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Cross-Reactivity of Myelin Basic Protein-Specific T Cells with Multiple Microbial Peptides: Experimental Autoimmune Encephalomyelitis Induction in TCR Transgenic Mice

Jane L. Grogan,* Achim Kramer, † Axel Nogai,* Liying Dong, † Manuela Ohde,* Jens Schneider-Mergener,‡ and Thomas Kamradt*‡

Activation of autoreactive T cells is a crucial event in the pathogenesis of autoimmune diseases. Cross-reactivity between microbial and self Ags (molecular mimicry) is one hypothesis that could explain the activation of autoreactive T cells. We have systematically examined this hypothesis in experimental autoimmune encephalomyelitis using mice bearing exclusively myelin basic protein (MBP)-specific T cells (designated T* α†). A peptide substitution analysis was performed in which each residue of the MBP Acl-11 peptide was exchanged by all 20 naturally occurring amino acids. This allowed the definition of the motif (supertope) that is recognized by the MBP Acl-11-specific T cells. The supertope was used to screen protein databases (SwissProt and TREMBL). By the search, 832 peptides of microbial origin were identified and synthesized. Of these, 61 peptides induced proliferation of the MBP Acl-11-specific transgenic T cells in vitro. Thus, the definition of a supertope by global amino acid substitution can identify multiple microbial mimic peptides that activate an encephalitogenic TCR. Peptides with only two native MBP-residues were sufficient to activate MBP Acl-11-specific T cells in vitro, and experimental autoimmune encephalomyelitis could be induced by immunizing mice with a mimic peptide with only four native MBP residues. The Journal of Immunology, 1999, 163: 3764–3770.

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* Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; pep, peptide; PT, pertussis toxin; SI, stimulation index.
for murine MBP-specific T cells was used: after careful definition of MHC and TCR contact residues within the immunodominant MBP epitope, database searches were performed that were based on these structural characteristics, allowing nonhomologous amino acids at the “non-contact-residues.” A number of microbial and viral peptides fulfilling the search criteria were identified, and some of these peptides induced EAE in mice (7, 24). However, this “knowledge-based” approach requires laborious analysis of the contact residues of an individual epitope with MHC and TCR. Therefore, we wished to examine an alternative approach to identifying microbial ligands for autoreactive TCRs. In earlier work, we had used the spot-synthesis technique for peptides (36, 37) to identify multiple ligands for mAbs (38). Here, we have used peptide spot synthesis for global amino acid replacements of the MBPAc1–11 epitope, which is immunodominant in mice of the H2d haplotype. We identified 61 microbial mimic peptides that activated MBPAc1–11-specific T cells. Several of these peptides induced EAE in mice that are transgenic for a MBPAc1–11-specific TCR (11).

Materials and Methods

Mice

Mice transgenic for a TCR that recognizes MBPAc1–11, bound to I-Au (11) were crossed onto TCR α-chain knockout mice (39), resulting in mice carrying only αβ T cells specific for MBPAc1–11 (“Tα”) (40, 41), and were obtained from Dr. Juan Lafaille (SKirball Institute, New York, NY). Mice were bred at our animal facility in specific pathogen-free conditions and checked for TCR expression by flow cytometry with anti-Vβ8-PE (MR5–2, PharMingen, San Diego, CA) and anti-CD4-FITC Abs (GK1.5). All animal experiments were performed according to institutional and state guidelines.

Peptides

Cellulose-bound peptides were prepared by automated spot synthesis (Abimed, Langenfeld, Germany; Software DIGEN, Jerini Biotools, Berlin, Germany) with the use of Whatman No. 50 cellulose membranes (Whatman, Maidstone, U.K.) as described before (36, 37). Peptides were N-terminally acetylated using acetylation and diisopropylethylamine. For synthetic reasons, the peptides contained an additional C-terminal glycine residue. Peptides were cleaved from the solid support by treating the cellulose with ammonia vapor for 5 h. Each spot was eluted in 200 μl double-distilled H2O resulting in an approximately 150–200 μM peptide solution. For titration experiments and in vivo analysis, peptides were conventionally synthesized according to standard Fmoc machine protocols with a multiple peptide synthesizer (Abimed). The following peptides were synthesized given in single letter code, with Ac denoting N-terminal acetylation: MBPAc1–11 (AcASQKRPSQRSK); pep200 (AcANMRQAPVTL; Escherichia coli, Salmonella typhimurium, Haemophilus influenzae, Buchnera aphidicola); pep378 (AcAASMNRPNVLAY; Mycobacterium tuberculosis); pep383 (AcAASMRSVPQVKL; E. coli, S. typhimurium); and pep387 (AcASQARQLADS; E. coli). Purity of the peptide was determined by HPLC and composition monitored by MALDI-TOF mass spectroscopy.

In vitro Tα+ spleen cell assays

Single-cell suspensions were prepared from spleens in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (complete RPMI, Sigma, St. Louis, MO) as described (14). For proliferation, cells were cultured in 96-well plates at 1 × 10^6/ml with 5 μl peptide spots (0.5–1.5 μM), with conventionally synthesized peptides at concentrations indicated, or with complete RPMI alone, at 37°C in 5% CO2. Proliferation was measured by an 18-h incorporation of 1 μCi [3H]thymidine on day 3. Stimulation indices (SI) were determined as cpm of peptides divided by cpm of cells cultured with medium alone (range for background values 1400–2100 cpm for the various experiments). SIs ≥15 were considered positive. For cytokine determination, cells were cultured in complete medium at 5 × 10^6/ml with 5 μl peptide spots, or at 1 × 10^6/ml for dose-response analysis with conventionally synthesized peptides as indicated. Supernatants were collected at 48 h for analysis by sandwich ELISA. IFN-γ, TNF-α, and TGF-β were determined with commercially available kits according to manufacturer’s instructions (Genzyme Diagnostics, Cambridge, MA). IL-4, IL-5, IL-10, and IL-2 were determined as described (42). The lower detection limit for each ELISA was as follows: IFN-γ, TNF-α, and IL-4, 50 pg/ml; IL-2 and TGF-β, 0.1 ng/ml; IL-5, 10 U/ml; and IL-10, 0.3 ng/ml.

Induction of EAE

Mice were injected s.c. at 2 sites at the base of the tail with 200 μg MBPAc1–11 or mimic peptides emulsified in CFA in a total volume of 0.2 ml. PT (200 ng; Life Technologies, Gaithersburg, MD) was injected i.v. 24 and 48 h after immunization. Age- and sex-matched control mice received PBS or CFA plus PT. Mice were examined every 1–2 days for clinical signs of EAE which was scored as follows: level 0, healthy; level 1, limp tail; level 2, partial hind leg paralysis; level 3, complete hind leg paralysis; level 4, front leg weakness; level 5, moribund. Data are represented as mean EAE of each group. Animals were sacrificed when their score reached 4–5, and their score was kept at 5 for the remainder of the experiment.

Results

Supertope definition by substitution analysis of MBPAc1–11 and identification of microbial peptides containing the MBP supertope

Peptides prepared by spot synthesis (37) were used for a substitution analysis of MBPAc1–11 in which each position of the peptide was substituted with all 20 naturally occurring amino acids. The resulting 220 peptides and synthesized spots of MBPAc1–11 were tested for induction of proliferation of Tα+ spleen cells in vitro.
 Identification of microbial peptides that activate MBP Ac1–11–specific T cells

The 832 peptides containing the supertope were prepared by spot synthesis and assayed for the induction of proliferation in T$^\dagger$α$^+$ cells in vitro. Of the 832 microbial peptides, 61 induced proliferation of the T$^\dagger$α$^+$ cells (SI ≥ 50; SI for MBP Ac1–11 = 80). The microbial mimic sequences, listed in decreasing order of SI, and the organism(s) containing the corresponding protein are shown in Table I. The mimic peptides had anywhere from 5 to 9 amino acid substitutions compared with MBP Ac1–11. There was no statistically significant correlation between the number of conserved amino acids and the SI (correlation coefficient, 0.08; p > 0.05). Therefore, other factors such as the topology of the peptide-MHC complex must determine the antigenic strength of the individual mimic peptides. Six peptides sharing only 2 amino acids with the original MBP Ac1–11 sequence activated the T$^\dagger$α$^+$ T cells (SI ≥ 50). Of the 61 mimics 52 had both R5 and P6 conserved and positions 8–11 could be replaced by any of the naturally occurring amino acids (Fig. 1B). The supertope was used to screen the SwissProt and TREMBL databases (software ExPasy) (43), and 832 peptides of microbial origin were identified that contained the supertope.

<table>
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<tr>
<th>Peptide</th>
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<th>Organism</th>
<th>Peptide</th>
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* The Swissprot and TREMBL databases were searched for microbial peptides containing the supertope depicted in Fig. 1. Sixty-one of the peptides identified induced proliferation in T$^\dagger$α$^+$ cells with SIs ≥ 50.

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**Table I. Microbial peptides that induce proliferation (SI ≥ 50) in T$^\dagger$α$^+$ cells**

(Fig. 1A). SIs ≥15 were considered positive. The substitutional analysis identified the amino acid substitutions tolerated at each position of the peptide. This revealed the binding motif (supertope) and thus the structural requirements for T cell recognition for the transgenic TCR (Fig. 1B). Arginine at position 5 (R5) could not be substituted with any other amino acid. At each of the other positions of the peptide, at least one substitution was tolerated (Fig. 1A). Alanine at peptide position 1 (A1) could be substituted only by serine (A1S) and P6 could be replaced only by glutamine. S2, Q3, and S7 could each be replaced by several other amino acids, and positions 8–11 could be taken by any of the naturally occurring amino acids (Fig. 1B). The supertope was used to screen the SwissProt and TREMBL databases (software ExPasy) (43), and 832 peptides of microbial origin were identified that contained the supertope.

**Dose-dependent activation of MBP Ac1–11–specific T cells by microbial peptides**

Next, we compared the dose requirements for the activation of MBP Ac1–11–specific T cells by microbial mimic peptides. Four peptides were selected for further analysis. On the basis of the results obtained with the peptides prepared by spot synthesis, we chose two highly stimulatory peptides (pep378 and pep383, SI ≥ 50; see Table I), and two peptides with low stimulatory capacity (pep200 and pep387, SI < 10; not included in Table I). These peptides and MBP Ac1–11 were synthesized conventionally and analyzed for the induction of proliferation and cytokine production in T$^\dagger$α$^+$ cells in a dose-response analysis. pep378 and pep383 induced proliferation comparable to that of MBP Ac1–11 (Fig. 2A). At concentrations ≥100 μg/ml, pep200 induced low proliferation of the T$^\dagger$α$^+$ cells, whereas pep387 did not induce proliferation of the...
T<sup>+</sup>α<sup>-</sup> cells. Thus, the results obtained with conventionally synthesized peptides confirmed the results obtained with these peptides prepared by spot synthesis. pep378 and pep383 induced stronger IL-2 production than MBPAc<sub>1–11</sub> (Fig. 2B), and neither pep200 nor pep387 induced IL-2 production in T<sup>+</sup>α<sup>-</sup> cells (Fig. 2B). IFN-γ was induced by MBPAc<sub>1–11</sub> and pep383 in similar amounts (Fig. 2C), the dose-response curve for pep378 was slightly shifted to higher concentrations, whereas neither pep200 nor pep387 induced IFN-γ production. Small amounts of TNF-α were induced by MBPAc<sub>1–11</sub>, pep378, and pep383 (Fig. 2D), whereas none of the peptides induced IL-4, IL-10, or TGF-β (data not shown).

**EAE induction by microbial peptides**

To test whether the microbial mimic peptides that activated T cells in vitro could also induce EAE, we immunized T<sup>+</sup>α<sup>-</sup> mice with these peptides. Mice were immunized with 200 μg of MBPAc<sub>1–11</sub>, pep383, pep378, pep200, or pep387. All mice received PT i.v. at 24 and 48 h after immunization and were observed for at least 35 days postimmunization for the development of EAE (Fig. 3). Mice immunized with MBPAc<sub>1–11</sub> showed clinical onset of EAE at day 8 (mean value; range, 7–9 days) and rapidly progressed to final stages by day 12. Immunization with the mimic peptides pep383 and pep378 induced EAE in 8 of 8 and 6 of 8 mice, respectively (data pooled from two independent experiments). Both onset and progression of disease with pep383 were delayed as compared with MBPAc<sub>1–11</sub> or pep378. Control mice received PBS in CFA and PT and remained healthy for the duration of the experiments (50 days). Of the two peptides identified that induced low proliferation of transgenic T cells in vitro, pep200 and pep387, neither induced EAE (even when the observation period was extended up to 60 days; data not shown). Pertussis toxin was necessary to facilitate EAE and mice that were immunized with MBPAc<sub>1–11</sub> or mimic peptides without PT did not develop EAE (data not shown). In agreement with other studies (11, 41, 44), we observed that all T<sup>+</sup>α<sup>-</sup> mice progressed to full EAE (score 5) rapidly after displaying clear signs of onset of EAE (score 2).

**Discussion**

Global amino acid substitution of the immunodominant encephalitogenic epitope MBPAc<sub>1–11</sub> allowed us to define the structural motif (supertope) recognized by the MBPAc<sub>1–11</sub>/I-A<sup>u</sup>-specific TCR transgenic, C<sub>a</sub>2/2 T lymphocytes used in this study (11, 41, 45). The supertope recognized by the T<sup>+</sup>α<sup>-</sup> T cells confirms and extends previous findings on the recognition of variants of the MBPAc<sub>1–11</sub> epitope by I-A<sup>u</sup>-restricted T cells. Using different T cell hybridomas, T cell clones, or intact mice, others have identified lysine at position 4 of the original peptide (K4) and R5 as the MHC contact sites of MBPAc<sub>1–11</sub> and Q3 and P6 as the putative TCR contact sites of the MBPAc<sub>1–11</sub>/I-A<sup>u</sup> complex (46–55). Most of this work was performed with alanine substitutions in the MBPAc<sub>1–11</sub> epitope. Our systematic analysis in which every residue of MBPAc<sub>1–11</sub> was replaced by every naturally occurring amino acid revealed that the MHC contact site R5 could not be replaced by any other amino acid without destroying recognition by the T<sup>+</sup>α<sup>-</sup> T cells, whereas L4 could be replaced by any of the amino acids. This is in agreement with earlier studies, which had demonstrated that substitution of L4 with several different amino acids can dramatically increase MHC binding (46, 49, 50, 53, 54).
However, increased MHC binding was not always associated with improved T cell activation in vitro, and several of the peptides with substitutions at position 4 abolished T cell activation of individual T cell hybridomas or clones (27, 46, 48, 50, 51, 53, 55). Thus, the T+α- T cells differ from some of the other T cell clones and hybridomas studied to date in that all the substitutions for K4 induced strong T cell proliferation (Fig. 1). As expected, only few substitutions were possible at the TCR contact sites. At position 5 P5Q was the only possible substitution. Moreover, only 9 of the 61 mimic peptides had the P5Q substitution, significantly less than expected for a chance distribution (p < 0.01; Table I). Similarly, whereas the supertope analysis had shown that phenylalanine, histidine, and methionine could each substitute for Q3, 31 of the 61 mimic peptides maintained glutamine at position 3, significantly more than expected for a chance distribution (p < 0.01). In addition to the known MHC and TCR contact residues, we also found A1 and S2 significantly (p < 0.01) more frequently conserved than expected by chance (Table I). In contrast, we found 16 different amino acids to be tolerated at position 7 and all naturally occurring amino acids at positions 8–11. Furthermore, no amino acid was overrepresented at any of these positions in the 61 mimic peptides. This is in agreement with earlier studies that had shown that alanine substitutions at positions 7–11 did not influence MHC binding or T cell recognition (27, 49, 54, 56).

A search of the SwissProt and TREMBL databases for peptides containing the supertope shown in Fig. 1B yielded 832 potentially cross-reactive peptides of microbial origin. However, only 61 of the 832 peptides induced proliferation of the T+α- T cells. Wucherpfennig et al. (5) used structural criteria to search a protein database for microbial mimics of MBP 89–94, the immunodominant epitope in HLA-DR2+ MS patients. Of 129 peptides fulfilling the set criteria that were synthesized, only 7 activated at least 1 of the 5 DR2-restricted human T cell clones tested in that study. Why do so many peptides that fulfill carefully designed structural criteria fail to induce T cell activation? One explanation is that some combinations of amino acid substitutions that are allowed individually will be “forbidden” when combined in one peptide sequence. This has been observed in a recent study in which MBP-specific human T cell clones were tested for reactivity with random peptide libraries (34). Furthermore, Reay et al. (28) have shown that changing residues apparently not involved in MHC or TCR contact can nevertheless have dramatic consequences on T cell activation. Thus, neither a detailed knowledge about the MHC and TCR contact sites of an epitope nor a global substitution analysis as performed in the work described here can exactly predict those peptides that will activate a cross-reactive TCR. Importantly, either of these approaches will not only predict T cell reactivity with peptides that are nonstimulatory but also miss several peptides that are stimulatory for the TCR in question. Substitutions that are “forbidden” if considered individually can be compensated for by additional substitutions at other positions of an antigenic peptide that enhance T cell activation (28, 34). Thus, it is very likely that our supertope analysis has missed some microbial peptides capable of stimulating the T+α- T cells.

25 of the 61 microbial mimic peptides that activated the T+α- T cells had four native MBP residues. Gautam et al. (49) have shown earlier that a peptide with only 4 native MBP-residues could activate T cell hybridomas specific for MBP 89–94/I-Aα. Extending these data, we found 18 peptides (see Table I) that had 3 native MBP residues and 6 peptides that had only 2 native MBP residues among those mimics that induced SI ≥50 in the T+α- T cells. Thus, in addition to viral peptides that have been shown earlier to activate MBP 89–94/I-Aα-specific or MBP 87–99/I-Aα-specific T cells (7, 24), we demonstrate here that bacterial peptides with as little as 2 or 3 conserved MBP residues can activate MBP 89–94/I-Aα-specific T cells. This is similar to findings obtained with human MBP 87–99-specific T cell clones: microbial mimics with as little as 3 native MBP-residues were shown to activate such clones (5). In one case, a peptide not sharing a single residue with the original MBP 87–99 sequence was found to stimulate human T cell clones raised against MBP (34).

The animal model EAE permits testing of microbial peptides for encephalitogenicity. Previous work had shown that some altered peptides could still induce T cell activation but not EAE when injected into susceptible mice (46, 47, 49). Previous findings had also indicated that at least 5 native MBP residues need to be present in a mimic peptide for the peptide to induce EAE after immunization of susceptible mice (24, 49). Extending these earlier reports, we found that a mimic peptide containing only 4 native MBP residues (pep 378) could induce EAE when injected into susceptible mice (Fig. 3). Both of the encephalitogenic peptides examined in our study had A1, S2, R5, and P6 conserved. Our findings that at least 61 microbial mimic peptides can activate the T+α- T cells in vitro and that a fraction of these mimic peptides can also induce EAE in the T+α- mice support recent evidence coming from extensive analyses of Ag recognition by individual T cells (5, 6, 27–32), or the structural analyses of TCR-peptide-MHC complexes (33), demonstrating that TCR recognition of Ag is degenerate. In fact, it has recently been suggested that a single TCR might productively interact with as many as 100 different ligands (57). How do these findings relate to the “molecular mimicry” hypothesis (8)? Our data presented here (and those of B. Maier and T. Kamradt, unpublished observations) and those of others (5, 6) indicate that peptide molecular mimicry at the level of T cell activation is a frequent event. We consider it very likely that T cell
cross-reactivity between a microbial peptide and a self peptide alone is not sufficient to induce autoimmune disease (9). In fact, in preliminary experiments we could not induce EAE in Tα- mice via infection with S. typhimurium, the bacterium from which the encephalitogenic mimic pep383 is derived (J. L. Grogan, U. E. Schaible, and T. Kamradt, unpublished observations). Such preliminary examinations, however, must be interpreted with great caution because all the peptides used in our studies were N-terminally acetylated. Earlier work had shown that N-terminal acetylation of MBP1–11 is essential for T cell recognition. It was proposed that the positively charged amino terminus revealed by removal of the N-terminal acetyl group was responsible for the observed elimination of the proliferative activity (58). Wraith et al. (46) found that unacylated MBP1–11 with a K4A substitution (MBP1–11[4A]) effectively activated T cell hybridoma 1934.4 despite its decreased binding to I-Aβ. Therefore, the N-terminal acetyl group is an important determinant in interactions with I-Aβ but not absolutely necessary for interaction with the TCR. This notion was further supported recently. Lee et al. reported on an unacylated but NH2-terminally extended MBP1–11 peptide (OVA-MBP). This peptide induced IL-3 production in an MBPAc1–11-specific T cell clone yet failed to trigger full T cell proliferation (54). Finally, acetylated MBP1–11 variants have been reported that induce T cell proliferation in vitro but not EAE in vivo (46, 47, 49). Therefore, it is impossible to predict from our in vitro and in vivo data that were obtained using N-terminally acetylated MBP1–11 whether the nonacetylated or N-terminally extended natural peptides would have similar effects. Current work in our laboratory addresses the questions whether the mimic peptide sequences are processed naturally and whether the naturally processed peptides are encephalitogenic. In addition to this aspect which is specific for the MBPAc1–11 system, a multitude of mechanisms usually prevents the induction of autoimmunity. For cross-reactive T cells to induce autoimmunity, neither the microbial peptide nor the self peptide should be a cryptic epitope (59); the self Ag must be present at high enough concentrations and the T cells at high enough numbers (60); the T cells must receive the “right” costimulatory signals (61), to produce the “right” set of cytokines (1, 40, 62), to migrate to the site where the self Ag is expressed (45, 63, 64), and must escape immunoregulation (41, 44). Nevertheless, molecular mimicry remains an attractive hypothesis for the pathogenesis of autoimmunity. It is, for example, conceivable that microbial Ags, even if they do not trigger disease directly, help maintain the memory T cell pool specific for a particular autoantigen. Furthermore, recurrent infections possibly even with different microbes could bring the number of autoreactive T cells over a critical threshold such that autoimmune disease will finally become manifest.

We have shown that the definition of a supertype by global amino acid substitution can identify multiple microbial mimic peptides that activate an encephalitogenic TCR. Peptides with only 2 native MBP-residues are sufficient to activate MBPAc1–11-specific T cells in vitro and EAE can be induced by immunizing mice with a mimic peptide with only 4 native MBP residues. The data show that molecular mimicry at the level of TCR cross-reactivity is a frequent event.

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