Glatiramer acetate (Copaxone®) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis

Petra W. Duda, Mascha C. Schmied, Sandra L. Cook, Jeffrey I. Krieger, and David A. Hafler

Laboratory of Molecular Immunology, Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA

Petra W. Duda's present address is: Neurology and Experimental Immunology, Department of Research, University Hospital Basel, 4031 Basel, Switzerland.

Address correspondence to: David A. Hafler, Center for Neurologic Diseases, Harvard Institutes of Medicine, Room 780, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA.
Phone: (617) 525-5330; Fax: (617) 525-5333; E-mail: hafler@cnd.bwh.harvard.edu.

Petra W. Duda and Mascha C. Schmied contributed equally to this work.

Received for publication November 23, 1999, and accepted in revised form February 15, 2000.

We examined the effect of glatiramer acetate, a random copolymer of alanine, lysine, glutamic acid, and tyrosine, on antigen-specific T-cell responses in patients with multiple sclerosis (MS). Glatiramer acetate (Copaxone) functioned as a universal antigen, inducing proliferation, independent of any prior exposure to the polymer, in T-cell lines prepared from MS or healthy subjects. However, for most patients, daily injections of glatiramer acetate abolished this T-cell response and promoted the secretion of IL-5 and IL-13, which are characteristic of Th2 cells. The surviving glatiramer acetate–reactive T cells exhibited a greater degree of degeneracy as measured by cross-reactive responses to combinatorial peptide libraries. Thus, it appears that, in some individuals, in vivo administration of glatiramer acetate induces highly cross-reactive T cells that secrete Th2 cytokines. To our knowledge, glatiramer acetate is the first agent that suppresses human autoimmune disease and alters immune function by engaging the T-cell receptor. This compound may be useful in a variety of autoimmune disorders in which immune deviation to a Th2 type of response is desirable.

J. Clin. Invest. **105**:967–976 (2000).

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) white matter. The high frequency of activated, myelin-reactive T cells in the circulation and cerebrospinal fluid of patients with MS is consistent with the hypothesis that an initiating event linked to an antecedent microbial infection in a genetically susceptible host eventually leads to an autoimmune-mediated destruction of myelin followed by the surrounding axons (1). After the initiating event(s), the CNS itself may become a potential depot of antigen and MHC, with expression of critical second signals required for T-cell activation such as B7-1 and CD40 (2, 3) leading to epitope spreading (4). MS is thought to be a Th1-mediated disease based largely on pathological resemblance to a delayed-type hypersensitivity response in the CNS and from observations made in the murine experimental autoimmune encephelomyelitis (EAE) model. However, direct cloning of myelin-reactive T cells from the blood of patients with MS suggests that the majority of T cells can secrete both Th1- and Th2-type cytokines (5).

A major goal in the treatment of autoimmune diseases has been the development of antigen-specific therapies that target autoreactive T cells. The discovery of epitope spreading in the EAE model (4, 6) and observations of diverse T-cell receptor repertoires in response to self-antigens have theoretically made this approach less attractive. Instead, the concept of bystander suppression has emerged in which autoreactive Th2 or Th3 T cells are generated that migrate to the inflamed target organ where they are antigen specifically reactivated, leading to the secretion of cytokines that downregulate inflammation in the local milieu in an antigen nonspecific mechanism (7). Two approaches have emerged for inducing immune deviation of autoreactive T cells: mucosal administration of antigen, which induces Th2 T-cell responses to the antigen (7), and altered peptide ligands (APLs), which, by inducing a weaker strength of signal, lead to Th2 deviation of cytokine secretion (8–11). Both approaches have been used in clinical trials to treat patients with MS, but to date, without success.

An alternative approach to the use of a single self-antigen that has been altered or given mucosally is the administration of peptide mixtures that contain many different antigen specificities. The use of random copolymers that contain amino acids commonly used as

MHC anchors and T-cell receptor (TCR) contact residues are possible "universal APLs." Glatiramer acetate (GA) (Copaxone; Teva Marion Partners, Kansas City, Missouri, USA) (12) is a random sequence polypeptide of the 4 amino acids alanine (A), lysine (K), glutamate (E), and tyrosine (Y) at a molar ratio of A/K/E/Y of 4.5:3.6:1.5:1, respectively, and an average length of 40–100 amino acids. Directly labeled GA efficiently binds to different murine H-2 I-A molecules and to the human counterparts, MHC class II DR, but not to DQ or MHC class I, molecules in vitro (13). Biochemical studies revealed that GA also binds directly and with high affinity to purified HLA-DR1, -DR2, and -DR4 (14), suggesting that GA contains multiple epitopes enabling it to bind promiscuously to MHC class II molecules, where it could potentially be recognized by CD4 T cells.

A "universal antigen" containing multiple epitopes would be expected to induce proliferation in vitro, as measured by [^{3}H]thymidine incorporation in naive T cells from the circulation, representing a high degree of cross-reactivity to other peptide antigens. In in vitro cultures of PBMCs from healthy humans, a strong dose-dependent proliferative response to GA has been reported (15). Similarly in our own studies, we found that GA elicits dose-dependent responses in all of more than 50 humans, including healthy subjects and patients with relapsing remitting (RR) and chronic progressive MS (P.W. Duda and D.A. Hafler, manuscript in preparation). The response to GA could be blocked by anti-DR antibodies and the restriction of GA-reactive CD4$^+$ T cells to a particular HLA DR molecule could be shown on a clonal level. The high proliferative and cytokine responses of naive PBMC CD4$^+$ T cells suggest a high frequency of circulating GA-reactive precursor T cells. Our own limiting dilution analysis suggests that the precursor frequency of GA-reactive T cells ranges from 1:5,000 to 1:100,000 PBMCs. Thus, GA appears to constitute a highly cross-reactive antigen preparation.

In animal models of MS, prophylactic subcutaneous administration of GA has been shown to prevent EAE induced by injection of purified myelin basic protein (MBP) (12), proteolipid protein (PLP) (16), or myelin oligodendrocyte glycoprotein (MOG) (17). Of greater importance, in a phase III clinical trial subcutaneous administration of GA has been shown to decrease the rate of exacerbations and to decrease the appearance of new lesions, based on magnetic resonance imaging (MRI), of patients with RR MS (18–20). This represents perhaps the first successful use of an agent that ameliorates autoimmune disease by altering signals presumably through the TCR.

The early observation that cyclophosphamide abrogated the beneficial effect of GA on EAE (21), suggested that the mechanism of action of GA involved the induction of regulatory T cells. Later, adoptive transfer of GA-specific T cells was found to inhibit EAE (22). It was originally thought that GA was structurally cross-reactive with MBP, although this has remained controversial. Recently, TCR antagonism has been suggested to occur in addition to competition for MHC binding (23). Stimulation of murine GA-reactive T-cell lines and clones with MBP was reported to induce the secretion of Th2 and Th3 cytokines to this cross-reactive antigen (24).

Taken together, these data led to the hypothesis that GA acts as an APL in vivo, leading to alterations of responses to myelin antigens by cytokine deviation of myelin-specific T cells and bystander suppression mediated by GA-reactive T cells. Here, we directly tested this hypothesis by investigating changes in antigen-specific responses in patients with MS who were undergoing treatment with daily subcutaneous injections of GA. T-cell reactivity to GA, the immunodominant MBP epitope 84–102 as a model myelin antigen, combinatorial libraries derived from the MBP 84-102 sequence, and a completely random 13mer sequence was examined in vitro before and during a year of treatment. Examination of proliferative responses to combinatorial libraries was deemed potentially informative based on the observation that combinatorial peptide libraries are a powerful tool to examine the degree of T-cell receptor degeneracy. That is, the degree to which a T-cell clone proliferates to a random combinatorial peptide library where all of the 13 amino acids are random, representing a total of 19^{13}independent peptides, to a first approximation provides information regarding the degree of degeneracy for that clone, i.e., the more peptides the clone can recognize, the more degenerate the T-cell receptor. Together, these experiments enabled us to examine whether daily subcutaneous injections of GA induced alterations of the T-cell immune response.

Methods

Patients. Patients with RR MS in early stages of the disease, with MRI findings consistent with the diagnosis, and who decided with their physicians to initiate GA treatment participated in the trial. No clinical exami-

Table 1
Characteristics of the study patients

Patient	Sex	DR	Age at onset of disease	Duration of disease (y)	EDSS[A]	Attacks[B]
1	F	3/13	42	7	1	4
2	M	2/4	48	10	3	0
3	F	2/4	36	2	1	3
4	F	3/4	35	1	2	1
5	M	1/11	28	2	1	2
6	F	2/4	45	1	1	2
7	F	2/8	24	2	0	0

[A]Assessed before initiation of treatment. [B]Number of attacks in the 2 years before initiation of treatment.

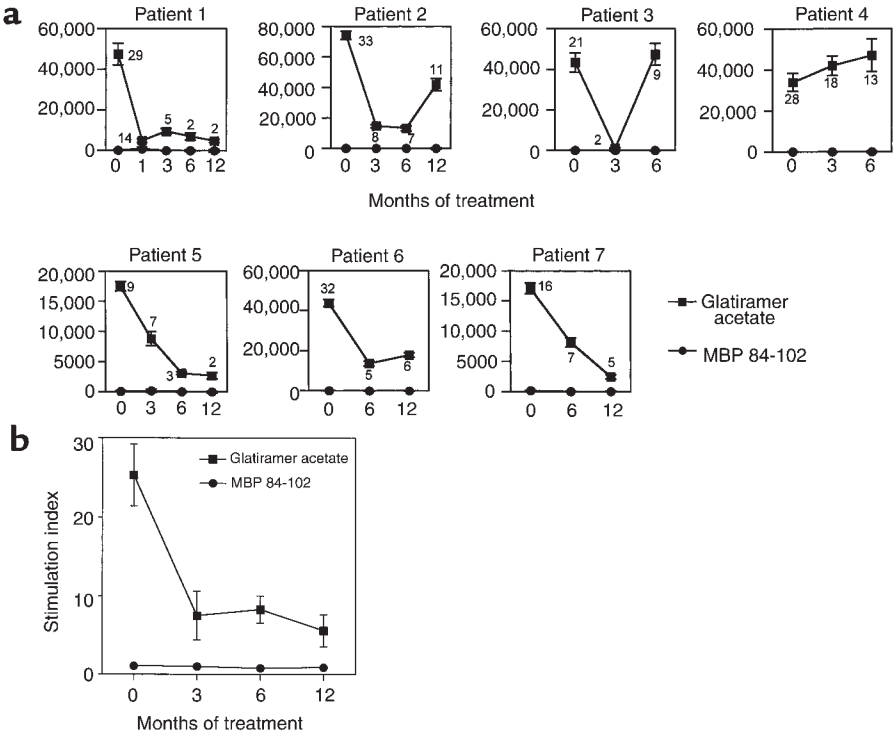

Figure 1
The proliferative response to GA is decreased on average after daily injections of GA. The antigen-specific proliferative response of 20 or 30 primary T-cell lines induced with 40 μg/mL GA as described in Methods was measured by split-well assay for each patient at each time point. Before and at 6 months of treatment, all 7 patients could be tested; at 3 and 12 months, data for 5 patients were obtained. (**a**) Each panel represents data from an individual patient. Squares represent mean ± SEM proliferation in Δ cpm of the GA-specific response compared with the no-antigen control. Background levels of the [^{3}H]thymidine incorporation for all patients of the no-antigen condition were 1,747 ± 111 before treatment, and 3,286 ± 175, 3,785 ± 262, and 3,509 ± 239 at 3, 6, and 12 months of treatment, respectively. The numbers in the figure indicate the SI over the no-antigen control for each time point. (**b**) The mean stimulation index SI ± SEM for all T-cell lines from all patients tested at each time point is shown.

nation other than routine follow-up in the clinic was performed, and no other preselection criteria were applied. Informed consent was obtained before enrollment, and the study was performed in compliance with the rules of the ethical guidelines for human experiments of the Institutional Review Board of the Brigham and Women's Hospital. Table 1 summarizes the patient characteristics.

Antigens. GA (Copaxone; lots 123211 and 123243) was supplied by Teva Marion Partners. MBP 84-102 (DENPVVHFFKNIVTPRTPP) and MBP 93R (ENPVVHFF**R**NIVTPR) peptides were synthesized by standard fmoc technology and HPLC-purified to greater than 99%. Combinatorial peptide library X13 was a 13mer randomized at each position, and combinatorial peptide libraries with random amino acids inserted at position X of the MBP 85-99 peptide (ENPVVHFFKNIVTPR) were: 90X (ENPVV**X**FFKNIVTPR), 91X (ENPVVH**X**FKNIVTPR), 93X (ENPVVHFF**X**NIVTPR), 90X93R (ENPVV**X**FF**R**NIVTPR), and 91X93R (ENPVVH**X**F**R**NIVTPR). All peptide libraries were obtained from Chiron Technologies (Raleigh, North Carolina, USA). Peptides were dissolved at 10 mg/mL in DMSO.

Generation of antigen-specific T-cell lines. PBMCs were isolated from fresh drawn heparinized blood by Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation according to manufacturer's protocol. Antigen-specific T-cell lines were generated by culturing 150,000 PBMCs per well in the presence of 40 μg/mL GA or MBP 84-102 peptide. Unless otherwise indicated, all cell cultures were done in 96-well U-bottom microtiter plates in 200 μL complete medium (RPMI 1640; BioWhittaker Inc., Walkersville, Maryland, USA) containing 2.5% heat-inactivated pooled human AB serum (PelFreeze, Brown Deer, Wisconsin, USA), sodium pyruvate, HEPES, nonessential amino acids, and glutamine) in a humidified 8% CO_2 incubator at 37°C. For patient 5, whole human MBP was used instead of the MBP 84-102 peptide throughout. On day 5 of culture, 120 μL of culture supernatants was removed and replaced by 140 μL complete medium containing 10% phytohemagglutinin-free (PHA-free) T-stim (Collaborative Biomedical Laboratories, Bedford, Massachusetts, USA). On day 7, each GA-induced T-cell line was transferred into 1 mL of complete medium containing 10% T-stim for further expansion. On day 12, split-well assays were performed to test for antigen-specific and cross-reactive proliferation and cytokine secretion. For patients 1, 2, 3, and 4, 40 T-cells lines to MBP 84-102 and 20 T-cell lines to GA were generated at each time point. For patients 5, 6, and 7, 30 T-cell lines were generated for each antigen.

Cross-reactivity assays. Equal aliquots of primary T-cell lines were stimulated with irradiated (33 Gy) autologous PBMCs that had been preincubated with antigen in 96-well U-bottom microtiter and ELISPOT plates at 37°C for 1 hour. The conditions tested with primary GA-reactive lines were 20 μg/mL GA, 100 μg/mL X13, 40 μg/mL MBP 84-102, 20 μg/mL 90X, 20 μg/mL 91X, 20 μg/mL 93X, 20 μg/mL 93R, 20 μg/mL 90X93R, 20 μg/mL 91X93R, and the no-antigen control. Primary MBP 84-102–induced lines were tested with 20 μg/mL MBP 84-102, 40 μg/mL GA, and no antigen.

Proliferation assay and cytokine measurement by ELISA. Equal aliquots of primary T-cell lines were stimulated with antigen-pulsed autologous PBMCs (100,000 per well). After 48 hours, 160 μL of supernatant was removed and frozen at –80°C for future cytokine analysis. The cells were pulsed with 1 μCi/well of [^{3}H]thymidine in 100 μL of complete medium. After a further 24 hours, cells were harvested onto filter paper, and incorporation of [^{3}H]thymidine was measured in a scintillation counter (Wallace, Gaithersburg, Maryland, USA). Supernatants were tested for cytokines in duplicate by performing standard sandwich ELISA using matched antibody pairs according to the manufacturer's protocol (Endogen Inc., Woburn, Massachusetts, USA).

ELISPOT assay. ELISPOT plates (Millipore Corp., Bedford, Massachusetts, USA) were coated with an optimal concentration of 100 μL of primary antibody diluted in 0.1 mM $NaHCO_3$ (pH 8.3) and incubated overnight at 4°C. Antibody pairs were the same as those used in the sandwich ELISA assay described earlier here. Plates were washed 3 times with PBS and blocked with 1% BSA in HBSS at 37°C for 1 hour. Plates were again washed with PBS 3 times, and antigen-presenting cells were added together with antigen and placed in a 37°C, 8% CO_2 incubator for 60 minutes. Responder T cells were added, and plates were placed in a 37°C incubator for 24 hours. The plates were then washed 3 times with TP buffer (0.05% Tween in PBS) and incubated with 100 μL of biotinylated secondary antibody in TP buffer overnight at 4°C. Plates were washed again 3 times with TP buffer and incubated at room temperature with 100 μL of a 1:1,000 dilution of streptavidin alkaline phosphatase conjugate Extravidin (Sigma Chemical Co., St. Louis, Missouri, USA) for 2 hours. Plates were washed, and viewing of spots was carried out with 100 μL BCIP/NBT substrate (Sigma Chemical Co.) prepared according to manufacturer's instructions and by developing in dark for up to 20 minutes. The reaction was stopped by washing the plates with distilled water.

Measurement of T-cell proliferation in primary in vitro culture. PBMCs, isolated from heparinized blood as already described here, were incubated at 50,000 PBMCs per well in the presence of PHA or at 150,000 PBMCs per well in the presence of tetanus toxoid (Massachusetts Biological Laboratories, Jamaica Plain, Massachusetts, USA). On day 6, 160 μL of culture supernatant was removed from each well and replaced by 100 μL of complete medium containing 1 μCi of [^{3}H]thymidine. After further incubation at 37°C for 18 hours, cells were harvested onto filter paper, and thymidine incorporation was measured by scintillation counting.

HLA typing. Determination of the HLA DR and DQ phenotypes of each patient was determined by standard PCR and hybridization methods.

Statistical analysis. Statistical analysis was performed using the STATISTICA for Macintosh package (StatSoft, Tulsa, Oklahoma, USA) as indicated. Unless otherwise indicated, results are given as mean ± SEM.

Results

The in vitro proliferative response of PBMCs to GA decreases upon in vivo administration of GA. PBMCs were isolated from 7 patients with RR MS before and at various times after subcutaneous administration of GA. At each time point tested, primary and secondary in vitro proliferation and cytokine assays in the presence and absence of GA were performed. We found that before treatment, there was a significant proliferative

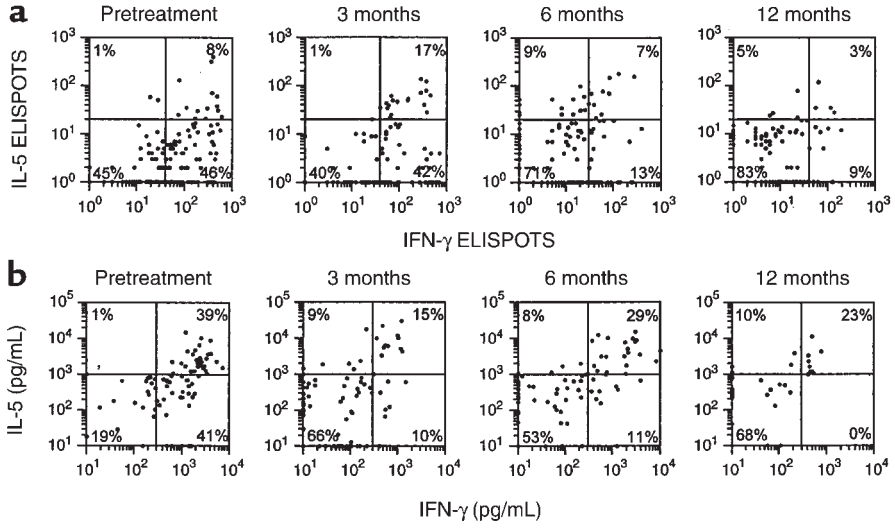

Figure 2
GA-specific secretion of cytokines is polarized toward a Th2 response after daily injections of GA. The GA-specific secretion of the cytokines IL-5 and IFN-γ was measured in T-cell lines by 2 methods: ELISPOT (**a**) and ELISA (**b**) assays. Each symbol represents the difference of spots counted or Δ pg/mL measured in split-well assays between the GA (20 μg/mL) condition and the no-antigen control. The limits of detection were 1 spot and 10 pg/mL, respectively. Numbers represent the percentage of T-cell lines in each quadrant with a minimum difference in spots of twice the SD of the negative controls for IL-5 and IFN-γ, respectively.

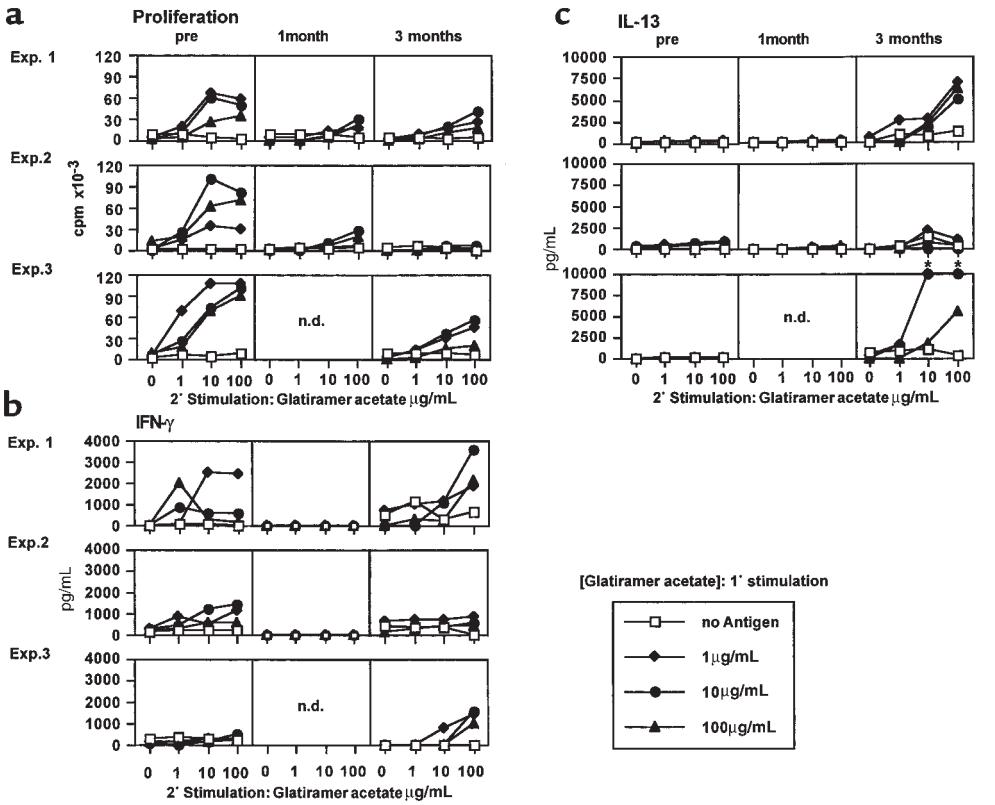

Figure 3
IL-13 secretion is increased after daily injections of GA. Primary T-cell lines were set up in 10 identical wells each in the presence of no antigen or with 1.0, 10, and 100 μg/mL GA and cultured as described in Methods. Identical wells were pooled on day 11, and 30,000 T cells each were restimulated with no antigen or 1.0, 10, and 100 μg/mL GA pulsed on 100,000 autologous APCs. Cytokines were measured in supernatants by ELISA after 48 hours as described, and proliferation was measured by [^{3}H]thymidine incorporation. Asterisks point at values that were above the upper limit of detection of the IL-13 assay of 10,000 pg/mL.

response as measured by [^{3}H]thymidine incorporation to GA in all 7 patients, with an average stimulation index (SI) in vitro of 24.8 ± 1.1; the average Δ cpm was 37,241 ± 1,766 cpm. Additionally, all of a total of 170 independently derived T-cell lines stimulated in primary in vitro culture with GA proliferated in response to the antigen (data not shown). After treatment with GA 20 mg subcutaneously daily for 3, 6 and 12 months, the proliferative response as measured by SI and Δ cpm significantly decreased (Figure 1a) (P < 0.001), although, as expected, individual patients varied in their response to GA (Figure 1b).

In vitro–generated GA-reactive T-cell lines deviate toward a Th2-cytokine profile upon treatment with GA. Having demonstrated that the proliferative response to GA changed after in vivo subcutaneous administration of GA, we next examined whether the cytokine profile also changed. The cytokine response was measured for the prototypic Th1 and Th2 cytokines IFN-γ and IL-5 by ELISPOT and sandwich ELISA in a total of 590 T-cell lines generated before and at various times after GA injection in all 7 patients. As shown in Figure 2, a and b, compared with the values detected before treatment, the average IFN-γ secretion to GA measured by either ELISA or ELISPOT was significantly decreased (P < 0.001 by Tukey's honest statistical difference test) after treatment for 3, 6, and 12 months, except for the measurement by ELISPOT at 3 months, which did not reach significance, and the ELISA measurement at 6 months, which only reached a significance level of P < 0.01. The GA-dependent IFN-γ secretion as determined by ELISPOT as Δ spots between the cells tested with antigen and the no-antigen control was 104 ± 10 before treatment, 132 ± 18 at 3 months, 28 ± 4 at 6 months, and 18 ± 3 at 12 months. IFN-γ secretion measured by sandwich ELISA in Δ pg/mL was 1,405 ± 150 before treatment, 222 ± 38 at 3 months, 797 ± 185 at 6 months, and 5.9 ± 46 at 12 months. When patients were analyzed individually, 5 of the 7 patients had statistically significant decreases in IFN-γ secretion (P < 0.001) by ELISPOT or sandwich ELISA (data not shown). The levels of IFN-γ secretion were correlated with the decreased proliferative capacity in these patients (r^2 of > 0.8 for ELISA values in all patients tested, measurement by ELISPOT correlated less well with r^2> 0.5 in 4 patients). This is in accordance with previous observations that the proliferative and IFN-γ responses are correlated (25).

GA-specific IL-5 secretion was, on average, not statistically significantly altered during treatment with GA. By ELISPOT assay, the average IL-5 secretion in Δ spots was 10 ± 3 before, 10 ± 2 at 3 months, 11 ± 2 at 6 months, and 6 ± 1 at 12 months after the initiation of treatment. When measured by sandwich ELISA as Δ pg/mL, IL-5 secretion was 1,484 ± 266 before treatment; 1,940 ± 554 at 3 months; 1,738 ± 343 at 6 months; and 1,146 ± 303 at 12 months after the initiation of treatment. One patient had a sustained statistically significant decrease in IL-5 (P < 0.001), and 1 had a sustained statistically significant increase (P < 0.001) in IL-5 secretion as measured by ELISPOT during treatment.

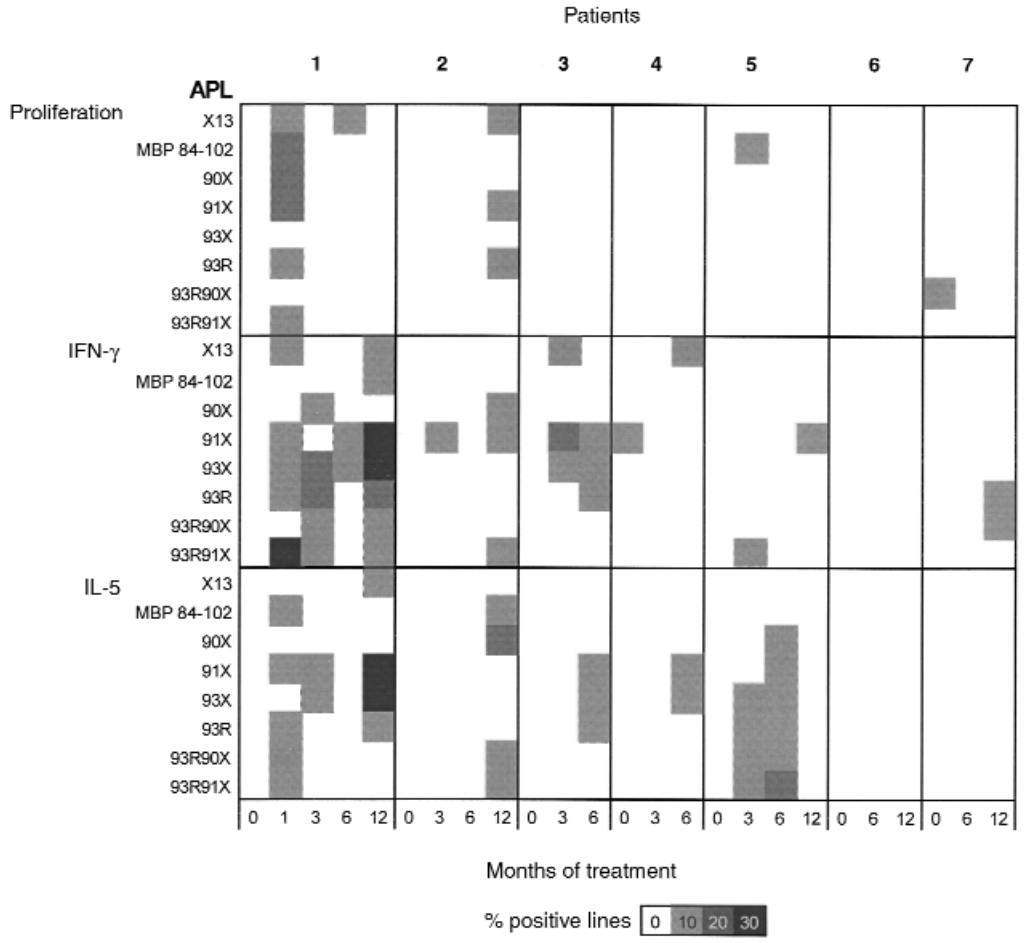

Figure 4
Cross-reactivity of GA-reactive T-cell lines is increased after daily injections of GA. Percentages of the GA-induced T-cell lines cross-reacting to each APL tested at each time point are shown for the 7 patients encoded by gray scale. Proliferative IFN-γ and IL-5 responses were examined for all T-cell lines and are represented separately in the top, middle, and lower third. A minimum SI of 2 and a difference of 2 SD over the background was required for classification as a cross-reactive T-cell line.

To examine further the cytokine pattern of PBMCs from patients before and after treatment with GA, T-cell lines from all subjects were grouped into Th0, Th1, and Th2 subsets, based on their cytokine profile. T-cell lines were considered positive for a cytokine when the difference of the GA condition was increased at least 2-fold over the SD of the no-antigen controls. Thus, values over background considered positive were 19 spots for IFN-γ and 10 spots for the IL-5 ELISPOT assay, and 138 pg/mL for IFN-γ and 485 pg/mL for the IL-5 ELISA assays. When measured by ELISPOT before treatment, 46% of all T cells evaluated were characterized as Th1 (Figure 2a). During the course of treatment, there was an increased proportion at 3 months of Th0-type T-cell lines and at 6 and 12 months of Th2-type T-cell lines. In parallel with the decreased proliferative response, an increasing proportion of T-cell lines that did not secrete either IFN-γ or IL-5 in response to GA was seen with treatment. Measurement by ELISA appears to be slightly more sensitive in this assay in which 39% of T-cell lines secreted Th0 cytokines and 41% secreted Th1 cytokines before treatment (Figure 2b). A similar shift toward a Th2 response as with the ELISPOT assay with a decrease of Th0 and a simultaneous increase of Th2-type T-cell lines at 3, 6, and 12 months of treatment was seen. When classifications of T-cell lines into Th0, Th1, and Th2 were performed under less-stringent conditions (minimum difference of 20 and 10 ELISPOTS for IFN-γ and IL-5, respectively, or 200 pg/mL in ELISA assays), a cytokine shift toward Th0/Th2 could also be confirmed (data not shown).

As the magnitude of the IL-5 response was uniformly low, especially as measured by ELISPOT, it was important to reconfirm the apparent Th2 deviation with GA treatment by a means other than measuring IL-5. Toward this end, 3 new patients with RR MS were recruited to the study, and serial measurements of IFN-γ and IL-13 secretion were made before and after the initiation of daily subcutaneous GA injections. IL-13 was chosen as the candidate Th2 cytokine because there are no IL-13 receptors on T cells to consume the secreted cytokine. Primary in vitro T-cell lines generated in the presence of no antigen and of 1.0, 10, and 100 μg/mL GA were examined at 30,000 PBMCs per well

each with the 4 antigen concentrations. Thus, equal numbers of cells were tested in the secondary stimulation, whereas in the previous assay with equal aliquots of primary cell lines, the number of cells tested decreased, on average, with treatment owing to lower expansion in the primary cultures. A marked increase in IL-13 secretion in 2 of 3 patients after 3 months of therapy was observed. IFN-γ secretion and the proliferative response were stable or decreased when compared with the pretreatment values (Figure 3).

Cross-reactivity of GA-reactive T-cell lines is increased upon treatment with GA. Cross-reactivity of GA-induced T-cell lines to combinatorial peptide libraries derived from the immunodominant MBP 84-102 peptide and a completely randomized 13mer library were performed to determine degeneracy of GA-specific T cells (Figure 4). Before treatment with GA, there was minimal cross-reactivity in either the proliferative or the cytokine responses. There was only 1 instance when antigen cross-reactivity was observed, in the proliferative response to the 93R90X combinatorial peptide library in patient 7. In striking contrast, 6 of the 7 patients demonstrated an increased number of cross-reactive T-cell lines after therapy to the combinatorial peptide libraries. However, no dominantly cross-reactive APL emerged from this analysis, consistent with the degenerate immune responses we observed.

In vitro T-cell reactivity to the immunodominant epitope MBP 84-102 is not significantly altered during GA therapy. In contrast to the results from primary in vitro cultures with GA, which is well approximated by a normal distribution of responses owing to the high precursor frequency of responsive T cells, the reactivity of primary T-cell lines to MBP 84-102 was low and as such not normally distributed. Therefore, nonparametric statistics were used for analysis. No significant change over time of treatment was seen for the MBP 84-102–specific proliferative responses (Figure 5a) by Mann-Whitney *U* tests. Further analysis included percentage of MBP 84-102–specific T-cell lines, which were not significantly changed over the course of treatment.

Analysis of cytokine secretion in response to MBP 84-102 by Mann-Whitney *U* test also did not reveal any significant differences during the course of treatment with one exception: IL-5 measured by ELISPOT at 12

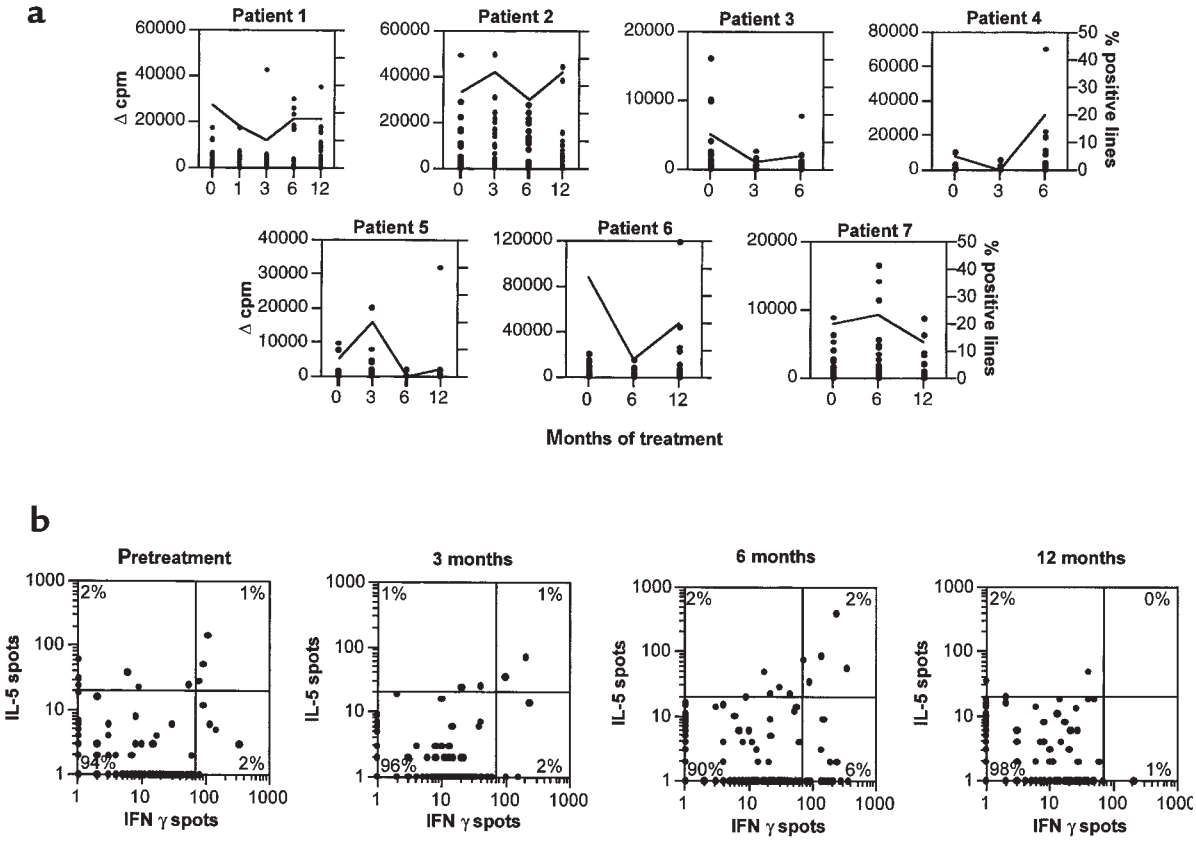

Figure 5

(**a**). The proliferative response to MBP 84-102 is not altered after daily injections of GA. A total of 900 T-cell lines were generated in response to MBP 84-102. The proliferative response in Δ cpm is given for each of 30 or 40 lines tested at each time point (circles, Δ cpm on left axes). On the right axes, the percentage of positive lines determined by a minimum 2.5-fold increase over background and a minimum difference of 1,500 cpm is shown (line). The mean background was 4,662 ± 138. (**b**). No changes occur in the cytokine response to MBP 84-102 after daily injections of GA. Lines having a minimum difference to the negative control of 2 SD, i.e., 70 spots for IFN-γ and 19 spots for IL-5, were considered positive for the respective cytokine.

months tested significantly decreased ($P < 0.01$) compared with pretreatment values. When the cytokines were analyzed for each patient individually, no significant change in the frequency of Th0-, Th1-, and Th2-cytokine–secreting lines was seen (Figure 5b), regardless of the stringency of the criteria used for classification. No cross-reactivity was seen between MBP 84-102–induced T cell lines and GA in vitro when judged by these criteria.

GA treatment does not change tetanus toxoid–specific in vitro T-cell responses. To examine the effects of GA treatment on the primary in vitro T-cell response to an unrelated recall antigen, PBMCs from 6 patients with RR MS who were undergoing GA therapy were analyzed with tetanus toxoid. A dose-dependent proliferative response to 0.3 μg/mL, 3 μg/mL, and 30 μg/mL of tetanus toxoid as well as to 0.1 μg/mL and 1 μg/mL of the PHA control was observed at all time points. Compared with the pretreatment values, no significant differences in the tetanus toxoid–specific proliferative responses were seen during treatment with GA as determined by the Student's *t* test (data not shown). Thus, as with MBP 84-102, subcutaneous treatment with GA did not alter immune responses to a common recall antigen.

Discussion

We examined the effect of daily subcutaneous injections of GA on antigen-specific T-cell responses in patients with MS. GA appears to function as a universal antigen, inducing primary in vitro proliferation of naive T-cell populations both in patients with MS and in normal healthy controls. Daily subcutaneous injections of GA caused a striking loss of in vitro responsiveness to the GA that was accompanied by immune deviation to a more Th2 type of response. The surviving GA-reactive T cells exhibited a greater degree of degeneracy as measured by cross-reactive responses to combinatorial peptide libraries. GA is, to our knowledge, the first agent effective in the treatment of an autoimmune disease that appears to alter immune function by engagement of the T-cell receptor and may be useful in a variety of autoimmune disorders in which immune deviation to a Th2 type of response may be desirable.

Perhaps the most striking observation in these investigations was the ability of GA to induce the proliferation of non-GA–primed T-cell populations, which then decreased with treatment. This was not a nonspecific mitogenic response, as MHC DR-restricted T-cell clones could be generated with in vitro antigen culture (P.W. Duda, and D.A. Hafler, manuscript in preparation). GA has been shown to directly bind different DR molecules without antigen processing, allowing many potential interactions with T-cell receptors (14). Although it had been thought that the recognition by the TCR of an MHC/peptide complex was highly specific, it has recently become clear that there can be extensive degeneracy in TCR recognition of antigen in the trimolecular complex (26, 27). Moreover, we recently demonstrated at a functional level that a TCR that may appear to be highly specific for 1 peptide/MHC complex may become significantly more degenerate in its recognition of antigen with subtle changes of amino acid side chains, particularly lysine engaging the TCR hydrophobic pocket created by α and β CDR3 loops (28). Thus the structure of GA may favor degenerate recognition by T cells.

Administration of GA in vivo resulted in a marked loss of subsequent in vitro proliferative responses to GA. The mechanism for the loss of this proliferative response is not known, although preliminary in vitro data suggest that it is highly dose dependent (M.C. Schmied and D.A. Hafler, unpublished data). These changes are consistent with deletion of high-affinity Th1 T cells by activation–induced cell death, as has been shown to occur with administration of high doses of antigen (29).

We did not detect any significant changes in MBP 84-102–specific proliferative and cytokine responses in the PBMCs from the study patients over the course of treatment. One obvious explanation for this result is that most MBP-reactive T cells in humans do not frequently cross-react with GA and are therefore not directly affected by the treatment. An alternative hypothesis is that these T cells are in a different state of activation in patients with MS than in healthy control individuals as has been shown previously (30–32). Alteration of the phenotype of committed memory T cells in vitro is only possible with strong signals such as combined IL-4 and anti–IL-12 treatment and is not likely to occur easily in vivo. This may also be reflected by the fact that the tetanus toxoid–specific memory responses were not altered during treatment. The difference in our findings from observations in the EAE model, where immunomodulatory effects of GA treatment on MBP reactive cells have been observed, may reflect the fact that treatment with GA before disease induction primarily targets naive T cells. These may be more readily influenced than MBP-specific memory T-cell populations in patients with MS.

Although there was essentially no cross-reactivity in MBP and GA-reactive T-cell lines before treatment, there was increased recognition of a multitude of peptides, including MBP 84-102 and the associated combinatorial peptide libraries, after treatment. Cross-reactive T cells have been shown in several animal models to protect from EAE. Injection of an APL of PLP (33), as well as adoptive transfer of APL-reactive T cells (11), could prevent disease induction by PLP in the SJL mouse. APLs of the immunodominant MBP peptide were effective in EAE prevention (9, 34). Adoptive transfer of GA-reactive T cells has also been shown to protect from EAE (22) and was thought to be mediated by a direct inhibitory effect by recognition of GA as APL for MBP-reactive T cells.

We observed an increased ratio of both IL-5 and IL-13 to IFN-γ secretion in GA-reactive T cells, corresponding to a cytokine immune deviation toward

Th2-cytokine secretion. These cytokines were chosen over measurement of IL-4, as IL-4 receptors on T cells hinder the measurement of this cytokine in T-cell culture supernatants; because receptors for both IL-5 and IL-13 are not expressed on T cells in detectable amounts, the problem of cytokine consumption is perhaps less important for these cytokines. Measurement of TGF-β in GA-reactive T-cell lines was examined but could not be detected.

In addition, as previously reported, we observed a correlation between IFN-γ secretion and [^{3}H]thymidine incorporation (25). This leads to the question of what the mechanism of action is by which GA affects the course of MS. Our data suggest that the remaining cross-reactive T cells, generated after in vivo GA administration may recognize GA as its own APL, with degenerate recognition of its random sequence of 4 amino acids. It has been shown that APLs recognized by T cells as weak or partial agonists can induce anergy (35, 36) and a shift toward Th2 (8, 10, 28) and Th3 cytokines (37). We hypothesize that GA mediates its beneficial effect on MS and perhaps EAE by induction of GA-specific Th2-polarized T cells that can enter into the activated target tissue. Some of these T cells may then recognize MBP or PLP or MOG as low-affinity APL. This broader specificity of GA-reactive T-cell lines became evident as responses were seen to the random 13mer library, in addition to the MBP-derived APLs and MBP 84-102 itself. Owing to the basic nature of GA, a T-cell repertoire with predominantly acidic residues in the CDR3 region will more likely be induced, with a potential to cross-react with a multitude of T-cell epitopes of naturally processed self-proteins as weak agonists (38) in the CNS. With an ensuing cytokine polarization toward Th2 responses by cross-reactive GA-specific T cells in MS lesions, myelin-reactive T cells could be inhibited in vivo by the mechanism of bystander suppression.

The direct immunomodulatory effects of GA, in contrast to nonspecific classic immunosuppressive therapies, only affect the relatively small (< 0.1%), albeit significant, proportion of GA-reactive T cells and would thus not be expected to alter responses to other antigens. As discussed earlier here, no changes were observed in T-cell responses to tetanus toxoid. However, the theoretical possibilities that other essential antigen-specific responses may be impaired or that untoward immunological reactions other than local irritation may be increased have not been reported.

In summary, GA does not appear to have a direct effect on the preexisting memory compartment of T cells, and it does not appear to be selectively cross-reactive with MBP. Instead, GA treatment induces a GA-specific Th2-polarized T-cell repertoire that recognizes antigen in a more degenerate fashion. This may be sufficient to mediate bystander suppression at many sites of inflammation in the CNS of patients with MS, leading to decreased disease activity.

Acknowledgments

We thank M. Ellefson for assistance in manuscript preparation, and G. Semana for performing the HLA typing on our subjects. P.W. Duda was supported by fellowships from the Novartis Foundation (former Ciba Geigy Foundation) and the Swiss Multiple Sclerosis Society. M.C. Schmied was supported by an Erwin Schroedinger Fellowship, FWF J1603-MED. This work was supported by grants from the National Institute of Health (RO1NS2424710, P01AI39671, and PO1NS38037 to D.A. Hafler), National Multiple Sclerosis Society grants RG2172B6 and RG2949A (to D.A. Hafler), and a grant from Teva Marion Partners.

1. Hafler, D.A. 1999. The distinction blurs between an autoimmune versus microbial hypothesis in multiple sclerosis. *J. Clin. Invest.* **104**:527–529.
2. Windhagen, A., et al. 1995. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J. Exp. Med.* **182**:1985–1996.
3. Grewal, I.S., et al. 1996. Requirement for CD40 ligand in costimulation induction, T cell activation, and experimental allergic encephalomyelitis. *Science.* **273**:1864–1867.
4. Lehmann, P.V., Forsthuber, T., Miller, A., and Sercarz, E.E. 1992. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature.* **358**:155–157.
5. Windhagen, A., et al. 1998. Cytokine secretion of myelin basic protein reactive T cells in patients with multiple sclerosis. *J. Neuroimmunol.* **91**:1–9.
6. Yu, M., Johnson, J.M., and Tuohy, V.K. 1996. A predictable sequential determinant spreading cascade invariably accompanies progression of experimental autoimmune encephalomyelitis: a basis for peptide-specific therapy after onset of clinical disease. *J. Exp. Med.* **183**:1777–1788.
7. Chen, Y., Kuchroo, V.K., Inobe, J., Hafler, D.A., and Weiner, H.L. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science.* **265**:1237–1240.
8. Evavold, B.D., and Allen, P.M. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science.* **252**:1308–1310.
9. Karin, N., Mitchell, D.J., Brocke, S., Ling, N., and Steinman, L. 1994. Reversal of experimental autoimmune encephalomyelitis by a soluble peptide variant of a myelin basic protein epitope: T cell receptor antagonism and reduction of interferon gamma and tumor necrosis factor alpha production. *J. Exp. Med.* **180**:2227–2237.
10. Pfeiffer, C., et al. 1995. Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J. Exp. Med.* **181**:1569–1574.
11. Nicholson, L.B., Murtaza, A., Hafler, B.P., Sette, A., and Kuchroo, V.K. 1997. A T cell receptor antagonist peptide induces T cells that mediate bystander suppression and prevent autoimmune encephalomyelitis induced with multiple myelin antigens. *Proc. Natl. Acad. Sci. USA.* **94**:9279–9284.
12. Teitelbaum, D., Meshorer, A., Hirshfeld, T., Arnon, R., and Sela, M. 1971. Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide. *Eur. J. Immunol.* **1**:242–248.
13. Fridkis-Hareli, M., et al. 1994. Direct binding of myelin basic protein and synthetic copolymer 1 to class II major histocompatibility complex molecules on living antigen-presenting cells: specificity and promiscuity. *Proc. Natl. Acad. Sci. USA.* **91**:4872–4876.
14. Fridkis-Hareli, M., and Strominger, J.L. 1998. Promiscuous binding of synthetic copolymer 1 to purified HLA-DR molecules. *J. Immunol.* **160**:4386–4397.
15. Brosnan, C.F., et al. 1985. Immunogenic potentials of copolymer I in normal human lymphocytes. *Neurology.* **35**:1754–1759.
16. Teitelbaum, D., Fridkis-Hareli, M., Arnon, R., and Sela, M. 1996. Copolymer 1 inhibits chronic relapsing experimental allergic encephalomyelitis induced by proteolipid protein (PLP) peptides in mice and interferes with PLP-specific T cell responses. *J. Neuroimmunol.* **64**:209–217.
17. Ben-Nun, A., et al. 1996. The autoimmune reactivity to myelin oligodendrocyte glycoprotein (MOG) in multiple sclerosis is potentially pathogenic: effect of copolymer 1 on MOG-induced disease. *J. Neurol.* **243**:S14–22S.
18. Johnson, K.P., et al. 1998. Extended use of glatiramer acetate (Copaxone) is well tolerated and maintains its clinical effect on multiple sclerosis relapse rate and degree of disability. Copolymer 1 Multiple Sclerosis Study Group. *Neurology.* **50**:701–708.
19. Johnson, K.P., et al. 1995. Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group. *Neurology.* **45**:1268–1276.

20. Comi, G., and Filippi, M. 1999. The effect of glatiramer acetate (Copaxone®) on disease activity as measured by cerebral MRI in patients with relapsing-remitting multiple sclerosis (RRMS): a multi-center, randomized, double-blind, placebo-controlled study extended by open-label treatment. *Neurology.* **52:**A289. (Abstr.)
21. Lando, Z., Teitelbaum, D., and Arnon, R. 1979. Effect of cyclophosphamide on suppressor cell activity in mice unresponsive to EAE. *J. Immunol.* **123**:2156–2160.
22. Aharoni, R., Teitelbaum, D., and Arnon, R. 1993. T suppressor hybridomas and interleukin-2–dependent lines induced by copolymer 1 or by spinal cord homogenate down-regulate experimental allergic encephalomyelitis. *Eur. J. Immunol.* **23**:17–25.
23. Aharoni, R., Teitelbaum, D., Arnon, R., and Sela, M. 1999. Copolymer 1 acts against the immunodominant epitope 82-100 of myelin basic protein by T cell receptor antagonism in addition to major histocompatibility complex blocking. *Proc. Natl. Acad. Sci. USA.* **96**:634–639.
24. Aharoni, R., Teitelbaum, D., Sela, M., and Arnon, R. 1997. Copolymer 1 induces T cells of the T helper type 2 that crossreact with myelin basic protein and suppress experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. USA.* **94**:10821–10826.
25. Fukaura, H., et al. 1996. Induction of circulating myelin basic protein and proteolipid protein specific TGF-β1 secreting T cells by oral administration of myelin in multiple sclerosis patients. *J. Clin. Invest.* **98**:70–77.
26. Sloan-Lancaster, J., and Allen, P.M. 1995. Significance of T-cell stimulation by altered peptide ligands in T cell biology. *Curr. Opin. Immunol.* **7**:103–109.
27. Hemmer, B., et al. 1997. Identification of high potency microbial and self ligands for a human autoreactive class II-restricted T cell clone. *J. Exp. Med.* **185**:1651–1659.
28. Ausubel, L.J., Krieger, J.I., and Hafler, D.A. 1997. Changes in cytokine secretion induced by altered peptide ligands of myelin basic protein peptide 85-99. *J. Immunol.* **159**:2502–2512.
29. Critchfield, J.M., et al. 1994. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. *Science.* **263**:1139–1143.
30. Allegretta, M., Nicklas, J.A., Sriram, S., and Albertini, R.J. 1990. T cells responsive to myelin basic protein in patients with multiple sclerosis. *Science.* **247**:718–721.
31. Zhang, J., et al. 1994. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J. Exp. Med.* **179**:973–984.
32. Bieganowska, K.D., et al. 1997. Direct ex vivo analysis of activated, Fas-sensitive autoreactive T cells in human autoimmune disease. *J. Exp. Med.* **185**:1585–1594.
33. Kuchroo, V.K., et al. 1994. T cell receptor (TCR) usage determines disease susceptibility in experimental autoimmune encephalomyelitis: studies with TCR V beta 8.2 transgenic mice. *J. Exp. Med.* **179**:1659–1664.
34. Marusic, S., and Tonegawa, S.M.S. 1997. Tolerance induction and autoimmune encephalomyelitis amelioration after administration of myelin basic protein-derived peptide. *J. Exp. Med.* **186**:507–515.
35. Sloan-Lancaster, J., Evavold, B.D., and Allen, P.M. 1993. Induction of T-cell anergy by altered T-cell–receptor ligand on live antigen-presenting cells. *Nature.* **363**:156–159.
36. Evavold, B.D., Sloan-Lancaster, J., Wilson, K.J., Rothbard, J.B., and Allen, P.M. 1995. Specific T cell recognition of minimally homologous peptides: evidence for multiple endogenous ligands. *Immunity.* **2**:655–663.
37. Windhagen, A., et al. 1995. Modulation of cytokine patterns of human autoreactive T cell clones by a single amino acid substitution of their peptide ligand. *Immunity.* **2**:373–380.
38. Jorgensen, J.L., et al. 1992. Mapping T-cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics. *Nature.* **355**:224–230.