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Presentation of a determinant by MHC class II can be prevented through competitive capture by a flanking determinant on a multideterminant peptide

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Abstract

Competitive capture is a process by which different determinants of an unfolding antigen compete for binding to the same MHC class II molecule. The “winning” determinant is then dominantly displayed. For self antigens, T cells with specificity for dominantly displayed determinants will be subject to strong tolerance induction. With this in mind we set out to characterize the determinant hierarchy of the junctional region of the Golli-MBP complex. Within this region the MBP 1–9 determinant is known to be a strong inducer of experimental autoimmune encephalomyelitis. We found that the Golli-MBP junctional region contains a triad of three overlapping determinants: LDVM1-5, MBP 1–9, and MBP 7–20. We demonstrate that these three determinants are unique and compete for binding to I-A^u and that a determinant hierarchy exists with MBP 7–20 being the most dominantly displayed determinant.

Keywords

antigen processing; autoimmunity; Golli-MBP; MBP; tolerance

Introduction

An aggressive immune response directed against a single self determinant is sufficient to initiate an autoimmune disease state [1]. For example, experimental autoimmune encephalomyelitis (EAE), an experimental animal model of multiple sclerosis, can be induced in mice of the H-2^u haplotype by immunizing with the N-terminal region of myelin basic protein (MBP) emulsified in complete Freund’s adjuvant (CFA). The mechanism that allows T cells specific for such self determinants to circumvent immunological tolerance remains a fundamental question. In the past it was believed that myelin antigens capable of inducing autoimmunity were *geographically* sequestered in an immunologically privileged site, behind the blood brain barrier. However, studies have demonstrated thymic expression of encephalitogenic proteins [2], and thymic expression of the autoimmune regulator (AIRE)

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allows for promiscuous gene expression, a mechanism to increase self antigen expression in the thymus [3]. Nevertheless, thymic expression alone does not guarantee sufficient display of all potential determinants within an antigen to induce tolerance; only certain processed determinants are displayed. Antigen processing results in a determinant display hierarchy on the surface of the antigen presenting cell (APC) and poorly processed self determinants are not displayed well enough to induce strong immunologic tolerance [4–6].

It has been hypothesized that the N-terminal region of MBP can bind to the I-A^u MHC class II molecule in multiple binding registers and that this competition for binding lowers the antigenic display of the encephalitogenic immunodominant determinant of MBP, Ac1-9 [7, 8]. The lowered display of the 1–9 determinant in turn allows for high affinity encephalitogenic Ac1-9-specific T cells to escape central tolerance induction. We have termed this type of competition “competitive capture”; different determinants of an unfolding antigen can compete for binding to the same MHC molecule.

Of importance to our understanding of autoimmunity in the MBP 1–9-induced model of EAE, we should note that the MBP 1–9 sequence is also expressed in the context of Golli-MBP. Analysis of the genetic region upstream to the “classical” MBP exons revealed a larger unit containing several novel exons, termed the “Golli” region (genes of the oligodendrocyte lineage) [9–11]. The 5' Golli exons are transcribed from their own upstream promoter in frame with the classical MBP exons and truncated products from this gene complex are found expressed at high levels in the fetal and adult thymus; meanwhile, in its classical form, MBP has never been detected in the early developing thymus. Interestingly, it has also recently been hypothesized that Golli-MBP plays a direct role in the immune response [12].

By generating T cell hybridomas specific to the three overlapping determinants--LDVM1-5, MBP:1–9, and MBP:7–20, we now clearly demonstrate the multiregister binding of the Golli-MBP region of the amino terminus of MBP. These results support competitive capture as a mechanism that protects encephalitogenic 1–9-specific T cells from central tolerance induction.

Methods

Mice

B10.PL mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our colony at the La Jolla Institute for Allergy and Immunology. Mice used in all experiments were age-matched, housed in filter-top cages and fed autoclaved chow.

Cell culture

The medium employed in all cell culture, was RPMI 1640 (GIBCO), supplemented with 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO), 4mM L-glutamine (GIBCO), 100 U/ml benzylpenicillin (GIBCO), 100 µg/ml streptomycin sulfate (GIBCO) and 10% heat-inactivated fetal bovine serum. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Peptides

Peptides were synthesized on an Advanced Chem Tech synthesizer and purified on a reverse phase column by high performance liquid chromatography. Purity was then determined by mass spectrometry and capillary electrophoresis. Amino acid sequences of peptides used in this study can be found in Table 1.

Antigen presenting cells (APC)

Individual spleens were aseptically removed and single cell suspensions were prepared in Petri dishes containing Hanks' balanced salt solution (HBSS). Large debris was removed by decanting, followed by two washes in HBSS before resuspension in complete RPMI (as described above) for use as APC in cultures. In some experiments antigen presenting cells were fixed as described [13]. Briefly, the APC were resuspended in 0.5 ml medium without serum and fixed by addition of 12.5 μ l glutaraldehyde 2% (end concentration 0.05%) for 30 s at room temperature. The reaction was stopped by adding 1 ml of 0.2 M L-glycine for an additional 45 s.

Hybridomas and antigenic display assays

The Ac1-9-specific T cell hybridomas 1934.4 and 172.10 were maintained in our laboratory. T cell hybridomas specific for LDVM1-9 (S12 & M72) and MBP:7–20 were also generated in our laboratory. Briefly, 4 B10.PL mice were immunized subcutaneously with 20 μ g of peptide emulsified in CFA and draining lymph nodes were removed on day 10. T cells were expanded for 7 days in the presence of peptide (10 μ g/ml) and then with IL-2 for an additional 4 days. Live cells were separated from dead cells with a Ficoll gradient. Before undergoing one more round of stimulation, cells were washed and pooled. T cells were then fused to BW5147 (TCR α -/ β -).

Peptide responsiveness of the hybridomas in both screening and subsequent peptide display assays was identified by IL-2 production. Briefly, 5×10^5 spleen cells were incubated as APC with the indicated amounts of peptide in round-bottomed 96-well, tissue culture-treated plates. After 4 hrs, 5×10^4 T hybridoma cells were added to each well and the plate was incubated for an additional 20 hrs. Culture supernatants were then collected. IL-2 was measured with an IL-2 ELISA.

Assays for hybridoma-activation

Briefly, 50 μ l samples of supernatant were incubated in wells of microtiter plates that had been previously coated with monoclonal anti-mouse IL-2 capture antibody (clone JES6-1A12). After washing, bound IL-2 was detected by adding monoclonal biotinylated anti-mouse IL-2 antibody directed to a non-overlapping epitope on the cytokine (clone JES6-5H4, both from BD Biosciences, San Diego, USA). Plates were developed using a horseradish peroxidase-based system. Absorbance values were read at 450 nm using a multiscan plate reader (LabSystems).

Results

Response to this triad determinant peptide, LDVM 1–16

In order to dissect the relationship between possible overlapping determinants within the Golli-MBP junctional region, we utilized T cell hybridomas with individual specificities for either LDVM1-9, Ac1-9, or MBP 7–20. Initial experiments sought to elucidate which of these determinants was most readily displayed from a longer LDVM1-16 peptide encompassing all three determinants. T cell hybridomas for each of the three specificities were incubated separately with splenocytic APC' pre-pulsed with the appropriate homologous peptide, or the LDVM1-16 sequence. Figure 1 illustrates results from one hybridoma of each specificity, clearly demonstrating that only those T cells specific for MBP7-20 were activated following incubation with LDVM1-16-pulsed splenocyte APCs. Although hybridomas specific for LDVM1-9 and Ac1-9 were readily activated by their homologous peptides, they failed to recognize that peptide from within the context of LDVM1-16. Similar results were observed with several hybridomas for each specificity (data not shown).

Variants of the long triad determinant peptide designed to diminish binding to I-A^d

Prior studies by our group and others have identified the MHC contact and TCR contact residues of the MBP1-9 sequence [14,15]. For example, it is known that substituting Y or M for K at position 4 (Ac-ASQYRPSQR or Ac-ASQMRPSQR) greatly increases MHC binding, by a factor of 10⁴. Utilizing this information, we then synthesized variants of LDVM1-16 containing substitutions at crucial binding residues designed to diminish or enhance the availability of each of the three MHC-binding registers (Table 1). Results from these experiments can be seen in Figure 2, where the clear preferential display of 7-20 from LDVM1-16 was further enhanced in peptides where the N-terminal determinant (LDVM1-5) had been rendered non-bindable to I-A^u, by altering the N-terminal and its #4 residues (SDVG1-16 peptide). Contrariwise, interfering with the C-terminal MHC-binding register (LDVM1-16(12D,13E)) dramatically enhanced display of the N-terminal LDVM1-5 determinant. While the Ac1-9 response remained largely unaffected in peptides containing the two sets of changes individually, a peptide containing *both* sets of substitutions demonstrated that the central 1-9 determinant could be a highly effective inducer of specific T cell activation. These results clearly suggest a hierarchy of peptide display as follows: MBP 7-20>LDVM1-5>1-9, revealing a scenario in which MBP:1-9 is consistently out-competed for binding to I-A^u by immediately flanking determinants.

Determinant selection within the long triad peptide occurs at the level of binding, not processing

We next asked if these patterns of selection arose from competition for binding of the peptide to MHC or whether parts of the peptide were destroyed by processing. By using fixed APCs, to repeat the above experiment, we could ask whether the APC were functioning as an MHC binding entity, eliminating the possibility of an actively metabolizing enzymatic processing component. Our results indicate that this determinant 'selection' does appear to occur at the level of binding, not processing, as fixed APCs present an identical, if somewhat diminished hierarchical pattern (Figure 3).

A finer characterization of the determinant cores

Lastly, we characterized the boundaries of these determinants at a finer level by making truncated analogs of the longer LDVM1-16 triad peptide. Figure 4A demonstrates that the N terminal LDVM-specific T cells can respond to the truncated LDVM1-9 and LDVM1-13 peptides. Thus, in the absence of amino acids 14-16 (ATA) the competing C-terminal register is lost allowing for display of the N-terminal register. Figure 4B reveals that the central register 1-9-specific T cells prefer Ac1-9 over the longer Ac1-20 peptide. This is further evidence in support of the dominance of the C-terminal 7-20 register. Lastly, the C-terminal-specific 7-20 hybridomas do not respond to LDVM1-13 but can respond to Ac1-20, 7-20, 7-16 and 9-20. Thus, the core of this determinant is likely 9-16. However, 7S and 8Q may also be important for recognition by some T cells.

Discussion

It has been argued that T cells directed against poorly expressed determinants are likely to be the most dangerous and pathogenic self-reactive population. Within the thymus, dominantly expressed determinants will usually induce tolerance, whereas less well-expressed determinants may fail to cause negative selection of high affinity/avidity T cell clones [4-6]. However, when a determinant has absolutely no ability to gain access to its MHC groove during central tolerance induction, specific T cells directed against that determinant will be protected against thymic or peripheral deletion, no matter what their avidity for antigen. The result of such escape from deletion is a repertoire with a broad spectrum of T cell receptor avidities. Even T cells with the highest avidity therefore remain in the repertoire, and may become the

dominant “driver” autoreactive clone, if sufficient peptide-MHC complexes containing the self determinant become available to initiate an immune response. In such cases, the T cell response against this weakly displayed determinant is competitively favored, owing strictly to the high avidity T cell clone(s) [16]. Meanwhile, high affinity clones directed against well-displayed dominant determinant will have been tolerized, and this is one reason why MBP 7–20-specific clones are not frequent contributors to responses to MBP. The expression of weakly displayed self-determinants may be increased if there is a local inflammatory response to a microbe. In this setting, inflammatory cytokines such as IFN- γ and IL-6 may upregulate MHC expression and antigen processing.

In the case of the B10.PL or PL/J H-2^u mouse strains, the amino terminal acetylated 1–9 (Acl-9) determinant is exactly the type described above. It is “dominant” in the context of the myelin basic protein (MBP) molecule, despite its precarious expression state, owing to its induction of high affinity T cells that affect immune tolerance. In other work, we have shown that the dominant T cell clone arising after immunization with MBP or Acl-9 has a CDR3 sequence DAGGGYEQY (BV8S2/BJ2S7) and is of relatively high affinity, as well as of the same specificity as the hybridoma 172.10 [16]. It is the lone clone driving the pathogenic response [17]; as the [B10.PL] mouse enters remission, the BV8S2/BJ2S7 (length 9) clone disappears from the lymph node and the spleen [17]. The other T cells in the repertoire appear to be unable to cause disease. It is therefore just such cases that may be particularly relevant in autoimmune disease pathology; thus, within the residual self repertoire, the high affinity T cells may be the major culprits, driving states of autoimmunity.

The premise that tolerance is directed towards dominantly displayed determinants is founded upon two sets of experiments conducted in the hen eggwhite lysozyme (HEL) system. In the first set, in the H-2^a B10.A strain, experimentally tolerized with HEL, only the co-dominant determinants were rendered tolerant. T cells directly against poorly expressed determinants were still available for induction in the residual repertoire. In the second set of experiments in H-2^d HEL-transgenic mice, HEL-specific immune responses were compared in Tg and non-Tg animals. T cells directed toward well-displayed “dominant” self determinants were preferentially tolerized. Interestingly, the degree of tolerance induction was again found to be directly related to the amount of HEL expressed in the HEL-Tg animal [18]. Thus, the residual responsiveness (to cryptic HEL determinants) in the transgenic animals was quite different from the HEL-specific T cell response in wild-type animals, which was directed against dominant determinants. Likewise, the myelin basic protein (MBP)-specific T cell responses in MBP-deficient and MBP-expressing congenic mice, have shown that the MBP-specific responses mounted by shiverer (MBP-deficient) mice are functionally different to those seen in MBP-expressing congenic animals [5,6,19]. The BALB/c (H-2^d) wild-type mouse, for instance, responds to MBP residues 59–76 [20]. In contrast, when primed with MBP-CFA, the BALB/c MBP-deficient shiverer animal mounts a vigorous MBP-specific T cell response directed towards MBP residues 89–101 [5]. Thus, the MBP-specific T cell repertoire of the wild-type BALB/c mouse has been shaped in part by the endogenous expression of MBP. In particular, it appears that endogenous MBP induces deletion or inactivation of the T cell repertoire directed towards the immunodominant determinant of the MBP-deficient animal, 89–101, making this determinant appear cryptic/subdominant in the wild-type, MBP-sufficient counterpart [5].

Interestingly, SJL mice mount a vigorous T cell response to the self-peptide proteolipid protein (PLP) 139–151 due to a high precursor frequency of 139–151-specific T cells. This intact repertoire of self-reactive T cells exists because a splice variant (DM20) of PLP, which lacks the 139–151 portion, is the form of PLP expressed in the embryonic thymus. Thus, tolerance is achieved to all potential determinants on PLP with the exception of this region, leaving a 139–151 repertoire particularly poised to cause autoimmunity [2]. A similar situation exists

with respect to the 85–99 determinant of MBP. Worthy of note, the display of this determinant is inversely related to the level of a cysteine protease, asparagine endopeptidase (AEP). Since AEP is well expressed in the thymus the presentation of 85–99 is low, allowing 85–99-specific T cells to escape tolerance induction [21,22].

The experiments performed in this manuscript demonstrate that there are three overlapping, I-A^u-restricted determinants in the embryonic form of MBP, Golli-MBP: LDVM1-5; 1–9; and 9–16. When this triad was synthesized into a single peptide, the interactions between each determinant and the I-A^u pocket for which they competed could be studied by altering crucial amino acid residues. In the presence of the two flanking determinants of higher affinity for the MHC, 1–9 appears to be completely excluded from gaining refuge within the I-A^u groove. When either of the two flanking determinants are altered so as to lose their binding affinity in a peptide comprised of the determinant triad, 1–9 is not presented. Only when both flanking determinants are deprived of their anchor residues is 1–9 then freed to bind to I-A^u. Once able to bind to I-A^u, 1–9 within the triad reveals itself, capable of inducing a strong response to Acl-9-specific T cells to secrete IL-2.

The peptide LDVMASQKR uses its methionine-4 to bind strongly to I-A^u and outcompetes ASQKRPSQR which has a lysine in the 4 position, a poor binding residue. Since LDVMASQKR has alanine as residue 5, a position recognized by the T cells, it was predicted that a T cell different than 172.10 would respond to this peptide, which requires arginine at this position. This was the case, and accordingly, when the peptide LDVMRSQKR was tested, it proved to be an inducer of 172.10 (8). The combined evidence suggests that 4M and 5R would represent adequate minimal residues in constituting a determinant for 172.10.

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Figure 1. Within LDVM1-16 the MBP 9–16 region is dominantly expressed

Hybridomas specific to LDVM1-9 (A), Ac1-9 (B) or 7–20 (C) were cultured with splenocytes pre-pulsed with either LDVM 1-16 (squares -v-) or homologous peptide (diamonds -v-) for 4 hours at given concentrations in μM . Cultures were then maintained for 20 hrs and supernatants were analyzed by ELISA for IL-2 production. Results are expressed as absorbance readings with no-antigen-containing wells subtracted, i.e. delta OD's. Each point represents a mean of duplicate wells. Although hybridomas specific for LDVM1-9 and Ac1-9 were readily activated by their homologous peptides (diamonds -v-), they failed to recognize their homologous peptide from within the context of LDVM1-16. Only the MBP7-20-specific T cell hybridoma was able to respond to the longer triad LDVM1-16 peptide (squares -v-).

Figure 2. Disruption of the flanking determinants results in MBP 1-9 display

Peptides were synthesized with substitutions engineered to disrupt either the N-terminal MHC-binding register (SDVG1-16, circles - λ -), the C-terminal MHC-binding register (LDVM1-16 (12D,13E), diamonds - ν -) or both (SDVG1-16(12D,13E), asterisk - Ω -). In addition, a peptide with enhanced binding of the central 1-9 determinant was also synthesized (LDVM1-16(Y4), triangles - σ -). Hybridomas raised specific to LDVM1-9 (A), Ac1-9 (B) or 7-20 (C) were cultured with splenocytes pre-pulsed with the peptides described above at the noted concentrations (μM). Cultures were maintained for 20 hrs and supernatants were analyzed by ELISA for IL-2 production. (B) The Ac1-9-specific hybridoma did not respond to LDVM1-16 (squares - ν -) nor to peptides with disruptions in a single MHC-binding register. However it could respond well to (SDVG1-16(12D,13E), which contains disruptions in both flanking determinants. Similarly, when the central register was enhanced in (LDVM1-16(Y4), Ac1-9-reponsiveness was restored. These results clearly suggest a hierarchy of peptide display as follows: MBP 7-20 > LDVM1-5 > Ac1-9, revealing a scenario in which Ac1-9 is consistently out-competed for binding by each of its immediately flanking determinants.

Figure 3. Fixed APCs display a similar antigen hierarchy

Peptides were synthesized with substitutions engineered to disrupt either the N-terminal MHC-binding register (SDVG1-16, circles -λ-), the C-terminal MHC-binding register (LDVM1-16 (12D,13E), diamonds -ν-) or both (SDVG1-16(12D,13E), asterisk -Ω-). In addition, a peptide with enhanced binding of the central 1-9 determinant was also synthesized (LDVM1-16(Y4), triangles -σ-). Hybridomas raised to LDVM1-9 (A) or 7-20 (B) were cultured with **fixed** splenocytes pre-pulsed with the peptides described above at the noted concentrations (μM). Cultures were maintained for 20 hrs and supernatants were analyzed by ELISA for IL-2 production. The results are identical to Figure 2. (A) The LDVM1-5-specific hybridoma could not respond to LDVM1-16 (squares -ν-) but could respond to the longer peptide LDVM1-16 (12D,13E) (diamonds -ν-), which contains a.a. substitutions designed to disrupt the C-terminal register.

Figure 4. Truncated peptides identify the existence of three different core registers within the longer LDVM1-16 peptide

LDVM 1-5(A), Ac1-9(B) and 7-20 (C) specific T cell hybridomas were incubated with different truncated peptides at the noted μM concentrations. Each graph represents responses of a unique T cell hybridoma. LDVM1-9-specific T cells respond to LDVM1-13 (diamonds -v-) and LDVM1-9 (squares -v-). Ac1-9-specific hybridomas (B) prefer Ac1-9 (dashes -) over Ac1-20 (triangles -σ-) and do not respond to LDVM1-13 (diamonds -v-). 7-20-specific hybridomas (C) respond to truncated peptides 7-16 (circles -λ-) and 9-20 (crosses +) but fail to respond to LDVM1-13 (diamonds -v-). Thus, the core of this determinant is likely a.a. 9-16.

Table I

Peptides used in this study

The listed peptide represent single, dual, and triple overlapping registers. The dominant register(s) found after incubating individual hybridomas with the peptide are shown enbloc in grey. For example, LDVM1-16 is a 20-mer with all three determinants represented: however, only the 7–16 determinant induces a response, owing to its competitive dominance in MHC binding. In LDVM1-16(Y4), the tyrosine substitution for lysine leads to strong binding by each of the registers and codominance.

Peptide Name	Sequence
Ac1-9	Ac-ASQKRPSQR
Ac1-20	Ac-ASQKRPSQRSKYLATASTMD
7-20	SQRSKYLATASTMD
7-16	SQRSKYLATA
9-20	RSKYLATASTMD
LDVM1-9	LDVMASQKRPSQR
LDVM1-13	LDVMASQKRPSQRSKYL
LDVM1-16	LDVMASQKRPSQRSKYLATA
LDVM1-16(Y4)	LDVMASQYRPSQRSKYLATA
	↓
LDVM1-16(12D,13E)	LDVMASQKRPSQRSKDEATA
SDVG1-16	SDVGASQKRPSQRSKYLATA
SDVG1-16(12D,13E)	SDVGASQKRPSQRSKDEATA

Legend
Dominant Register
Y ↓	Increased MHC binding
SGDE * * * *	Decreased MHC binding