in shiverer and C3H mice can be drawn from the data in Fig. 2 *A* (along with the peptide dose required for immunization). T cell activation (including the induction of cytokine production) occurs when a critical number of MHC–peptide ligands is available for the TCR binding (25), a number that is dependent on the TCR's intrinsic affinity for this MHC–peptide complex (26) and that can be modulated by variations in TCR and/or coreceptor densities (27). Following the classic definition of avidity for polyvalent ligand-receptor interactions, functional T cell avidity can be defined as the concentration of peptide that leads to half the maximal activation of T cells in a given population for a constant number of APCs. According to this definition, the functional avidity of the T cell response in shiverer mice was at least three orders of magnitude greater than that in C3H mice. MBP:79-87–reactive T cells yielded identical dose response curves when activated by APCs derived from C3H and shiverer mice (data not shown); this shows that the differences in the responses do not reflect differences in the APC compartment but in the avidity of the T cells themselves. These data strongly argue for the functional inactivation of the high avidity MBP:79-87–reactive T cell repertoire in the MBP-bearing mice.

Using a third independent approach to assess and compare the avidity of MBP-reactive T cells in shiverer and C3H mice, we studied the dose response characteristics of MBP:79-87–reactive T cell hybridomas generated from both mouse strains. Fusing T cells from mice immunized with 50 μg MBP:79-87, we obtained 346 hybrid clones from shiverer mice and seven hybrid clones from C3H mice (a number that reflects the frequency of peptide-reactive T cells detected in the freshly isolated draining LN, Fig. 2 *A*). All seven T cell hybridomas from C3H mice required concentrations of MBP:79-87 in excess of 10^{-5} M to be activated (Fig. 2 *B*). In contrast, the 25 shiverer mouse T cell hybridomas that we randomly selected for closer characterization fell into three categories. Nine of these clones were triggered by peptide concentrations as low as 10^{-10} M, as exemplified in Fig 2 *B* by clone 2G9.4. Seven clones responded only to peptide concentrations in excess of 10^{-6} M (exemplified in Fig. 2 *B* by clone 1B2.5). The third type of clone (9 of 25) showed intermediate results similar to those seen with the clone 3D8.5 shown in Fig. 2 *B*. According to Tonegawa's algorithm (26), which stipulates that the peptide concentrations at which T cell activation occurs is inversely proportional to the hybridoma's TCR affinity, the differences in the concentrations required to activate the clones reflect differences in the affinity of the clones, while the plateau, because of the limitations in this assay, reflects the maximum CTLL proliferation rather than the maximum production of IL-2. Importantly, the T cell hybrids from all three categories and from both mouse strains responded comparably to anti-CD3 stimulation. Because of the similarity of these responses to antibody-mediated TCR ligation, and because these cells were grown under the same conditions, in long-

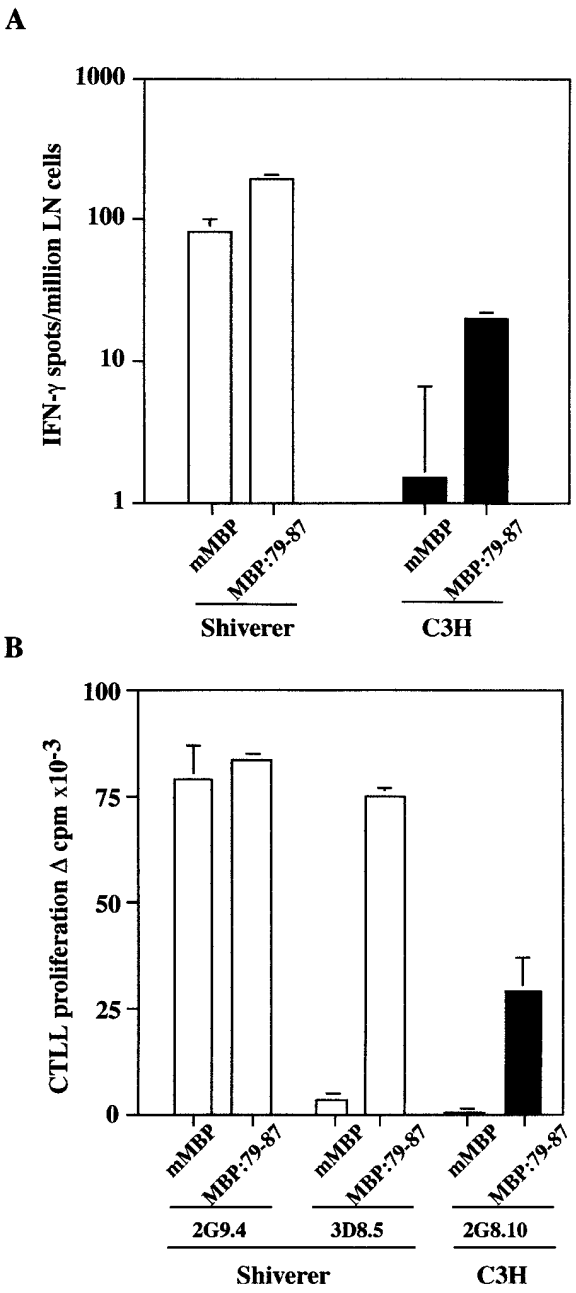

Figure 3. Recognition of naturally processed MBP and the MBP:79-87 peptide after immunization with this peptide. (*A*) The IFN-γ–recall response in primary draining LN cells measured by ELISA spot. Shiverer or C3H mice were immunized with 50 μg MBP:79-87 and tested on day 9 for reactivity to the peptide (5×10^{-5} M) and to MBP (50 μg/ml). The data show responses of individual mice and are fully representative of 20 mice tested in five experiments. (*B*) The IL-2 response of mMBP:79-87–reactive T cell hybridomas. Hybridoma clones generated from MBP:79-87–immunized shiverer or C3H mice (see legend to Fig. 2 *A* and the text) were tested in a standard CTLL assay for their response to 5×10^{-5} M of peptide 79–87 and 50 μg/ml mMBP protein. Two response patterns were seen in shiverer mice, represented by clones 2G9.4 and 3D8.5, respectively. All seven C3H-derived clones displayed the response pattern of the 2G8.10 clone shown. The same results were obtained in *A* and *B* when the MBP concentration was increased by as much as 10-fold, to 500 μg/ml (data not shown).

term culture and in the absence of MBP, which could have affected their function, the differences in their MBP:79-87 response characteristics could not have resulted from differences in the functional states of the cells, but must reflect differences in TCR-affinities for this peptide. The fact that the repertoire of T cell hybridomas obtained from shiverer mice consists of clones with high, intermediate, and low avidities is consistent with the notion that this repertoire has not undergone negative selection. In contrast, the fact that only low avidity T cell hybrids were obtained from C3H mice points towards a repertoire that has been depleted of precursor cells that have high and intermediate avidity for MBP:79-87.

MBP:79-87 Is a Cryptic Determinant in the MBP$^{+/+}$ Mice. We tested freshly isolated MBP:79-87 peptide-primed T cells and the T cell hybridomas obtained after immunization with this peptide to see if they could be stimulated by the native MBP protein. In shiverer mice, the peptide-primed primary cells gave a vigorous recall response when cultured with MBP the magnitude of which was comparable to the response recalled by the peptide itself (Fig. 3 *A*). Similarly, 12 of the 25 T cell hybridomas obtained from these mice responded to the protein as well as the peptide, as exemplified in Fig. 3 *B* by clone 2G9.4. The remaining thirteen shiverer-derived clones were stimulated only by the peptide, as was the clone 3D8.5 in this figure. In C3H mice, the MBP:79-87 peptide-induced response was not recalled by mMBP when freshly isolated cells were tested (Fig. 3 *A*) and none of the seven hybridomas obtained from such immunization was stimulated by the native antigen (Fig. 3 *B*). Because the MBP:79-87–specific repertoire in shiverer mice consisted of clones that recognized type-A and type-B conformations (28) of the MBP:79-87 determinant, these data also suggest that the repertoire of these mice had not been negatively selected. (The type-B conformation is assumed only when the peptide directly binds to MHC molecules on the cell surface; type-A conformation is assumed after natural processing of protein.) In contrast, the MBP:79-87–reactive repertoire in C3H mice was heavily biased towards the recognition of the type B determinant.

Discussion

The extent to which homologous self peptides contribute to the positive selection of T cells has been controversial. Early experiments suggested that the selecting peptide for a given T cell might need to be related in sequence to the antigenic peptide (29–31). Recently, it was demonstrated that a CD4$^+$ T cell repertoire selected in the thymus for a single peptide could react with an array of different peptides (32). Similar observations were made in mice whose thymic peptide diversity was extremely limited by the prevalence of the CLIP peptide (33), which also suggests that the peptide that triggers an immune response can be of a sequence unrelated to that of the peptide(s) that select(s) the clone. Our data strongly support the latter notion. The MBP-deficient shiverer mice responded vigorously to MBP (Table 1), showing that positive selection by endogenous MBP is not required to generate an MBP-specific repertoire. On the contrary, with all shiverer mice tested, the response to MBP involved a three- to eightfold greater clonal size than did the response to the "foreign" antigens, HEL and OVA (Table 1) to both of which C3H mice are high responders. It would seem that, if endogenous lysozyme or albumin were to contribute to the shaping of responses specific for HEL and OVA, it would be by negative rather than positive selection of the preimmune repertoires.

Although antigens like HEL and MBP include several sequences that can bind a given class II allelic product, frequently only one of these potential determinants actually induces a response after immunization with the protein (16). This immunodominance of a single determinant is a poorly understood feature of T cell responses. It could be the result of unique aspects of antigen presentation, e.g., MHC-guided processing (34–36), or it could be the result of repertoire limitations imposed by the homologous autoantigen. For example, sequences shared by the foreign (e.g., HEL) and self (e.g., mouse lysozyme) antigens could contain several immunodominant determinants, but because these determinants would negatively select the repertoire, only a fraction of the potential determinants, those that are species-foreign, would be available to elicit a response. The fact that *MBP*$^{-/-}$ shiverer mice recognize a single determinant (Fig. 1 *A*) strongly argues against negative selection of the repertoire being a major defining factor for the single-determinant dominance of this response.

On the other hand, the fact that the immunodominant determinant in the *MBP*$^{-/-}$ mice behaved as a cryptic determinant in the *MBP*$^{+/+}$ mice suggests that the repertoire selected by the endogenous antigens contributes to the defining of the determinant hierarchy. Unlike the situation with foreign antigens, where peptides with the highest affinity for MHC are likely to be the most immunogenic (17), with self antigens, such peptides are prone to induce tolerance most efficiently (18, 19), thus making them appear cryptic.

Our data suggest that the determinant that is actually immunodominant on MBP induces profound clonal inactivation in mice that express MBP. Although immunization with peptide MBP:79-87 triggered a response in C3H mice, the clones induced were of low avidity (Fig. 2) and recognized only the peptide (Fig. 3). Apparently, the vast majority of high avidity cells capable of recognizing the endogenous protein did encounter it before the immunization and the outcome was functional inactivation. This tolerance might have resulted from clonal deletion or from the induction of an anergic state, or both, possibly affecting T cells with different avidities for MBP differently. TCR-transgenic models using TCRs with different avidities for MBP might be required to clearly distinguish between these possibilities. Because MBP messenger RNA is expressed in the thymus (1–3) and because it has recently been demonstrated that there is sufficient antigen presentation of MBP in this organ to stimulate MBP-reactive T cell

hybridomas (4), the development of central tolerance may account for the clonal inactivation that we observed. Therefore, MBP does not appear to be a sequestered antigen, as once thought, but one that causes negative selection of the high avidity repertoire (24).

The TCR transgenic approach to studies of self tolerance to MBP suggested that MBP is ignored by MBP-specific T cells (5–7). Although ignorance was also seen in several other TCR transgenic models in which neo-self antigens were expressed in peripheral tissues (37–42), we propose that this mechanism applies only for low avidity MBP-reactive T cells. If the peripheral repertoire in MBP-expressing hosts consists of T cells that have escaped negative selection due to their low avidity for MBP (T cell clones of which were used to construct TCR transgenic mice), the transgenic T cells are likely to be ignorant of the autoantigen as well.

MBP:79-87 qualifies as cryptic determinant in C3H mice because it does not recall a response after the immunization with native MBP (Fig. 1 *A*) but is immunogenic when injected as a peptide (Figs. 2 *A* and 3 *A*). Because all seven hybrids that we obtained from C3H mice recognized the MBP:79-87 peptide, but not the native protein, the MBP:79-87–reactive T cell repertoire in these mice seemed to be biased towards recognition of the type B determinant (28). It is unclear whether such T cells are capable of mediating autoimmune pathology. For type B determinants to constitute a target in the CNS there would have to be extracellular peptide available for direct cell surface binding to APC, and, because of the apparent low avidity of these T cells, the concentration of endogenous free peptide would need to be high.

We found C3H mice to be resistant to MBP-induced and MBP:79-87–induced EAE when using protocols that cause severe disease in EAE-susceptible mouse strains. Even grafting lymphoid cells from naive shiverer mice into C3H mice did not render C3H mice susceptible to the induction of active disease. Neither could we induce passive disease by injecting MBP:79-87–primed T cells from shiverer into C3H mice, nor with primary cells or T cell lines. Although this outcome suggests that the shiverer/C3H model might not qualify for studies of EAE, it sends a novel and surprising message about disease susceptibility. Whereas the primary focus of research addressing susceptibility to T cell–mediated autoimmune disease has been on the ability to generate a vigorous type-1 response to the autoantigen (MHC-linked Ir gene effects, T cell repertoires, type-1/type-2 biases), these data suggest that even the most vigorous, unipolarly type 1-biased T cell responses to MBP do not cause clinical disease if they occur in a host that lacks the genetic susceptibility to develop downstream inflammatory pathology. In such mice, in addition to central tolerance, powerful peripheral tolerance mechanisms seem to prevent T cell–mediated immunopathology of the central nervous system.

Because of the EAE-resistance of C3H mice, we could not address the issue of whether the low avidity T cells that escape negative selection can be pathogenic. Previous studies performed in the H-2u model might provide clues to this question showing that peptide, MBP:121-140, a cryptic determinant in $MBP^{+/+}$ $H\text{-}2^{u}$ mice, is codominant with MBP:Ac1-11 in the congenic $MBP^{-/-}$ mice (43). These data can be interpreted as reflecting similar avidity-repertoire selections, as we do here. If this were the case, the fact that MBP:121-140 is encephalitogenic in $H\text{-}2^{u}$ mice (15) might be seen as evidence that negative selection for a dominant determinant is incomplete and can cause autoimmune pathology.

The inactivation of T cells that are specific for the dominant MBP determinant is likely to occur in EAE-susceptible mice too, but even if it were as profound in those strains as we observed it to be in C3H mice, for theoretical reasons it is unlikely to be complete. A central question in the understanding of autoimmune disease will be how autoreactive T cells that escaped negative selection and have the autoantigen presented to them at a subthreshold level can be induced to mediate autoimmune pathology. Like T cell activation itself, negative selection is threshold-dependent (24), that is, T cells will be deleted only if their TCRs bind a critical number of MHC–peptide ligands, a number defined by the affinity of the TCR for the peptide (26). The MBP-specific clones that escape negative selection will, therefore, fall into two broad categories: first, T cells with receptors having a low affinity for an abundantly presented peptide (T cell crypticity); second, T cells that have high, low and intermediate avidities for peptides that are presented in low copy numbers on the APC (determinant crypticity). Because the autoantigen is presented at subthreshold levels to both types of clones, they are ignorant of it. This activation threshold seems to be lowered after the naive cell is primed to a memory/effector state. Studies of TCR-transgenic cells showed that the activation of naive T cells requires peptide concentrations up to 50 times higher than those needed by primed/memory T cells (44). If the activation threshold of T cells, however, is not constant for an individual clone but varies as a function of its state of activation, environmental priming by cross-reactive antigens, or by immunization in adjuvant could make the number of endogenous MHC/peptide ligands that was substimulatory to the naive cell stimulatory to the memory cell. The autoantigen that was ignored by the naive cells will be attacked by the memory cells.

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