Brief Definitive Report

A Polyalanine Peptide with only Five Native Myelin Basic Protein Residues Induces Autoimmune Encephalomyelitis

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Summary

The minimum structural requirements for peptide interactions with major histocompatibility complex (MHC) class II molecules and with T cell receptors (TCRs) were examined. In this report we show that substituting alanines at all but five amino acids in the myelin basic protein (MBP) peptide Ac1-11 does not alter its ability to bind $A\alpha^uA\beta^u$ (MHC class II molecules), to stimulate specific T cells and, surprisingly, to induce experimental autoimmune encephalomyelitis (EAE) in (PL/J × SJL/J)F_1 mice. Most other amino acid side chains in the Ac1-11 peptide are essentially irrelevant for T cell stimulation and for disease induction. Further analysis revealed that binding to $A\alpha^uA\beta^u$ occurred with a peptide that consists mainly of alanines and only three of the original residues of Ac1-11. Moreover, when used as a coimmunogen with MBP Ac1-11, this peptide inhibited EAE. The finding that a specific in vivo response can be generated by a peptide containing only five native residues provides evidence that disease-inducing TCRs recognize only a very short sequence of the MHC-bound peptide.

Specific interactions between TCRs and peptides bound to MHC molecules are crucial for T cell stimulation (1). Class II molecules of the MHC bind peptides and present them to CD4+ Th cells (1, 2). Much of our understanding of peptide MHC interactions and their subsequent recognition by specific T cells has relied on the use of single amino acid substitutions in peptides to identify key amino acids required for binding to various MHC molecules and for stimulating antigen-specific T cells in vitro (3–7). To date, there is no evidence that antigen-specific in vitro and in vivo responses can be initiated by a peptide containing only a few principal MHC and TCR contact residues. To determine the minimum MHC class II binding and T cell recognition requirements for peptide, we studied presentation of the immunodominant N-terminal myelin basic protein (MBP) peptide Ac1-11, which induces experimental autoimmune encephalomyelitis (EAE) in (PL/J × SJL/J)F_1 mice (8). EAE is an inflammatory and demyelinating disease of the central nervous system that serves as a model for the human disease multiple sclerosis. Responses to Ac1-11 are restricted by $A\alpha^uA\beta^u$ (MHC class II) (5, 8). Critical amino acids in Ac1-11 have been identified for binding to $A\alpha^uA\beta^u$ and for recognition by the TCR (see below) (3, 4). Using peptide analogs of Ac1-11 consisting mainly of alanines, we have examined the minimum residues in Ac1-11 for establishing specific binding to $A\alpha^uA\beta^u$, for stimulation of specific T cells in vitro, and, more importantly, for inducing EAE. The data presented in this report imply that the overall conformation of the whole peptide (bound to MHC class II molecules) may not be crucial for T cell recognition in vitro and in vivo.

Materials and Methods

Animals. PL/J and (PL/J × SJL/J)F_1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Peptide Antigens. Peptides were synthesized by standard Fmoc chemistry, purified if necessary (90–99% pure), and structure confirmed by amino acid analysis and mass spectroscopy. Peptide Ac3.5 does not dissolve in PBS especially at higher concentrations. OVA 323–339 was biotinylated by a short chain NHS-biotin (*N*-hydroxy-succinimidebiotin) from Pierce Chemical Co. (Rockford, IL), as described (9). All amino acids are indicated in single letter codes.

Hybridoma Assay. The T cell hybridoma 1934.4 was established from the T cell clone PJR-25 (3). Activation of 1934.4 cells was assessed by measuring IL-2 production as described previously (3). In experiments where competition for antigen presentation was examined, competitor peptides were coincubated with Ac1-11, APCs, and 1934.4 for 24 h. Supernatants were harvested and assayed for IL-2 as above.

$A\alpha^uA\beta^u$ Transfectants. $A\alpha^uA\beta^u$ transfectants, in which the class II negative cell line M12.C3 (10) was used as a recipient, were prepared as described (9). One cell line expressing $A\alpha^u$ and $A\beta^u$

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was subcloned using FACS® (Becton Dickinson & Co., Mountain View, CA) by selecting the top 5% brightest cells and plating one cell per well. Clone 622.18 was selected and used in this study.

Cell-surface Peptide Binding Assay. A total of 10^5 cells (622.18), expressing $A\alpha^uA\beta^u$ were incubated either alone with biotinylated OVA 323–339 peptide (10 μM), or with competitor peptides (400 μM) and biotinylated OVA 323–339 (10 μM) together for 18–20 h. After washing, cells were incubated (30 min at 4°C) with streptavidin Texas red (Pierce Chemical Co.) (1 mg/100 ml cell suspension), and analyzed by flow cytometry (9). To measure the relative amount of streptavidin Texas red bound, the mean fluorescence was determined for at least 5,000 propidium iodide negative cells. The background, measured in the absence of biotinylated peptide, was subtracted from each signal. Specific binding is expressed as percent inhibition of the control binding of biotinylated OVA 323–339 in the absence of competitors using the following formula:

$$\text{percent inhibition} = 100 - \left[\left(\frac{\text{Signal with competitors-background}}{\text{Signal without competitors-background}} \right) \times 100 \right]$$

Induction of EAE. EAE was induced by injecting MBP Ac1-11 emulsified in CFA subcutaneously at the base of the tail in a total volume of 0.1 ml. For coimmunization, the inhibitor peptides were mixed in the same emulsion. 200 ng of pertussis toxin (JRH Biosciences, Woodland, CA) was injected intravenously at the time of immunization and again 2 d later. Mice were examined daily as described previously (3) and in figure legends.

Results

Peptide Containing only the Key MHC and TCR Residues Stimulate Ac1-11-specific T Cells In Vitro. Peptide analogs of Ac1-11 with multiple alanine substitutions were synthesized (Fig. 1 *A*). When the peptides were analyzed for stimulation of the Ac1-11-specific and $A\alpha^uA\beta^u$-restricted T cell hybridoma 1934.4, surprisingly few of the native amino acids were required (Fig. 1 *B*). The substituted peptides Ac3.5.6.10 and Ac3.5.6 stimulated 1934.4 better, and Ac3.4.5.6 stimulated less than wild type Ac1-11. All other peptides failed to stimulate the hybridoma 1934.4 (Fig. 1 *B*). The enhanced T cell stimulation by Ac3.5.6 and Ac3.5.6.10 is probably due to alanine at position four, which we know increases binding to $A\alpha^uA\beta^u$ (3, 4). These results demonstrate that only a minimum of four native amino acids (at positions 1, 3, 5, and 6) in Ac1-11 are required to establish sufficient contact with the MHC class II and the TCR to stimulate Ac1-11-specific T cells in vitro (see peptide Ac3.5.6). However, recognition by the TCR of a critical residue appears to be very specific since a conservative change at position 3 (glutamine to asparagine) in both Ac1-11 and Ac3.5.6 abrogates recognition of these peptides, although these peptides still bind to $A\alpha^uA\beta^u$ (data not shown).

To determine the minimum number of residues in Ac1-11 that bind $A\alpha^uA\beta^u$, the I-A negative cell line M12.C3 transfected with $A\alpha^uA\beta^u$ genes was used in a cell-surface binding assay (9, and see legend for Fig. 1 *C*). Binding of the wild type and substituted Ac1-11 peptides was assessed by competition with the biotinylated (OVA) peptide 323–339 by flow

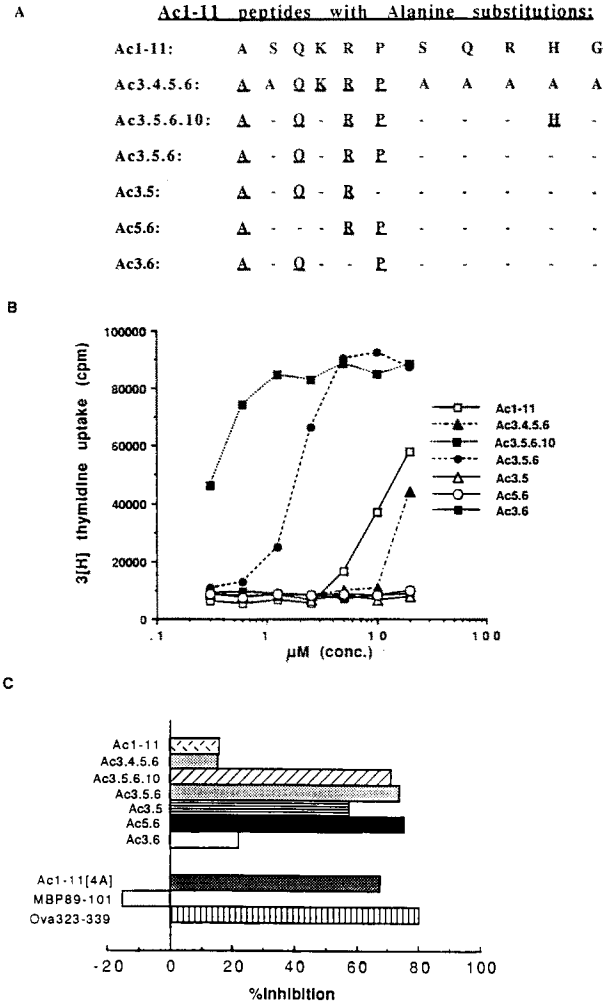

Figure 1. T cell responses to alanine analogs of Ac1-11. (*A*) Peptide analogs of Ac1-11. (*Top*) Sequence of Ac1-11 (single letter amino acid codes). (–) Alanine in place of the original residues. (*Underlined*) Residues in alanine peptides represent original amino acids. (*B*) Stimulation of an Ac1-11-specific hybridoma 1934.4 by Ac1-11 and alanine-substituted peptides. A total of 2×10^4 T cell hybridoma 1934.4 cells, peptides (0.3–20 μM), and 2.5×10^5 spleen cells from PL/J mice as APCs were incubated for 18–20 h. Culture supernatants were analyzed for IL-2 using the HT-2 cell line as described in Materials and Methods. (*C*) Cell surface binding assay. $A\alpha^uA\beta^u$ expressing cells (622.18) were incubated with 10 μM biotinylated OVA 323–339 either alone or with competitor peptides (400 μM) as in Materials and Methods. Inhibition of biotinylated OVA 323–339 binding to cell surface $A\alpha^uA\beta^u$ on a B cell transfectant by competitor peptides is shown (see Materials and Methods). Background for this experiment was 1.695, and signal with biotinylated OVA 323–339 was 8.643. Experiments were repeated at least three times with a similar pattern of inhibition of binding by competitor peptides.

cytometry (9). Binding of OVA 323–339 is specific, as only cell lines expressing $A\alpha^uA\beta^u$ and $A\alpha^dA\beta^d$ bind the peptide, but cell lines expressing $A\alpha^kA\beta^k$ or no class II do not (9). When used as competitors in this binding assay, the alanine-substituted peptides (Fig. 1 *A*) Ac3.5.6, Ac3.5.6.10, Ac3.5 (this peptide is poorly soluble in culture medium and PBS), and Ac5.6 efficiently competed for the cell-surface binding of biotinylated OVA 323–339. On the other hand, Ac3.4.5.6,

Ac3.6, and Ac1-11 consistently competed poorly (Fig. 1 *C*) (Ac1-11 has been shown to be a poor binder to $A\alpha^uA\beta^u$ [3]). These results indicate that the positively charged argenine at position 5 in Ac1-11 is important in binding to $A\alpha^uA\beta^u$, presumably interacting with a negatively charged residue in the MHC molecule. As controls, Ac1-11[4A] and OVA 323–339, which are known to bind $A\alpha^uA\beta^u$, inhibited binding of biotinylated OVA 323–339, but MBP89-101, a nonbinder, did not (Fig. 1 *C*). For the most part, binding of these alanine analogs correlated well with T cell stimulation in vitro. However, peptides Ac5.6 and Ac3.5, which failed to stimulate specific T cells, showed strong binding to $A\alpha^uA\beta^u$ in the cell-surface binding assay. Further evidence that peptides Ac5.6 and Ac3.5 bind to $A\alpha^uA\beta^u$ is shown by a functional competition assay in which these peptides compete with Ac1-11 for antigen presentation to T cells (Fig. 2). Therefore, inability of peptides Ac5.6 and Ac3.5 to stimulate T cells appears to be due to absence of the T cell recognition residue glutamine at position 3 in Ac5.6 and proline at position 6 in Ac3.5. Both glutamine and proline have previously been shown to be important for T cell recognition of Ac1-11 (3, 4).

Polyalanine Peptides with Key MHC and TCR Residues Induce EAE. The minimum length required for various peptides (including Ac1-11) to induce in vivo responses has been determined (5–7). However, how many amino acids in these peptides (self or nonself) are directly involved in initiating immune responses is not known. This may have direct implications for the induction of autoimmune diseases since self peptides (with only a few crucial recognition residues in the peptide) may be presented by susceptibility MHC class II molecules and recognized by pathogenic T cells. If a similar sequence containing these critical residues is found in a nonself protein (e.g., viral), this sequence then might trigger the onset of autoimmunity through molecular mimicry (11, 12). To examine this issue, we were particularly interested in determining whether peptides with multiple alanines could initiate responses in vivo. Since Ac3.5.6.10, Ac3.5.6, and Ac3.4.5.6 stimulated the T cell hybridoma 1934.4 (Fig. 1 *B*), they were used to induce EAE in (PL/J × SJL/J)F_1 mice. Data summarized from three separate experiments are shown (Table 1). Only peptide Ac3.4.5.6 was able to induce EAE with a similar incidence and severity as Ac1-11. Thus, we have shown that most other side chains are not necessary for specific functions such as MHC class II binding, T cell activation, and for EAE induction.

Nonstimulatory Polyalanine Peptide Inhibits EAE. Finally we examined whether the $A\alpha^uA\beta^u$-binding nonstimulatory alanine-substituted peptides (Ac5.6 and Ac3.5) could modulate EAE. Such modulation would indicate that these peptides may compete for MHC class II binding in vivo. Various studies have suggested that in vivo T cell responses, including EAE, can be inhibited by peptides that bind to MHC class II molecules (3, 9, 13–17). Since peptide Ac5.6 contains the minimum required amino acids for binding to $A\alpha^uA\beta^u$ (Fig. 1 *C*) and also competes well with Ac1-11 for presentation in vitro (Fig. 2), we asked whether it would inhibit EAE.

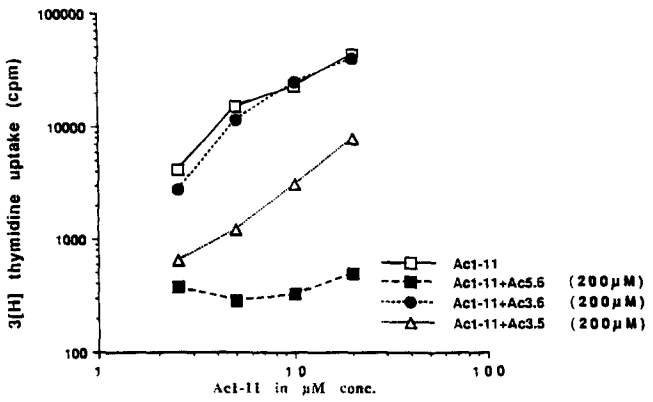

Figure 2. Competition for antigen presentation between Ac1-11 and nonstimulatory alanine-substituted peptides. Antigen presentation assays were performed as described in Fig. 1 *B*. Ac1-11 (2.5–20 μM) was coincubated with or without competitor peptides (200 μM) for the duration of the experiment. Supernatants were harvested and assayed for IL-2 as described in Fig. 1 *B*. Data is representative of at least three experiments.

Table 1. *Induction of EAE by Alanine-substituted Peptides*

Groups	Percent incidence	Mean clinical scores	Day of onset
Ac1-11	87 (27/31)	3.8	12.4
Ac3.4.5.6	80 (20/25)*	3.05	14
Ac3.5.6.10	15.7 (3/18)	0.51	15.75
Ac3.5.6	0	0	0

EAE in (PL/J × SJL)F_1 mice was induced as described in Materials and Methods. 100 nmol of peptides emulsified in CFA with 400 μg *Microbacterium tuberculosis* was injected subcutaneously at the base of the tail in a total volume of 100 μl for each peptide. Mice were examined daily for 30–35 d and were graded as follows: 1, limp tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, total paralysis; and 5, moribund.

* Chi-Square = 0.013, $P = 0.7$ when compared with Ac1-11 group.

Table 2. *Inhibition of EAE by Ac5.6*

Groups	Percent incidence	Mean clinical scores	Day of onset
Ac1-11	92 (25/27)	3.4	12.75
Ac1-11 + Ac5.6	40 (10/25)*	1.2	14.3
Ac1-11 + Ac3.6	100 (18/18)	3.5	13.3

EAE was induced as described in Table 1. For coimmunization, the competitor peptides (500 nmol) were mixed with Ac1-11 (25 nmol) in the same emulsion. Mice were scored daily as in Table 1. Data pooled from three separate experiments is shown.

* Chi-square = 14.0, $P = 0.0002$ when compared with Ac1-11 group.

When used as a coimmunogen with Ac1-11 in the induction of EAE, Ac5.6 had a substantial effect in reducing the incidence of EAE in (PL/J × SJL/J)F_1 mice (Table 2). Since Ac3.6, which is a weak binder (Fig. 1 *C* and Fig. 2) did not inhibit the disease (Table 2), the data suggest that Ac5.6 inhibits EAE by competing for the MHC class II binding of Ac1-11. Such inhibition of EAE by competitive binding of Ac5.6 contrasts markedly with disease prevention by Ac1-11[4A] (3, 17). Unlike Ac1-11[4A], Ac5.6 is non-crossreactive with Ac1-11. A recent report indicates that substituted peptide analogs can also act as antagonists for TCRs (18). The possibility that Ac5.6 may be inhibiting T cell responses in vitro and EAE in vivo by acting as an antagonist for TCRs is not ruled out.

Discussion

We have shown in this report that some Ac1-11 alanine-substituted peptide analogs, consisting mainly of alanines with just a few critical native amino acids, bind to $A\alpha^uA\beta^u$, stimulate specific T cells in vitro and most importantly, induce EAE. Previously, MHC class I mediated lysis has been inhibited by using competitor peptides substituted with either polyglycine or polyproline (19). Binding of a polyalanine peptide to HLA-DR1 has also been shown (20). However, these studies did not examine induction of T cell responses using these peptides. Our experiments demonstrate not only that MHC class II-restricted T cells can be stimulated in vitro, but also that EAE can be induced by a peptide that consists mainly of alanines. This suggests that very few amino acids need to interact with MHC class II molecules and with TCRs to initiate specific responses in vivo resulting in autoimmune disease. Moreover, by defining key residues in Ac1-11, we have also identified a peptide that binds $A\alpha^uA\beta^u$ but fails to stimulate Ac1-11–specific T cells. When used as a competitor, this peptide inhibits EAE in mice.

Overall conformation of the peptide-MHC complex has been implicated as a structure recognized by TCRs (1, 7, 21). Our data strongly suggest that, a peptide unrelated to the immunizing peptide can fulfill this requirement as long as the peptide contains the critical MHC and TCR contact residues. Molecular mimicry has been suggested as a mechanism for autoimmunity (12, 13). Since only four or five native residues in a peptide were able to induce EAE, it is conceivable that a pathogen (e.g., viral) with limited homology to self at few amino acid residues may trigger autoimmune disease.

Finally, we do not know whether a similar pattern of T cell stimulation in vitro and in vivo will be found in other antigen systems. Nevertheless, in the absence of a detailed structure of class II MHC-peptide complexes, this report provides a useful experimental system to study binding and T cell recognition of peptides, and to understand peptide conformation and orientation in the binding groove of MHC class II molecules.

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