Brief Definitive Report

Prevention of Experimental Autoimmune Encephalomyelitis by Antibodies Against Interleukin 12

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Summary

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease of the central nervous system that can be transferred to naive mice via CD4+ T cells isolated from appropriately immunized mice. We have evaluated the effects of recombinant murine interleukin 12 (rmIL-12), a potent inducer of interferon γ (IFN-γ) and promoter of Th1 T cell development, on the course of adoptively transferred EAE. The transfer of lymph node cells (LNC) isolated from proteolipid protein (PLP)-primed animals and stimulated in vitro with PLP to naive mice resulted in a progressive paralytic disease culminating in complete hind limb paralysis in the majority of the recipients. When mice were injected with LNC that had been stimulated in vitro with PLP in the presence of rmIL-12, the subsequent course of disease was more severe and prolonged. The addition of rmIL-12 during the in vitro stimulation with PLP resulted in a 10-fold increase in IFN-γ and a 2-fold increase in tumor necrosis factor (TNF) α in the supernatants, relative to LNC stimulated with PLP alone. However, neutralization of IFN-γ or TNF-α in vitro with specific antibodies did not abrogate the ability of rmIL-12 to exacerbate the subsequent disease. Similarly, mice treated with rmIL-12 in vivo after the transfer of antigen-stimulated LNC developed a more severe and prolonged course of disease compared with vehicle-treated control animals. In contrast, treatment of mice with an antibody to murine IL-12 after cell transfer completely prevented paralysis, with only 40% of the mice developing mild disease. These results demonstrate that in vitro stimulation of antigen primed LNC with PLP and rmIL-12 enhances their subsequent encephalitogenicity. Furthermore, inhibition of endogenous IL-12 in vivo after LNC transfer prevented paralysis, suggesting that endogenous IL-12 plays a pivotal role in the pathogenesis of this model of autoimmune disease.

Experimental allergic encephalomyelitis (EAE) is a T cell–mediated autoimmune disease of the central nervous system (CNS). Disease can be induced in susceptible strains of mice by immunization with CNS myelin antigens or, alternatively, disease can be passively transferred to susceptible mice via antigen-stimulated CD4+ T cells (1). Elimination of CD4+ cells, but not CD8+ cells, prevents disease transfer, thus confirming the role of CD4+ T cells in disease induction. The clinical course of disease is characterized by weight loss and hind limb weakness, which commonly progresses to bilateral hind limb paralysis. In most species, the disease remits spontaneously, with the sequence of recovery being the reverse of that of onset. Acute disease is associated with the infiltration of CD4+ lymphocytes followed by the recruitment of monocytes and macrophages into the CNS lesions (2). Analysis of cytokine profiles in the CNS by mRNA expression after the transfer of myelin basic protein (MBP)-stimulated lymph node cells (LNC) demonstrated that the onset of disease is associated with cytokine expression typical of a Th1-type immune response, with high levels of IL-2 and IFN-γ mRNA (3). In contrast, immunohistochemical analysis demonstrated that IL-4, a cytokine produced by Th2 cells, appeared transiently later in the course of disease as symptoms began to resolve (2). Recovery from disease is also associated with a decline in IL-2 and IFN-γ mRNA and a concomitant rise in IL-10 mRNA consistent with a Th2-type response (3). These changes in cytokine profiles suggest that the onset of disease and subsequent spontaneous recovery are associated with a switch from a predominantly Th1 type response to a more typical Th2-type response within the inflammatory foci.

The differentiation pathway that leads to the generation of CD4+ Th1 and Th2 cells is regulated at least in part by cytokines released from lymphocytes and accessory cells, with IFN-γ and IL-4 inducing differentiation to Th1 and Th2 cells, respectively (4–6). In addition to IL-4 and IFN-γ, IL-10 and IL-12 appear to play primary roles in the differentiation of CD4+ T helper cells (7, 8). IL-12 is a novel heterodimeric cytokine that enhances NK-mediated cytotoxicity and induces IFN-γ production by NK cells and T lymphocytes (9, 10). The ability of IL-12 to induce IFN-γ– and promote Th1-specific immune responses has been demonstrated both in vitro (11)

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and in vivo (12, 13). In the latter case, administration of IL-12 following *Leishmania major* infection rendered susceptible BALB/c mice, which normally respond to *L. major* with preferential expansion of IL-4– and IL-10–producing Th2 cells, resistant to leishmaniasis. Examination of LNC from these IL-12–cured mice showed induction of Th1 T cells that produced high levels of IFN-γ (12).

Given the association between the onset of EAE and a Th1 effector T cell population, we evaluated the role of recombinant murine (rm) IL-12 on the course of adoptively transferred EAE using LNC stimulated with a synthetic peptide of myelin proteolipid protein (PLP) in SJL/J mice. Here we report that IL-12 is a key regulator of immune responsiveness in this model of autoimmune disease.

Materials and Methods

Adoptive Transfer of PLP-sensitized LNC. Female SJL/J mice (7–10 wk) were purchased from The Jackson Laboratory (Bar Harbor, ME), housed five to a cage, and fed standard rodent chow diet with water ad libitum. Mice were immunized in two sites on the flank with 75 μg (150 μg total) of mouse PLP peptide comprising residues 139–151 (provided by G. Brown, Genetics Institute). PLP was administered in 200 μl of CFA containing 2 mg/ml *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI). On the day of immunization, mice were injected intravenously with 0.75×10^{10} *Bordetella pertussis* bacilli (Massachusetts Public Health Laboratories, Boston, MA). 10 d after immunization, lymph nodes (popliteal, axillary, and brachial) were harvested and the cells resuspended in RPMI-1640 containing 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 5×10^{-5} M 2-ME, 100 μg/ml streptomycin, and 100 U/ml penicillin. PLP was added to the cultures at 2 μg/ml. After incubation for 96 h, the cells were harvested, washed twice, and 30×10^6 cells LNC were injected intraperitoneally into naive SJL/J mice.

Clinical Evaluation of Disease. After the transfer of cultured LNC, recipient mice were monitored for clinical signs of EAE and scored on the following scale: 0.5, distal limp tail; 1.0, complete limp tail, 1.5, limp tail and hind limb weakness (unsteady gait); 2.0, partial hind limb paralysis; and 3.0, complete bilateral hind limb paralysis. Mean cumulative scores were calculated by the addition of daily EAE scores for individual mice during the course of disease.

In Vitro Administration of rmIL-12 Before Cell Transfer. Recombinant murine IL-12 (20 ng/ml) (Genetics Institute) was added during the in vitro stimulation of LNC with PLP before cell transfer. After 96 h the cells were washed twice, and 30×10^6 cells were transferred to naive SJL/J mice, which were monitored for signs of disease. In separate experiments, LNC were cultured with either antigen alone, antigen plus rmIL-12 (20 ng/ml), or antigen plus rmIL-12 plus either a neutralizing antibody to IFN-γ (5 μg/ml) (Endogen, Inc., Boston, MA) or 200 μl of a polyclonal rabbit anti-mouse TNF-α antibody (Genzyme Corp., Cambridge, MA). At the end of the culture period, supernatants were collected (pooled from three flasks) and IFN-γ and TNF-α measured by ELISA (Genzyme Corp.). 30×10^6 cells from each culture condition were transferred to naive mice, which were monitored for signs of disease.

In Vivo Administration of rmIL-12 and Anti-IL-12 Antibody After the Transfer of PLP-stimulated LNC. rmIL-12 (0.3 μg/mouse, 200 μl i.p.) was administered to mice after transfer of 30×10^6 PLP-stimulated LNC. rmIL-12 was administered on days 0, 1, and 2 after cell transfer. Control mice received an equal volume of vehicle alone. To determine if IL-12 is involved in the induction of disease after transfer of PLP-stimulated LNC, mice were treated with 200 μg of a sheep polyclonal antibody against murine IL-12 (provided by V. VanCleave, Genetics Institute; 200 μl i.p.) on alternate days for either 6 or 12 d total after cell transfer and monitored for clinical signs of disease. Control mice received an equal amount of sheep IgG.

Results

The Addition of rmIL-12 During the In Vitro Stimulation of LNC with PLP Exacerbates Clinical Signs of EAE After Cell Transfer. LNC from PLP-immunized mice were stimulated in vitro with either PLP alone or PLP and rmIL-12 (20 ng/ml) for 96 h, after which time they were tested for their ability to transfer disease to naive SJL/J mice. Control mice that received LNC stimulated in vitro with PLP developed clinical signs of disease between days 6 and 8. All seven control mice reached scores ≥2, with four out of seven mice progressing to complete hind limb paralysis, which lasted between 1 and 4 d (Fig. 1). All the control mice recovered by day 19. In contrast, mice that received LNC cultured in vitro with PLP and rmIL-12 developed severe EAE with rapid onset of clinical signs (Fig. 1). By day 6, four out seven mice had clinical scores of ≥2, and all mice developed complete hind limb paralysis by day 8. In this particular experiment, five out of seven mice failed to recover from the paralysis.

To determine the effects of rmIL-12 on cytokine production during the in vitro stimulation with antigen, LNC from PLP-primed mice were cultured with either PLP alone or PLP and rmIL-12 (20 ng/ml). After 96 h in culture, IFN-γ and TNF-α in the supernatant were measured by ELISA. The addition of rmIL-12 during the in vitro stimulation of LNC with PLP resulted in a >10-fold increase in IFN-γ and a 2-fold increase in TNF-α compared with cells cultured with PLP alone (Table 1). To determine if stimulation of either IFN-γ

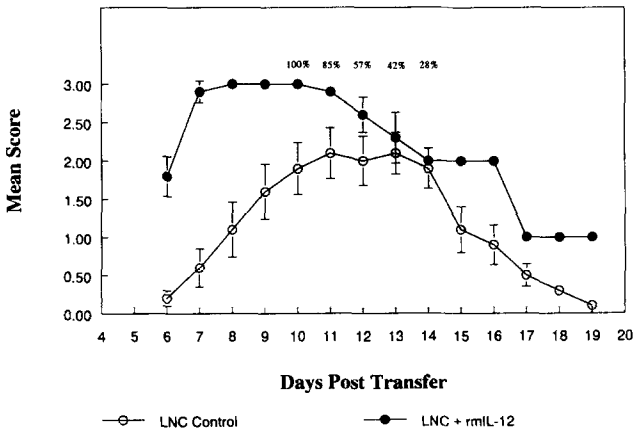

Figure 1. Adoptive transfer of EAE via LNC stimulated in vitro with PLP and rmIL-12. Lymph nodes were harvested from mice 10 d after immunization with PLP and stimulated in vitro with antigen alone (*open circles*) or antigen and 20 ng/ml rmIL-12 (*solid circles*) as described in Materials and Methods. The results are presented as mean score ± SEM for surviving animals ($n = 7$ mice). The percentage of surviving animals in the IL-12 group is indicated above the curve. All control mice recovered from disease. The data are representative of at least three separate experiments.

Table 1. *IFN-γ and TNF-α Production After In Vitro Stimulation with PLP and rmIL-12*

	IFN-γ	TNF-α	OD	CHP	MCS
	ng/ml	*pg/ml*			
LNC + PLP	5.0 ± 0.5	100 ± 1.1	6.1 ± 1.2	4/7	16.2 ± 6.4
LNC + PLP + rmIL-12	65.3 ± 1.3	195 ± 13.4	4.5 ± 0.5	6/6	35.3 ± 4.5
LNC + PLP + rmIL-12 + anti-IFN-γ	bdl*	183 ± 2.4	5.2 ± 0.5	3/4	30.6 ± 16.5
LNC + PLP + rmIL-12 + anti-TNF-α	79.8 ± 8.3	bdl	4.0 ± 0	4/5	30.3 ± 11.2

LNC from PLP-immunized mice were stimulated in vitro with PLP alone, PLP and rmIL-12 (20 ng/ml), or PLP and rmIL-12 in the presence of neutralizing antibodies to IFN-γ or TNF-α for 96 h as described in Materials and Methods. At the end of the culture period, supernatants from individual flasks were pooled and assayed for the presence of IFN-γ and TNF-α by ELISA.
Data represent mean cytokine determinations from multiple dilutions (± SD).
After in vitro stimulation with PLP, 30 × 10^6 LNC were transferred to naive mice, which were monitored for signs of disease.
* Below detection limit.
CHP, complete hind limb paralysis (incidence); *MCS*, mean cumulative score (± SD); *OD*, day of onset of disease (± SD).

or TNF-α was an essential component of the in vitro effects of rmIL-12 on T cell encephalitogenicity, LNC were stimulated with PLP and rmIL-12 in the presence of neutralizing antibodies to IFN-γ or TNF-α. The addition of the neutralizing antibody to IFN-γ during stimulation with PLP and rmIL-12 completely neutralized assayable IFN-γ but had no effect on the increase in TNF-α in the supernatants (Table 1). Similarly, neutralization of TNF-α during stimulation with PLP and rmIL-12 had no effect on the increase in IFN-γ (Table 1). Furthermore, cells stimulated in vitro with PLP and rmIL-12 in the presence of neutralizing antibodies to either IFN-γ or TNF-α still induced severe disease in naive mice with the same kinetics and duration as that seen after the transfer of cells stimulated with PLP and rmIL-12 alone (Table 1).

The Effect of In Vivo Administration of IL-12 on Disease Progression. Mice were treated with rmIL-12 (0.3 μg/mouse, i.p.) or saline for 3 d after the transfer of 30 × 10^6 PLP-stimulated LNC and monitored for clinical signs of disease. The onset of disease in the saline treated control mice ranged between days 6 and 8 after cell transfer, and 80% of the mice progressed to full bilateral hind limb paralysis. Peak disease in the control mice lasted ~3 d, after which time the mice spontaneously recovered (Fig. 2). In contrast, although the onset of clinical signs in mice treated with rmIL-12 was only slightly earlier (day 5), the subsequent progression to peak disease was accelerated, and all mice displayed full hind limb paralysis by day 8. The duration of paralysis was also significantly extended, lasting up to 14 d (range, 11–14).

The Effects of Anti-IL-12 Antibody Administration on the Course of Disease. To determine if endogenous IL-12 is required for the successful transfer of disease, mice were treated with 200 μg of a sheep polyclonal antibody to murine IL-12 every other day for either 6 or 12 d after cell transfer. Control mice received an equal amount of sheep IgG. The onset of clinical signs in the sheep IgG–treated control mice was similar to that seen in untreated mice that received PLP-stimulated LNC (day 6–7, Fig. 3 *a*). All control mice developed signs of disease graded ≥2 (70% developed full hind limb paralysis). Administration of anti-IL-12 antibody during the first 6 d after cell transfer delayed the onset of clinical signs by ~7 d, but had no effect on the ultimate severity of disease, with all mice reaching a score of ≥2 (80% developed full hind limb paralysis). To determine if this delay of disease after cell transfer could be sustained by prolonged administration of anti-IL-12 antibody, mice were treated every other day for 12 d after transfer of PLP primed LNC. This extended treatment with anti-IL-12 antibody not only delayed the onset of disease, but also dramatically reduced the severity of disease, with no mice developing hind limb paralysis and only two out of five mice developing mild signs of disease (Fig. 3 *b*).

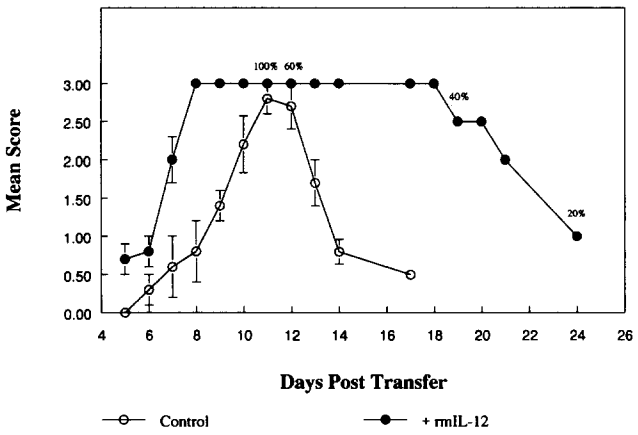

Figure 2. The effects of in vivo administration of IL-12 on the adoptive transfer of EAE via PLP-stimulated LNC. LNC from PLP-immunized mice were cultured in vitro with antigen as described in Materials and Methods and transferred to naive mice. rmIL-12 (0.3 μg/mouse) was administered on days 0, 1, and 2 after cell transfer (*solid circles*). Control mice received an equal volume of saline (*open circles*). The data are expressed as mean score ± SEM for surviving animals (n = 5 mice). The percentage of surviving animals in the IL-12 group is indicated above the curve. All control mice recovered from disease. The data are representative of three separate experiments.

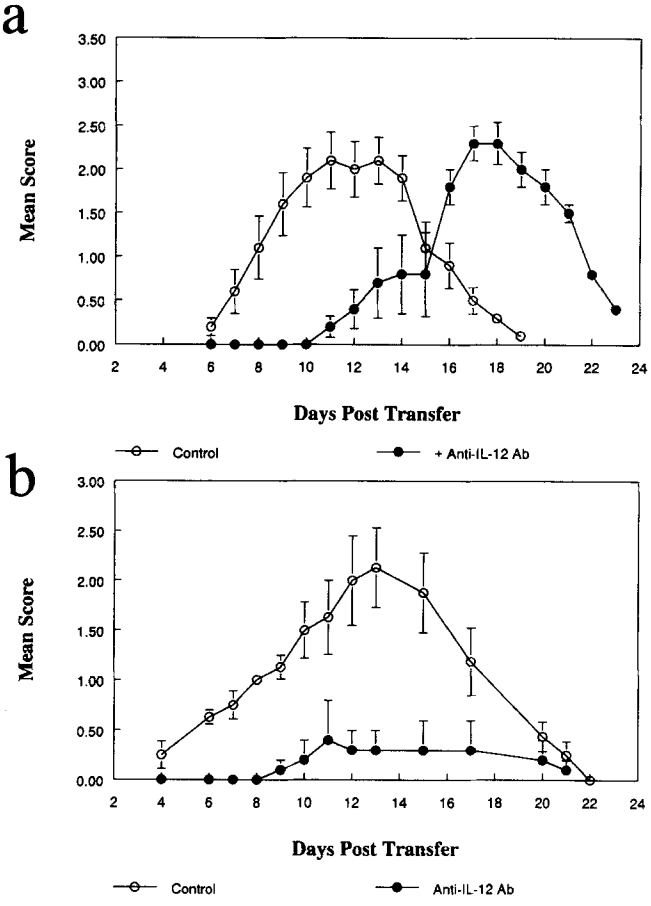

Figure 3. The effects of in vivo administration of anti-IL-12 antibody on the adoptive transfer of EAE via PLP-stimulated LNC. LNC from PLP-immunized mice were cultured in vitro with antigen as described in Materials and Methods. Anti-IL-12 antibody (sheep anti-mouse polyclonal antibody, 200 μg/mouse) was administered intraperitoneally starting on the day of cell transfer (*solid circles*). Control mice received an equivalent amount of sheep IgG (*open circles*). (*a*) Mean clinical score ± SEM after administration of αIL-12 antibody every other day from day 0 to day 6. (*b*) Mean clinical score ± SEM after administration of anti-IL-12 antibody every other day from day 0 to day 12 (n = 5–7 mice). The data are representative of two (*a*) and three (*b*) separate experiments, respectively.

Discussion

In this study, we examined the effects of rmIL-12 administration on the development of adoptively transferred EAE after stimulation of LNC with a synthetic peptide corresponding to mouse myelin PLP residues 139–151. This peptide has been used previously for the active induction of EAE in SJL/J mice (14). Using LNC isolated from PLP-immunized mice, the addition of rmIL-12 during the in vitro stimulation with antigen exacerbated the subsequent clinical signs of disease after cell transfer. A similar effect on disease severity was seen when spleen cells were used to transfer disease (data not shown). These results suggest that in vitro stimulation with rmIL-12 had direct effects on T cell effector function and/or number, ultimately rendering these cells more encephalitogenic.

Previous studies have demonstrated that MBP-specific $CD4^+$ T cells secreting cytokines associated with a Th1 phenotype effectively transferred EAE (15). Consistent with this finding, we observed increases in both IFN-γ and TNF-α in the supernatant of LNC cultures after stimulation in vitro with PLP and rmIL-12 compared with cells cultured with antigen alone. Since the production of these secondary cytokines after stimulation with rmIL-12 could have effects on accessory cells, we added neutralizing antibodies against IFN-γ or TNF-α to our in vitro cultures. The observation that neutralization of these cytokines did not affect the ability of rmIL-12 to exacerbate disease after cell transfer supports the conclusion that rmIL-12 has a direct effect on T cell effector function and/or number and argues against secondary effects mediated by IFN-γ or TNF-α during the in vitro stimulation with PLP. In addition, these antibody blocking experiments indicate that, in this setting, the stimulation of either IFN-γ or TNF-α by rmIL-12 is independent of the production of the other. Although the data strongly suggest a direct effect of rmIL-12 during the in vitro stimulation with PLP, this cannot be unequivocally stated, as it is possible that some bioactive cytokine is present in the cultures despite the absence of detectable levels of IFN-γ and TNF-α by ELISA.

Although there was a marked increase in both IFN-γ and TNF-α production in cultures treated with rmIL-12, it is not clear if this reflects the preferential expansion of Th1 effector cells, or, alternatively, results from an increase in the production of these cytokines on a per cell basis. To address the effects of rmIL-12 on the in vitro responsiveness to PLP, proliferation was measured by [^{3}H]thymidine incorporation of LNC after PLP stimulation. We were unable to demonstrate a difference in antigen-stimulated proliferation in the presence of rmIL-12 when PLP was added at the concentration used to transfer disease (2 μg/ml). There was, however, an enhanced proliferative response at low concentrations of PLP (0.01 μg/ml) in the presence of rmIL-12 (data not shown), suggesting that rmIL-12 could enhance the expansion of PLP-responsive T cells in vitro. The observation that rmIL-12 did not enhance proliferation at higher doses of antigen may be due to increased production of other cytokines in these cultures, particularly IL-2, which could mask the effects of rmIL-12 on a subset of T cells.

A profound effect on the course of EAE was also seen when rmIL-12 was administered in vivo after the transfer of PLP-stimulated LNC. This in vivo treatment with rmIL-12 accelerated the progression to complete hind limb paralysis and resulted in a severe and prolonged clinical course of disease. Histological and immunohistochemical evaluation of the inflammatory foci of mice killed at peak disease confirmed the presence of multiple perivascular lesions typical of the clinical pathology associated with EAE (data not shown). Although there were no dramatic differences between rmIL-12–treated and control mice in either the cellular composition of the lesions or in the relative extent of IFN-γ staining, preliminary results suggest an increase in the number of inflammatory lesions in rmIL-12–treated mice, which could account for the enhanced disease severity (Waldburger, K., unpublished observation). An extensive histological analysis to determine the nature of the inflammatory response during the course of EAE after rmIL-12 administration is being conducted.

The in vivo effects of rmIl-12 on disease severity are also independent of secondary IFN-γ stimulation, as the combination of rmIL-12 and a neutralizing anti–IFN-γ antibody in vivo after cell transfer resulted in a rapid progression of disease, which proved lethal in all mice (Leonard, J. P., K. E. Waldburger, and S. J. Goldman, unpublished observations). Taken together with the observation by us and others that treatment with a neutralizing anti-IFN-γ antibody alone exacerbates disease (16), these data suggest that rmIL-12 directly activates encephalitogenic T cells in vivo, although we cannot exclude the contribution of secondary effects mediated by TNF-α.

The most compelling evidence for an immunoregulatory role of IL-12 in the pathogenesis of EAE came from experiments in which endogenous IL-12 was blocked after the transfer of PLP-stimulated LNC. In these studies, treatment of mice with anti-IL-12 antibody during the first 6 d after cell transfer significantly delayed the onset of clinical signs; however, the ultimate severity and duration of disease was not affected. In contrast, extending the period of antibody treatment to 12 d completely prevented the development of hind limb paralysis. These data suggest that endogenous IL-12 is essential for the effective transfer of disease and demonstrate that prolonged inhibition of endogenous IL-12 can prevent paralysis and substantially reduce the incidence and severity of clinical signs. Preliminary experiments have demonstrated some beneficial effects of anti-IL-12 antibody when given therapeutically; anti-IL-12 antibody treatment commencing at the time of onset of disease (1 wk after transfer) and continuing for a period of 6 d resulted in a reduction in the rate of disease progression, although the ultimate severity was not changed (Leonard, J. P., unpublished observation).

Inhibition of endogenous IL-12 by an anti-IL-12 antibody produced changes in the kinetics of disease transfer very similar to those seen after inhibition of integrin-mediated cell adhesion (15). Interactions between lymphocytes and endothelial cells appear to play a major role in regulating the entry of antigen specific cells into the CNS (17, 18). By use of PLP-specific T cell clones, the expression of the $\alpha 4\beta 1$ integrin (very late antigen 4 [VLA-4]) was found to be a prerequisite for the successful transfer of EAE (19). In addition, antibodies directed against VLA-4 blocked both lymphocyte and monocyte binding to vessels on EAE brain slices (20) and delayed the onset of disease after transfer of MBP-specific T cell clones (15). Similarly, antibodies against vascular cell adhesion molecule 1, the endothelial cell ligand for VLA-4, delayed the onset of disease after the transfer of MBP-specific T cell clones or MBP-stimulated LNC (15). Interestingly, the continued administration of anti-VLA-4 antibody delayed the onset of clinical signs and reduced the severity of disease (15) in a manner similar to that seen by use of an antibody against murine IL-12. In our studies, the slightly earlier onset of disease and rapid progression to complete hind limb paralysis after rmIL-12 administration in vivo, as well as the delayed onset of disease after administration of an antibody to IL-12 in vivo, are consistent with modulation of integrin adhesion function as a potential component contributing to the effects of IL-12 in this model.

The requirements for successful transfer of EAE via either antigen-stimulated primary LNC or antigen-specific $CD4^+$ T cell lines or clones are complex and incompletely understood. The results of our experiments with an adoptive transfer model of EAE support a major role for IL-12 in regulating the T cell response to PLP and, consequently, the severity of transferred disease. More importantly, the data show that IL-12 is required in vivo for efficient effector function in this model. The ability of IL-12 to stimulate Th1 T cells and its pivotal role in regulating effector function may have significant implications for the etiology of autoimmune diseases such as multiple sclerosis.

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