

A Single Amino Acid Substitution in the Autoantigenic Peptide Alters the Cytokine Profile Inducing Protective T Cells

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Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease model where T cells attack myelin, the membrane that covers the axons of neurons. EAE is induced by injecting proteolipid protein (PLP), one of the major myelin proteins, with adjuvant into the animal. This induces Th1 T cells to migrate to the brain and secrete the cytokines IFN- γ and IL-2, leading to inflammation with loss of neurologic function. In contrast, PLP reactive T cells secreting Th2 cytokines, IL-4 and IL-10, suppress EAE, indicating that a balance between the autoreactive Th1 and Th2 cells is important for inhibiting pathologic autoimmune responses. T cell functions can be altered by substituting single amino acid side chains at T cell receptor (TCR) contact sites on the peptide antigen. This experiment was performed to determine whether a pathogenic PLP reactive Th1 clone's function could be switched to a protective PLP reactive Th2 type clone by a peptide generated by a single amino acid substitution of the native encephalitogenic PLP peptide. PLP reactive clones were investigated from mice immunized with either the native PLP peptide 139-151 or the same PLP peptide altered at TCR contact residues. The altered PLP peptide switched the cytokines secreted by the responding T cells from an inflammatory to a suppressive nature. This change could inhibit the function of the autoaggressive T cell. The design of altered peptide antigens that switch T cell function could be used to treat autoimmune diseases.

Introduction

The immune response to antigens is regulated by T cells, which comprise approximately 60% of mononuclear cells.¹ T cells do not recognize free antigen, but rather require macrophages, which process antigen into small peptides of 12-25 amino acids in length. The peptides are transported to the major histocompatibility complex (MHC), a molecule on the surface of macrophages. The MHC binds processed antigen in a form such that T cells, using a specific receptor on its surface, contact amino acid side chains of the peptide antigen.^{2,3} Thus, the peptide should possess residues that bind to the MHC molecules (MHC contact residues) and also have residues that have affinity for the T cell receptors (TCR contact residues). Upon recognizing antigen in the context of MHC, the T cell secretes growth factors known as cytokines.

Cytokines can induce T cells to grow and multiply in number to eliminate antigen^{4,6} or under other circumstances regulate the immune response.^{6,7} Naive T lymphocytes, triggered by antigen, differentiate into two subpopulations each producing its own set of cytokines and mediating distinct effector functions. Th1 cells secrete interleukin 2 (IL-2) and

interferon- γ (IFN- γ), leading to activated macrophages and inflammatory immune responses with tissue destruction. In marked contrast, Th2 cells secrete IL-4 and IL-10, suppressing inflammatory responses induced by Th1 T cells.⁷

While T cells can protect us from infections by recognizing foreign proteins expressed on viruses and bacteria, there are also populations of T cells that recognize self-proteins and induce autoimmune diseases.⁸ These autoreactive T cells, which are present in all mammals, are normally in a resting state and thus do not induce autoimmune destruction of self-organs. However, when T cells recognizing self-proteins are activated to become autoaggressive, they secrete Th1 cytokines (IFN- γ and IL-2), which induce autoimmune disease in the organ where the self-antigen peptide is located.⁹

Experimental autoimmune encephalomyelitis (EAE) is one such animal model of autoimmune disease where T cells attack myelin, the membrane that covers the axons of neurons and facilitates nerve impulses. EAE is also thought to be a model of the human autoimmune disease multiple sclerosis. The experimental disease model can be induced in mice by injecting proteolipid protein (PLP), one of the major myelin proteins, with adjuvant into the animal.^{10,11} The Th1 T cells, induced by the PLP immunization, migrate to the brain and secrete the cytokines IFN- γ and IL-2, which induce inflammation and myelin destruction resulting in a loss of neurologic function. In contrast, PLP-reactive T cells secreting Th2 cytokines, IL-4 and IL-10, suppress EAE, indicating that a balance between the autoreactive Th1 and Th2 cells is important for inhibiting autoreactivity.^{10,12} Similarly, it is postulated that myelin-reactive activated Th1 T cells are the primary cause of the autoimmune disease multiple sclerosis.

Allen and coworkers recently demonstrated that T cell functions can be altered by stimulating T cell clones with peptides that have been substituted by single amino acid side chains at the T cell receptor (TCR) contact sites.¹³ For example, stimulation of a T cell clone with altered peptide antigens can induce IL-2 receptor expression and cytolytic T cell replication.^{14,15} Altered peptides also have been shown to act as TCR-antagonists, inducing non-responsiveness in T cell clones.¹⁶⁻¹⁸ Based on the previous work by Allen, experiments were performed to determine whether a peptide generated by a single amino acid substitution of the native encephalitogenic PLP peptide could alter the function of autoreactive T cells. Mice were immunized with either the native PLP peptide 139-151 or the same PLP peptide altered at TCR contact residues.^{19,20} These clones generated against PLP were stimulated with a series of different altered peptide antigens with substitutions at TCR contact points. Cytokine secretion was measured to determine whether a Th1 clone generated against PLP could be switched by altered peptide antigen to a Th2 clone. The identification of altered PLP antigens that switch the T cell clone's function could be useful therapeutically in the cure of EAE and have potential utility in the treatment of multiple sclerosis.

Methods

Peptides. Peptide antigens were synthesized on a Milligen model 9050 synthesizer using Fmoc chemistry. Milligen PAL amide resins were used to generate peptides with C terminal amides.²⁰ As determined by high pressure liquid chromatography (HPLC), peptides were more than 90% pure.

The PLP peptide 139-151 W(144) induces EAE in mice. By using a panel of alanine substituted peptides at each residue, it has been shown that the tryptophan (W) at position 144 is the primary TCR contact point, and histidine (H) at position 147 and leucine (L) at position 141 are the secondary TCR contact points.^{19, 20} A series of peptides were synthesized with single amino acid substitutions at the primary TCR contact point W(144). Substitutions consisted of a change from the nonpolar tryptophan W(144) to: (1) the nonpolar but less bulky leucine L(144); (2) to the uncharged but polar glutamine Q(144); and (3) to the charged and polar arginine R(144). Table 1 summarizes the sequences of the peptides used in these experiments.

Generation of T Cell Lines and Clones. T cell clones were previously made in the laboratory as briefly described here.²¹ T cell clones were generated from the lymph node cells of mice immunized with PLP 139-151 and PLP 139-151 with a substitution to Q at position 144, which will be referred to as Q(144). The lymph node cells 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, Bedford, MA) and 0.06% recombinant IL-2. Cells were fed every 2-3 days and restimulated every 10 to 18 days by the immunizing antigen (20µg/ml) plus irradiated syngeneic spleen cells (5x10⁶ cells/ml) as a source of antigen presenting cells (APCs). By repeated stimulation with antigen, specific short-term T cell lines were generated.

Clones were obtained from the lines by culturing T cells with the peptide Q(144) at limiting dilution. 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 (5x10⁶ cells/ml) after 10 days. Four days later wells that contained growing cells were identified and the cells were transferred to 48 well plates (Sumilon, Sumitomo Bakelite Co, Tokyo) fed with medium containing T cell growth factors every 2-3 days, and expanded by activation with antigen and APCs every 2 to 4 weeks.

Measuring Proliferation of T Cell Clones. T cell clones (4x10⁵ per well) were cultured in triplicate in 96 well round bottom plates (Falcon, Becton Dickinson, Lincoln Park, NJ), in the presence of antigen for 48 hours, and then ³H-thymidine (1 µCi/well) was added for the last 16 hours before harvesting the cells. Mean ³H-thymidine incorporation was determined in a Beckman scintillation counter (model LS 5000; Beckman Instruments, Fullerton, CA).

Cytokine Production. T cell clones were cultured at 4-5x10⁶ cells/ml in the presence of PLP peptide at the concentrations shown. Supernatants were collected at the time point specified and assayed for cytokines. The amounts of IL-4, IFN-γ, and IL-10 were measured by cytokine Enzyme Linked Immunosorbent Assay (ELISA).²² The results shown are the mean concentration of cytokine detected in 3 wells. Standard errors were always less than 10%. When no cytokine was detectable in supernatants from cells cultured without antigen the baseline value was taken as the lower limit of detection of the assay (IFN-γ 100 pg/ml; IL-2 and IL-10 50 pg/ml).

Cytokine Measurements. For each ELISA, 96 well plates (Immulon 4, Dynatech Laboratories, Chantilly, VA) were coated with the appropriate coating antibody (1 µg/ml in carbonate buffer, pH 8.6). For IFN-γ the coating antibody was monoclonal anti-mouse IFNγ, the primary antibody was rabbit polyclonal anti-mouse IFN-γ (1:500) (both from Endogen, Cambridge, MA), and the secondary antibody was goat anti-rabbit immunoglobulin-horseradish peroxidase (1:50,000) (Biosource International, Camarillo, CA). For IL-4 and IL-10, paired antibody sets (PharMingen, San Diego, CA) were used according to manufacturer's directions, followed by avidin-peroxidase (1:2000) (Sigma, St. Louis, MO). The peroxidase substrate was TMB substrate (KPL, Gaithersburg, MD). Absorbance was read at 450 nm on a BioRad microplate reader (Richmond, CA).

Results

Proliferative Response and Cytokine Production in Response to PLP Peptides. Short-term T cell lines, generated in the laboratory, were specific for either the native peptide or the peptide Q(144). The T cell lines were stimulated *in vitro* with a series of altered peptide ligands to measure the proliferative responses and cytokine production. As indicated in Figure 1A, the T cell lines specific for the native peptide W(144) exhibited the highest proliferative

PROTEOLIPID PROTEIN ALTERED PEPTIDE ANTIGEN SEQUENCES

PLP Peptides	139	140	141	142	143	144	145	146	147	148	149	150	151
<i>TCR contact</i>			↑			↑			↑				
W(144) Native	H	S	L	G	K	W	L	G	H	P	D	K	F
<i>MHC contact</i>							↓			↓			
Q(144)	H	S	L	G	K	Q	L	G	H	P	D	K	F
L(144)	H	S	L	G	K	L	L	G	H	P	D	K	F
R(144)	H	S	L	G	K	R	L	G	H	P	D	K	F

Table 1. PLP sequences used in these investigations are shown. PLP W(144) is the native sequence. Position 144 is the primary TCR contact residue while positions 141 and 147 are secondary TCR contact residues (reference 25).

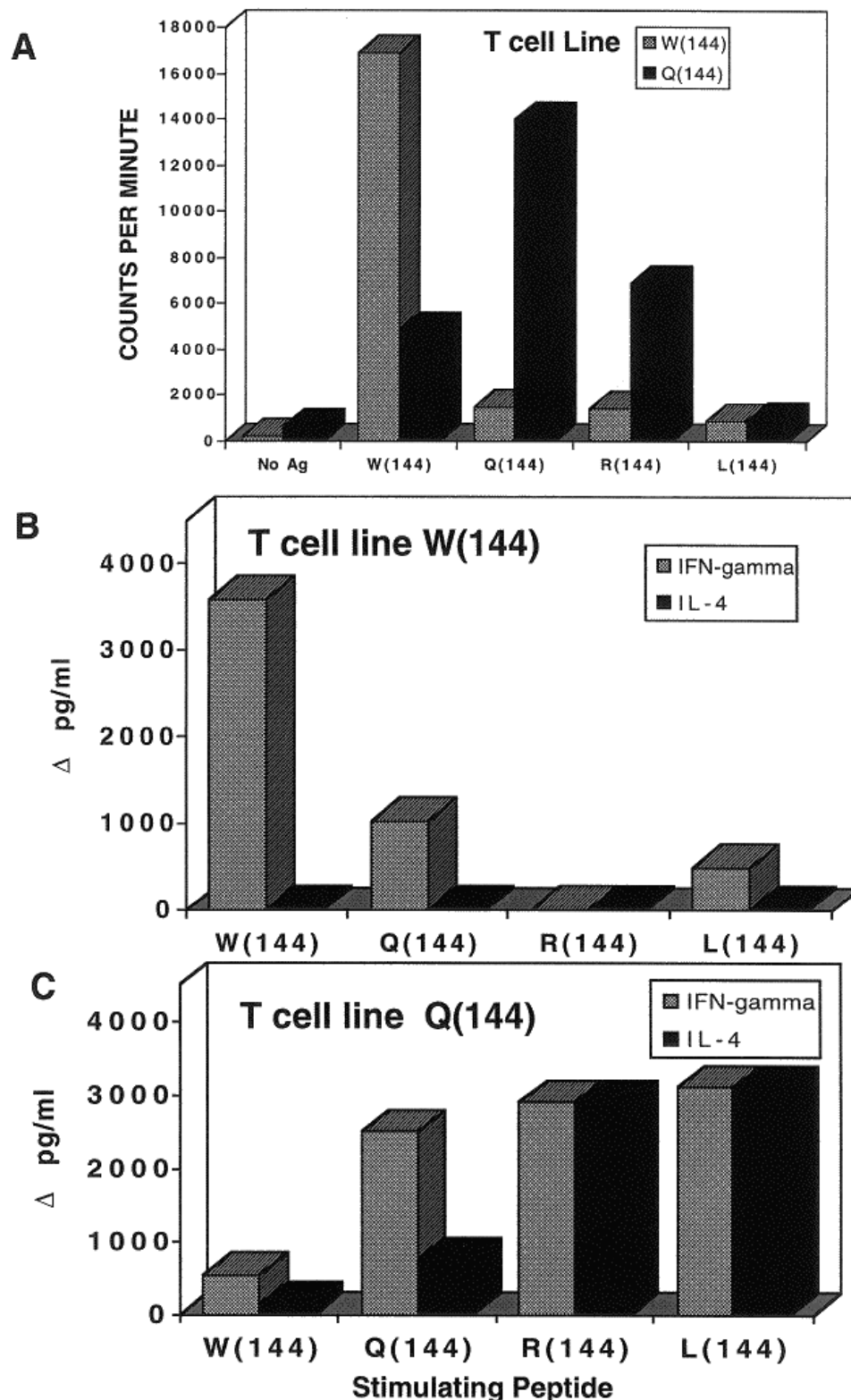


Figure 1A. There is more cross-reactivity with T cell lines specific for PLP Q(144) as compared to the native W(144) peptide. Short-term T cell lines specific for either the native PLP 139-151 W(144) peptide or the altered peptide antigen PLP 139-151 Q(144) were tested for proliferation to assess peptide recognition. T cell lines specific for the native peptide W(144) exhibited the highest proliferative response with *in vitro* stimulation by W(144), with little cross-reactivity to the other peptides. In contrast, T cell lines specific for the altered peptide antigen Q(144) exhibited the highest proliferative response with *in vitro* stimulation by Q(144), with significant proliferative responses to R(144), less so to W(144). **Figure 1B and 1C.** Altered PLP peptide induces IL-4 secretion. T cell lines specific for native peptide ligand PLP 139-151 W(144) and the altered peptide ligand PLP 139-151 Q(144) were stimulated *in vitro* with a series of PLP peptides substituted at position 144, and the cytokines IFN- γ and IL-4 were measured. There was predominantly a Th1 response with IFN- γ and without IL-4 secretion with *in vitro* stimulation of T cell lines specific for W(144) peptides. In marked contrast, T cell lines specific for the altered peptide antigen Q(144) secreted both IFN- γ and IL-4 with *in vitro* stimulation of T cell lines specific for altered PLP peptides.

Frequency Of Cross-Reactive T Cell Clones By Proliferation (³H-Thymidine Incorporation) After Immunization With PLP Q(144) Peptide

Proliferative response of Q(144) clones to the peptide W(144)	Number of clones	% of clones
Equal to response to peptide Q(144)	29	64%
2-fold or greater than response to Q(144)	14	31%
One-half or less than response to Q(144)	2	4%

Table 2. Forty-five T cell clones were examined for proliferative responses to the PLP 139-151 Q(144) altered peptide and to the native PLP peptide W(144) after *in vitro* inoculation with PLP Q(144). Counts per minute (CPM, ³H-thymidine incorporation) were measured. These data indicate that T cell clones, generated after immunization with altered peptide antigens, can recognize native self-antigens.

response with *in vitro* stimulation by W(144) with little cross-reactivity to the other peptides. In contrast, the short-term T cell lines specific for the peptide Q(144) exhibited the highest proliferative response with *in vitro* stimulation by the peptide Q(144) with significant proliferative responses to R(144), less so to W(144). These data indicate that while T cells generated against the native peptide were highly specific for the native peptide, the T cell lines specific for the Q(144) peptide showed greater cross-reactivity.

Cytokine Response. The T cell lines specific for the native W(144) peptide and the altered peptide Q(144) were stimulated *in vitro* with a series of PLP peptides substituted at position 144, and the cytokines IFN- γ (Th1 type T cells) and IL-4 (Th2 type T cells) were measured. The data from the native W(144) specific T cell line in response to PLP peptides, as shown in Figure 1B, indicate a predominantly Th1 response with IFN- γ without IL-4 secretion. In marked contrast, as illustrated in Figure 1C, the T cell lines specific for the altered peptide antigen Q(144) secreted both IFN- γ and IL-4 with *in vitro* stimulation by the altered PLP peptides. Thus, native W(144) specific T cell lines exclusively induces IFN- γ producing Th1 T cells, whereas the altered peptide antigen Q(144) induces both IFN- γ (Th1) and IL-4 (Th2) producing T cells.

Cloning of T Cells Specific for PLP 139-151 Q(144). A T cell line specific for the peptide Q(144) was cloned as described in the *Methods* section. These clones were examined for proliferative responses to the altered peptide Q(144) and to the native peptide W(144). Approximately 55% of the clones cross-reacted equally with the altered peptide Q(144) and native peptide W(144), indicating that immunization with the altered peptide Q(144) could generate T cell clones capable of recognizing the native peptide W(144) (Table 2). These data also indicate that T cell clones generated after immunization with altered peptide antigens can recognize native self-antigen. One cross-reactive clone, 1B6 was expanded *in vitro* for detailed investigation to determine whether an altered peptide antigen can switch the cytokines secreted by an individual autoreactive T cell clone.

Modulation of Cytokine Production by Altered Peptides. To determine whether the cytokine profile of the clone could be switched with stimulation by altered PLP peptides, the 1B6 clone was stimulated with peptides at different concentrations, and IFN- γ secretion was compared to IL-4 secretion and T cell proliferation (Δ CPM) (Figure 2). The clone secreted both IFN- γ and IL-4 when stimulated with the altered

peptide Q(144), which was used to generate the clone. The altered peptides R(144) and L(144) also induced high amounts of both IFN- γ and IL-4 secretion. In striking contrast, the native peptide W(144) induced IFN- γ but no IL-4 secretion. Regarding proliferation, it was of interest that the peptide R(144) stimulated clone 1B6 at lower concentrations than either the altered peptide Q(144) or the native peptide, whereas the peptide L(144) did not activate the T cell clone at the concentrations tested.

Differences in the kinetics of cytokine secretion induced by the altered peptide antigens may explain the changed patterns of IFN- γ , IL-2 and IL-4 secretion for the 1B6 clone. As shown in Figure 3, the decreased IL-4 secretion with stimulation by the native peptide W(144) as compared to the altered peptide Q(144) was not due to different kinetics of secretion. That is, there was little or no IL-4 secretion (maximal < 50 pg/ml) at 8, 16, or 42 hours after stimulation with the native peptide. When tested at the same time points, the peptide Q(144) induced a large amount (about 1,000 pg/ml) of IL-4 at 42 hours after activation. Together, these data show that a T cell clone recognizing self-antigen can be differentially induced to secrete IL-4 with stimulation by peptides altered at TCR contact points. The clone that produces mostly Th1 cytokines (IFN- γ and IL-2) upon activation with the native W(144) peptide can produce IL-4 in addition to IFN- γ upon activation with the altered peptide Q(144).

Discussion

Myelin PLP-reactive T cells generated by the inoculation with the native peptide W(144) with adjuvant induces Th1 type cells secreting IL-2 and IFN- γ which lead to the experimental autoimmune disease EAE. As it has recently been shown that T cell function can be modified by stimulating T cells with antigenic peptides altered by substituting single amino acid side chains at the TCR contact sites, the immunologic effects of altering the primary TCR contact residue of the autoantigen of PLP 139-151 peptide were examined. A T-cell clone secreting IFN- γ in response to the native peptide was generated from lymph node cells after immunization of the altered peptide Q(144). This T cell clone, recognizing self-antigen, could be differentially induced to secrete the suppressor cytokine IL-4 with stimulation by peptides altered at the position 144 TCR contact point. As PLP-reactive Th2 T cells secreting IL-4 can inhibit EAE, these data suggest that altered peptide antigens may be of use therapeutically to treat experimental autoimmune disease.

Changes in Cytokine Secretion and Proliferation Induced by PLP 139-151 Altered Peptide Antigens

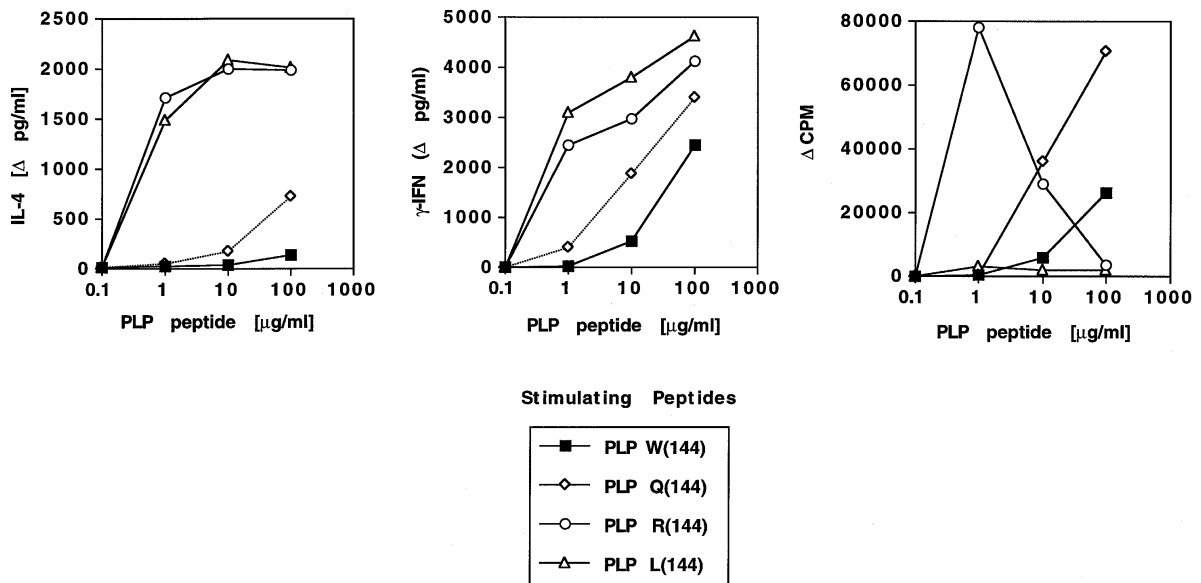


Figure 2. Cytokine secretion with native PLP W(144) vs. the altered PLP Q(144) peptide. The clone 1B6, which was cross-reactive with PLP W(144) and Q(144) as shown in Table 2, was investigated to determine whether cytokine secretion could be switched with stimulation by altered PLP peptides. Different concentrations of peptides were used to stimulate the clone, and IFN- γ secretion was compared to IL-4 secretion and ^3H -thymidine incorporation (ΔCPM). The clone secreted both IFN- γ and IL-4 when stimulated with the altered PLP 139-151 Q(144) peptide, which was used to generate the clone. The altered PLP 139-151 R(144) and L(144) peptides also induced high amounts of both IFN- γ and IL-4 secretion. In striking contrast, the native PLP peptide W(144) induced IFN- γ but no IL-4 secretion (closed symbols).

Kinetics of Cytokine Secretion induced by T cell Clones Recognizing PLP 139-151 W(144) vs. Q(144)

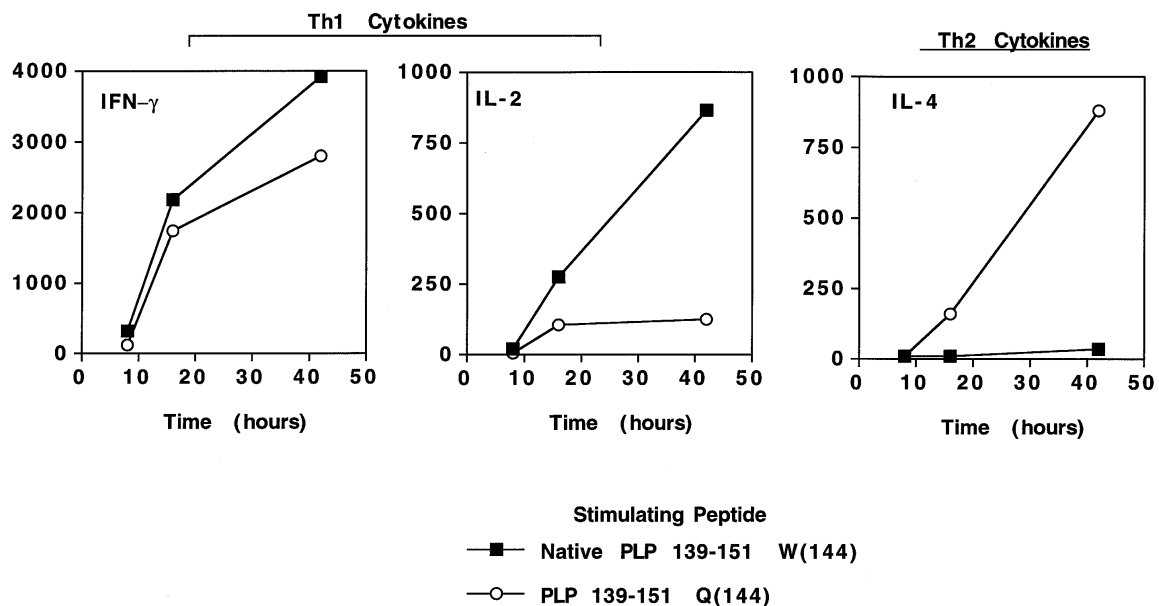


Figure 3. Kinetics of the IFN- γ , IL-2 and IL-4 secretion for T cell clone 1B6. The kinetics of cytokine secretion induced by the altered peptide antigens were examined at different time points after antigen stimulation *in vitro*. 100 μg of PLP peptide, a concentration providing optimal stimulation determined from previous experiments, was used. There was little or no IL-4 secretion at 8, 16, or 42 hours after stimulation with the native peptide. Thus, the decreased IL-4 secretion with stimulation by the native peptide as compared to the Q144 peptide was not due to different kinetics of secretion. Together, these data show that a T cell triggered by native antigen can be differentially induced to secrete IL-4 with IFN- γ with altered peptide antigen stimulation.

Based on the data from the experiments presented here, studies were performed to determine whether T cells induced by immunization with the peptide Q(144) would be able to confer protection from EAE. Naive mice were immunized with either the native peptide, the altered peptide Q(144) or a non-encephalitogenic control peptide, PLP 190-209. Short-term T cell lines were generated from lymph node cells prepared from mice 10 days after immunization and activated *in vitro* with immunizing or control peptides. T cell lines generated from mice immunized with the peptide Q(144) and activated *in vitro* with Q(144) conferred protection from clinical disease associated with EAE.²⁶ This demonstrates that immunization with Q144 induces regulatory cells which protect mice from EAE.

A series of T cell clones, generated after immunization with PLP Q(144), were cross-reactive with other altered peptide antigens. Similarly, examination of short-term T cell lines specific for the altered peptide Q(144) demonstrated more cross-reactive T cells as compared to T cell lines specific for the native peptide W(144). Position 144 is the primary TCR contact point of the native W(144) peptide and peptides with substitutions at this residue are not recognized by a panel of W(144)-reactive T cell clones.²⁰ 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 Q(144)-reactive T cells are derived from different precursors than T cells which are induced with the native peptide, since TCRs recognize a different part of the peptide. Alternatively, naive T cells specific for W(144), with the potential to become Th1 cells if activated by the native peptide, might differentiate into Th2-like T cells if activated by Q(144).

Exposure to cross-reactive peptides that are altered at TCR contact points might occur as a consequence of viral or bacterial infections. For this reason T cells have been stimulated with the peptides R(144) and L(144) to determine the cytokine response to a set of peptides similar to cross-reactive viruses. In this regard, it has been shown that viruses can trigger myelin-reactive T cells to proliferate and can induce EAE.²³ Attention has also recently been drawn to the potential importance of cross-reactivity in maintaining long-term immunological memory,²⁴ and the same phenomenon may be important in the development and regulation of autoimmune disease. Moreover, viruses may mutate in attempts to mimic self and avoid detection by the immune system, thus further leading to cross-reactive antigens with self-proteins.

The mechanism by which alterations of TCR contact residues can modify T cell cytokine secretion is not known. As mentioned above, it has been previously demonstrated that modification of antigenic peptides at TCR contact points¹³ can alter T cells' functions. A number of altered peptide antigens are capable of binding to the TCR but are incapable of triggering a response and thus act as pharmacological antagonists;²⁵ other altered peptides may interact with the TCR, preventing responding cells from proliferating later;¹⁵ furthermore, some altered peptide antigens have been shown to partially activate T cells.¹³ The results from the experiments presented here suggest that the Q(144) peptide affects T cell differentiation *in vivo* and induces T cells that are functionally like Th2 cells, though they secrete both IL-4 and IFN- γ , rather than autoaggressive disease inducing Th1 cells.

In summary, Th1 myelin-reactive T cells secreting IL-2 and IFN- γ induce experimental autoimmune demyelinating diseases, while disease recovery is associated with the presence of Th2 myelin-reactive T cells secreting IL-4. Thus, one potential approach to the treatment of autoimmune diseases such as multiple sclerosis may be to selectively generate IL-4-secreting autoreactive T cells. The results of the present experiments suggest that immunization with autoreactive peptides altered at TCR contact points can potentially switch T cell function and alter the course of human autoimmune diseases.

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