The nature of cryptic epitopes within the self-antigen myelin basic protein

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Abstract

Mechanisms that allow potentially autoreactive T cells to escape central tolerance and persist in the peripheral lymphoid organs of healthy individuals are poorly defined. It has been proposed that such cells are specific for epitopes which normally are not well presented to the immune system or, in other words, are cryptic. We have used synthetic peptides to define potential T cell epitopes within the N-terminal portion of myelin basic protein (MBP). These were defined in terms of their relative affinity for the MHC-restriction element I-Au and their ability to activate T cells in mice of the H-2b haplotype. Three epitopes were identified, one of which corresponded to the known dominant N-terminal epitope (Ac1-9). The other two epitopes (9-20 and 5-20) bound to their MHC-restriction element with relatively high affinity but were cryptic, as defined by the poor response to these epitopes following immunization with intact MBP. Even the longer of these two epitopes did not induce autoimmune encephalomyelitis in H-2b mice. These results demonstrate that antigen processing can control both the induction of and effector function of autoreactive T cells, and is therefore a principal mechanism involved in limiting the autoreactive T cell repertoire.

Introduction

Mechanisms governing the immunodominance of T cell epitopes within proteins have been established using well characterized foreign antigens (1). Dominant epitopes are those that are presented at a high level, in the context of a particular MHC protein, and are recognized by a complementary TCR with the appropriate affinity to signal a response. One of the central mechanisms involved in this process is affinity between MHC and the T cell epitope, peptides from foreign antigens having been shown to display high affinity for their restriction element (2,3). The epitopes involved in autoimmunity should, however, display different properties. Their presentation to the immune system must be an inefficient process allowing autoreactive T cells to escape tolerance induction during selection of the T cell repertoire or, in other words, such self-determinants should behave as cryptic epitopes (1). Some mechanisms by which epitopes for autoreactive T cells remain cryptic have been proposed (1), and these include physical sequestration and ineffective antigen processing. One additional mechanism has been suggested by recent studies with the N-terminal epitope of myelin basic protein (MBP) in a murine model of experimental autoimmune encephalomyelitis (EAE). This self-antigenic epitope displays surprisingly low affinity for MHC (4,5) and this property has been shown to allow T cells specific for the epitope to escape tolerance induction (6). In normal H-2b mice and also in mice transgenic for an encephalitogenic TCR (6), T cells specific for the N-terminal epitope remain silent unless mice are injected with a high dose of antigen in a potent adjuvant. Since the induction of tolerance depends on the interaction of antigenic peptides with MHC, it is proposed that those T cells that recognize self-peptides binding with high affinity will be more susceptible to tolerance induction while T cells specific for low-affinity peptides, such as the N-terminal epitope of MBP, remain silent (4). This hypothesis has gained support from the analysis of some T cell epitopes associated with autoimmune disease, which were shown to bind with low to moderate affinity (7), but not others which were shown to bind with high affinity (8,9). This apparent discrepancy most
Table 1. Sequences of peptides used in this study

<table>
<thead>
<tr>
<th>Wild Type Peptides:</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Ac1-9: Ac-ASQKRPSSQR</td>
<td></td>
</tr>
<tr>
<td>Ac1-11: Ac-ASQKRPSSRKS</td>
<td></td>
</tr>
<tr>
<td>Ac1-13: Ac-ASQKRPSSQRSKYL</td>
<td></td>
</tr>
<tr>
<td>Ac1-15: Ac-ASQKRPSSQRSKYLATS</td>
<td></td>
</tr>
<tr>
<td>Ac1-18: Ac-ASQKRPSSQRSKYLATSADT</td>
<td></td>
</tr>
<tr>
<td>Ac1-20: Ac-ASQKRPSSQRSKYLATSMD</td>
<td></td>
</tr>
<tr>
<td>5-20: RPSQRSKYLATSMD</td>
<td></td>
</tr>
<tr>
<td>9-20: RSKYLATSMD</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptide Analouges:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac1-9(4Y): Ac-ASQYRPSQR</td>
</tr>
<tr>
<td>Ac1-20(11A): Ac-ASQKRPSSARYLATSMD</td>
</tr>
<tr>
<td>Ac1-20(13K): Ac-ASQKRPSSARYLATSMD</td>
</tr>
<tr>
<td>Ac1-20(4Y,12K): Ac-ASQYRPSRRSLLATSMD</td>
</tr>
<tr>
<td>Ac1-20(12K): Ac-ASQKRPSSRRSLLATSMD</td>
</tr>
<tr>
<td>Ac1-20(4K): Ac-ASQYRPSRRSLLATSMD</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biotinlated Peptides:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biol-9(4Y): Bio-YGASQYRPSQR</td>
</tr>
<tr>
<td>Ac1-9-Bio: Ac-ASQKRPSSQYR</td>
</tr>
<tr>
<td>Ac1-20(11A)-Bio: Ac-ASQKRPSSQYRSLATSMD</td>
</tr>
</tbody>
</table>

Letters in bold represent deviations from the wild-type sequence. Letters in bold italic identify the positions of residues that have had biotin covalently linked to the ε amino group.

probably arises from the technical difficulty involved in identifying naturally processed T cell epitopes, which was not ascertained in either of these studies. It is important to emphasize that it is not possible to interpret the influence of peptide–MHC affinity on self-tolerance unless it is clear that the peptide in question is identical to a natural product of antigen processing. Only peptides produced by antigen processing will be presented by antigen-presenting cells (APC), under physiological conditions, and only these peptides will influence the developing or adult T cell repertoire.

It has been possible to predict the length of the naturally processed epitope for the N-terminal epitope of MBP. Peptides based on this epitope and extending beyond 12 amino acids in length do not activate encephalitogenic T cells in vitro without further antigen processing (4). Moreover, all of the peptides displaying antigenic activity in vitro, ranging in length from nine to 11 amino acids, generate unstable peptide–MHC complexes. While these data lend clear support to a mechanism by which T cells specific for certain self-antigens may escape tolerance, they do not fully explain the mechanism of epitope selection since the affinity of longer, non-antigenic peptides was not measured nor was the role of antigen processing elucidated.

The studies described in this paper clarify the mechanism of determinant selection at the N-terminus of MBP. T cells from mice primed with MBP, and therefore specific for naturally processed epitopes, preferentially recognize short peptides. The antigenic Ac1-9 peptide displays relatively low affinity for MHC whereas longer, non-antigenic peptides bind with high affinity. Despite their high affinity for MHC, these epitopes are not generated by natural processing of the intact protein, indeed they are ‘absolute’ cryptic epitopes according to the definition of Sercarz et al. (1). Injection of mice with one such cryptic epitope fails to induce disease indicating that cryptic self-epitopes may remain effectively ‘silent’ if T cells specific for them are unable to recognize the naturally processed self-antigen.

**Methods**

**Animals**

PL/J mice were bred under specific pathogen-free conditions in isolators in the Pathology Department, Cambridge University and were housed under filters in a specific pathogen-free facility.

**Hybridomas**

The B cell hybridoma, PL-8, was derived from the fusion of lipopolysaccharide-stimulated B cells from PL/J mice with the class II+ cell line, M12C3 (10). The hybridoma expresses high levels of I-A^d^ and acts as a competent APC. Derivation of the 1934.4 T cell hybridoma has been described fully elsewhere (11). A25, 172.10 and HY9.0 hybrids, likewise specific for Ac1-9 in the context of I-A^d^, were kindly provided by Dr Vipin Kumar (UCLA). All hybridomas were maintained in Iscove’s modified Dulbecco’s medium (Gibco, Paisley, UK) supplemented with 5% FCS (Flow, Irvine, UK), 2 mM L-glutamine (Gibco), 5x10^-5 M 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin.

**Spinal cord homogenate (SCH)**

Spinal cords were flushed from spines using a water-filled syringe. The freshly isolated cords obtained from various strains of inbred mice were homogenized, frozen at -80°C and lyophilized. The lyophilized powder was stored at 4°C.

**Peptides**

All peptides were based on the N-terminal sequence of murine MBP (Ac-ASQKRPSSQRSKYLATSMD). Synthesis of such peptides made use of F-moc chemistry on either a multiple peptide synthesis BT7400 block (Biotech, Luton, UK) or an LKB-Biotynx 4175 synthesizer and has been described in detail previously (12). Biol1-9(4Y) was based on the 1-9 sequence but with the addition of glycine and tyrosine spacer residues at the N-terminus. The extended peptide was biotinylated prior to cleavage from the resin using a long-chain derivative of NHS-biotin (Calbiochem, La Jolla, CA), thereby inserting a 14 atom spacer between the biotin and the N-terminal amino group. Ac1-9 and Ac1-20(11A) were biotinylated following cleavage from the resin using a biotinylation reagent.
kit (Amersham International, Amersham, UK) according to the manufacturer's instructions. Table 1 shows the sequences of all peptides employed in this study.

**Antigen-presentation assay**

Presentation of peptides to T cell hybridomas was measured as a function of IL-2 production. PL-8 cells were fixed in 0.5% paraformaldehyde (BDH, Poole, UK), as described (4), and were plated at 5x10^4 cells/well of a 96 well flat-bottomed plate. T cell hybridomas were plated at the same density in the presence of varying concentrations of peptide. After incubation for 18 h at 37°C, culture supernatants were collected and assayed for the presence of IL-2 by means of ^[3]H]thymidine incorporation by the HT-2 cell line (13), as described previously (4).

**Peptide binding assays**

The binding of peptides to I-A<sub>u</sub> was measured directly by incubating live PL-8 cells with varying concentrations of Bio1-9(4Y), Ac1-9-Bio or Ac1-20(11A)-Bio in complete medium for 2 h at 37°C. Cells were washed free of unbound peptide and stained on ice for 30 min with ExtrAvidin-FITC (Sigma, Poole, UK) in PBS containing 1% FCS and 2 mM NaN<sub>3</sub>. Cells were washed and fixed in 1% formaldehyde (BDH) prior to analysis on a Becton Dickinson FACScan using FACSMate software. The extent of peptide binding was plotted as mean channel fluorescence.

Direct binding of unbiotinylated peptides of varying length was assessed by means of a competition binding assay. Aliquots of PL-8 cells were incubated in serial 2-fold dilutions of the Bio1-9(4Y) probe in 96-well microtitre plates. Competitor peptides were added to all cultures at a fixed concentration of 400-800 µg/ml and the cells incubated for 2 h at 37°C. Binding of the biotinylated probe was visualized, as before, by the addition of ExtrAvidin–FITC.

**Induction of EAE**

EAE was induced in 8- to 12-week-old mice by s.c. injection of 200 µg peptide in 0.1 ml of an emulsion consisting of equal volumes of complete Freund's adjuvant (Difco, West Molesey, UK) and PBS containing heat-killed Mycobacterium tuberculosis, strain H37RA (Difco), at 4 mg/ml (CFA). Pertussis toxin (Porton Products, Maidenhead, UK) (200 ng) was given i.p. at the same time as peptide and again 2 days later. Mice were monitored for disease daily and EAE scored as follows: (i) flaccid tail, (ii) partial hind limb paralysis and impaired righting reflex, (iii) total hind limb paralysis, (iv) fore and hind limb paralysis, and (v) moribund.

**Lymph node proliferation assay**

Mice were primed s.c. at the base of the tail with CFA containing either Ac1–20, 9–20 at 200 µg per mouse, a mixture of Ac1–9 and 5–20 containing 100 µg of each peptide per mouse or SCH, as a source of MBP, at 1 mg/mouse. On day 10 after priming, inguinal lymph nodes were harvested and disrupted through a sterile wire mesh. Cells were washed, resuspended in Iscove's modified Dulbecco's medium (Gibco, Paisley, UK) and plated in triplicate, with or without antigen, at 0.2 ml/well in 96-well flat-bottomed microtitre plates. Lymph node cells were cultured at a concentration of 8X10<sup>5</sup> cells/well in Iscove's modified Dulbecco's medium supplemented with 0.5% normal mouse serum. After 72 h, 0.5 µCi [^3]H]thymidine (Amersham, Little Chalfont, UK) was added to each well and wells were harvested 24 h later for assessment of thymidine incorporation.

**Results**

Properties of the dominant encephalitogenic epitope in H-2<sup>u</sup> mice

Recent studies of the foreign antigen, cytochrome c, have revealed that peptides increasing in length beyond the minimal T cell epitope elicit progressively enhanced responses from an antigen-specific T cell hybridoma, a peptide of 23 residues stimulating the strongest response (14). In contrast, our own experiments, using the dominant, N-terminal epitope of murine MBP, have previously revealed an inverse relationship between length and stimulatory capacity (4). Since these observations were based on the response of a single T cell hybridoma, however, we have extended our investigations to a panel of Ac1–9-specific hybridomas derived from two strains of mice susceptible to EAE.

As shown previously, the T cell hybridoma 1934.4, isolated from PLJ mice primed with whole MBP, responded optimally to the minimal T cell epitope, Ac1–9, when presented by PL-8 cells fixed with paraformaldehyde to prevent processing (4). Peptides of 15 residues or longer failed to stimulate, while those of intermediate length elicited suboptimal responses. A second hybridoma, A25, derived from B10.PL mice similarly primed with whole MBP, displayed an identical pattern of stimulation (Fig. 1a), as did two further hybrids, 172.10 and HY9.0 (data not shown), confirming our original findings.

One possible explanation for the lack of response to longer peptides, from within the Ac1–20 sequence, is that these peptides do not bind to the appropriate restriction element (I-A<sup>u</sup>) as a result of steric hindrance. Alternatively, the longer peptides may bind to I-A<sup>u</sup> but in an altered conformation to that adopted by shorter peptides such as Ac1–9. These possibilities were resolved through the use of a competitive binding assay. A high-affinity analogue of Ac1–9, bearing tyrosine at position 4, was synthesized with a biotin replacing the acetyl group at the N-terminus. Binding of the resulting peptide [Bio1–9(4Y)] to I-A<sup>u</sup> could be detected at the surface of PL-8 cells by flow cytometry upon addition of ExtrAvidin–FITC. The non-stimulatory peptides were therefore used to compete for binding of the biotinylated probe to PL-8. As shown in Fig. 1b, while Ac1–9(4Y) strongly inhibited binding, Ac1–9A<sup>u</sup> competed little better than PBS, confirming previous reports of the relatively low affinity of this epitope for I-A<sup>u</sup> (4,5). In contrast, peptides of 15 residues or longer strongly inhibited binding of Bio1–9(4Y), displaying an affinity equal to or greater than Ac1–9(4Y). These results show that the failure of the longer peptides to elicit T cell responses cannot be attributed to their inability to bind I-A<sup>u</sup>. Paradoxically, therefore, encephalitogenic T cells from two strains of mice preferentially recognize those peptides derived from the N-terminus of MBP which display minimal affinity for class II.

**Identification of two registers for binding to I-A<sup>u</sup> within Ac1–20**

The difference in affinity between short and long peptides from the N-terminal sequence is most readily explained by
of Lys4 and Tyr12 were reversed to test whether moving the Ac1-20 peptide. In the first of these analogues, the positions binding in the conformation recognizable by encephalitogenic position 4.

putative anchor residue to position 4 would favour peptide act as the anchor residue in peptides of 13 amino acids or

\[ (4,5) \]

we reasoned that Tyr12 might optimize binding to I-A\textsuperscript{u} strongly hydrophobic amino acids. Since the substitution of longer, in preference to the naturally-occurring lysine at position 4, has been shown to Ac1-9(4Y) (data not shown).

\[ \text{Ac1-20} \]

Ac1-11 and Ac1-13. While Ac1-11 failed to compete for binding to I-A\textsuperscript{u}, addition of residues 12 and 13 rendered Ac1-13 of comparable affinity to Ac1-9(4Y) (data not shown). Residues 12 (Tyr) and 13 (Leu) of murine MBP are both strongly hydrophobic amino acids. Since the substitution of lysine with tyrosine at position 4 of Ac1-9 has been shown to optimize binding to I-A\textsuperscript{u} (4,5), we reasoned that Tyr12 might act as the anchor residue in peptides of 13 amino acids or longer, in preference to the naturally-occurring lysine at position 4.

To address this possibility we synthesized variants of the Ac1-20 peptide. In the first of these analogues, the positions of Lys4 and Tyr12 were reversed to test whether moving the putative anchor residue to position 4 would favour peptide binding in the conformation recognizable by encephalitogenic T cells. Analogues were also synthesized in which either tyrosines or lysines occupied both positions 4 and 12 in order to assess the contribution of other residues to the peptide binding motif.

Figure 2(a) shows that while 1934.4 failed to mount a response to the wild-type peptide (Ac1–20), when presented by fixed APC, the hybridoma responded in an enhanced fashion to Ac1–20(4Y,12K) compared to the optimal length peptide, Ac1–9. Identical patterns of stimulation were observed with hybridomas A25, 172.10 and HY9.0 (data not shown). The magnitude of this response was equivalent to that elicited by the high-affinity analogue Ac1–9(4Y), suggesting that Ac1–20(4Y,12K) was stably bound to I-A\textsuperscript{u} at position 4, rendering residues 3 and 6 accessible to the TCR. Interestingly, a similar response was obtained to Ac1–20(4Y), in which both positions 4 and 12 were equally favoured as anchor residues. This result suggests that other residues in the 1–9 sequence contribute more to the stability of peptide–MHC interactions than those surrounding position 12.

Accordingly, Arg5 has been shown to represent a second MHC-binding determinant in the Ac1–9 epitope (12,15). In addition to Arg5 there are clearly other residues towards the C-terminus of peptide Ac1–20 that contribute to binding. For example, Ac1–20(12K) failed to stimulate the hybridoma to a level comparable to Ac1–9 (Fig. 2b), suggesting that a significant proportion of this peptide remained bound in a non-permissive conformation despite substitution of residue 12. A similar level of stimulation could be recovered from the wild-type peptide, Ac1–20, by substituting Leu13 for a lysine residue (Fig. 2b), implying that leucine contributes subtly to the binding of peptides anchored via Tyr12.

The observation that Ac1–20 binds to I-A\textsuperscript{u} via Tyr12 in preference to Lys4 suggests that the N-terminus of the 20-mer peptide might lie outside the peptide-binding groove. To test this hypothesis directly, we synthesized a further analogue of Ac1–20 in which Lys11 was replaced by alanine. This analogue was prepared in order to leave a single free amino group at position 4 for biotinylation. The Ac1–20(11A) peptide was indistinguishable from the wild-type Ac1–20 peptide with respect to its high affinity for I-A\textsuperscript{u} and poor stimulatory capacity when presented by fixed APC (data not shown).

Viable PL-8 cells incubated with the biotinylated peptide, Ac1–20(11A)-Bio, followed by ExtrAvidin–FITC, were stained in a dose-dependent fashion (Fig. 3a). Whereas Ac1–20(11A)-Bio was readily detected at the cell surface by flow cytometry, cells treated with Ac1–9, likewise biotinylated at position 4, failed to emit a level of fluorescence above background. The inability of Ac1–9–Bio to stain PL-8 cells suggests that this peptide binds with such low affinity as to defy detection or that, once bound, the biotin is buried within the peptide-binding groove where it is inaccessible to avidin. In either case, the dose-dependent staining of PL-8 with Ac1–20(11A)-Bio suggests that the longer peptide binds with a relatively high affinity and is anchored via a residue beyond the 1–9 sequence such that a substantial proportion of the peptide hangs outside the groove where it may interact with avidin. Since one of the TCR-contact residues, Gln3, lies N-terminal to the biotin group, this determinant is clearly unavailable for ligation of the TCR: that Pro6 is separated from the biotinylated
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Fig. 2. (a and b) Relative stimulatory capacity of various peptide analogues increasing in length from the N-terminus of murine MBP. Peptides were added to mixed cultures of PL-8 and 1934. Experimental details are as described for Fig. 1(a).

Fig. 3. (a) Influence of peptide length on binding of two biotinylated peptides to PL-8 cells. Peptide binding was assessed by incubating cells with peptide for 2 h, washing and staining with ExtrAvidin-FITC before analysis by flow cytometry. (b) Relative affinity of two peptides from the N-terminus of murine MBP when compared with the high-affinity peptide analogue Ac1-9(4Y). PL-8 cells were incubated with Bio1-9(4Y) and the competitor peptides, at a fixed concentration of 800 μg/ml, for 2 h. Binding of the biotinylated peptide was revealed by staining the cells with ExtrAvidin-FITC before analysis by flow cytometry.

lysine by a single amino acid, suggests that this determinant is likewise inaccessible.

In order to confirm that the peptide sequence C-terminal to Ac1-9 contains a motif for high-affinity binding to I-A\(^d\), Ac1-9(4Y) was compared with a peptide spanning residues 9-20 in a competition binding assay. As shown in Fig. 3(b), peptide 9-20 bound to I-A\(^d\) with a relative affinity approaching that of Ac1-9(4Y) and similar to the longer analogues of Ad-20 (Fig. 1b). Similar results were obtained with peptide 5-20 from MBP.

Ac1-20 contains three independent T cell epitopes

We wished to know whether the high-affinity MHC-binding peptides contained within the N-terminal 20-mer peptide of MBP could act as T cell epitopes. If so, are these epitopes generated by antigen processing or are they cryptic? Lymph node T cells from H-2\(^b\) mice were primed by s.c. injection with peptide Ac1-20 emulsified in CFA. After 10 days the draining lymph nodes were collected and lymph node cells cultured with various peptide antigens including Ac1-9, 5-20, 9-20 and Ac1-20 itself. The Ac1-20 primed cells displayed a hierarchy of responses to this set of peptides (Fig. 4a). A strong response was mounted against the priming antigen Ac1-20, the greater part of which could be accounted for by T cells specific for 5-20 with a lesser response being noted against Ac1-9. This indicates that processing of the Ac1-20 antigen reveals both the known encephalitogenic epitope, Ac1-9, and an adjacent epitope 5-20.

Cells from mice primed with peptide Ac1-20 did not respond well to peptide 9-20 (Fig. 4a), despite the high affinity for I-A\(^d\) displayed by this peptide in vitro (Fig. 3b). Cells from mice primed with this peptide alone, however, responded well to the same peptide in vitro but failed to respond to Ac1-9 (Fig. 4b). Peptide 9-20, therefore,
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Fig. 4. Lymph node responses to myelin antigens and peptides. Groups of mice were primed with (a) Ac1-20/CFA, (b) 9-20/CFA, (c) spinal cord homogenate/CFA or (d) a mixture of Ac1-9 and 5-20/CFA. After 10 days the draining lymph nodes were excised for assessment of their T cell proliferative capacity using the indicated peptides as stimulating antigens.

possesses the properties of a cryptic epitope since there are T cells capable of responding to this peptide in the H-2\(^d\) mouse (Fig. 4b), yet they are not recruited following priming with the longer Ac1-20 antigen (Fig. 4a).

A further degree of epitope selection was revealed by priming H-2\(^d\) mice with SCH as a source of mouse MBP. In this case the hierarchy of responses altered dramatically. Mice primed with SCH responded poorly, if at all, to either peptide 5-20 or 9-20 (Fig. 4c). Lymph node cells from these mice responded equally well, however, to peptides Ac1-9 and Ac1-20. We predict that the response to Ac1-20 arises from Ac1-9 reactive cells recognizing shorter fragments of Ac1-20 generated by antigen processing in the lymph node cultures. Evidently this response is specific for the acetylated N-terminal amino acids of the peptide since there is no significant response to 5-20. In marked contrast, mice primed with a mixture of Ac1-9 and 5-20 respond to both peptides but the response to 5-20 is dominant (Fig. 4d). Peptide 5-20 is clearly capable of stimulating T cell responses to itself in vivo.

The results shown in Fig. 4 distinguish three epitopes within the Ac1-20 peptide. Peptide 9-20 is cryptic in both Ac1-20 and MBP, response to this epitope being revealed only by priming with the isolated peptide. Peptide 5-20 is cryptic in SCH-primed animals but is dominant over Ac1-9 in mice primed with either Ac1-20 (Fig. 4a) or a mixture of the two peptides (Fig. 4d). Peptide Ac1-9 dominates the response in SCH-primed mice even though this response is relatively low in mice primed with Ac1-20.

A cryptic epitope fails to induce EAE in H-2\(^d\) mice

It has been suggested that T cells specific for cryptic epitopes, such as 5-20 of MBP, lie dormant in the normal individual but may become activated and autoaggressive if the epitopes are presented at higher concentrations (16). In order to test this hypothesis, H-2\(^d\) mice were injected with either the dominant N-terminal epitope (Ac1-9) or the adjacent cryptic epitope (5-20) and pertussis toxin in order to induce acute EAE. While all of the mice injected with Ac1-9 developed EAE, none of the mice primed with 5-20 displayed signs of EAE when monitored for up to 60 days (Table 2). The cryptic epitope, 5-20, is not encephalitogenic despite the fact that the epitope alone is highly immunogenic (Fig. 4d).

Discussion

The studies described in this paper demonstrate that various mechanisms exist to enable self-antigenic T cell epitopes to remain hidden from the immune system. The dominant epitope of MBP binds with low affinity to its MHC restriction element...
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Table 2. Summary of encephalomyelitis in H-2k mice

<table>
<thead>
<tr>
<th>Peptide antigen used to induce disease</th>
<th>Disease incidence</th>
<th>Mean day of onset</th>
<th>Mean maximal disease</th>
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<tbody>
<tr>
<td>Ac1-9</td>
<td>10/10</td>
<td>9.9 ± 1.1</td>
<td>2.3 ± 1.5</td>
</tr>
<tr>
<td>5-20</td>
<td>0/13</td>
<td>-</td>
<td>0</td>
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Mice were immunized by injecting 200 µg peptide in CFA, giving pertussis toxin at the same time as peptide and again 2 days later, and were monitored for signs of EAE for 60 days.

thus allowing ‘escape from tolerance’. Other epitopes from within the N-terminal twenty amino acids of MBP bind with high affinity to MHC. Nevertheless, these epitopes are immunologically silent since they are not generated by antigen processing.

The N-terminal 20-mer peptide of MBP can associate with the I-Au molecule in two registers, one of which generates a high-affinity peptide from the N-terminal epitope because of its low affinity for I-Ak. In the normal H-2k mouse the number of Ac1-9/I-Ak complexes does not reach the threshold required to produce either T cell tolerance or T cell activation. This belief has been confirmed by recent studies involving PUJ mice transgenic for a TCR specific for peptide Ac1-9 (6).

Thymocytes from adult, transgenic mice are not affected by repeated i.p. injections of the wild-type peptide Ac1-9 at high concentration. A single injection of higher affinity analogues of Ac1-9, however, causes rapid deletion of double-positive thymocytes. Furthermore, these transgenic mice do not develop encephalomyelitis spontaneously despite expression of an autoreactive TCR on all of their T cells. Only when the wild-type epitope is injected at high concentration in CFA, with associated slow release of antigen and up-regulation of co-stimulatory molecules, does triggering of autoreactive T cells occur.

The second epitope, peptide 9-20, is, on the other hand, a high-affinity MHC-binding peptide. This epitope follows the definition of an ‘absolute’ cryptic epitope (1) since it is not generated by antigen processing of either MBP or Ac1-20. Mice immunized with Ac1-20, however, respond well to the third epitope, peptide 5-20 of MBP. T cells specific for 5-20 are, nevertheless, effectively silent since they fail to respond to MBP in vivo and are unable to produce clinical signs of EAE.

We can speculate as to why peptides 9-20 and 5-20 of MBP are cryptic. Both peptides 9-20 and Ac1-20 bind to I-Ak with high affinity. The fact that injection of Ac1-20 does not prime cells reactive with 9-20 in vivo suggests that the conformation of the 9-20 region may be different depending on the length of peptide bound. The implication is that processing of Ac1-20 is not able to generate the 9-20 epitope as it is seen by T cells from 9-20 primed mice. Either an epitope of the correct length cannot be generated by antigen processing or, once processed, it adopts a conformation which is different to the synthetic 9-20 peptide. Evidence in favour of the latter possibility has been provided by recent studies of a processed I-Ek-restricted epitope from hen eggwhite lysozyme by Viner et al. (20). These authors demonstrated that just such an epitope could be immuno-
The N-terminal peptide of MBP would, on the other hand, fall into the category of a 'latent' cryptic epitope evading conditions of antigen processing. Epitopes 5-20 and 9-20 of MBP fall under the definition of 'absolute' cryptic epitopes. Recognize the intact antigen either at high dose or in altered antigen itself, but the responding cells are then able to are only generated by immunizing with the peptide fragment itself and the cells elicited by such epitopes do not then (1) who define cryptic self-epitopes as either Sercarz antigens will not necessarily play a role in disease. In the conditions. It is clear then that all cryptic epitopes of self-activated by injection with 5-20 do not cause encephalomyelitis, however, because they do not see this epitope epitope 5-20 of MBP. This is a cryptic epitope which is present this otherwise cryptic epitope. A similar mechanism of selective antigen processing in the thyroid gland has been invoked to explain the induction of experimental autoimmune thyroiditis in rats with the synthetic peptide 2495-2511 of thyroglobulin (23). Furthermore, injection of cryptic epitopes derived from small nuclear ribonucleoproteins can initiate lupus autoimmunity in normal mice (24). Arising from these studies, it might be assumed that any cryptic epitope could induce autoimmune disease if injected at a concentration high enough to break tolerance. This is evidently not true for epitope 5-20 of MBP. This is a cryptic epitope which is highly antigenic as an isolated synthetic peptide. The T cells activated by injection with 5-20 do not cause encephalomyelitis, however, because they do not see this epitope following processing of MBP under normal physiological conditions. It is clear then that all cryptic epitopes of self-antigens will not necessarily play a role in disease. In the case of self-antigens it is useful to use the nomenclature of Sercarz et al (1) who define cryptic self-epitopes as either ‘absolute’ or ‘latent’. Responses to ‘absolute’ cryptic epitopes are only generated by immunizing with the peptide fragment itself and the cells elicited by such epitopes do not then respond to the intact antigen. ‘Latent’ cryptic epitopes may be revealed by immunization with the epitope rather than the antigen itself, but the responding cells are then able to recognize the intact antigen either at high dose or in altered conditions of antigen processing. Epitopes 5-20 and 9-20 of MBP fall under the definition of ‘absolute’ cryptic epitopes. The N-terminal peptide of MBP would, on the other hand, fall into the category of a ‘latent’ cryptic epitope evading recognition through low affinity for MHC. Only under conditions when the antigen is injected at high concentration in adjuvant does activation of autoreactive cells occur in the H-2^k mouse. The cryptic epitopes 1158–1180 of IRBP and 2495–2511 of thyroglobulin are also ‘latent’ cryptic epitopes whose recognition may depend on altered conditions of antigen processing.

The major conclusion to be drawn from this study is, therefore, that antigen processing of myelin antigens serves as the principal mechanism involved in selection of encephalitogenic determinants in the H-2^k mouse. This finding serves as a note of caution to those searching for motifs for preferential peptide binding to autoimmune disease associated MHC alleles (25). Not only will antigen processing limit the number of potential self-epitopes presented to the immune system but the affinity with which these epitopes bind to their MHC restriction element may even correlate inversely with disease.

Our results enable us to delineate two separate mechanisms involved in the maintenance of self-tolerance to potential T cell epitopes within the N-terminus of MBP. The two distinct, high-affinity epitopes, 5–20 and 9–20, are silent because they are not generated by antigen processing. The immuno-dominant epitope of MBP (Ac1–9), on the other hand, is evidently generated by antigen processing and yet remains silent in the normal H-2^k mouse. It behaves as though it were cryptic through low affinity for MHC.

The results with 5–20 of MBP are notable in light of recent results arising from other autoimmune models. It has been shown, for example, that a cryptic epitope contained within the interphotoreceptor retinoid binding protein (IRBP) can cause disease when injected into naive animals (22). It is believed that T cells, primed by injection with this peptide, are pathogenic because the local retinal tissue provides a unique environment in which IRBP can be processed to present this otherwise cryptic epitope. A similar mechanism of selective antigen processing in the thyroid gland has been invoked to explain the induction of experimental autoimmune thyroiditis in rats with the synthetic peptide 2495–2511 of thyroglobulin (23). Furthermore, injection of cryptic epitopes derived from small nuclear ribonucleoproteins can initiate lupus autoimmunity in normal mice (24). Arising from these studies, it might be assumed that any cryptic epitope could induce autoimmune disease if injected at a concentration high enough to break tolerance. This is evidently not true for epitope 5–20 of MBP. This is a cryptic epitope which is highly antigenic as an isolated synthetic peptide. The T cells activated by injection with 5–20 do not cause encephalomyelitis, however, because they do not see this epitope following processing of MBP under normal physiological conditions. It is clear then that all cryptic epitopes of self-antigens will not necessarily play a role in disease. In the case of self-antigens it is useful to use the nomenclature of Sercarz et al (1) who define cryptic self-epitopes as either ‘absolute’ or ‘latent’. Responses to ‘absolute’ cryptic epitopes are only generated by immunizing with the peptide fragment itself and the cells elicited by such epitopes do not then respond to the intact antigen. ‘Latent’ cryptic epitopes may be revealed by immunization with the epitope rather than the antigen itself, but the responding cells are then able to recognize the intact antigen either at high dose or in altered conditions of antigen processing. Epitopes 5–20 and 9–20 of MBP fall under the definition of ‘absolute’ cryptic epitopes. The N-terminal peptide of MBP would, on the other hand, fall into the category of a ‘latent’ cryptic epitope evading recognition through low affinity for MHC. Only under conditions when the antigen is injected at high concentration in adjuvant does activation of autoreactive cells occur in the H-2^k mouse. The cryptic epitopes 1158–1180 of IRBP and 2495–2511 of thyroglobulin are also ‘latent’ cryptic epitopes whose recognition may depend on altered conditions of antigen processing.

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Abbreviations

APC antigen-presenting cell
CFA complete Freund’s adjuvant
EAE experimental autoimmune encephalomyelitis
IRBP interphotoreceptor retinoid binding protein
MBP myelin basic protein
SCH spinal cord homogenate

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