Bystander Modulation of Chemokine Receptor Expression on Peripheral Blood T Lymphocytes Mediated by Glatiramer Therapy

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Background: Glatiramer acetate therapy is thought to be effective for multiple sclerosis (MS) by promoting T_H2 cytokine deviation, possibly in the brain, but the exact mechanism and site of action are incompletely understood. Determining the site of action and effect of glatiramer on cell trafficking is of major importance in designing rational combination therapy clinical trials.

Objective: To determine whether glatiramer therapy will also act in the peripheral blood through bystander modulation of chemokine receptor (CKR) expression and cytokine production on T lymphocytes.

Design: Before-and-after trial.

Setting: A university MS specialty center.

Patients: Ten patients with relapsing-remitting MS.

Interventions: Treatment with glatiramer for 12 months and serial phlebotomy.

Main Outcome Measures: Cytokine production, CKR expression, and cell migration.

Results: The glatiramer-reactive T cells were T_H2 cytokine biased, consistent with previous studies. We found a significant reduction in the expression of the T_H1 inflammation associated with the CKRs CXCR3, CXCR6, and CCR5 on glatiramer- and myelin-reactive T cells generated from patients with MS receiving glatiramer therapy vs baseline. Conversely, expression of the lymph node–homing CKR, CCR7, was markedly enhanced on the glatiramer-reactive T cells derived from patients with MS undergoing glatiramer therapy. There was a reduction in the percentage of $CD4^+$ glatiramer-reactive T cells and an increase in the number of $CD8^+$ glatiramer-reactive T cells.

Conclusions: Glatiramer may suppress autoreactive $CD4^+$ effector memory T cells and enhance $CD8^+$ regulatory responses, and bystander modulation of CKRs may occur in the periphery.

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THE MECHANISM OF ACTION of glatiramer acetate is only partially understood.[1] Several recent studies[1] have demonstrated that, in vitro, glatiramer causes a marked polyclonal expansion of T_H2 cells that inhibit the T_H1 response thought to be pathogenic in multiple sclerosis (MS). Furthermore, glatiramer-reactive T_H2 cells secrete brain-derived neurotrophic factor, which could be neuroprotective.[2,3] In experimental autoimmune encephalomyelitis, glatiramer-reactive T cells have been demonstrated in the brain, and they cross-react with myelin antigens.[4] In humans, there are no data regarding the capacity of glatiramer-reactive T cells to penetrate the central nervous system (CNS). Previous studies[5,6] have shown that glatiramer can cross-react with myelin-reactive T cells, and because glatiramer itself likely does not access the CNS, this would have to occur systemically. Indeed, it has been suggested that bystander modulation may occur in the periphery.[7,8]

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Herein, we sought to determine whether glatiramer-reactive T cells derived from patients with MS receiving this therapy have a significant reduction in chemokine receptors (CKRs) associated with effector cell homing to sites of inflammation but enhancement of a more naive lymph node–homing phenotype. This would support the possibility that glatiramer may also act peripherally in the lymph nodes through bystander suppression.

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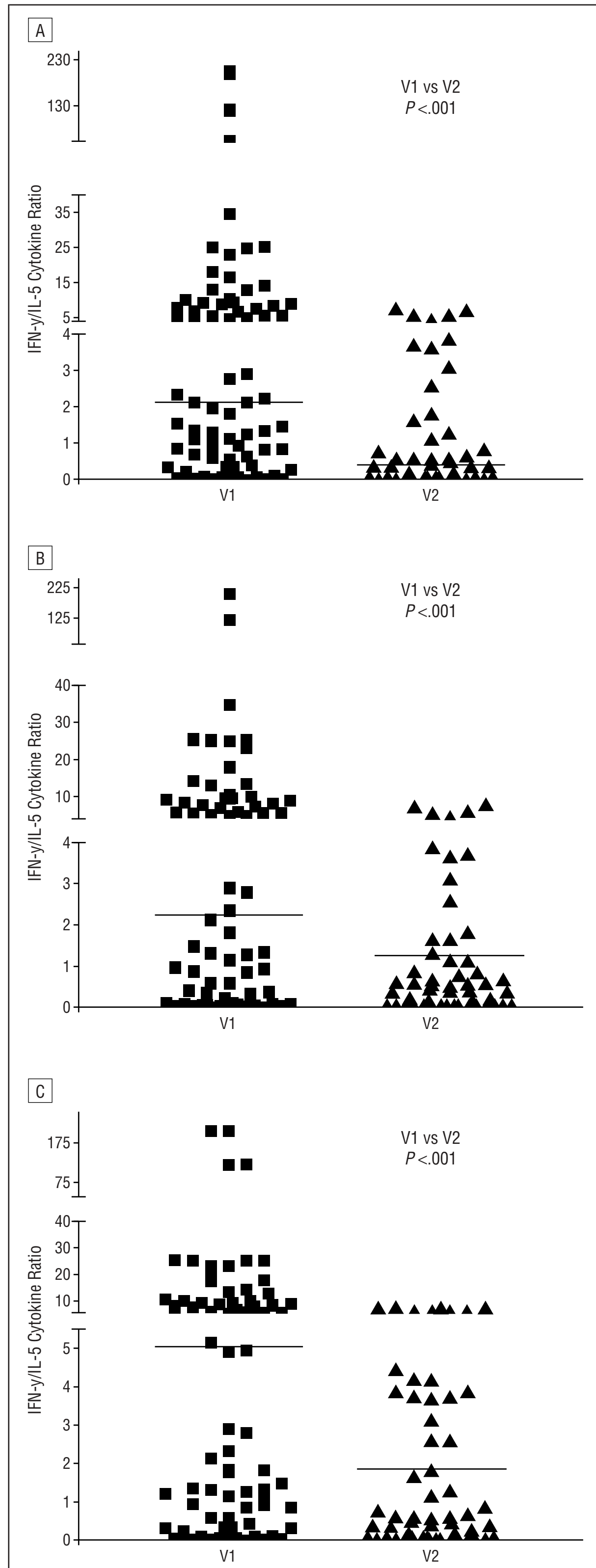

Figure 1. Interferon γ (IFN-γ) (T_H1)/interleukin 5 (IL-5) (T_H2) cytokine ratios on glatiramer acetate–specific (A), myelin basic protein–specific (MBP) (B), and tetanus toxoid (TT)–specific (C) T cells at visit 1 (V1) (baseline, before glatiramer therapy) and visit 2 (V2) (12 months after initiation of glatiramer therapy). A total of 372 (122 glatiramer, 122 MBP, and 128 TT) T-cell lines were examined (see the "Methods" section). Horizontal bars represent median ratios. Significant decreases in the ratio were determined using Mann-Whitney *U* tests (nonparametric data).

METHODS

Human peripheral blood mononuclear cells were obtained from 10 patients with MS before and at various times after therapy with glatiramer after obtaining informed consent according to an institutional review board–approved protocol at the University of Maryland, Baltimore. Patients had not been previously treated with any immune-modulating drugs. Antigen-specific T-cell lines (TCLs) were generated from peripheral blood mononuclear cells using a split-well assay and were then studied using 4-color fluorescent-activated cell sorter analysis on day 7 after restimulation (21 total days in culture), as reported previously.[9] Supernatants were collected for cytokine measurements after antigen restimulation and were frozen until assayed. Interferon γ (IFN-γ) and interleukin 5 levels were measured as markers of T_H1 and T_H2 phenotypes using a sandwich enzyme-linked immunosorbent assay according to the manufacturer's recommendations (Biosource International, Camarillo, Calif). Cell migration in response to chemokines was measured using 24-well chambers separated by a polycarbonate filter with a 5-μm pore size (Transwell; Corning Costar, Cambridge, Mass). The cells that transmigrated into the lower compartment were counted in a hemacytometer. Results are expressed as a migration index that is the ratio between the numbers of migrating cells in the chemokine-containing chamber and in the medium control chamber, which is normalized to a value of 1. All migration conditions were run in triplicate.

Statistical analyses were performed using a software program (Prism; GraphPad Software, San Diego, Calif). Comparisons between times or culture conditions were made using either a 2-tailed *t* test (parametric data) or a Mann-Whitney *U* test (nonparametric data). In serial analyses, significance was determined using 1-way analysis of variance with the Dunnett multiple comparison test. Significance was defined as $P<.05$.

RESULTS

We first determined the IFN-γ and interleukin 5 cytokine profiles of 372 antigen-specific (122 glatiramer, 122 myelin basic protein [MBP], and 128 tetanus toxoid [TT]) TCLs at visit 1 (baseline, before initiation of therapy) and at visit 2 (after 12 months of treatment with glatiramer). In vivo treatment of patients with MS taking glatiramer resulted in a significant T_H2 bias in cytokine production not only from glatiramer-reactive T cells but also from a dampening of the MBP- and TT-reactive T_H1 cell cytokine release (**Figure 1**).

Next we analyzed the CKR profiles of these TCLs (**Figure 2**). At baseline, glatiramer-reactive TCLs expressed high levels of the T_H1-associated CKRs CXCR3, CXCR6, and CCR5. This was not significantly different from the levels of expression of the CKRs on MBP- or TT-reactive TCLs. However, after 12 months of therapy with glatiramer, the ex vivo–generated glatiramer-reactive T_H2-biased T cells had significant reductions in the expression of all of these T_H1-associated CKRs (Figure 2A, C, and D and **Table**). Conversely, there was markedly elevated expression of the lymph node–homing CKR CCR7 (Figure 2B). We also examined the expression of 1 CKR, CCR4, that has been linked to T_H2 effector cells, and found that it too was reduced after glatiramer therapy (Figure 2E).

A critical finding was that the reduction in CKR expression was not specific for glatiramer-reactive T cells

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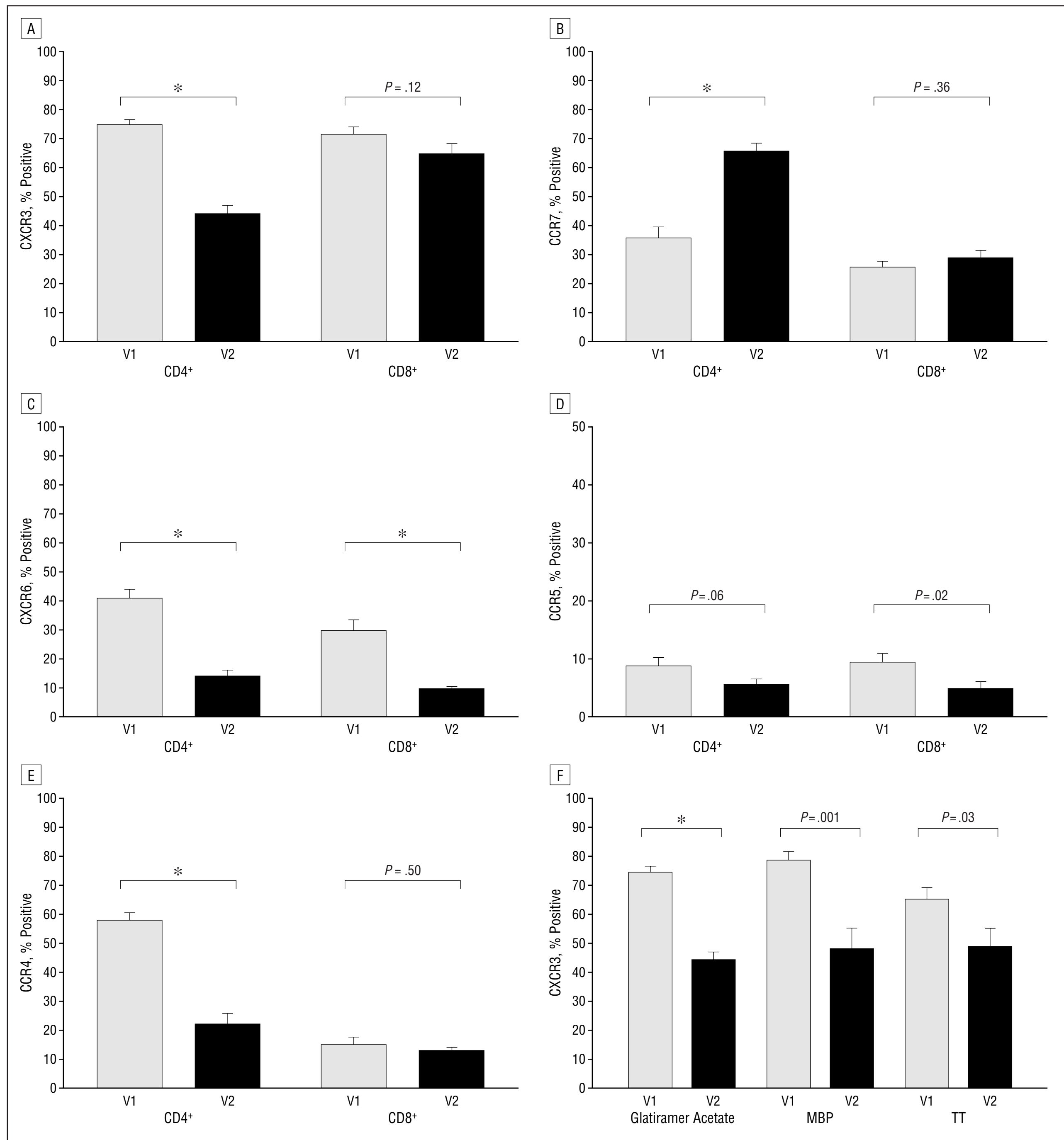

Figure 2. Mean chemokine receptor (CKR) expression on $CD4^+$ and $CD8^+$ subsets of antigen-specific T-cell lines (TCLs) at visit 1 (V1) and visit 2 (V2) as determined using flow cytometric analysis of TCLs (see the "Methods" section). The CKRs on glatiramer acetate–reactive TCLs are shown in CXCR3 (A), CCR7 (B), CXCR6 (C), CCR5 (D), and CCR4 (E). The CXCR3 expression on glatiramer TCLs compared with myelin basic protein (MBP)– and tetanus toxoid (TT)–specific TCLs is also shown (F). Two-tailed unpaired *t* tests (parametric data) were used to measure significant differences between CKR expression at V1 vs V2. Asterisk indicates $P < .001$; error bars, SEM.

because MBP- and, to some extent, even TT-reactive TCLs also had decreased expression of effector CKRs (Figure 2F and Table). This effect was most notable in CD4 subsets, but in some circumstances, CD8 cells had significant and concomitant shifts in CKR expression (Figure 2 and Table).

We next performed a serial examination of CKR expression across time to determine the kinetics of this response and whether the changes were sustained. The reduction in CXCR3 expression could be seen at 6 months, but the increase in CCR7 expression was not seen until 1 year, suggesting that these switches may be dissociated (**Figure 3**).

The functional relevance of the observed shifts in CKR expression was confirmed using migration assays (Transwell) (**Figure 4**). At baseline, glatiramer-reactive TCLs migrated best to the chemokine CXCL-10 (CXCR3 ligand), consistent with their high expression

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Table. Chemokine Receptor Expression on Antigen-Specific T-Cell Lines Before (V1) and After 12 Months of (V2) Glatiramer Therapy*

	CXCR3/CD4	CXCR3/CD8	CCR7/CD4	CCR7/CD8	CXCR6/CD4	CXCR6/CD8	CCR5/CD4	CCR5/CD8
Glatiramer acetate								
V1	74.4 ± 1.9	71.3 ± 2.6	35.9 ± 3.6	25.7 ± 1.7	40.7 ± 3.5	29.6 ± 3.7	8.7 ± 1.4	9.4 ± 1.5
V2	44.2 ± 2.4	64.9 ± 3.1	65.6 ± 2.9	28.6 ± 2.6	13.8 ± 2.2	9.1 ± 1.0	5.5 ± 1.0	4.8 ± 1.3
P value†	<.001	.12	<.001	.36	<.001	<.001	.06	.02
MBP								
V1	78.5 ± 2.9	76.9 ± 5.0	29.8 ± 6.2	24.6 ± 3.5	41.2 ± 3.6	30.6 ± 3.9	12.0 ± 3.6	9.4 ± 2.6
V2	47.8 ± 7.3	70.7 ± 5.6	57.4 ± 7.4	18.7 ± 3.3	14.4 ± 4.0	9.8 ± 1.3	10.1 ± 4.8	6.2 ± 4.3
P value†	.001	.42	.01	.24	<.001	.001	.75	.53
TT								
V1	64.9 ± 3.8	84.2 ± 1.9	37.3 ± 4.5	31.7 ± 4.9	26.8 ± 3.9	38.4 ± 2.6	14.8 ± 3.9	24.3 ± 7.5
V2	48.5 ± 6.1	44.8 ± 6.5	34.4 ± 7.8	11.1 ± 2.2	32.8 ± 7.5	9.0 ± 1.8	14.3 ± 4.3	4.6 ± 2.6
P value†	.03	<.001	.76	.001	.48	<.001	.94	.02

Abbreviations: MBP, myelin basic protein; TT, tetanus toxoid; V1, visit 1; V2, visit 2.
*Data are given as mean ± SEM percentages. CXCR3, CXCR6, CCR5, and CCR7 are chemokine receptors.
†For V1 vs V2.

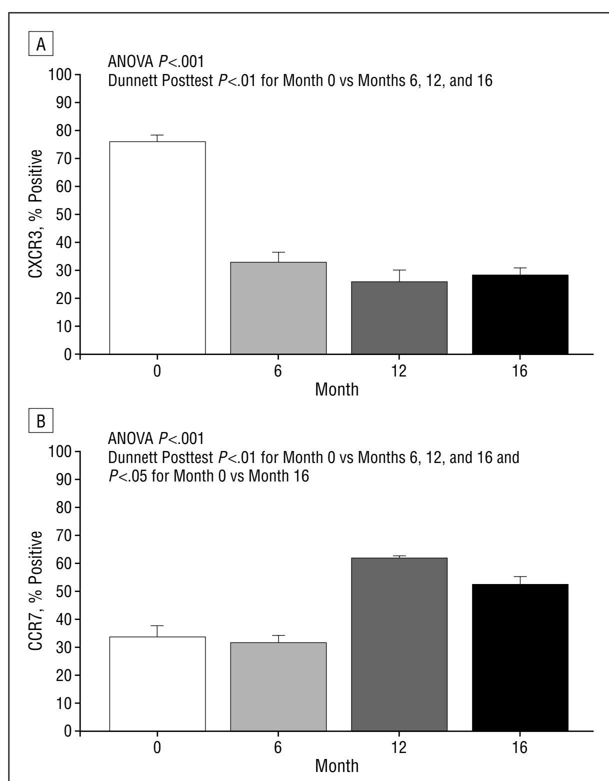

Figure 3. Serial analysis of mean CXCR3 (A) and CCR7 (B) (chemokine receptors) expression on $CD4^+$ glatiramer acetate–specific T-cell lines at baseline (month 0) and at months 6, 12, and 16 after initiation of glatiramer therapy. Significant differences were determined using 1-way analysis of variance (ANOVA), and posttest analysis of change from baseline was made using the Dunnett multiple comparison test. Error bars represent SEM.

of CXCR3. They also exhibited some capacity to migrate to CCL19 (CCR7 ligand). In comparison, the glatiramer-reactive TCLs obtained from patients undergoing glatiramer therapy migrated significantly better to the CCR7 ligand MIP-3β but exhibited reduced migration capacity to the CXCR3 ligand IP-10, consistent with the switches in expression of these CKRs after therapy. Glatiramer-reactive TCLs exhibited insignificant migration to the alternative strong T_H1-associated chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted) (CCR5 ligand) at either time.

Finally, we observed a significant reduction in glatiramer-reactive $CD4^+$ cells at visit 1 vs visit 2 (mean ± SEM: 79% ± 2% vs 62% ± 3%; $P<.001$) and an increase in $CD8^+$ cells after initiation of glatiramer therapy (mean ± SEM [visit 1 vs visit 2]: 20% ± 2% vs 35% ± 3%; $P<.001$) (**Figure 5**). Although there was a trend toward decreased proliferation as measured by the stimulation index, this was not significant (mean ± SEM [visit 1 vs visit 2]: 7% ± 1% vs 5% ± 1%; $P=.15$).

COMMENT

We demonstrated that in vivo therapy with glatiramer causes a statistically significant reduction in the expression of CKRs associated with T_H1 cell homing to tissue sites of inflammation. Specifically, CXCR3 and CCR5, which have been demonstrated on pathogenic infiltrating T cells in MS brain tissue, and CXCR6, which is expressed on T_H1 cells in the periphery, were reduced on not only the glatiramer TCL but also the MBP TCL, suggesting that bystander modulation of CKRs occurs in the peripheral blood compartment of glatiramer-treated patients with MS. Alternatively, the lymph node–homing CKR, CCR7, was significantly elevated after 1 year of therapy. The observed switch in CKR expression is reminiscent of the recently described paradigm of effector memory T cells being $CD45RA^-/CCR7^-$ and central memory T cells being $CD45RA^-/CCR7^+$.[10] Our results are consistent with the concept that glatiramer may bias against effector memory T cells through continued weak affinity interactions with cross-reactive T-cell receptors such as MBP, as has been previously demonstrated.[5,6] Because glatiramer is a large copolymer and does not access the CNS itself, it has been postulated that glatiramer-reactive cells mediate bystander suppression in the CNS through cross-reactivity with myelin antigens being presented by microglial cells; indeed, this has been shown

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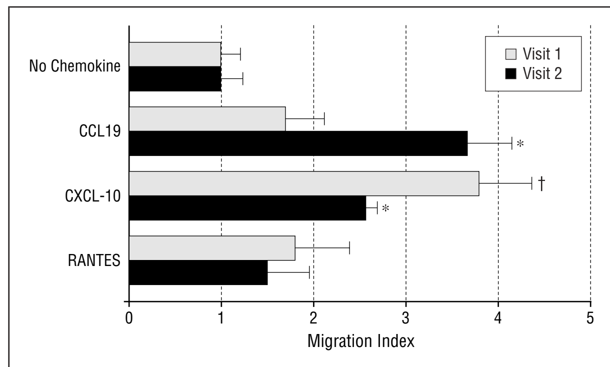

Figure 4. Migration of glatiramer acetate–reactive T-cell lines in response to CCL19 (CCR7 ligand), CXCL-10 (CXCR3 ligand), and RANTES (regulated upon activation, normal T-cell expressed and secreted) (CCR5 ligand). Glatiramer T-cell lines established from patients before glatiramer therapy (visit 1) or after 12 months of glatiramer therapy (visit 2) were stimulated for 48 hours with plate-bound αCD3 (1 μg/mL) plus soluble αCD28 (1 μg/mL) and then were incubated in Transwell chambers for 3 hours. The number of T cells migrating across 5-μm pore filters into lower chambers containing 500 ng/mL indicated chemokines and was quantified and expressed as the migration index (see the "Methods" section). Two-tailed unpaired *t* tests (parametric data) were used to measure significant differences in migration with and without chemokine. Asterisk indicates $P < .01$; dagger, $P < .05$. Error bars represent the SE of triplicate wells.

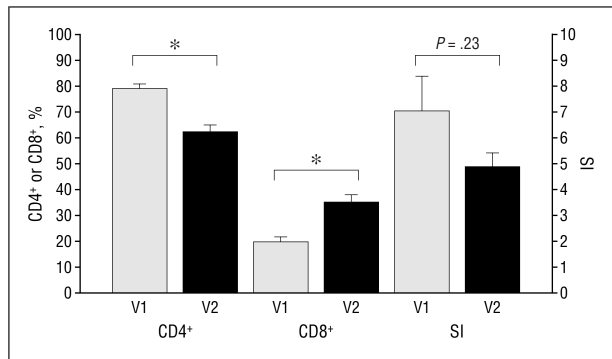

Figure 5. Mean percentage of $CD4^+$ or $CD8^+$ glatiramer acetate–reactive T cells as measured by flow cytometry at visit 1 (V1) (baseline, before glatiramer therapy) and visit 2 (V2) (12 months after initiation of glatiramer therapy). Stimulation indices (SIs) of glatiramer-reactive T-cell lines at V1 and V2 are also shown. Two-tailed unpaired *t* tests (parametric data) were used to measure significant differences between surface expression and SIs at V1 vs V2. Asterisk indicates $P < .001$; error bars, SEM.

in experimental autoimmune encephalomyelitis.[4] Our data provide an explanation for how glatiramer itself could suppress the number of myelin-specific effector memory T cells migrating into the CNS through bystander modulation in the peripheral lymph nodes. The timing of the observed CKR conversion is also consistent with a delayed immunologic mechanism of action and a radiologic response, as was seen in the serial magnetic resonance imaging–based clinical trial.[11] Long-term follow-up[12] of glatiramer-treated patients with MS suggests that an unusually high number of patients exhibit sustained disease quiescence after years of therapy. The data herein support the possibility that glatiramer could induce tolerance to pathogenic myelin-reactive T cells that results in sustained remissions in some patients. Tolerance may occur through bystander modulation by T_H2-secreting $CD4^+$ T cells, regulatory $CD8^+$ T cells, or both, as was recently demonstrated.[13] It is also possible that either the T_H2 milieu or even a direct effect of glatiramer on antigen-presenting cells could promote a T_H2 bias of naive-responding T cells with cognate T-cell receptor for myelin antigens, as has been seen by other investigators.[8,14]

Several recent studies[15-17] have focused on ways of enhancing T_H2 immune deviation with agents that are known to act systemically, including IFN-τ, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), and albuterol. Furthermore, a large double-masked, placebo-controlled trial of combination therapy with IFN-β and glatiramer acetate is planned. Our data provide further rationale for combination therapy with IFN-β or other agents for 2 reasons.[18] A peripheral mechanism of action characterized by selective inhibition of T_H1 effector memory T cells would be complementary to the effects of IFN-β, which acts systemically and at the blood-brain barrier. In addition, in cases in which IFN-β does not completely eliminate gadolinium-enhancing lesions and blood-brain barrier permeability, it would be preferable to have T_H2 influx with brain-derived neurotrophic factor–secreting cells that may diminish the likelihood of T1 black hole formation, as opposed to the case with IFN-β, in which T1 black hole formation seems to be unaffected when an enhancing lesion forms.[19]

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REFERENCES

1. Dhib-Jalbut S. Mechanisms of action of interferons and glatiramer acetate in multiple sclerosis. *Neurology*. 2002;58(suppl 4):S3-S9.
2. Chen M, Valenzuela RM, Dhib-Jalbut S. Glatiramer acetate–reactive T cells produce brain-derived neurotrophic factor. *J Neurol Sci*. 2003;215:37-44.
3. Ziemssen T, Kumpfel T, Klinkert WE, Neuhaus O, Hohlfeld R. Glatiramer acetate–specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy. *Brain*. 2002;125:2381-2391.
4. Aharoni R, Teitelbaum D, Leitner O, Meshorer A, Sela M, Arnon R. Specific Th2 cells accumulate in the central nervous system of mice protected against experimental autoimmune encephalomyelitis by copolymer 1. *Proc Natl Acad Sci U S A*. 2000;97:11472-11477.
5. Dhib-Jalbut S, Chen M, Said A, Zhan M, Johnson KP, Martin R. Glatiramer acetate–reactive peripheral blood mononuclear cells respond to multiple myelin antigens with a Th2-biased phenotype. *J Neuroimmunol*. 2003;140:163-171.
6. Duda PW, Schmied MC, Cook SL, Krieger JI, Hafler DA. Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis. *J Clin Invest*. 2000;105:967-976.

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7. Racke MK, Lovett-Racke AE. Bystander suppression in experimental autoimmune encephalomyelitis: where and how does it occur? *Res Immunol*. 1998; 149:820-827.
8. Kim HJ, Ifergan I, Antel JP, et al. Type 2 monocyte and microglia differentiation mediated by glatiramer acetate therapy in patients with multiple sclerosis. *J Immunol*. 2004;172:7144-7153.
9. Calabresi PA, Yun SH, Allie R, Whartenby KA. Chemokine receptor expression on MBP-reactive T cells: CXCR6 is a marker of IFNγ-producing effector cells. *J Neuroimmunol*. 2002;127:96-105.
10. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 1999;401:708-712.
11. Comi G, Filippi M, Wolinsky JS; European/Canadian Glatiramer Acetate Study Group. European/Canadian multicenter, double-blind, randomized, placebo-controlled study of the effects of glatiramer acetate on magnetic resonance imaging–measured disease activity and burden in patients with relapsing multiple sclerosis. *Ann Neurol*. 2001;49:290-297.
12. Johnson KP, Brooks BR, Ford CC, et al; Copolymer 1 Multiple Sclerosis Study Group. Sustained clinical benefits of glatiramer acetate in relapsing multiple sclerosis patients observed for 6 years. *Mult Scler*. 2000;6:255-266.
13. Karandikar NJ, Crawford MP, Yan X, et al. Glatiramer acetate (Copaxone) therapy induces CD8(+) T cell responses in patients with multiple sclerosis. *J Clin Invest*. 2002;109:641-649.
14. Vieira PL, Heystek HC, Wormmeester J, Wierenga EA, Kapsenberg ML. Glatiramer acetate (copolymer-1, Copaxone) promotes Th2 cell development and increased IL-10 production through modulation of dendritic cells. *J Immunol*. 2003; 170:4483-4488.
15. Soos JM, Ashley TA, Morrow J, Patarroyo JC, Szente BE, Zamvil SS. Differential expression of B7 co-stimulatory molecules by astrocytes correlates with T cell activation and cytokine production. *Int Immunol*. 1999;11:1169-1179.
16. Youssef S, Stuve O, Patarroyo JC, et al. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature*. 2002;420:78-84.
17. Makhlouf K, Weiner HL, Khoury SJ. Potential of β2-adrenoceptor agonists as add-on therapy for multiple sclerosis: focus on salbutamol (albuterol). *CNS Drugs*. 2002;16:1-8.
18. Dhib-Jalbut S, Chen M, Henschel K, Ford D, Costello K, Panitch H. Effect of combined IFNβ-1a and glatiramer acetate therapy on GA-specific T-cell responses in multiple sclerosis. *Mult Scler*. 2002;8:485-491.
19. Brex PA, Molyneux PD, Smiddy P, et al. The effect of IFNβ-1b on the evolution of enhancing lesions in secondary progressive MS. *Neurology*. 2001;57:2185-2190.

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