Cutting Edge: Myelin Basic Protein-Specific Cytotoxic T Cell Tolerance Is Maintained In Vivo by a Single Dominant Epitope in H-2K Mice

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Multiple sclerosis (MS) is believed to be an autoimmune disease mediated by T cells specific for CNS Ags. MS lesions contain both CD4+ and CD8+ T lymphocytes. The contribution of CD4+ T cells to CNS autoimmune disease has been extensively studied in an animal model of MS, experimental autoimmune encephalomyelitis. However, little is known about the role of autoreactive CD8+ cytotoxic T cells in MS or experimental autoimmune encephalomyelitis. We demonstrate here that myelin basic protein (MBP) is processed in vivo by the MHC class I pathway leading to a MBP79–87/Kk complex. The recognition of this complex by MBP-specific cytotoxic T cells leads to a high degree of tolerance in vivo. This study is the first to show that the pool of self-reactive lymphocytes specific for MBP contain MHC class I-restricted T cells whose response is regulated in vivo by the induction of tolerance. The Journal of Immunology, 1999, 163: 1115–1118.

The ability to activate autoreactive T cells in the periphery of healthy animals in models of autoimmune disease demonstrates that central and peripheral tolerance is incomplete. This is illustrated in experimental autoimmune encephalomyelitis (EAE),3 an animal model of multiple sclerosis (MS) (1) that is induced by immunization with myelin basic protein (MBP) or by adoptive transfer of activated, MBP-specific T cells into naive recipients (2–5). Although MS lesions contain both CD4+ and CD8+ T lymphocytes (6), studies of EAE focus on the role of autoreactive CD8+ cytotoxic T cells in the development and manifestation of EAE.

Previous studies suggested that CD8+ T cells might participate as effector or regulatory cells in EAE (7–10). The existence of MHC class I-restricted T cells specific for naturally processed MBP in vivo has not been demonstrated, although human CD8+ T cells specific for a peptide of MBP have been isolated in vitro (11). These issues motivated us to study the MHC class I-restricted immune response to MBP and examine the potential role of endogenous MBP in shaping the CTL repertoire specific for this Ag.

Materials and Methods

Mice

C3HeB/FeJ and C3HeB/FeJ-MBPshic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MBPshic and MBPshib (MBPshic) mice (12–14) were identified by PCR (15) and whole body tremor.

Construction of a recombinant adenovirus expressing MBP

An E1 inserted, Ad5 recombinant adenovirus expressing MBP (Ad/MBP) was generated by inserting the plasmid pXCL.1 containing an MBP cDNA (16) into an E1-deficient Ad5 adenovirus pM17 (17, 18). In addition, a L929 cell line expressing MBP (L/MBP) was generated using the expression vector pCDNA3.1 (Invitrogen, Carlsbad, CA) containing the MBP cDNA (16). Expression of MBP mRNA was detected in cells infected with Ad/MBP and L/MBP cells by RT-PCR (19). A recombinant vaccinia virus expressing MBP (Vac/MBP) (20), was obtained from The rion Biologics (Cambridge, MA).

Synthetic peptides

Peptides were synthesized using TBOC chemistry on a model 430A peptide synthesizer (Applied Biosystems, Foster City, CA). The peptides were purified by reverse-phase HPLC, and all peptides were analyzed for purity by mass spectrometry.

Infection of mice and generation of MBP-specific T cell lines and clones

Mice were infected with 10⁷ pfu Ad/MBP virus i.p. or 10⁶ pfu Vac/MBP i.v. After 3 wk infection, immunized mice were harvested and 3 × 10⁷ splenocytes were stimulated in vitro in 10-ml cultures with 1 × 10⁶ irradiated target cells in RPMI 1640 media supplemented with 10% FCS. All procedures have been approved by the animal care committee at the University of Washington.

31Cr release assays

Target cells were infected with virus at a multiplicity of infection of 10 and incubated for 72 h for adenovirus and 12 h for vaccinia virus in growth media before labeling with chromium. Target cells were then incubated with 100 μCi of [51Cr]O₄⁻ (Amersham, Arlington Heights, IL) for 60 min, washed, and incubated with effector cells in a standard 4-h 51Cr release assay. The percent lysis was calculated as (51Cr release in the presence of
MBP-specific CTL responses are generated in MBP<sup>−/−</sup> but not MBP<sup>+/+</sup> mice. C3H MBP<sup>−/−</sup> (A) and C3H MBP<sup>+/+</sup> (B) mice were i.v. infected with 10<sup>6</sup> pfu Vac/MBP. Spleens were removed after 3 wk and stimulated in vitro with irradiated L cells infected with Vac/MBP. B gal, β galactosidase.

**Results and Discussion**

**MBP-specific cytotoxic T cells are generated in vivo**

C3H MBP<sup>−/−</sup> and MBP<sup>+/+</sup> mice were infected i.p. with Ad/MBP. Splenocytes from infected mice were harvested and stimulated in vitro for 5 days with L/MBP. The stimulated splenocytes were then tested for their ability to lyse syngeneic target cells expressing MBP. From 14 of 16 MBP<sup>−/−</sup> mice, MBP-specific killing was observed. In contrast, no MBP-specific killing by T cells from wild-type mice was observed (0 of 15 mice; data not shown).

Because adenovirus has a restricted tropism, a second protocol was used to assess MBP-specific cytotoxic T cell responses. Splenocytes from mice infected with Vac/MBP were stimulated in vitro with irradiated L cells infected with Vac/MBP. From all MBP<sup>−/−</sup> and MBP<sup>+/+</sup> mice, a potent vaccinia-specific T cell response was generated. In contrast, an MBP-specific cytotoxic T cell response was generated only in MBP<sup>−/−</sup> mice (13 of 13) but not in MBP<sup>+/+</sup> mice (0 of 15) (Fig. 1, A and B).

**MBP<sub>79-87</sub> (DENPVVHFF) is the core MBP-specific CTL epitope**

Twenty-one MBP-specific T cell clones were established from three MBP<sup>−/−</sup> mice after one in vitro stimulation by limiting dilution cloning. All clones were of the αβ TCR<sup>+</sup>, CD8<sup>+</sup> lineage (data not shown). The fine specificity of three MBP-specific T cell clones was determined using target cells pulsed with a panel of overlapping peptides comprising the entire MBP protein. All three clones specifically lysed target cells pulsed with MBP<sub>68-91</sub> but no other peptides (Fig. 2A). The remaining 18 T cell clones were tested and specifically lysed target cells expressing this peptide (data not shown).

To assure that the exclusive specificity of the T cell clones for MBP<sub>68-91</sub> was representative of all CTLs specific for MBP, T cell clones derived from MBP<sup>−/−</sup> mice after one in vitro stimulation were tested for their ability to lyse target cells coated with the panel of overlapping MBP peptides. Only T cells derived from MBP<sup>−/−</sup> mice were able to specifically lyse MBP-expressing targets. The MBP-specific response was directed solely at targets coated with MBP<sub>68-91</sub>, indicating that the dominant MBP epitope is contained in this region. (Fig. 2B).

To identify the core 9-mer epitope that is targeted by MBP-specific CTLs, we first evaluated the sequence within MBP<sub>68-91</sub> for an H-2<sup>k</sup> class I binding motif (21). The overlapping 9-mer peptides MBP<sub>78-86</sub>, MBP<sub>79-87</sub>, and MBP<sub>80-88</sub> were tested. All MBP-specific CTL clones recognized target cells coated with the MBP<sub>79-87</sub> peptide epitope in a dose-dependant manner (Fig. 3, and data not shown). These results indicate that MBP<sub>79-87</sub> represents the naturally processed MBP-specific CTL epitope in C3H mice.

**MBP-specific CTLs are restricted by the MHC class I allele H-2<sup>k</sup>**

To identify the H-2<sup>k</sup> allele that presents MBP<sub>79-87</sub> to CD8<sup>+</sup> cytotoxic T cells, Con A blasts from the B10 H-2<sup>k</sup> congenic strains
B10.A(4R), B10.MBR, and C3H were used as target cells. The results suggested that the MBP<sub>79–87</sub> peptide is presented by the MHC class I allele K<sup>b</sup> (data not shown). To confirm this result, we tested the ability of RMA-S cells and RMA-S cells transfected with K<sup>b</sup> to present the MBP epitope. RMA-S-K<sup>b</sup> cells were able to present the MBP epitope, while untransfected RMA-S cells were not (Fig. 4).

**MBP-specific T cells are tolerized in the periphery of wild-type mice**

The results described above demonstrate that MBP-specific, MHC class I-restricted cytotoxic T cells are present in the periphery of MBP-deficient mice and that these T cells undergo tolerance in wild-type mice that express endogenous MBP. These observations raised the question of where tolerance to MHC class I epitopes of MBP occurs in vivo. To address this issue, we used two independent protocols to assess whether peripheral mechanisms are responsible for tolerance of MBP-specific CTLs. Results are shown in Table I. In the first experiment, MBP-specific T cells (group 1) and vaccinia-specific T cells (group 2) were transferred into SCID mice to test for retention of Ag-specific responses. <sup>51</sup>Cr release assays were performed with the T cell lines just before transfer to confirm their CTL activity and the recipient SCID mice were bled 1 wk after transfer to assure survival of T cells after transfer (data not shown). After 4 wk, spleens from recipient mice were harvested and tested in CTL assays. Vaccinia-specific T cells were easily detected in recipients of vaccinia-specific T cells. In contrast, MBP-specific CTL activity was not detected in any recipients of MBP-specific CTLs. In a separate approach, we asked whether naive peripheral T cells from MBP<sup>−/−</sup> mice that have not been exposed to MBP would undergo tolerance when transferred into the periphery of MBP<sup>+/+</sup> mice. SCID mice were reconstituted with naive lymphocytes isolated from MBP<sup>−/−</sup> (group 3) and MBP<sup>+/+</sup> (group 4) mice. The mice were bled 1 wk after transfer to assure that the lymphocytes had reconstituted all mice equally. Four weeks after reconstitution, mice were infected with either Ad/MBP or Vac/MBP. Potent vaccinia-specific responses were generated in mice that received either MBP<sup>−/−</sup> or MBP<sup>+/+</sup> lympocyes when Vac/MBP was used both as the immunogen and to restimulate the T cells in vitro. We attempted to generate MBP-specific CTL responses by immunizing the mice with either Ad/MBP or Vac/MBP. However, no responses were detected in SCID mice reconstituted with lymphocytes from either MBP<sup>−/−</sup> or MBP<sup>+/+</sup>. Control MBP<sup>−/−</sup> mice (group 5) but not MBP<sup>+/+</sup> mice (group 6) infected and restimulated using these protocols at the

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**Table I. MBP-specific T cells are tolerized in the periphery of wild-type mice**

<table>
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<tr>
<th>Group</th>
<th>Mouse</th>
<th>Transferred Cells</th>
<th>Immun.</th>
<th>Restim.</th>
<th>% Specific Lysis</th>
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<tr>
<td>1</td>
<td>SCID</td>
<td>MBP T cell line&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Vac/MBP</td>
<td>8,2</td>
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<td>2</td>
<td>SCID</td>
<td>MBP T cell line&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/A</td>
<td>Vac/MBP</td>
<td>1,3</td>
</tr>
<tr>
<td>3</td>
<td>SCID</td>
<td>Vac T cell line&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N/A</td>
<td>Vac/MBP</td>
<td>4,6</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>SCID</td>
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<td>MBP&lt;sub&gt;79–87&lt;/sub&gt;</td>
<td>2,2</td>
</tr>
</tbody>
</table>

<sup>a</sup>T cells were used at an E:T ratio of 50:1 and 12:1. This experiment was repeated twice with similar results.

<sup>b</sup>A total of 3 x 10<sup>7</sup> MBP or vaccinia-specific primary T cells were transferred i.v. after one in vitro stimulation.

<sup>c</sup>A total of 2 x 10<sup>8</sup> spleen and lymph node cells from nonmanipulated MBP<sup>−/−</sup> or MBP<sup>+/+</sup> mice were transferred i.v.
same time as the recipient mice generated MBP-specific CTL responses. Therefore, although these data do not exclude a role for central tolerance mechanisms, they indicate that peripheral mechanisms eliminate functional MBP-specific CTL responses from mice expressing endogenous MBP.

Tolerance in MBP-specific CTLs could be maintained in wild-type mice by encountering the MBP<sub>79–87</sub> epitope in the periphery. The MBP gene locus is complex and contains at least two additional promoters 5′ of the classical promoter, which transcribe a unique family of gene products termed golli-MBP (19, 22, 23). These genes are encoded by a combination of exons from classical MBP as well as exons 5′ of classical MBP. Transcripts and protein expression of the golli-MBP isoforms, which contain the MBP<sub>79–87</sub> epitope, have been found outside of the nervous system in both lymphoid tissue and in major organs (19, 22–24).

Recently, it has been demonstrated that some MHC class II-restricted T cells specific for MBP are also efficiently tolerized in wild-type mice (15, 25). The tolerogenic CD4<sup>+</sup> T cells specific for MBP are also efficiently tolerized in both lymphoid tissue and in major organs (19, 22–24).

The studies reported here describe a model system in which MHC class I-restricted T cells specific for MBP can be generated and analyzed for their contribution to autoimmune disease. The identification of naturally occurring MHC class I-restricted epitopes allows monitoring of MBP-specific CTL responses during the course of disease. Therefore, the ability of MBP-specific CTLs to be activated by (or contribute to) determinant spreading can be investigated (26–28). This model system provides a novel approach to define the role of CNS Ag-specific CD8<sup>+</sup> CTLs in the pathogenesis of autoimmune disease.

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References


