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An Altered Peptide Ligand Mediates Immune Deviation and Prevents Autoimmune Encephalomyelitis

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Summary

In experimental autoimmune encephalomyelitis (EAE) induced with myelin proteolipid protein (PLP) peptide 139–151, we have previously shown that the disease is mediated by Th1 cells, which recognize tryptophan 144 as the primary TCR contact point. Here we describe an altered peptide ligand (APL), generated by a single amino acid substitution (tryptophan to glutamine) at position 144 (Q144), which inhibits the development of EAE induced with the native PLP 139–151 peptide (W144). We show that the APL induces T cells that are cross-reactive with the native peptide and that these cells produce Th2 (IL-4 and IL-10) and Th0 (IFNγ and IL-10) cytokines. Adoptive transfer of T cell lines generated with the APL confer protection from EAE. These data show that changing a single amino acid in an antigenic peptide can significantly influence T cell differentiation and suggest that immune deviation may be one of the mechanisms by which APLs can inhibit an autoimmune disease.

Introduction

The paradigm of T helper cell differentiation from naive precursors into type 1 helper T cells (Th1) and Th2 cells has proven to be a powerful tool in understanding the outcome of host responses to infection and autoimmune diseases (Powrie and Coffman, 1993). Naive $CD4^+$ T lymphocytes triggered by antigen differentiate into at least two subpopulations, each producing its own set of cytokines and mediating distinct effector functions. Th1 cells produce interleukin 2 (IL-2), tumor necrosis factor-β, and interferon-γ (IFNγ), activate macrophages, and induce delayed type hypersensitivity (DTH) responses. Th2 cells produce IL-4, IL-5, and IL-10, stimulate the production of mast cells, eosinophils, and immunoglobulin E (IgE) antibodies, and regulate cell-mediated immunity (Mosmann and Coffman, 1989). IL-4 and IFNγ show reciprocal inhibition and IL-10 inhibits the production of IFNγ and other Th1 cytokines by interfering with antigen presentation by macrophages (Paul and Seder, 1994). These two cell populations cross-regulate one another because their respective cytokines act antagonistically (Swain, 1993; Street and Mosmann, 1994).

The autoreactive T cells that induce organ-specific autoimmune diseases including experimental autoimmune encephalomyelitis (EAE) generally display a Th1 phenotype (Miller and Karpus, 1994). We (Kuchroo et al., 1992) and others (Ben-Nun and Cohen, 1982; Zamvil and Steinman, 1990; Baron et al., 1993) have generated T cell clones specific for different encephalitogenic epitopes of the myelin antigens, myelin basic protein (MBP), or myelin proteolipid protein (PLP). The adoptive transfer of these myelin antigen-reactive Th1 but not Th2 cells can induce EAE. In addition, during progression of EAE, Th1 cytokines are present in the inflammatory EAE lesions in the CNS, whereas Th2 cytokines are absent, strongly suggesting that Th1 cytokines play a role in the pathogenesis of the disease (Khoury et al., 1992; Merrill et al., 1992). On the other hand, regulatory T cells that suppress the development of EAE produce cytokines that correspond to the Th2 profile (Karpus et al., 1992; Chen et al., 1994) and recovery from EAE in mice and rats is associated with an increase in the presence of Th2 cells and cytokines in the CNS (Khoury et al., 1992; Kennedy et al., 1992). These findings, along with the observation that Th2 cytokines can inhibit the actions of inflammatory Th1 cytokines, suggest that the induction and activation of Th2 cells may potentially prevent EAE and other autoimmune diseases mediated by Th1 cells. Indeed, Racke et al. (1994) have recently shown that cytokine-induced immune deviation can be used as a therapy in inflammatory autoimmune diseases, which supports this hypothesis. To generate Th2 responses, they administered IL-4 to animals that were immunized for the development of EAE. This treatment resulted in amelioration of clinical disease, the induction of MBP-specific Th2 cells, and the inhibition of proinflammatory cytokines in the CNS.

Human T cells elaborating either Th1 or Th2 type cytokines have also been described, and it has been proposed that in leprosy, AIDS, and other human diseases these subtypes may affect the clinical outcome of disease (Salgame et al., 1991; Romagnani, 1994; Mosmann, 1994). Because of the importance of Th differentiation in a number of diseases, considerable effort is being devoted to elucidating the factors that influence the process. In a transgenic model of spontaneous autoimmune disease, non-major histocompatibility complex (MHC)-encoded genes influence T cell differentiation and this correlated with the susceptibility or resistance to disease (Scott et al., 1994). Similarly, in murine models of Leishmaniasis, the elaboration of IL-4 or IFNγ in mice of different genetic backgrounds correlates with disease progression or resolution (Reed and Scott, 1993; Belosovic et al., 1989; Sadick et al.,

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1990). A variety of experimental models have shown that the production of IL-12 by macrophages and IFNγ by natural killer cells promotes the differentiation of naive T cells into Th1 cells and inhibits their differentiation into Th2 cells. Conversely, IL-4 is necessary for Th2 differentiation and inhibits the development of IFNγ-secreting cells (Hsieh et al., 1993; Paul and Seder, 1994). The route of administration of antigen also influences T cell differentiation and the outcome of disease. In a mouse model of EAE, oral administration of MBP leads to the generation of Th2 cells and these animals are resistant to the development of disease (Chen et al., 1994). Finally, the concentration of peptide antigen has been shown to affect the type of immune response, suggesting that ligand density may have a role in determining the outcome of differentiation (Murray et al., 1992). In an extension of these studies, it has been shown that following immunization with a human collagen IV peptide or altered peptides derived from it, IL-4 mRNA is detectable by polymerase chain reaction (PCR) after activation of $CD4^+$ T cells derived from animals immunized with the altered peptide ligand (APL), but not following activation of $CD4^+$T cells from animals immunized with the native peptide (Pfeiffer et al., 1995).

To investigate whether antigen structure could alter T cell differentiation, and affect the development of an autoimmune disease, we have used a well-characterized autoimmune model in which SJL mice ($H\text{-}2^s$) immunized with PLP 139–151 (W144) in complete Freund's adjuvant (CFA) develop EAE (Sobel et al., 1994). Immunization with this peptide generates PLP-specific Th1 cells, which can induce EAE when transferred into naive animals. The disease differs from that caused by MBP in that the PLP 139–151-specific T cell clones show a greater diversity of T cell receptor (TCR) usage (Kuchroo et al., 1994a). Despite this diversity, the majority of T cell clones recognize the peptide in the same way, with tryptophan (W) at position 144 as the primary TCR contact residue, and histidine (H147) and leucine (L141) as the secondary residues (Kuchroo et al., 1994b). Since W144 is the critical residue for normal recognition of the peptide, we made a large panel of peptides with single amino acid substitutions at this position. A peptide in which the tryptophan was changed to glutamine (Q144) induced a T cell response, which was strikingly different from that induced by the native peptide and also prevented the development of EAE induced by the native peptide.

Results

The APL Q144 Protects Mice from EAE

To determine whether the altered peptide itself can induce EAE, animals were immunized with Q144 in CFA. No clinical disease was detected up to 42 days following immunization with this peptide, although all animals concurrently immunized with the native peptide, as expected, developed severe EAE (data not shown). We next investigated whether coimmunization of Q144 peptide with the native PLP 139–151 would affect the induction of EAE. Results from a representative experiment (Figure 1) show that

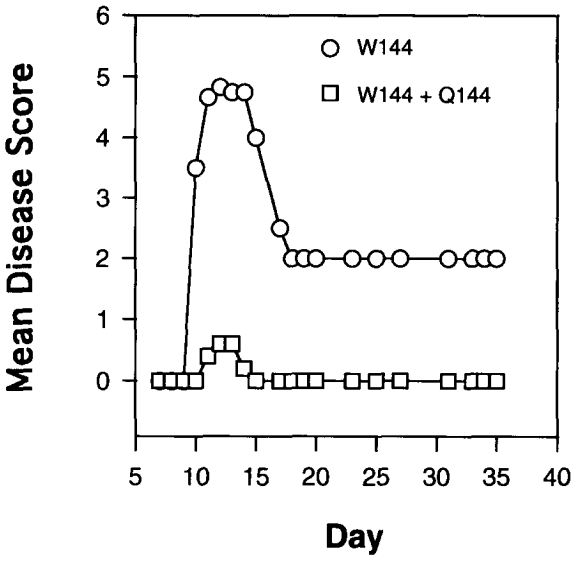

Figure 1. Coimmunization with Q144 Ameliorates EAE

Animals were immunized with the native peptide W144 alone (open circles) or coimmunized with W144 and Q144 at a ratio of 1:5 (open squares) and assessed daily for signs of clinical disease.

coimmunization of altered peptide with the native peptide reduced the incidence and mean severity of disease. The effect of the altered peptide on EAE was then tested in a larger series of experiments in which Q144 was either given before (preimmunization) or together with (coimmunization) the encephalitogenic PLP 139–151 peptide. The results show that most of the animals (about 70%) preimmunized with Q144 were protected from disease and in those animals that became sick there was a significant reduction in the clinical disease (Table 1). The mean day of onset of disease was later in the group preimmunized with Q144 than in the control group (no preimmunization) or animals immunized with L144 or T144. Coimmunization of Q144 with the native peptide (W144) also protected animals from disease (5 of 10), decreased disease severity but did not delay disease onset, and in the mice that did have clinical signs reduced the amount of CNS inflammation.

The Peptide Q144 Is Not an MHC Blocker but Induces T Cells that Are Cross-Reactive with the Native Peptide

A number of studies have shown that unrelated synthetic peptides or altered self-peptides may bind to MHC class II molecules more efficiently than the autoantigenic peptide inhibiting its binding and preventing the development of autoimmune disease (Lamont et al., 1990). To study whether Q144 functions by a similar mechanism, we tested the lymph node cells (LNCs) from SJL mice immunized with Q144, the native PLP 139–151 peptide, or a mixture of both for in vitro proliferative responses against various antigens (Figure 2). The LNCs from animals immunized with the native peptide proliferated in vitro to the immunogen but not to a control PLP peptide (PLP 190–209), or to other peptides with a single substitution at posi-

Table 1. Immunization with Altered Peptide Ligands Protects Mice from EAE

Peptide		Clinical Disease			Histological Disease: Number of Inflammatory Lesions	
Preimmunized	Immunized	Incidence	Mean day of onset	Mean maximum severity	With clinical disease (n)	Without clinical disease (n)
Preimmunization						
None	W144	13/13	15.1 ± 1.4	3.3 ± 0.3	74 ± 25(4)	–
Q144	W144	4/13[a]	23.8 ± 6.5	2.3 ± 0.5[c]	61 (1)	11 ± 2 (3)
L144	W144	4/7	16.0 ± 1.3	3.2 ± 0.6	136 ± 6 (3)	24 (1)
T144	W144	4/4	14.5 ± 0.5	2.3 ± 0.5	35 ± 10(4)	–
Coimmunization						
None	W144	9/10	10.3 ± 0.2	4.4 ± 0.5	100 ± 25(5)	ND
None	Q144	0/14	–	–	–	ND
None	W144 + Q144 (1:5)	4/10[b]	12.0 ± 0.6	2.1 ± 0.4[c]	7 (1)	1 ± 0.5 (4)
None	W144 + L144 (1:5)	4/5	12.5 ± 0.3	2.1 ± 0.4[c]	37 ± 8 (4)	0 (1)

[a] $p < 0.001$
[b] $p < 0.05$ by Fisher's exact probability test (compared with controls)
[c] $p < 0.001$ by t test (compared with controls)
EAE was induced in SJL mice by immunization with W144 (native PLP 139–151) emulsified in CFA as described. Mice were preimmunized with 100 μg of altered peptide emulsififed in CFA 20–40 days prior to the induction of disease or coimmunized with W144 and the altered peptide at the ratio shown. ND, not determined.

tion 144 (Q144 or A144), or a peptide with a double substitution at positions 144 and 147 (L144/R147). In contrast, LNCs from animals immunized with Q144 showed a much more degenerate response in that they proliferated to the same extent when stimulated in vitro with the native peptide W144 or Q144, and responded even more vigorously to a mixture of native peptide and Q144. When animals were immunized with the native peptide and Q144 peptides together, the LNCs responded well both to W144 and to a mixture of native peptide and Q144, but the response to Q144 alone was reduced. These data suggest that the Q144 peptide does not inhibit the generation of T cells specific for the native PLP peptide. Coimmunization of the native peptide with Q144, however, lowers the T cell responses specific for Q144. This may be due to competition for I-A^s binding during the initial T cell induction, since the native W144 peptide binds to I-A^s more efficiently than does Q144 (Kuchroo et al., 1994b).

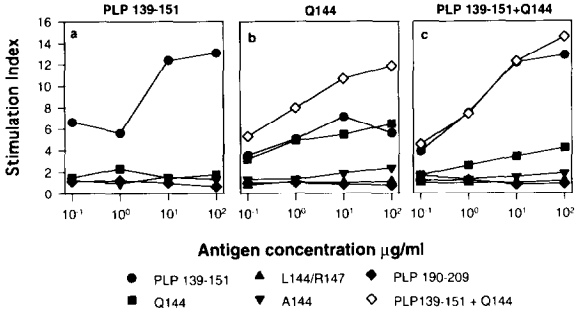

Figure 2. Proliferative Response of LNC from Mice Immunized with W144, Q144, or W144 plus Q144

Mice were immunized with W144 (a), Q144 (b), or W144 plus Q144 (c). LNCs were stimulated in vitro 10 days following immunization with the antigens indicated. Mice immunized with a single peptide received a total of 100 μg of antigen; mice immunized with a mixture of PLP 139–151 and Q144 received 100 μg of native peptide and either 100 μg or 300 μg of Q144. There were no significant differences when the peptides were used at either of these ratios and the data are pooled in (c). In these experiments, when a mixture of peptides was used to activate LNCs the total peptide concentration was the sum of the individual peptide concentrations. The results are expressed as a stimulation index (SI) defined as mean cpm with antigen/mean cpm with medium alone. The results shown are the average SI of three to five independent experiments.

Immunization with Q144 Alters the Pattern of Cytokine Production

To compare the type of T cell response generated in mice immunized with Q144 with that induced by the native peptide, the cytokines elaborated by LNCs taken from these animals 10 days after immunization were measured (Table 2). LNCs from animals immunized with native W144 peptide proliferated and produced significant amounts of IL-2 and IFNγ in vitro when stimulated with the native peptide but not when stimulated with Q144 or A144. This Th1 pattern of cytokine production is consistent with the pattern of response seen in T cell clones derived from mice immunized with W144 (Kuchroo et al., 1993), and with the known effects of CFA upon the outcome of T cell differentiation (Janeway et al., 1988). In contrast, LNCs from animals immunized with Q144 proliferated quite vigorously when activated with either Q144, native W144, or A144. When these LNCs were activated by Q144, significant levels of IL-2, IFNγ, and IL-10 were detected. IL-4 was measured by enzyme-linked immunosorbent assay (ELISA) or using CT4S cells and was less than 2× background in all assays. The data suggests that immunization with Q144 alters the phenotype of the T cells induced, leading to cells producing IL-10 in addition to cells producing IL-2 and IFNγ.

To determine the type of cells involved in the antigen-specific proliferation and production of various cytokines

Table 2. Cytokine Production by Lymph Node Cells from Mice Immunized with the Native Peptide W144 or the Altered Peptide Q144

		Antibody Treatment							
		Control				Anti-CD4			
			pg/ml				pg/ml		
Immunization	Activation	ΔCPM	IL-2	IFN γ	IL-10	ΔCPM	IL-2	IFN γ	IL-10
W144	W144	**109 100**	**170**	**4250**	75	**13 300**	<50	**1300**	<50
	Q144	**13 900**	60	260	<50	4000	<50	380	<50
	W144 + Q144	**84 500**	70	**1030**	<50	**12 300**	<50	600	<50
	A144	**8600**	<50	<100	<50	3500	<50	270	<50
	p190	300	<50	<100	<50	3600	<50	240	<50
Q144	W144	**21 300**	<50	270	<50	**5700**	<50	260	<50
	Q144	**91 300**	**150**	**4450**	**160**	**32 300**	<50	**1270**	<50
	W144 + Q144	**83 400**	120	**2760**	140	**23 200**	<50	**1300**	<50
	A144	**21 100**	<50	<100	60	**4800**	<50	170	<50
	p190	0	<50	170	<50	1500	<50	<100	<50

Groups of SJL mice were immunized with 100 μg/mouse of either the native W144 peptide or Q144 in CFA. LNCs were harvested 10 days after immunization and activated with 10–50 μg/ml of the relevant peptide in the presence of control antibody (KJ-16) or anti-CD4 antibody (GK1.5). When peptides were mixed, the total peptide concentration was kept constant and the ratio of peptides was 1:1. Supernatants were collected 24 and 40 hr after activation and cytokine concentrations were determined as described. Values >3× baseline are highlighted. One representative experiment of four is shown.

following in vivo immunization with the altered peptide Q144, monoclonal antibodies (MAbs) specific for anti-CD4, anti-CD8, and anti-TCR Vβ8.1/Vβ8.2 (as an isotype-matched control antibody) were added to the LNC at the time of in vitro activation. Data with the control anti-Vβ8.1/Vβ8.2 MAb and anti-CD4 antibody are shown (Table 2), the results obtained with the anti-CD8 antibody were similar to the control MAb (data not shown). In the LNCs taken from mice immunized with the native PLP peptide there was a dramatic inhibition (88%) of proliferation and IFNγ production in the presence of anti-CD4 MAb. In the LNCs from mice immunized with the altered peptide Q144 and activated with Q144 peptide, addition of anti-CD4 antibody inhibited proliferation by 65% and IFNγ production by over 70%. IL-2 and IL-10 were completely inhibited by anti-CD4 antibody. These data suggest that the immune deviation (with increase in IL-10 production) mediated by the altered peptide Q144 is dependent upon the antigen-specific activation of $CD4^+$ helper/inducer T cells.

T Cells Specific for Q144 Cross-React with the Native Peptide and Produce Th0 or Th2 Cytokines

To analyze the cells induced by Q144 further, T cell lines were generated from SJL mice immunized with W144 or Q144. A T cell line derived from animals immunized with native peptide W144 (WL.1) has properties that recapitulate those found in primary LNC cultures, with a dominant proliferative response to W144 and production of the Th1 cytokine IFNγ (Figure 3). The T cell line WL.1 also produced IFNγ upon activation with the altered peptides Q144 and A144 and, in some experiments, low levels of IL-10 were detected when the line was stimulated with Q144. The T cell line derived from animals immunized with Q144 (QL.1) demonstrated greater cross-reactivity in that it showed a significant proliferative response to the Q144, native, and A144 peptides and lower responses to the L144/R147 peptide (Figure 3), but showed no response to an unrelated PLP peptide (data not shown). The line produced high levels of both IFNγ and IL-10 in response to these antigens.

To analyze whether IL-10 and IFNγ were produced by the same cell, the Q144-specific T cell line (QL.1) was cloned by limiting dilution and screened for cytokine production (Figure 4). Of the 48 clones that were established, 27 produced significant levels of cytokine following activation with Q144. IL-10 was the commonest cytokine detected, produced by 22 of 27 (81%) clones. Nine clones produced only IL-10, five produced IL-4 and IL-10, four

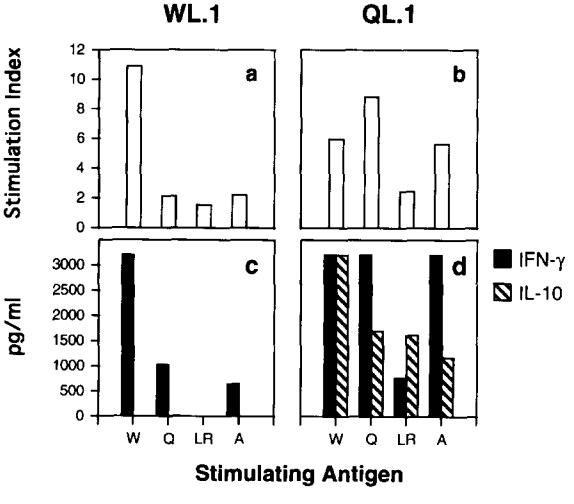

Figure 3. Proliferative Response and Cytokine Production by T Cell Lines Specific for native W144 (WL.1) or Q144 (QL.1) Peptides

(a, b) Proliferative response.

(c, d) Cytokine production.

The T cell lines were activated with various antigens (50 μg/ml): W (W144, the native peptide), Q (Q144), LR (L144/R147), and A (A144). The proliferative response was measured in triplicate and expressed as a mean stimulation index calculated as described in Figure 2. Cytokine concentrations in culture supernatants taken at 40 hr were measured by ELISA.

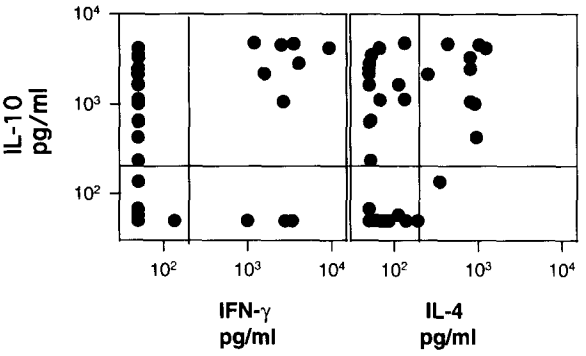

Figure 4. T Cell Clones Specific for Q144 are Th2- and Th0-Like

Clones (48) derived from QL.1 were activated with 20 μg/ml of Q144. The supernatants from the clones were taken at 40 hr and tested for the production of Th1 (IFNγ) and Th2 (IL-10, IL-4) cytokines. The amount of cytokine produced by each clone is displayed in terms of IFNγ versus IL-10 and IL-4 versus IL-10 to determine whether the Q144-specific clones were of Th0 (IFNγ, IL-10) or Th2 (IL-4, IL-10) phenotype. The righthand panel shows that 9 clones made high levels of IL-4. Of these, 7 were classified as Th2 and 2 as Th0. The lefthand panel shows that 7 clones secreted IL-10 and IFNγ together. Of these, 3 also secreted other cytokines.

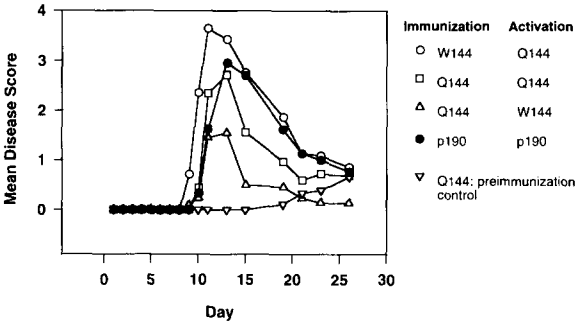

Figure 5. Transfer of Protection by Short-Term T Cell Lines

SJL mice were immunized with W144, Q144, or PLP 190–209 in CFA. LNC were harvested 10 days later and activated in vitro with 20 μg/ml of the relevant peptide. Live cells were purified 4 days after activation by Ficoll–Hypaque centrifugation, resuspended in phosphate-buffered saline, and transferred into naive mice as described. Mice were simultaneously immunized with PLP 139–151/CFA for disease. The results presented are the mean disease scores of each group from three independent experiments.

produced IL-10 and IFNγ, and four clones produced a mixture of three or four cytokines (IL-10, IL-4, IL-2, and IFNγ). Of the remaining five clones, three produced only IFNγ, one produced IL-2, and one produced IL-4. These results clearly show that immunization with the APL has resulted in the development of predominantly Th2/Th0 cells producing high levels of IL-4 and IL-10, and in the generation of a population of cells simultaneously secreting IL-10 and IFNγ. A panel of 16 Q144-specific T cell clones was selected at random and tested for cross-reactivity to the native W144 peptide. As assessed by T cell proliferation assay 44% (7 of 16) of these Q144 specific T cell clones cross-reacted with W144 (data not shown).

T Cell Lines Induced by Q144 Protect Mice from EAE

To determine whether T cells induced by immunization with Q144 were able to transfer protection, naive SJL mice were immunized with either W144, Q144, or a nonencephalitogenic peptide PLP 190–209. Short-term T cell lines were generated from LNCs prepared from mice 10 days after immunization and activated in vitro with immunizing or control peptides. LNCs from mice immunized with W144 and Q144 were also activated with Q144 and W144, respectively, to generate cross-reactive T cell lines. After 4 days in culture, T cell blasts were harvested and transferred into naive mice, which were then actively immunized with W144/CFA to induce EAE. The course of the disease was followed for 26 days (Figure 5). As a positive control, mice preimmunized with Q144 were further immunized with PLP 139–151 and followed for disease progression in the same experiment.

Transfer of T cell lines generated from the LNCs of mice immunized with native W144 and activated with W144 (data not shown) or Q144 accelerated the onset of clinical disease by 1–2 days and enhanced the maximum disease severity when compared with the control mice that were injected with a T cell line specific for an unrelated PLP peptide 190–209. In contrast, the T cell lines generated from mice immunized with Q144 and activated in vitro with Q144 conferred some protection from clinical disease but the greatest protection was seen after transfer of the T cell line generated from mice immunized with Q144 and activated in vitro with W144. This demonstrates that immunization with Q144 induces cells that protect mice from EAE, but that these T cells are cross-reactive and need to be activated by the native peptide to mediate protection.

Discussion

We have shown that immunization with an autoantigenic peptide altered at the primary TCR contact point (Q144) protects mice from the development of EAE. This protection is not due to MHC blockade. Rather, immunization with the altered peptide induces T cells of a Th0 and Th2 phenotype. Furthermore, the T cells generated by immunization with the altered peptide are degenerate in their recognition in that they are activated by not only the immunizing antigen (Q144) but also the native peptide W144 and several analogs of this peptide. The transfer of short-term lines derived from mice immunized with the altered peptide protects animals from the induction of disease by the native peptide. Although APLs have been shown to have multiple functions, including induction of anergy, TCR antagonism and partial activation (Evavold et al., 1993; Sette et al., 1994), our data suggest that APLs can also influence T cell differentiation and thereby affect the overall disease course.

Mechanism by which Unrelated Synthetic Peptides or Altered Self-Peptides Inhibit Development of Autoimmune Diseases

A number of studies have shown that some peptides can prevent the development of autoimmune disease by MHC blockade. These peptides compete with self-peptides for binding to MHC class II molecule(s) and inhibit disease

(Lamont et al., 1990; Sakai et al., 1989). Several lines of evidence show that Q144 does not inhibit the generation of W144-specific T cells, and suggest that the Q144 does not inhibit disease in vivo by MHC blockade. First, preimmunization of mice with Q144, 4–6 weeks prior to the immunization with the native peptide prevents EAE. Second, transfer of T lines that are derived from mice immunized with Q144 only and then activated in vitro with W144 (conditions under which there is no chance of MHC blockade) transfer protection (Figure 5). Third, the relative binding affinity of Q144 and W144 to I-A^s is not significantly different (Kuchroo et al., 1994b). And fourth, in vitro, LNCs from animals immunized with W144 plus Q144 are activated almost equally by W144 alone or a mixture of W144 and Q144 (Table 2).

Analogs of disease-inducing self-peptides have been demonstrated to inhibit the disease process in several experimental autoimmune diseases (Gautam et al., 1992; Wauben et al., 1994). These analogs are structurally similar to the native peptide and MHC blockade is not the only mechanism responsible for disease inhibition (Janeway, 1989; Wauben et al., 1992). A number of peptide analogs that inhibit clinical disease have been described but the actual mechanism(s) contributing to the inhibition of disease have been difficult to define (Wraith et al., 1989; Smilek et al., 1991). Our data offer an explanation for these results and suggest that some peptide analogs may mediate their protective effects by immune deviation.

Recently, it has been demonstrated that both modification of antigenic peptides at TCR contact points (Evavold et al., 1993) and subtle changes in MHC molecules (Racioppi et al., 1993) can generate altered TCR ligands that have multiple functions. Some of the peptide analogs are capable of binding to the TCR but are incapable of triggering a response and thus act as pharmacological antagonists (TCR antagonists) (De Magistris et al., 1992; Kuchroo et al., 1994b; Franco et al., 1994); some may interact with the TCR and anergize the responding cells (Sloan-Lancaster et al., 1993); and some analogs have been shown to activate T cells partially (Evavold and Allen, 1991). These studies indicate that an altered TCR ligand can interact with the TCR and induce changes in the responding cells without evoking a full repertoire of effector functions. Our results suggest that the Q144 peptide does not act as an MHC blocker and does not have TCR antagonist activity (data not shown). Instead, this APL affects T cell differentiation in vivo and induces T cells that are of a Th0 or Th2 phenotype rather than autoaggressive disease-inducing Th1 cells. Although IL-4 protein is undetectable in the primary culture, cells secreting IL-4 may be present in the mixed population that responds to Q144, but remain inhibited in their expression of cytokine by the high concentration of IFNγ. This implies that analogs themselves may regulate immune responses and adds a novel mechanism by which the altered self-peptides may function in vivo.

Role of Antigen in T Cell Differentiation

T cell differentiation is a complex process that is known to be influenced by the microenvironment. Cytokines are clearly an important part of this milieu but other factors may also have a role (Scott et al., 1994; Kuchroo et al., 1995). In the present experiments, we have demonstrated that the alteration of a single amino acid leads to a change in the phenotype of the responding T cell. This result is particularly surprising since all immunizations were carried out with peptide emulsified in CFA, an adjuvant which strongly favors the generation of Th1 cells (Janeway et al., 1988). The generation of cells secreting IFNγ and IL-10 together may reflect the combined influence of the high levels of IL-12 known to be induced by mycobacteria, in the presence of an APL that favors the generation of Th2 cells, since this phenotype is neither Th1 nor Th2. Whether these cells are terminally differentiated or whether they represent a form of Th0 cell type has yet to be established.

The molecular mechanisms by which altered antigen affects T cell differentiation are unknown. Q144 binds I-A^s with approximately half the affinity of the native peptide (Kuchroo et al., 1994b) and substitution at position 144 could significantly affect its ability to stabilize MHC complexes, a property which has been shown to influence immunogenicity (Nelson et al., 1994). These differences might lead to changes in ligand density, MHC-peptide conformation, or both, which could alter the pattern of cytokines secreted or the expression of adhesion and costimulatory molecules by the antigen-presenting cell (APC).

Alternatively, an APL may induce differential signaling through the TCR of naive T cells (Evavold et al., 1993; Sloan-Lancaster et al., 1994; Madrenas et al., 1995). This differential signaling through the TCR in vivo might lead to the release of cytokines (such as IL-4) from Th precursor cells, which act upon the T cell in an autocrine fashion, committing T precursor cells to a Th2 lineage even in a microenvironment that favors the generation of Th1 cells. In support of our data, APLs have recently been shown to affect T cell differentiation and induce IL-4 mRNA in LNC culture, although IL-4 was not detectable in culture supernatant (Pfeiffer et al., 1995). Similarly, it has also been shown that human T cell clones may change their cytokine profile when activated in vitro with analogs of the native autoantigenic MBP peptide, providing further evidence of a physiological role for this process (Windhagen et al., 1995). Another possibility is that the native antigen may induce qualitatively different intracellular signals than those elicited by the peptide analogs, leading directly to differences in lineage commitment. One prediction of this model would be that differentiation and lineage commitment can occur independently of the cytokine environment. Several possible mechanisms have been proposed to explain how the TCR may transduce differential signals, including ligand density and conformational changes within the TCR itself (Janeway and Bottomly, 1994), but their role in T cell differentiation has not been tested.

The APL Q144 Induces T Cells that Are Degenerate in their Recognition and Transfer Protection

Immunization with Q144 produced a population of T cells that is more cross-reactive than the T cells induced by immunization with the native peptide W144. Position 144 is the primary TCR contact point of the native peptide and

peptides with substitutions at this residue are not recognized by a panel of W144-specific T cell clones (Kuchroo et al., 1994b). One explanation for the greater cross-reactivity of the Q144-specific cells is that they may use another residue as the primary TCR contact point, which is common between Q144 and the native peptide, and therefore substitutions at position 144 are tolerated. This would suggest that Q144-specific T cells are derived from different precursors than cells induced with the native peptide, since TCRs recognize a different part of the peptide. Alternatively, naive T cells specific for W144 and with the potential to become Th1 cells if activated with the native peptide, might differentiate into Th2-like cells when activated by Q144. Recently, attention has been drawn to the potential importance of cross-reactivity in maintaining long-term immunological memory (Matzinger, 1994), and the same phenomenon may be important in the development and regulation of autoimmune disease.

In this study, we directly demonstrate the generation by immunization with Q144 of T cells that protect animals from EAE. Cells from animals immunized with Q144 and activated in vitro by W144 were the most effective in this regard, confirming the importance of cross-reactive T cells in this system. These experiments support the hypothesis that antigen can influence the outcome of T cell differentiation and suggest a novel mechanism by which APLs can protect animals from EAE. This protection arises because the Q144-specific T cells have at least two important properties: they cross-react with the native peptide and can therefore be activated by the native antigen in the CNS, and they secrete Th2 cytokines upon activation with either native or Q144 peptide. Th2 cells are known to inhibit Th1 responses via the production of IL-4 and IL-10 and, moreover, adoptive transfer of PLP 139–151-specific Th2 cells can protect mice from EAE (Kuchroo et al 1995). In a preliminary experiment, we have examined the effect of anti-IL-4 antibody on protection mediated by Q144 in vivo and found that this treatment abrogated protection (L. B. N. and V. K. K., unpublished data). This provides further support for the hypothesis that Q144 mediates protection by inducing regulatory Th2 cells in vivo. The ability of altered peptides to modify the immune response from a pathogenic to a protective one may have significant implications for autoimmunity. In addition, the peptide analogues will be useful tools for studying intracellular signaling and for dissecting the influence of antigen on the process of T cell differentiation.

Experimental Procedures

Animals

Female (4- to 6-week-old) SJL mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and housed under virus-free conditions. They were maintained in accordance with the guidelines of the Committee on Animals of Children's Hospital and Harvard Medical School, Boston.

Antigens

Peptide antigens for in vitro studies were synthesized by Dr. R. Laursen on a Milligen model 9050 synthesizer using Fmoc chemistry. Milligen PAL amide resins were used to produce peptides with C terminal amides. Most peptides were >90% pure, as determined by high pressure liquid chromatography (HPLC), and were not purified further. For disease induction, HPLC-purified peptide PLP 139–151, obtained from Alkermeres, Incorporated (Cambridge, Massachusetts) was used in some experiments. The peptides used in these experiments were PLP 139–151 (HSLGKWLGHPDKF), Q144 (HSLGKQLGHPDKF), A144 (HSLGKALGHPDKF), T144 (HSLGKTLGHPDKF), L144/R147 (HSLGKLLGRPDKF), and PLP 190–209 (SKTSASIGSLCADARMYGVL). Single letter abbreviations for the amino acids are the following: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Induction and Assessment of EAE

Mice were injected subcutaneously at two sites with W144 emulsified in CFA and supplemented with Mycobacterium tuberculosis H37 RA (500 μg/mouse; Difco, Detroit, Michigan). On day 0 and 3, each mouse was also injected intravenously with 10^9 heat-killed Bordetella pertussis bacilli (pertussis vaccine lot number 264, Massachusetts Public Health Biological Laboratories, Boston, Massachusetts). The concentration of W144 was titrated in each set of experiments to give optimal disease and was between 50–100 μg/mouse. In the preimmunization experiments, SJL mice were initially immunized at two sites with peptide emulsified in CFA supplemented with M. tuberculosis H37 RA (400 μg/mouse; Difco) or not preimmunized (controls). In the coimmunization experiments, mice were immunized with the peptide or mixture of peptides shown, keeping the concentration of disease-inducing peptide constant. Mice were examined daily beginning on day 9 for disease, which was assessed clinically according to the following criteria: 0, no disease; 1, limp tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb plus forelimb paralysis; 5, moribund or dead. When animals were moribund or at the end of the experiment (day 30–40), mice were sacrificed and brains and spinal cords were fixed, processed for histologic analysis, and evaluated as described (Kuchroo et al., 1994b).

Culture Media

DMEM supplemented with 0.1 mM nonessential amino acids, sodium pyruvate (1 mM), L-glutamine (2 mM), MEM essential vitamin mixture (1 ×), penicillin (100 U/ml), streptomycin (100 U/ml), gentamicin (0.1 mg/ml), 10% heat-inactivated fetal bovine serum (BioWhittaker, Incorporated, Walkersville, Maryland), asparagine (0.1 mM), folic acid (0.1 mg/ml), and 2-mercaptoethanol (5×10^{-5} M) (Sigma, St Louis, Missouri) was used for the culture of LNCs. For the expansion of T cell lines and clones, this medium was supplemented with 0.6% T cell growth factor (T-Stim, Collaborative Biomedical Research, Bedford, Massachusetts) and 0.06% recombinant IL-2.

In Vitro Proliferation Assays

Mice were injected subcutaneously at five sites with antigen emulsified in CFA (Difco) containing a total of 250 μg M. tuberculosis H37 RA. Mice immunized with a single peptide received a total of 100 μg of antigen; mice immunized with a mixture of W144 and Q144 received 100 μg of W144 and either 100 μg or 300 μg of Q144 (i.e., a total of 200 or 400 μg of antigen per mouse). On day 10, lymph nodes were removed and LNCs prepared from them. LNCs (4×10^5 per well) were cultured in triplicate in 96-well round-bottomed plates (Falcon, Becton Dickinson, Lincoln Park, New Jersey), in the presence of antigen, for 48 hr and then [^{3}H]thymidine (1 μCi/well) was added for the last 16 hr before harvesting the cells. The [^{3}H]thymidine incorporation was determined in a Beckman scintillation counter (model LS 5000; Beckman Instruments, Fullerton, California). The stimulation index (SI) was calculated as mean cpm with antigen/mean cpm with medium plus APCs alone.

In Vitro Cytokine Assays

Supernatants were collected from LNCs (4×10^5 per well), T cell lines (5×10^4 T cells plus 5×10^5 syngeneic irradiated spleen cells per well) or T cell clones 24 or 40 hr after activation in vitro. The concentration of IL-4 in the supernatants of LNC was measured by ELISA or by using CT4S cells (a gift from Dr. A. K. Abbas), which were maintained in culture in medium supplemented with recombinant IL-4. Prior to assay, CT4S cells were kept overnight in IL-4-free medium, harvested, and washed three times, then resuspended at 1×10^5 cells/ml. Aliquots (50 μl) were dispensed into 96-well flat-bottomed plates and incubated

either with known concentrations of recombinant mouse IL-4 (Pharmingen, San Diego, California) or supernatants from stimulated LNCs. After 24 hr, the plates were incubated for 16 hr with added [^{3}H]thymidine, harvested, and the radioactivity counted. Standard curves were derived from cells exposed to known concentrations of IL-4.

The concentrations of IFNγ, IL-2, IL-10 (and IL-4 in the supernatants collected from T cell lines or T cell clones) were measured by quantitative capture ELISA according to the guidelines of the manufacturers. In brief, purified rat MAb to mouse cytokine IL-2 (clone JES-1A12), IL-4 (clone BVD4-1D11), IL-10 (clone JES5-2A5), and IFNγ (clone R4-6A2)) were obtained from Pharmingen (San Diego, California) and used to coat ELISA plates (Immulon 4, Dynatech Laboratories, Chantilly, Virginia). Recombinant mouse cytokines (IL-2, IL-4, IL-10, and IFNγ; Pharmingen) were used to construct standard curves and biotinylated rat MAb to mouse cytokine IL-2 (clone JES6-5H4), IL-4 (clone BVD6-24G2), IL-10 (clone SXC-1), and IFNγ (clone XMG1.2) (Pharmingen) were used as the second antibody. Assays were developed with TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories, Maryland) and read after the addition of stop solution at 450 nm using a model 2550 Microplate Reader (Bio-Rad Laboratories, California).

Derivation of T Cell Lines and Clones

T cell lines were generated from LNCs from mice immunized with W144 (WL.1) or Q144 (QL.1). LNCs were prepared and cultured in syngeneic serum with the appropriate antigen (20 μg/ml) for 5 days. T cell blasts were purified over a Ficoll–Hypaque gradient and fed with culture medium containing 0.6% T cell growth factor (T-Stim, Collaborative Biomedical Research) and 0.06% recombinant IL-2. Cells were fed every 2–3 days and restimulated every 10–18 days by the immunizing antigen (20 μg/ml) plus irradiated syngeneic spleen cells (5 × 10^6 cells/ml) as a source of APCs. Clones were obtained by culturing cells from QL.1 at limiting dilution as previously described (Kuchroo et al., 1992). Cells were fed with culture medium plus T cell growth factors every 2–3 days and restimulated with a mixture of antigen (20 μg/ml) plus irradiated syngeneic spleen cells (5 × 10^6 cells/ml) after 10 days. Wells that contained growing cells were identified 4 days later and the cells transferred to 48-well plates (Sumilon, Sumitomo Bakelite Company, Tokyo, Japan), fed with medium containing T cell growth factors every 2–3 days, and expanded by activation with antigen and APCs every 2–4 weeks.

Adoptive Transfer of T Cell Lines

Mice were immunized using the same protocol as the in vitro proliferation assays. Lymph nodes were removed on day 10 and LNCs were resuspended at a concentration of 6–10 × 10^6 cells per ml in culture medium containing 0.5% syngeneic serum in place of fetal bovine serum. Cells were cultured in the presence of various antigens (20 μg/ml) for 4 days, then harvested and purified over a Ficoll–Hypaque gradient. Cells were resuspended in phosphate-buffered saline at 25 × 10^6 cells per ml and injected intravenously into recipient animals (0.2 ml, 5 × 10^6 cells per mouse), then recipient mice were immunized with the native peptide PLP 139–151 and CFA as described to induce active EAE.

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