Quantitative Analysis of Peptides from Myelin Basic Protein Binding to the MHC Class II Protein, I-A^u, Which Confers Susceptibility to Experimental Allergic Encephalomyelitis

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ABSTRACT

Background: An important issue in autoimmune diseases mediated by T cells, such as experimental allergic encephalomyelitis (EAE), is the affinity of the disease-inducing determinants for MHC class II proteins. Tolerance, either due to clonal deletion or anergy induction, is thought to require high-affinity interactions between peptides and MHC molecules. Low-affinity binding is compatible with the hypothesis that breaking tolerance to self proteins does not have to occur for onset of disease. In contrast, a high-affinity interaction implies that an event leading to a breakdown of tolerance is central to the autoimmune process.

Materials and Methods: Detergent-solubilized and affinity-purified I-A^u was incubated with varying concentrations of a set of peptides from myelin basic protein and a biotinylated peptide agonist. The specific complexes were separated from excess peptide by capture on antibody-coated plates, and the affinity of the peptides was measured by adding europium-labeled streptavidin and measuring the resultant fluorescence.

Results: The immunodominant and encephalitogenic determinant, Ac 1–11, was shown to bind to I-A^u relatively poorly (IC_{50} = 100 μM), demonstrating that in this protein, immunodominance did not correlate with high-affinity binding. In contrast with the natural sequence, the ability of shorter analogs to induce EAE did correlate with their apparent affinity.

Conclusions: The dominance of the natural determinant does not arise from a high-affinity interaction with the MHC class II molecule. This suggests that other mechanisms are operative and that the specific T cell for this peptide/MHC ligand is of high affinity.

INTRODUCTION

The ability of MHC class II molecules to bind and display fragments of proteins on the surface of antigen-presenting cells both selects the T cell repertoire in the thymus and determines which $CD4^+$ T cell clones are expanded or anergized in the periphery (1). A detailed understanding of the molecular basis of peptide binding to HLA-DR molecules has been derived from the solution of the crystal structure of an HLA-DRB1*0101:peptide complex and biochemical analyses of binding (2–7). The majority of the free energy of binding arises from the formation of hydrogen bonds between the backbone of the peptide ligand and conserved residues composing the binding site (3). The hydrogen bonding network orients the vast majority of peptides in closely related conformations so that the sidechains are directed into multiple subsites, whose topology and chemical composition vary between alleles.

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These principles are likely to hold for other human and murine MHC class II proteins, primarily because many of the residues shown to form hydrogen bonds to the peptide ligands in the HLA-DRB1*0101 crystal are conserved in most isotypes and alleles (3,8,9). However, the chemical composition and topology of the binding site will vary depending on the sequence of the class II protein.

I-A^u confers susceptibility to experimental allergic encephalomyelitis (EAE), which is a possible animal model for multiple sclerosis (MS) (10,11). Previous studies have defined the amino-terminal 11 amino acids of myelin basic protein (MBP Ac 1–11) to be both immunodominant and encephalitogenic in H-2^u mice (12). Some of the amino acids interacting with the class II protein and the T cell receptor have been defined by assaying a variety of structural analogs of MBP Ac 1–11 in T cell proliferation and qualitative cell surface binding assays (13,14).

To investigate whether there is a correlation between the ability of MBP-derived peptides to bind I-A^u and their capacity to induce EAE in mice of the H-2^u haplotype, the apparent affinity of a number of peptides of myelin basic protein and other proteins has been determined.

MATERIALS AND METHODS

Purification of I-A^u Proteins

The procedures used for detergent solubilization and affinity purification of I-A^u molecules from the cell line MUD-45 (kindly provided by Dr. D. Zaller), which expresses I-A^u α- and I-A^u β-chains, were similar to those described previously by Babbitt et al. (15).

Binding and Inhibition Assays

For direct binding assays, an optimal concentration of affinity-purified I-A^u (35 nM) was incubated with serial dilutions of an ovalbumin peptide corresponding to residues 323–339 modified with long-chain biotin at the amino terminus (NLCB ova 323–339) in phosphate-buffered saline containing 0.02% dodecyl maltoside, pH 5.5, in 96-well polypropylene plates (Costar, Cambridge, MA, U.S.A.) for 16–20 h at 37°C. In studies optimizing the assay, only between 5 and 10% of the I-A^u molecules were capable of binding added peptide. Therefore, the effective concentration of I-A^u was approximately 3.5 nM. The conditions of the assays were shown to be in ligand excess, because a 2-fold reduction of these class II concentrations did not change the measured ED_{50} values. The I-A^u:peptide complexes (50 μl) were transferred, in duplicate, to wells of a 96-well microtiter plate precoated with 10.3.6.2 (16) and blocked with heat inactivated fetal calf serum. Europium-labeled streptavidin (LKB–Wallac, Turku, Finland) was added and incubated overnight. After washing, complexes were measured by the addition of 0.1 M acetate/phthalate buffer, pH 3.2, containing 0.1% Triton X-100, 15 μM 2-naphthoyltrifluoroacetone, and 50 μM tri-N-octylphosphine oxide, which released the chelated europium from streptavidin and formed a highly fluorescent micellar solution. The resultant fluorescence was measured using a fluorescent plate reader (DELPHIA, LKB–Wallac, Turku, Finland). The data was analyzed using a four-parameter logistical curve fit program (SigmaPlot) that calculated the concentration of biotinylated peptide giving a half-maximal signal (ED_{50}).

The effects of pH on I-A^u binding of ovalbumin 323–339 were explored by performing assays over a range from 4.0 to 9.0. The lowest ED_{50} values were observed between pH 5.0 and 6.5, consistent with previous reports (17). Both lower IC_{50} values and higher occupancy were observed when dodecyl maltoside was used compared with Tween 20, octyl glucoside, NP-40, octanoyl-N-methylglucamide, and Triton X-100.

The inhibition assay format was identical to the procedure described above with the exception that the unlabeled peptide antagonist was serially diluted and incubated with constant concentrations of NLCB ovalbumin 323–339 (5 nM) and the I-A^u protein. The concentration of unlabeled peptide that prevented 50% of the labeled peptide from binding was the IC_{50} value. The concentration of the biotinylated ovalbumin 323–339 used in each assay was experimentally determined to be at least one-sixth of its measured ED_{50} in order to assure the inhibition was primarily measuring the binding characteristics of the competitor peptide. This was confirmed by demonstrating that the IC_{50} of unlabeled ovalbumin 323–339 was equal to the measured ED_{50} of the labeled peptide, demonstrating that the IC_{50} solely measured the affinity of the antagonist for the receptor and was independent of the presence of the agonist. However, the IC_{50} and ED_{50} values are referred to as being equivalent to the $K_d^{apparent}$ and not a true K_d, because of the known decomposition of MHC class II molecules

during the course of the assay (R. Cubbon et al., submitted). Therefore, the incubation time was chosen to permit sufficient complex to be formed to allow ease of detection, but was minimized to limit the amount of decomposition of the receptor. The presence of endogenous peptides make the assay an exchange reaction, rather than a simple binding event. Consequently, the inherent characteristics of the MHC molecules prevent the assay from ever attaining true equilibrium. However, by balancing the time of incubation, the $K_d^{apparent}$ can approximate the true K_d.

Peptide Synthesis

Peptides were synthesized using solid-phase techniques (18), either using an Applied Biosystems Peptide synthesizer or an Advanced Chemtech robotics system utilizing FastMOC chemistry with commercially available Wang resins, and Fmoc protected amino acids as previously described (6). All peptides were purified using reverse-phase HPLC and their structures were confirmed by amino acid analysis and fast atom bombardment mass spectrometry.

RESULTS

Design of Binding and Inhibition Assays

Detergent solubilized and affinity purified I-A^u was incubated with varying concentrations of a peptide corresponding to residues 323–339 of hen egg ovalbumin modified at the amino terminus with long-chain biotin. The amount of specific complexes were quantitated by addition of europium-labeled streptavidin, and measuring the resultant fluorescence. The concentration of agonist that resulted in the half-maximal signal (ED_{50}) was 10 nM (Fig. 1A). Consistent with a variety of other binding studies of MHC class II proteins, only 6% of the I-A^u molecules bound this peptide.

To convert the direct binding assay into a competitive assay using unlabeled antagonists, a sufficiently low concentration of the biotinylated agonist was chosen and incubated with varying concentrations of unlabeled ovalbumin peptide with affinity-purified I-A^u. After capture and detection of the complexes on microtiter plates, the half-maximal signal (IC_{50}) of the resultant inhibition curve was identical, within experimental error, to the ED_{50} (Fig. 1B). Because the IC_{50} was equivalent to the ED_{50} value, the measured IC_{50} values were independent of the structure or composition of the agonist. Because empty MHC class II proteins decompose during the incubation period of the assay (R. Cubbon et al., submitted), which results in a change in the concentration of the functional receptor in the assay, the IC_{50} and ED_{50} were not equated with equilibrium binding constants (K_d). However, because the incubation times and conditions of the assays were strictly reproduced, structural data on binding can be derived from the comparison of the measured IC_{50} and ED_{50} values.

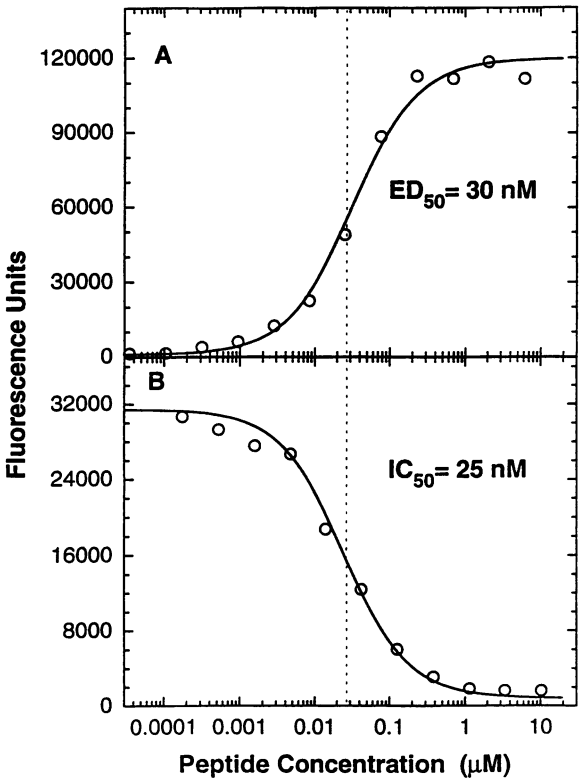

FIG. 1. Demonstration that the conditions used in the inhibition assays are sufficient to conclude that the measured IC_{50} values are representative of the apparent affinity of the inhibitor for I-A^u and independent of the presence or composition of the agonist

(A) Binding curve to I-A^u and the calculated ED_{50} value of a peptide corresponding to residues 323–339 of ovalbumin modified with long chain biotin at its a amino group. (B) Inhibition curve and the measured IC_{50} value of the same peptide lacking biotin.

Common Features of Peptides Binding I-A^u with High Affinity

To determine the structural requirements for the formation of I-A^u:peptide complexes, a series of

TABLE 1. Measured IC_{50} values of a variety of peptides corresponding to regions of rat myelin basic protein (MBP), rabies glycoprotein (rabies-derived peptides [RDP]), and a peptide from α1-antitrypsin (AT) binding to I-A^u

		I-A^u IC_{50} (nM)
MBP Ac 1–11	AcASQKRPSQRHG	>100,000
MBP 1–11	ASQKRPSQRHG	>100,000
MBP Ac 9–20	AcRHGSKYLATAST	20
MBP 11–23	SKYLATASTMDHA	80
MBP 31–47	RHRDTGILDSLGRFFSG	>100,000
MBP 35–47	TGILDSLGRFFSG	>100,000
MBP 42–54	GRFFGGDRGAPKRG	6,100
MBP 68–80	YGSLPQKSHGRTQ	5,300
MBP 84–106	VVHFFKNIVTPRTPPPSQGK	>100,000
MBP 89–101	VHFFKNIVTPRTP	>100,000
MBP 141–156	KGVDAQGTLSKIF	8,500
MBP 142–167	KGVDAQGTLSKIFKLGGRDSRSGS	3,800
MBP 152–165	SKIFKLGGRDSRS	>100,000
MBP 158–170	IFKLGGRDSRSGSP	12,000
Ovalbumin 323–339	ISQAVHAAHAEINEAGR	25
al AT 148–163	QAVHKALTIDETG	>100,000
RDP-1	QVVALKPAIAAAA	100
RDP-2	AAQVVKLKEEIKVDQ	>100,000
RDP-3	AAQVKSLKPEKIVDQ	6,000
RDP-4	VVSLKPEIIVDQYEY	>100,000

peptides corresponding to regions of rat myelin basic protein, rabies glycoprotein, and a peptide from α1-antitrypsin were screened for binding (Table 1). I-A^u bound the peptides with a wide range of IC_{50} values. Several peptides did not display any inhibition at concentrations as high as 100 μM, whereas others, such as ovalbumin 323–339, MBP Ac 9–20, MBP 11–23, and a peptide composed principally of alanine originally based on a sequence from the surface glycoprotein of rabies virus, bound with IC_{50} values less than 100 nM. Assuming that the better binding peptides all shared a small number of critical sidechains, they were directly compared, as shown in Table 2. In all cases, the sequences contained either a hydrophobic amino acid followed by a basic residue, or a pair of hydrophobic amino acids, separated by one amino acid from either alanine, serine, or threonine. When the relative position and composition of these amino acids were compared with the sequence of a well-studied peptide, known to bind I-A^u, Ac 1–11 of myelin basic protein, an interesting similarity was seen. In MBP Ac 1–11, substitution of lysine at the fourth position with alanine generated more potent analogs when assayed by T cell recognition, cellular inhibition assays, and qualitative binding assays (13,14). Analogs containing a tyrosine at this position bound I-A^u even better (10). This was confirmed when these analogs were assayed using the fluorescent binding assay. The natural sequence, Ac 1–11, bound I-A^u poorly, exhibiting an IC_{50} value close to 100 μM (Table 2). The measured IC_{50} value for the analog with alanine substituted for lysine was almost two orders of magnitude lower than the parent sequence (2.5 μM). Replacement with tyrosine resulted in a peptide that was bound with approximately 20 times higher affinity than the peptide containing alanine (IC_{50} = 100 nM). Previous studies have shown that a single T cell hybridoma can recognize each of these analogs (19), consistent with the substitutions not grossly affecting the orientation of the peptide in the binding site. Comparing the sequence of the analog of MBP Ac 1–11 containing tyrosine at the

TABLE 2. Peptides known to bind I-A^u aligned to emphasize a common motif postulated to be important in binding

		I-A^u IC_{50} (nM)
MBP Ac 9–20	AcRHGSK***YL***A***T***AST	20
Ovalbumin 323–339	QA***VH***A***A***HAEINEAGR	25
MBP 11–23	SK***YL***A***T***ASTMDHA	80
RDP-1	QVVA***LK***P***A***IAAAA	100
MBP Ac 1–11	AcASQ***KR***P***S***QRHG	>100,000
MBP Ac 1–11 A_4	AcASQA***R***P***S***QRHG	2,500
MBP Ac 1–11 Y_4	AcASQ***YR***P***S***QRHG	70

fourth residue with the other peptides that bound I-A^u well revealed that it also contained the postulated motif of a hydrophobic amino acid, tyrosine, followed by a basic residue, arginine, separated from a small residue, serine, by one amino acid (Table 3).

Minimal Requirements for High-Affinity Binding

If tyrosine and arginine were the most important sidechains in binding, the majority of residues not only in the MBP sequence, but also in any peptide should be able to be replaced with alanine, as was done for a hemagglutinin peptide binding to HLA-DRB1*0101 (4) (Table 4). This premise was supported by demonstrating a 13–amino acid alanine backbone containing an tyrosine and arginine at adjacent positions bound I-A^u with IC_{50} values less than 50 nM. Placement of three amino acids from an ovalbumin peptide including an adjacent valine histidine pair within a polyalanine sequence resulted in an even higher affinity peptide. The position of the pair of amino acids relative to the amino and carboxyl termini was not equivalent, and yet both bound with high affinity. A possible explanation was that the sidechains occupied the same subsites in the binding site, and the end groups of the two peptides were not in the same location in the binding site. If true, then peptides shorter than 13 amino acids might bind with equal or higher affinity.

Support for the assumption that peptides shorter than 11 amino acids could bind I-A^u well was the previous study that several shorter, simplified analogs of the amino terminus of rat myelin basic protein were recognized by T cell clones and bound well to I-A^u using a less sensitive binding assay (20). When peptides corresponding to the first six, seven, eight, or eleven amino acids of MBP were assayed for binding, a similar pattern in their measured IC_{50} values was observed regardless of length (Table 5). All peptides containing a tyrosine at the fourth position bound significantly better than analogs containing alanine at this residue, which in turn bound with lower IC_{50} values than analogs containing lysine. This result was consistent with the hexamers, heptamers, octamers, and undecamers all

TABLE 3. Demonstration that only a few amino acid sidechains present in natural sequences are necessary for high-affinity binding to I-A^u

		I-A^u IC_{50} (nM)
MBP Ac 1–11 Y_4	AcASQ**YR**PSQRHG	100
	AAAA**YR**AAAAAAA	31
Ovalbumin 323–339	ISQA**VH**A**A**HAEINEAGR	25
	AA**VH**AA**H**AAAAAA	4

TABLE 4. Comparison of the ability of peptides of varying length, based on the amino terminal sequence of myelin basic protein, to bind I-A^u

		I-A^u IC_{50} (nM)
MBP Ac 1–11 $A_{2,7-11}$	AcAAQKRPAAAAACOO$^-$	36,000
MBP Ac 1–11 $A_{2,4,7-11}$	AcAAQARPAAAAACOO$^-$	240
MBP Ac 1–11 $A_{2,7-11}Y_4$	AcAAQYRPAAAAACOO$^-$	80
MBP Ac 1–8 $A_{2,4,7,8}$	AcAAQARPAACOO$^-$	500
MBP Ac 1–8 $A_{2,7,8}Y_4$	AcAAQYRPAACOO$^-$	60
MBP Ac 1–7 $A_{2,7}$	AcAAQKRPACOO$^-$	>100,000
MBP Ac 1–7 $A_{2,4,7}$	AcAAQARPACOO$^-$	5,500
MBP Ac 1–7 $A_{2,7}Y_4$	AcAAQYRPACOO$^-$	60
MBP Ac 1–6 A_2	AcAAQKRPCOO$^-$	>100,000
MBP Ac 1–6 $A_{2,4}$	AcAAQARPCOO$^-$	>100,000
MBP Ac 1–6 A_2Y_4	AcAAQYRPCOO$^-$	>100,000
MBP Ac 1–7 $A_{2,7}Y_4$	AcAAQYRPACONH$_2$	300

binding in an equivalent location in the class II binding site to allow the fourth amino acid to contact the same subsite.

TABLE 5. Sidechain specificity was similar between peptides of varying length and composition

		I-A^u IC_{50} (nM)
S-2	AcASKYFDACOO$^-$	35
A-2	AcAAKYFDACOO$^-$	61
T-2	AcATKYFDACOO$^-$	4,712
Y-2	AcAYKYFDACOO$^-$	>100,000
P-2	AcAPKYFDACOO$^-$	>100,000
Y-4	AcAAKYFDACOO$^-$	27
F-4	AcAAKFFDACOO$^-$	229
M-4	AcAAKMFDACOO$^-$	745
R-4	AcAAKRFDACOO$^-$	>100,000
G-4	AcAAKGFDACOO$^-$	>100,000
W-5	AcAAKYWDACOO$^-$	49
F-5	AcAAKYFDACOO$^-$	98
Y-5	AcAAKYYDACOO$^-$	117
R-5	AcAAKYRDACOO$^-$	1,189
P-5	AcAAKYPDACOO$^-$	>100,000
T-5	AcAAKYTDACOO$^-$	>100,000
I-7	AcAAKYFDICOO$^-$	94
A-7	AcAAKYFDACOO$^-$	98
V-7	AcAAKYFDVCOO$^-$	462
K-7	AcAAKYFDKCOO$^-$	35,349

Only when the peptide was reduced to six amino acids was detectable inhibition lost. A slightly different pattern of IC_{50} values was seen for peptides of varying lengths containing alanine or tyrosine at the fourth position. In the former case, the IC_{50} values increased as the peptides were shortened from 11 down to seven amino acids, consistent with the additional peptide bonds making contacts with the binding site that increased affinity. In contrast, analogs containing tyrosine at the fourth residue bound I-A^u equivalently regardless of whether the peptide was eleven, eight, or seven amino acids in length. Evidently, the presence of the tyrosine compensated for the loss of the contacts provided by the carboxyl-terminal amino acids. The α-carboxyl group was beneficial for binding, because replacement with an amide resulted in a 5-fold increase in the IC_{50} value (Table 5). An acetylated heptamer, containing eight peptide bonds, a tyrosine at the fourth, arginine at the fifth, and alanine at the seventh position, bound I-A^u with an IC_{50} value of 60 nM, providing support that these characteristics were the minimal requirements for high-affinity binding.

DISCUSSION

The first unusual aspect of this investigation was that when a set of peptides corresponding to much of the sequence of rat myelin basic protein was assayed for binding to I-A^u, only residues

11–23 bound with an IC_{50} value less than 100 nM. The immunodominant and encephalitogenic epitope in H-2^u mice, Ac 1–11, bound with an IC_{50} value greater than 100 μM. This is in direct contrast with the results of a similar study showing that the encephalitogenic determinant in H-2^s mice, MBP 84–98, bound the restricting I-A^s molecules with high affinity (21). In addition, DRB1*1501 and DRB5*0101 of the MS-associated DR2 haplotype bound candidate autoantigen peptides from the MBP 84–102 region with high affinity (22).

As an initial step in understanding the biochemical basis of this paradox, which presumably involves details of antigen processing, establishment of the T cell repertoire, and T cell tolerance, the structural details of I-A^u:peptide interactions were explored. A detailed knowledge of the requirements for the formation of I-A^u:peptide complexes is a prerequisite for the investigation of the more complex biological mechanisms involved in the biological and chemical basis of immunogenicity of autoantigens.

Comparison of the primary sequences of the peptides that bound I-A^u with low IC_{50} values revealed a loose motif of either a pair of hydrophobic amino acids or a hydrophobic followed by a positively charged residue separated from a small amino acid by one position. This pattern was consistent with the previously reported beneficial substitution of either an alanine or a tyrosine for a lysine in MBP Ac 1–11 (10,13). When the analogs with alanine substituted for each amino acid in this sequence were assayed for binding to I-A^u, only substitutions at the fourth and fifth residues significantly increased the affinity of the peptide.

The importance of these three amino acids was supported further by substitution of these residues into simplified polymers of alanine. This backbone hides the importance of a small amino acid at the second and seventh position, but the observed low IC_{50} values of these simplified peptides verified the importance of these sidechains in binding. However, their position in the polyalanine backbone relative to the charged amino and carboxyl termini did not appear to be critical, indicating that shorter analogs will also bind with high affinity. Low IC_{50} values were observed for analogs as short as seven amino acids. In each of the sets of peptides, analogs containing tyrosine bound better than those with alanine, which in turn bound better than peptides containing lysine, providing strong evidence that in all cases the peptides were located in equivalent position in the binding site with the fourth amino acid contacting the identical subsite regardless of the length of the peptide. The demonstration that MBP Ac 1–7 could stimulate an I-A^u–restricted T cell hybridoma specific for MBP Ac 1–11 (20) further supports this premise. Data in this paper also emphasize that the important sidechain contacts dictate the register of binding, regardless of whether they are in the context of autoantigenic determinant or a simplified polyalanine peptide.

The observation that the immunodominant and principal encephalitogenic determinant of MBP, Ac 1–11, binds I-A^u with an IC_{50} value greater than 100 μM is consistent with the theory that autoreactive T cell clones escape tolerance induction because of their relative low binding affinity for the MHC class II protein (24). In contrast, T cell clones specific for regions of MBP that bind I-A^u well, such as residues 11–23 and 142–167, should be tolerized. However, recent experiments have shown that H-2^u mice are not tolerant to 11–23 and 142–167, but also that these regions of MBP are nonencephalitogenic and appear to be processed less efficiently from the intact protein than Ac 1–11 (L. Fugger and H. O. McDevitt, unpublished data). Furthermore, the amino terminal acetyl group of Ac 1–11, may make this peptide less susceptible to exopeptidases than other MBP-derived peptides. Consequently, the local concentration of Ac 1–11 at the site(s) where I-A^u:peptide complexes are formed may be far greater than other MBP peptides with higher apparent affinity. These complexes are most likely recognized by T cell receptors with both high specificity and affinity, because a hexamer containing only five of the native residues in the parent MBP Ac 1–11 is encephalitogenic (20). Therefore, the molecular basis for why a low-affinity peptide such as MBP Ac 1–11 is the immunodominant and principal disease-inducing epitope in MBP, might be a combination of efficient processing of this determinant from MBP and the induction of clones expressing T cell receptors with extraordinarily high affinity for the I-A^u:MBP Ac 1–11 complex.

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