Brief Definitive Report

Inhibition of Leukotriene B_4–Receptor Interaction Suppresses Eosinophil Infiltration and Disease Pathology in a Murine Model of Experimental Allergic Encephalomyelitis

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Summary

Leukotriene B_4 (LTB_4) is a chemotactic and cell-activating factor present at inflammatory sites in a variety of autoimmune diseases including multiple sclerosis (MS). In this study, we used a murine model of MS, experimental allergic encephalomyelitis (EAE), to assess the potential role of LTB_4 on cell infiltration and paralysis. Injection of encephalogenic T cells into naive animals induced paralysis and weight loss that was completely inhibited by treatment with the selective LTB_4 receptor antagonist CP-105,696 (ED_{50} = 8.6 mg/kg orally). Although migration of lymphocytes into the central nervous system was unaffected, the efficacious effects of CP-105,696 correlated with up to a 97% decrease in eosinophil infiltration into the lower spinal cord as determined by light and electron microscopy and quantitated by levels of the specific enzyme marker eosinophil peroxidase. These results demonstrate that eosinophil recruitment in EAE is dependent on LTB_4 receptor ligation and further reveal a previously unrecognized role for eosinophils in the pathogenesis of this disease.

Experimental allergic encephalomyelitis (EAE) is an inflammatory disease of the central nervous system (CNS) myelin with pathological similarities to multiple sclerosis (MS) (1–3). The immunological effector cells responsible for initiating the disease have been shown, with depletion experiments, to be $CD4^+$ lymphocytes (4). Once these encephalogenic T cells migrate into the CNS, they are stimulated to release proinflammatory cytokines (5) and chemokines (6), which recruit inflammatory cells, compromise the integrity of the blood-brain barrier, and stimulate the release of mediators that break down myelin, resulting in impaired nerve conduction and paralysis (1, 3).

The mediators involved in the pathogenesis of EAE and MS are unclear. Products of the 5-lipoxygenase pathway, specifically leukotriene B_4 (LTB_4) (7, 8), have been detected in the spinal fluid of MS patients (9) and have been suggested to play a role in the pathogenesis of EAE (10). In addition to having direct chemoattractant activity (11, 12), LTB_4 can modulate the activity of lymphocytes (7), raising the possibility that it might act on encephalogenic T cells in the CNS and enhance their ability to release mediators important for disease progression. In this study, we determined the effects of the specific LTB_4 receptor antagonist CP-105,696 (13–15) on disease pathology and cell-specific infiltration into the spinal cord of mice injected with antigen-specific encephalogenic T cells.

Materials and Methods

Reagents. The LTB_4 receptor antagonist CP-105,696 was synthesized as previously described (15). RPMI medium supplemented with 10 mM Hepes buffer, 100 U/ml penicillin–streptomycin, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.05 mM 2-ME was used for culturing T cells. All components were obtained from BioWhittaker Inc. (Walkersville, MD).

Encephalogenic Protein. A previously described encephalogenic peptide (acetyl-FFKNIVTPRTPPP-amide) (16) corresponding to regions 91–103 of guinea pig myelin basic protein was prepared by the Protein Chemistry Division at Pfizer, Inc. All reagents were characterized by electrospray mass spectra (Finnegan TSQ-700) and amino acid analysis (ABI 420A) and were found to have had a single peak by reverse-phase HPLC.

Animals. Female SJL mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6–8 wk of age. Animals were acclimated in-house for a minimum of 2 wk before use and were allowed food and water *ad lib.* Animals were routinely checked for pathogens and were always kept in microisolator cages.

Passive Transfer Model of EAE. EAE was induced in animals using the passive transfer model as previously described (17). Mice were immunized in three separate areas on the lower back once a week for 2 wk with an emulsion containing 30 μg H37RA

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Mycobacterium tuberculosis (3114-33-8; Difco Laboratories Inc., Detroit, MI), 75 μl PBS, 75 μl IFA (0639-60-6; Difco Laboratories Inc., Detroit, MI) and 75 μg of the encephalogenic peptide. Spleens or lymph nodes (inguinal, brachial, and axillary) were collected 7 d after the last immunization. A single-cell suspension was made, and cells were placed into culture using T-225 flasks (Costar Corp., Cambridge, MA) for 4 d at a density of 3×10^6 cells/ml in RPMI medium containing 10% FCS and 5 μg/ml of the encephalogenic peptide. Cells were also plated into 96-well plates to determine proliferative responses to the antigen over the 4-d period using [^{3}H]thymidine incorporation as a guide. After the culture period, the cells were collected, layered over Histopaque (1077-1; Sigma Chemical Co., St. Louis, MO), and centrifuged at 800 *g* for 15 min. Lymphocytes were then collected, washed, adjusted to 2.5×10^8 lymphoblasts/ml in PBS (the lymphoblasts were confirmed by FACS® analysis to be $CD4^+$ $IL\text{-}2R^+$ cells). 200 μl of the cell suspension was then injected intraperitoneally into naive mice. Unless otherwise indicated, CP-105,696 was orally administered on a daily basis beginning at the time of T cell injection.

Paralysis was monitored on a daily basis according to the following criteria: (*a*), limp tail; (*b*), hind limb weakness; (*c*), one hind limb paralyzed; (*d*), both hind limbs paralyzed; (*e*), moribund; (*f*), death. Each group contained a minimum of five animals and were allowed access to food and water at the cage base. Moribund animals were killed. The mean disease severity for each group was plotted over the study period, and the area under the curve was calculated. The percent inhibition of disease severity was then calculated compared with the placebo-treated controls. The animal weight was also determined at various time points during the course of disease.

Biochemical Measurements. The selective eosinophil enzyme, eosinophil peroxidase (EPO), was measured as previously described (18). Spinal cords were homogenized, subjected to two freeze–thaw cycles, and centrifuged at 1,000 *g* for 30 min. As a standard, serial dilutions of a lysed suspension of 2.5×10^4 eosinophils/ml were used.

Microscopic Studies. Samples for light microscopy were prepared by removing brain and selected portions of the spinal cord and fixing by immersion in 10% neutral-buffered formalin for 12–18 h. The fixed specimens were trimmed, processed, and embedded in paraffin using standard techniques. 5-μm thick sections were cut on a microtome (Autocut; Reichert Jung, Vienna, Austria), stained with hematoxylin and eosin, and viewed with a microscope (FXA; Nikon Inc., Melville, NY).

Samples for electron microscopic examination were prepared by removing small portions of the formalin-fixed spinal cord and fixing for 1 h by immersion in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, 4°C). The tissue was then processed and embedded in epoxy resin. Thin sections were cut on a ultramicrotome (model FC-4, Reichert Jung), stained with uranyl acetate, and viewed on an electron microscope (model 1200; JEOL USA, Peabody, MA).

Statistics. The results are expressed as the mean ± 1 SD. Statistical analysis was done using the Student's *t* test and the Mann-Whitney U test. A *P* value of <0.05 was considered significant. The area under the curve was calculated using the trapezoidal rule.

Results

The paralysis induced in mice after the administration of encephalogenic T cells could be completely prevented by the administration of a 20-mg/kg dose of the LTB_4 receptor antagonist CP-105,696 (Fig. 1). At a lower dose of 10 mg/kg, disease severity was reduced by up to three disease scores or 65% at day 38. The serum level of CP-105,696 18 h after the seventh daily oral treatment with 10 mg/kg was

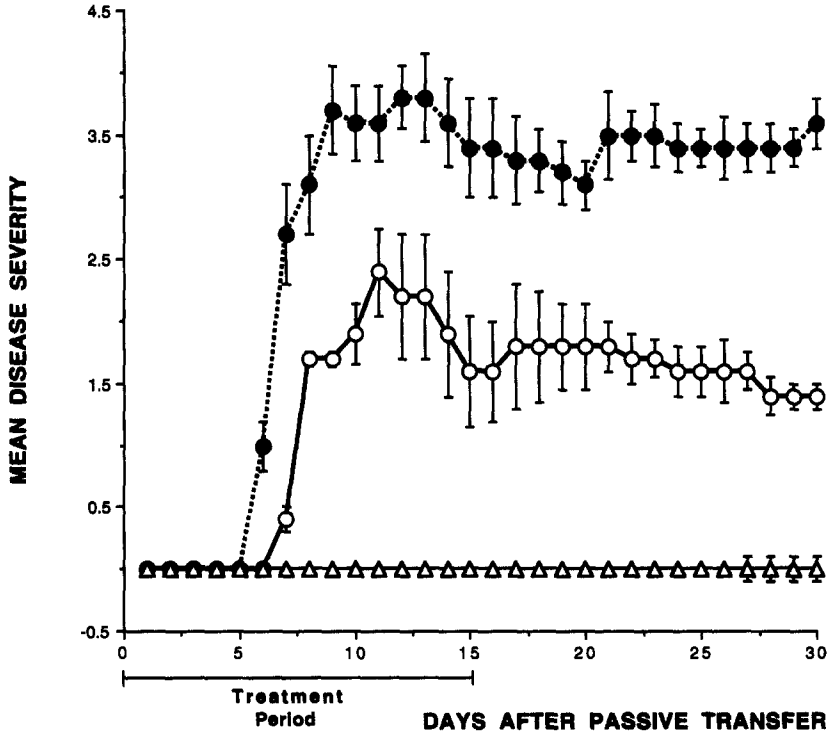
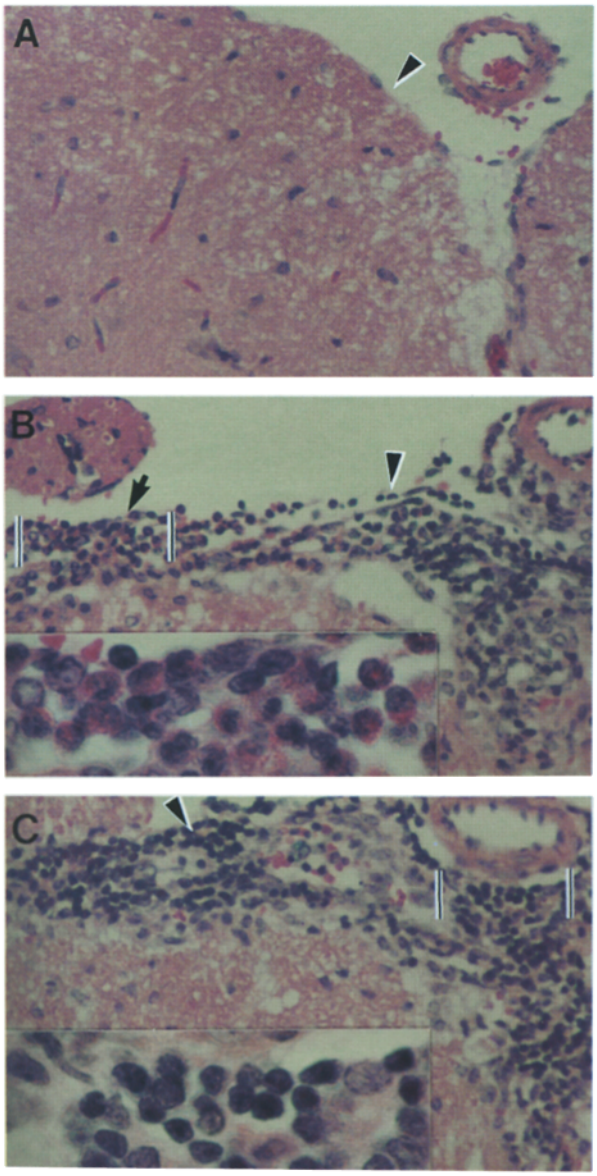


Figure 1. Effect of CP-105,696 on the development of EAE. Naive mice were injected intraperitoneally with encephalogenic T cells. Immediately after injection, animals received 15 daily oral treatments with CP-105,696. Disease was monitored for 30 d. This study is representative of three studies using five animals per group. Solid circles, placebo; open circles, 10 mg/kg CP-105,696; open triangles, 20 mg/kg CP-105,696. Significance was obtained beginning on day 6.

Table 1. *Effect of CP-105,696 on the Development of EAE after Passive Transfer*

Dose	Inhibition of Disease	Day of onset	Maximum severity
mg/kg	%		
20.0	91.4 ± 7.4*	>18 ± 8*	0.6 ± 0.6*
10.0	50.0 ± 5.0*	7.3 ± 0.7	2.6 ± 0.5
1.0	No effect	6.8 ± 0.8	3.4
0.1	No effect	7.0 ± 0.8	3.3
Placebo	0	7.5 ± 2.0	3.7 ± 1.3

The data represent a total of three studies using a minimum of five animals per group. Treatments were administered per os daily for 15 d beginning at the time of passive transfer.
*$P < 0.05$.



90–100 μg/ml. This level of drug blocks LTB_4 (100 nM)-mediated upregulation of the complement receptor (CD11b/CD18) on neutrophils in mouse whole blood by >95%. In addition, although rare, in cases where some death was observed in the control group, CP-105,696 was also able to prevent mortality (data not shown).

As shown in Table 1, the efficacious effects of CP-105,696 could be observed using three different parameters of disease scoring: a decrease in total disease over the 40-d period (area under the curve), a decrease in maximum disease severity, and a prolongation of the day of disease onset. The ED_{50} for the effects of CP-105,696 when administered immediately after the passive transfer of T cells was calculated to be 8.6 mg/kg based on total disease, 9.5 mg/kg based on the day of onset, and 12.6 mg/kg based on maximum disease severity. These protective effects of CP-105,696 were observed by following disease over a 40-d period but using only a 15-d course of treatment and were also reflected by a decrease in weight loss in the CP-105,696–treated group (data not shown). Similar results were obtained when treatment with CP-105,696 was initiated up to 4 d after the passive transfer of encephalogenic T cells. However, when treatment was initiated after the onset of symptoms, when high numbers of eosinophils are already present, no protective effects were observed (data not shown).

Histological examination of the brain and upper segment of the spinal cord of EAE animals revealed cellular infiltrates consisting primarily of lymphocytes at the light microscopic level. In contrast, the composition of the cellular infiltrate was different in the lower spinal cord near the cauda equina, where a large number of eosinophils in addition to lymphocytes were observed (Fig. 2 *B*). In this area of the spinal cord, the leukocytes appeared to penetrate the neural tissue along the course of the supplying vessels. In addition, large numbers of leukocytes and degenerating axons were observed in the surrounding nerve roots. Fig. 3 illustrates a section of the lower area of the spinal cord examined at the electron microscopic level. The characteristic eosinophil granules can be observed, which in many instances had been released within the extracellular matrix. In

Figure 2. Light microscopy of the caudal spinal cord from a naive control animal (*A*), an animal injected with encephalogenic T cells (*B*), and an animal injected with encephalogenic T cells and treated daily with 10 mg/kg of CP-105,696 (*C*). Samples were taken 10–14 d after the injection of T cells. The histological sections are representative of three separate experiments. No inflammatory cells were observed surrounding the spinal cord (*arrowhead*) in the naive controls (*A*). In contrast, a large influx of inflammatory cells were observed surrounding the spinal cord (*arrowhead*) in the vehicle-treated animal injected with encephalogenic T cells (*B*). Some of these cells were eosinophils (*arrow*), which were better observed in the high magnification insert of the region within the bars. Treatment with CP-105,696 (*C*) resulted in a slight reduction in the T cell influx (*arrowhead*), but more importantly, it resulted in near total blockage of the influx of eosinophils. In the high magnification insert of the region within the bars, a representative field of the surrounding T cells can be seen. ×280; inset ×840.

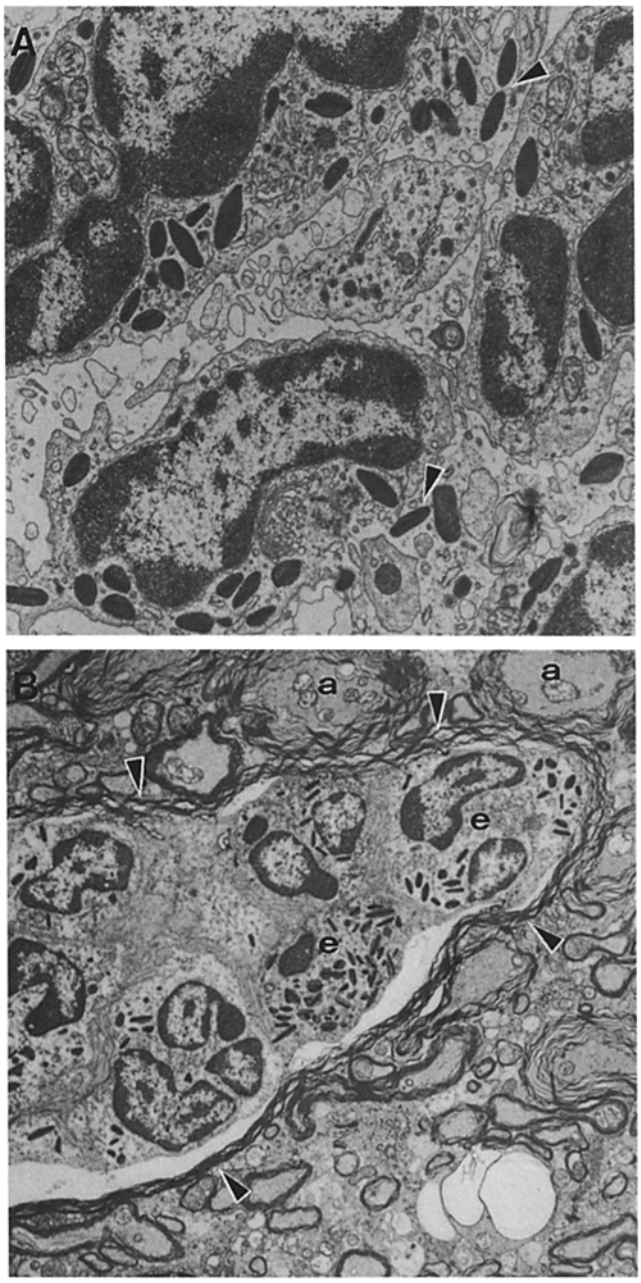


Figure 3. Electron microscopy of the caudal spinal cord from animals injected with encephalogenic T cells and treated with vehicle. Samples were taken 10-14 d after the injection of T cells. The histological sections are representative of three separate experiments. (*A*) A representative field containing numerous eosinophils releasing their granules (*arrowheads*) can be seen. ×9,750. Several axons (*a*) within their encasing myelin sheath can be observed. In the longitudinal section of the large fiber (*arrowheads*), numerous eosinophils (*e*) are present within the myelin sheath, apparently replacing the axon. ×4500.

addition, we also observed eosinophils within apparently intact myelinated sheaths replacing the axon (Fig. 3 *B*) in the nerve rootlets surrounding the lower spinal cord, suggesting a compromise in integrity upstream from the section. In animals treated with CP-105,696, a decrease in eosinophil infiltration was observed at both the light (Fig. 2

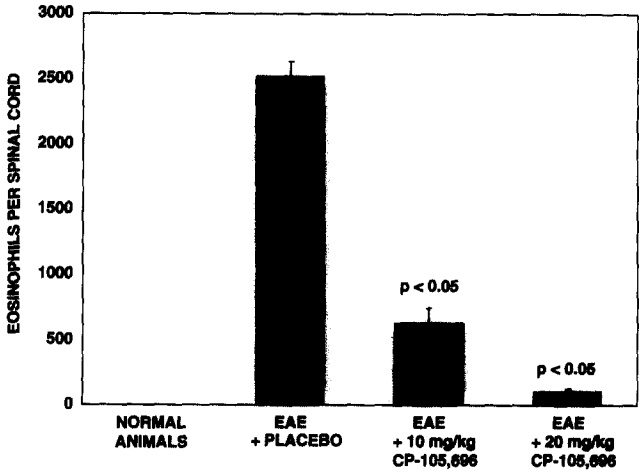


Figure 4. Effect of CP-105,696 on eosinophil infiltration as determined by EPO. Mice were given encephalogenic T cells intraperitoneally and then treated orally with placebo or CP-105,696. Spinal cords were removed on day 14, and the level of EPO was determined. The number of eosinophils was then calculated from a standard curve. The detection limit based on the dilution of the spinal cord homogenate used was 100 eosinophils/tissue. The graph represents a summary of five experiments.

C) and electron (data not shown) microscopic level. In contrast, the effects of CP-105,696 on lymphocyte infiltration were minimal and did not reflect the protective effects observed with this agent on paralysis.

To better quantify the reduction in eosinophil infiltration, the amount of EPO was measured. EPO levels are detectable at times coincident with the appearance of paralysis in animals. When animals were treated with CP-105,696, up to a two-log reduction in the level of EPO in the spinal cord was observed compared with vehicle-treated animals (Fig. 4). Animals that did not receive encephalogenic cells had no detectable cellular infiltration into the spinal cords and no detectable EPO levels.

Discussion

Previous studies have indicated the important role for $CD4^+$ T cells in the pathogenesis of EAE (4). Although lymphocytes may be responsible for initiating the disease, the actual disease pathology may be mediated by various inflammatory cells. We demonstrated that by blocking the LTB_4 receptor, the development of paralysis and the recruitment of eosinophils into the spinal cord was prevented. These studies indicate that eosinophil infiltration was dependent on LTB_4 receptor ligation and reveal an important role for eosinophils in this disease as illustrated by the ability of CP-105,696 to inhibit disease coincident with a decrease in eosinophil, but not lymphocyte, numbers in the spinal cord. Although it could be argued that CP-105,696 is inhibiting additional factors necessary for eosinophil infiltration, previous studies have indicated the selectivity for this agent for the LTB_4 receptor, as indicated by its inability to inhibit the activity of other G protein–

receptor agonists, including IL-8, C5a, and PAF (13). It also has no effect on leukotriene or prostaglandin biosynthesis nor on IL-1–induced neutrophil accumulation in vivo (14).

There are two possible ways that LTB_4 might stimulate eosinophil infiltration. The first is by direct chemotactic effects on the eosinophil LTB_4 receptor. LTB_4 has been shown to induce eosinophil chemotaxis (11), and CP-105,696 has been demonstrated to block this effect (13). However since LTB_4 is also chemotactic for neutrophils, which were not observed in tissue, a direct cell-recruiting effect is unlikely. The second, perhaps more likely, mechanism is by stimulating encephalogenic T cells (7) to release eosinophil-chemotactic agents. Recently, encephalogenic T cells have been shown to express message levels for several chemokines (6), including the eosinophil chemoattractants macrophage inflammatory protein (MIP) 1α and RANTES (19, 20). Although this mechanism cannot be tested at this time since neutralizing antibodies and specific ELISAs to these chemokines are not commercially available, a report has indicated that neutralizing antibodies to MIP-1α will prevent paralysis in EAE (21).

Although they have been observed in the spinal fluid of MS patients (22, 23), eosinophils have not been previously implicated in promoting the disease pathology associated with EAE. Whereas lymphocytes clearly are present in the CNS, our studies indicate that decreasing eosinophil but not lymphocyte infiltration was associated with inhibition of paralysis. EAE is an ascending disease of the CNS such that damage occurs first to the lower area, where the myelinated axons supplying the tail and hind limbs originate. As such, damage to this area would more closely correlate with hind-limb paralysis. Therefore the role of eosinophils in EAE may have been underestimated in previous studies, which have usually concentrated on the upper areas of the spinal cord and brain (24). In addition, studies illustrating selective inhibition of eosinophil rather than lymphocyte recruitment have not been done. A decrease in the number of eosinophils shown histologically and enzymatically correlated with a favorable outcome in animals treated with CP-105,696. Our electron microscopic observations demonstrating eosinophils within the myelin sheath further support the hypothesis that these cells may be involved in axonal atrophy and paralysis. In addition, as a proof of concept, an eosinophil-derived neurotoxin has been reported to induce EAE-like paralysis when injected into naive animals (25).

In summary, LTB_4 receptor ligation was responsible for mediating eosinophil influx into the lower spinal cord of animals during EAE. These eosinophils are important mediators of paralysis, since treatment with the specific LTB_4 receptor antagonist CP-105,696 selectively decreased eosinophil infiltration into the CNS and concomitantly prevented disease symptoms. The lack of effects of CP-105,696 when treatment was initiated after the onset of symptoms and when large numbers of eosinophils are already present suggests that continued eosinophil infiltration is not important for the progression of disease. Studies are in progress to assess the pathological role of eosinophils on secondary relapses.

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