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Prevention and Treatment of Experimental Autoimmune Encephalomyelitis by CNI-1493, a Macrophage-Deactivating Agent

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Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are characterized by episodic neurologic dysfunction, perivascular mononuclear cell inflammation occurring mainly in white matter, and demyelination. Strong circumstantial evidence supports the conclusion that macrophage activation and local production of proinflammatory cytokines are necessary for disease induction and lesion formation. We now report that CNI-1493, a small m.w. compound, which inhibits macrophage activation and subsequent proinflammatory cytokine production, suppresses EAE induced in the genetically susceptible SJL/J mouse. Treatment with 5 mg/kg/day completely suppressed mild disease (clinical index of 1.6 ± 0.5 in the untreated group as compared with 0.0 ± 0.0 for the treated group) and significantly reduced acute disease (clinical index of 4.3 ± 0.7 in the untreated group as compared with 0.5 ± 0.3 for the treated group). Suppression of clinical manifestations of the disease correlated with a significant decrease in histopathology and proinflammatory cytokine expression at the lesion site. Moreover, drug treatment during the chronic phase resulted in amelioration of clinical signs. The data presented here should prove useful in developing novel chemotherapeutic approaches for the treatment of MS. The Journal of Immunology, 1998, 160: 5588–5595.
phosphorylation of p38 mitogen-activated protein kinase, which plays a key role in posttranscriptional regulation of proinflammatory cytokine synthesis (29). When given at an estimated therapeutic to toxic ratio of 40:1, CNI-1493 suppresses LPS-stimulated production of macrophage TNF, IL-1, IL-6, MIP-1α, MIP-1β, and NO production. In contrast, no effect is found on the constitutive synthesis of TGF-β or MHC class II up-regulation by IFN-γ (30). Furthermore, CNI-1493 retains its cytokine-suppressive activities even in the presence of IFN-γ, in contrast to the immunosuppressive activities of glucocorticoids such as dexamethasone (30). A beneficial effect of CNI-1493 has been noted in several models of macrophage-mediated toxicity including: reduced edema formation in carrageenan-induced inflammation in the footpad; protection against the toxic effects of LPS; a reduction in infarct volume in cerebral ischemia; protection against adult respiratory distress syndrome by cecal ligation and puncture; reduction in cardiac signs in adjuvant arthritis; and a prolongation of cardiac allograft survival (reviewed in Ref. 31). The efficacy of this compound in protecting against diseases of macrophage-derived toxicity, combined with its low toxicity and short elimination half-life, makes it an attractive new therapeutic compound.

In this study we have addressed the potential efficacy of this potent inhibitor of macrophage activation in an inflammatory condition of the CNS in which proinflammatory cytokines have been shown to play a central role. The model system that we have chosen is EAE in the SJL/J mouse induced by the passive transfer of T cells sensitized to MBP. We show that i.p. administration of CNI-1493 successfully prevents clinical manifestations in mild EAE and significantly reduces severe EAE. The clinical improvement was accompanied by a significant decrease in related histopathology and a decrease in proinflammatory cytokine expression at the lesion site. In addition, treatment with CNI-1493 was found to significantly inhibit the chronic phase of EAE. Finally, we show that CNI-1493 has no effect on T cell proliferation at the therapeutic concentrations used in this study.

Materials and Methods

Induction and clinical evaluation of EAE

Female SJL/J (H-2b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used between the ages of 6 and 8 wk. Mice were housed and maintained in a federally approved animal facility at the Albert Einstein College of Medicine (Bronx, NY) in accordance with National Institutes of Health guidelines. For induction of passive transfer EAE, doxorubicinized mitomycin C-treated syngeneic spleen cells (R&D Systems, Cambridge, MA) emulsified with an equal volume of CFA (Difco Laboratories, Meriden, CT) was administered i.p. at a dose of 1 or 5 mg/kg in a volume of 100 μl of saline to the desired concentration in sterile saline. Drug or vehicle was administered daily by the i.p. route at a dose of 1 or 5 mg/kg in a volume of 100 μl of saline.

The statistical significance of differences in clinical index between groups was analyzed using Student’s two-tailed t test. Significance was accepted if p < 0.05.

Histopathology

Animals were anesthetized by ether inhalation and perfused intracardially with 20 ml ice-cold PBS. Sections of the brain and lumbar spinal cord were removed and either flash frozen in liquid nitrogen and stored at −80°C, or immersion-fixed in Trump’s fixative (4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). For histopathologic analysis, fixed tissue was dehydrated through a graded series of ethanol, cleared in propylene oxide, and embedded in Epon 812 (EM Sciences, Fort Washington, PA). For each animal, four to six 1-μm epony sections were stained with toluidine blue and examined by light microscopy. Coded slides were scored according to density of inflammatory cells in perivascular CNS compartments using the following scale: 1, perivascular inflammation of less than three cells thick; 2, perivascular inflammation of more than 3 cells thick; 3, parenchymal inflammation. The histopathologic score was then calculated for each animal by adding all the scores for the individual.

RNase protection assay

Three animals per group were sensitized as described above, killed, and perfused intracardially with saline. The lumbar spinal cord was dissected out and snap-frozen in liquid nitrogen. RNA was prepared from the samples using Tri-Reagent per manufacturer’s instructions (Molecular Research Center, Cincinnati, OH). Twenty micrograms of RNA was subjected to RPA using Ribobiquant MCK-2 probe set (PharMingen, San Diego, CA). The assay was performed using the Ambion RPA II kit essentially as described by the manufacturer. Briefly, 50 ng of the MCK-1 probe mixture was used as the template for transcription of antisense RNA probes using T7 RNA polymerase and [α-32P]UTP. Labeling was quantitated by liquid scintillation counting, and labeling efficiency was typically greater than 90%. Twenty micrograms of total RNA were hybridized to 3.5 × 105 cpm of probe overnight at 45°C. Unhybridized probe was digested with RNase A and T1, and hybrids were precipitated. Protected fragments were resolved on a 5% polyacrylamide/8 M urea gel. The gel was fixed, dried, and bands were visualized by autoradiography on Fuji film RX. messenger RNA levels for each of the cytokines was quantitated by densitometry of autoradiographs using the NIH-Image software package. Sample loading was normalized by comparison of L32 and glyceraldehyde-phosphate dehydrogenase mRNA levels.

T cell proliferation assays

Anti-CD3-mediated proliferation. Proliferative responses were assessed with T cells purified from naive spleens using high affinity negative selection mouse T cell enrichment columns (R&D Systems, Cambridge, MA). Ninety-six-well plates were coated for 3 h at 37°C in a humidified chamber with hamster anti-murine CD3 (PharMingen, San Diego, CA) at 2 μg/ml. Plates were then washed three times with water. T lymphocytes were added at a density of 0.5 to 5 × 104 cells/ml in the presence or absence of CNI-1493 at various dilutions in 0.2 ml final volume. Plates were then incubated for 2 days at 37°C in a CO2 incubator and 8 h before harvest pulsed with 1 μCi of [3H]Thymidine (NEN-Dupont, Boston, MA) per well. Cells were harvested and incorporated radioactivity was quantitated on a Top Count Microplate Scintillation Counter (Packard Instrument, Meriden, CT).

Anti-CD4 proliferative reaction. BR6.9 is an I-Aβ-restricted CD4 T cell clone responsive to the 146–162 peptide of the nicotinic acetyl choline receptor derived from C57BL/6 (B6) mice and was obtained from Dr. Anthony J. Infante of the University of Texas Southwestern Medical Center, San Antonio, TX. Nicotinic acetyl choline receptor peptide (1 μg/ml) was added to 2.5 × 105 responder cells and incubated with 2.5 × 105 APCs (mitomycin C-treated syngeneic spleen cells) for 3 days. [3H]Thymidine (1 μCi) was added during the last 6 h of culture, and cells were harvested onto glass fiber mats and counted for radioactivity in a Betaplate counter.

CNI-1493 and treatment protocol

CNI-1493 (Cas. Reg. No. 164301-51-3) was synthesized and purified as previously described (32). The purity was >99% as estimated by melting point, nuclear magnetic resonance, elution from HPLC, and elemental analysis. For each experiment, CNI-1493 was freshly prepared as a 1 mM working stock solution in sterile, endotoxin-free distilled water, and diluted to the desired concentration in sterile saline. Drug or vehicle was administered daily by the i.p. route at a dose of 1 or 5 mg/kg in a volume of 100 μl of saline.

**Note:** For adoptive transfer, naive syngeneic animals were injected i.p. at a dose of 3 × 106 cells/ml. The lower number of cells resulted in a mild disease course, while the higher number resulted in the severe form of the disease. Before transfer, cells were washed extensively in HBSS. To obtain a clinical index (CI), mice were scored on a scale of 0 to 5 according to standard procedures as follows: 0, no clinical disease; 1, limp/flaccid tail; 2, hind limb weakness (ataxia) with incomplete paralysis; 3, complete paralysis of one or two hind limbs; 4, forelimb weakness or partial paralysis; 5, moribund. Intermediate scores were assigned if neurologic signs were of lower severity than typically observed. Onset of disease was determined by loss of tail tone. Recovery was defined as a clear clinical improvement of at least one grade following a paralytic incident. Relapses were defined as worsening of the clinical index by 1.0 grade for two consecutive days.
**Supernantigen-specific proliferation.** BR6.9 cells express Vβ6 and are thus responsive to the endogenous murine mammary tumor retroviral supernantigen Mls1 expressed by cells from DBA12 mice. To determine whether CNI-1493 affected supernantigen responses, 2.5 × 10^6 BR6.9 cells were coincubated with 2.5 × 10^3 mitomycin C-treated DBA12 spleen cells for 3 days in the presence or absence of CNI-1493. [3H]thymidine (1 μCi) was added during the last 6 h of culture, cells were harvested onto glass fiber mats and counted for radioactivity in a Betaplate counter.

**TFN ELISA.** Immunlon II (Dynatech, Chantilly, VA) ELISA plates were coated with 5 μg/ml hamster anti-murine TNF mAb (Genzyme Diagnostics, Cambridge, MA). The plates were blocked with Superblock blocking buffer (Pierce, Rockland, IL) and washed in Tris-buffered saline (TBS)/0.05% Tween-20. Culture supernatants and TNF standards (tTNF, R&D Systems, Minneapolis, MN) were diluted in TBS/0.2% Tween-20 and incubated overnight at 4°C. After washing, rabbit anti-murine TNF antiserum (BAS 16.248) was added at a dilution of 1:800 using 1× TBS, 0.2% Tween-20, and 1% goat serum, and incubated for 2 h at room temperature. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN) was added at a 1:3000 dilution after washing the plate in TBS/0.05% Tween-20. After a 1-h incubation, the substrate (0.5 mg/ml of p-nitrophenyl-phosphate pNPP) in 10 mM diethanolamine/0.5 mM MgCl_2, pH 9.5) was added to the wells and incubated in the dark for 30 min at room temperature. After development, the plate was then read at 405 nm using a Dynatech ELISA reader. Unknown samples were compared against a standard curve between 1,000 and 60,000 pg/ml. The data were plotted with the Biotynx program (Dynatech) using a sigmoidal, log/linear scales with tails curve fitting.

**Results**

**Suppression of mild EAE by CNI-1493**

Animals sensitized by the passive transfer of MBP-reactive T cells develop a relapsing-remitting disease course that is characterized clinically by an ascending paralysis and pathologically by focal areas of submeningeal and perivascular inflammation and demyelination, localized preferentially to the spinal cord. The acute phase of the disease usually starts 5 to 7 days posttransfer (dpt), peaks approximately 11 to 12 dpt, and is followed by a period of remission lasting 10 to 12 days. Most animals then relapse with peak clinical signs occurring approximately 28 to 40 dpt. The pattern of subsequent relapses and remissions is variable and is linked to the severity of early events in the disease process, with more severe episodes of the acute disease associated with more frequent relapses and a gradual progression into a chronic disease state.

To determine the efficacy of CNI-1493 to suppress disease, we initially tested its ability to suppress mild EAE. Sensitized animals were divided into coded groups and injected i.p. on a daily basis with 5 mg/kg CNI-1493 or vehicle alone commencing immediately before, and continuing through, the peak of the disease. Clinical expression of disease was assessed on a daily basis through day 40. Control animals treated with saline developed a typical disease course (Fig. 1A). In two separate experiments, CNI-1493 treatment at 5 mg/kg from days 4 to 10 completely suppressed both the acute and chronic phases of the disease. In the representative experiment illustrated (Fig. 1A), the mean clinical index (MCI) at peak of disease (9 dpt) was 1.6 ± 0.5 in untreated and 0.0 ± 0.0 for the treated group (mean ± SD, n = 5 per treatment group).

**Suppression of severe EAE by CNI-1493**

Having ascertained that the compound inhibited mild EAE, we then tested its effect on a more severe form of the disease. Animals were again divided into coded groups and injected daily i.p. with two doses (5 and 1 mg/kg) throughout the course of the acute phase (12 days). The results of one representative experiment are shown in Figure 1B. In animals treated with either saline or 1 mg/kg CNI-1493 a typical disease course was observed with severe paralysis noted during the acute phase of the disease (days 7–9), which was followed by a remission (days 14–23) and subsequent relapse (day 25 on). In contrast, animals treated with 5 mg/kg CNI-1493 showed only mild clinical signs during the acute phase of the response that was followed by a delayed and more attenuated chronic phase of the disease (Fig. 1B). The MCI at the peak of disease (7 dpt) was 4.3 ± 0.7 in controls animals as compared with 0.5 ± 0.3 for treated animals (mean ± SD, n = 4, p < 0.05). This dose is well below the LD50 of 90 mg/kg i.p. in mice (33). The onset of the relapse, defined as an increase in MCI of 0.5 grade for more than 2 days, was delayed in animals receiving the 5 mg/kg dose as compared with animals injected with vehicle alone (24–26 dpt vs 27–29 dpt, p < 0.05). Similar results were obtained with a treatment course of 6 days overlapping the onset of disease. Five animals per group were treated for 6 days after the
first sign of clinical illness, as described above. The untreated group had a MCI at peak disease of 3.5 ± 0.0, while the treated group had a MCI of 0.2 ± 0.2.

Effect of the treatment regimen on chronic-relapsing EAE

We then examined the effect of treatment with CNI-1493 at different stages of the acute clinical episode (Table I). When given before disease onset (defined as the first day of clinical signs) no clinical efficacy was noted (Table I, Expt. 1). Similarly, when given after peak expression of disease (defined as the height of clinical signs), no effect was noted on either the clinical expression of the disease or on the kinetics of the recovery process (Table I, Expt. 2). Interestingly, however, when a single dose was given at the onset of disease, a 50% decrease in clinical disease was noted (Table I, Expt. 3).

Inhibition of chronic EAE by CNI-1493

Having ascertained that CNI-1493 suppressed the acute phase EAE, we then tested the effect of drug treatment on the chronic phase of the disease. EAE was induced by adoptive transfer as before and the animals monitored for clinical signs. Following the acute phase of the disease, the animals went into remission followed by a relapse. At the time of relapse, 10 animals were paired by clinical index, coded, and one animal from each pair was given 5 mg/kg/day for 6 days (25–31 dpt) and the other animal vehicle alone. The cumulative data, with the MCI expressed as a percentage of the control animal, for all pairs is shown in Figure 2A. Representative data from this experiment are shown in Figure 2B. As shown in Figure 2, animals that received CNI-1493 had a significantly improved disease course compared with those that received saline alone. Figure 2 also shows that animals continued to improve clinically for an extended period of time (25 days) after treatment was terminated.

In an additional experiment, treatment was delayed until the animals had entered a late unresolving chronic phase (>60 days with CI >3.0). Animals were paired, coded as above, and given CNI-1493 i.p. for 11 days (74–84 dpt). No effect on clinical expression of disease was noted (data not shown).

Histopathology of mice treated with CNI-1493

To determine the effect of CNI-1493 on pathologic expression of EAE during the acute phase of the disease, two additional experiments were performed for histopathologic analysis. In untreated, control animals (CI = 3.5), focal submeningeal areas of perivascular

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<sup>a</sup> Chronic relapsing-EAE was induced as described in Materials and Methods. A total of 5 mg/kg CNI-1493 or vehicle was injected i.p. for 4 days prior to onset of disease (Expt. 1), 7 days after peak of disease (Expt. 2), or 1 day, at onset of disease (Expt. 3).

<sup>b</sup> Number of animals per group.

<sup>c</sup> Day of relapse: experiment 1 = day 41; experiment 2 = day 27; experiment 3 = day 21.

<sup>d</sup> One animal died at peak disease; therefore, n for relapse is four animals.

<sup>e</sup> Statistical significance (Student’s two-tailed t test, assuming equal variances): p = 0.006 at disease peak and p = 0.023 at relapse.

Figure 2. Accelerated recovery from the chronic phase of EAE by CNI-1493 treatment. At the time of relapse, 10 animals were paired according to clinical score and one animal from each pair was given 5 mg/kg CNI-1493 and the other was given vehicle alone for 6 days. Animals were then scored for clinical expression of disease by an investigator blinded to the experimental protocol. A, Cumulative data from all of the animals tested are shown. Results are expressed as the percentage of change from the controls, as determined from the MCI of both groups of animals (mean ± SD of five experimental pairs). B, Representative data for four pairs. The solid lines represent the CI for the control (saline)-injected animals and the dashed line for the CNI-1493-treated animal in each group. The day of first injection and duration of treatment is indicated by the bar. Asterisk represents death.
Inflammatory cytokines IL-1, IL-6, IL-2, and IFN-γ.

In untreated, control animals, high levels of mRNA of the proinflammatory cytokine mRNA by RNAse protection assay. Two additional experiments were performed for analysis of levels of proinflammatory cytokine expression in EAE lesions during the acute phase of the disease, a significant reduction in disease expression is obtained. In contrast, treatment before disease onset or after peak clinical expression of disease is ineffective. These results suggest that there is a CNI-1493-sensitive event at disease onset. Consistent with this notion are the data that show that a single injection of CNI-1493 given at this time also significantly reduces clinical expression of mononuclear cell inflammation and demyelination in white matter were detected in the lumbar and cervical regions of the cord. Edema, as determined by evidence of perivascular accumulations of proteinaceous exudate, was also present particularly in association with vessels in the anterior fissure. In contrast, in animals treated with CNI-1493 (CI = 0.5), no demyelination was detected and only minimal evidence of perivascular inflammation and edema formation was observed. A histologic index determined from coded slides gave a value of +3.0 (n = 3) for the control animals and +0.5 (n = 3) for the CNI-1493-treated animals. Representative pathology from these animals is shown in Figure 3.

Down-regulation of inflammatory cytokine mRNA levels at lesion site of mice treated with CNI-1493

To determine the effect of CNI-1493 on the inflammatory cytokine expression in EAE lesions during the acute phase of the disease, two additional experiments were performed for analysis of levels of proinflammatory cytokine mRNA by RNAse protection assay. In untreated, control animals, high levels of mRNA of the proinflammatory cytokines IL-1, IL-6, IL-2, and IFN-γ were detected at the lesion site. Animals that received CNI-1493 had reduced levels of proinflammatory cytokine levels at the lumbar spinal cord (Fig. 4). RNA levels for IL-1, IL-2, and IFN-γ were reduced ~50%, while IL-6 message was reduced by about 75%.

No effect of CNI-1493 on T cell activation

Since EAE is known to be caused by neuroantigenic-specific CD4+ T cells, we sought to determine whether CNI-1493 had any effect on T cell proliferative responses. In vivo pharmacologic studies have determined that the maximal possible blood levels of CNI-1493 after i.p. injection of 5 mg/kg in mice is 0.025 μg, corresponding to a concentration of ~30 nM (33). T cell proliferation was induced by cross-linking of CD3 in the presence of various concentrations of CNI-1493. As can be seen in Figure 5A, CNI-1493 concentrations more than 10-fold higher than 30 nM had no effect on T cell proliferation. We then tested the effects of CNI-1493 on specific T cell responses to neuroantigen (nicotinic acetyl choline receptor, NACR) and superantigen (murine mammary tumor retroviral superantigen, Mls1+). Again, concentrations more than 10-fold higher than 30 nM had no effect on specific T cell proliferation to NACR peptide (Fig. 5B) or to superantigen (Fig. 5C). Finally, no suppression of TNF responses by T cells was observed in any of these experiments (Fig. 5D). Similar results were obtained using MBP as the antigenic stimulus and T cells derived from SJL mice (data not shown). Therefore, our results suggest little, if any, effect of CNI-1493 on infiltrating T cells.

Discussion

In this study we have established the efficacy of the novel macrophage-deactivating agent CNI-1493 in preventing the clinical manifestations of EAE, an animal model for MS. The results show that when CNI-1493 is given over a period that spans the onset of the first clinical episode, or during the early phases of the chronic phase, a significant reduction in disease expression is obtained. In contrast, treatment before disease onset or after peak clinical expression of disease is ineffective. These results suggest that there is a CNI-1493-sensitive event at disease onset. Consistent with this notion are the data that show that a single injection of CNI-1493 given at this time also significantly reduces clinical expression of disease.
Disease and that no effect of this drug was noted on T cell responses, as determined by mitogen-, Ag-, or superantigen-reactive assays. In studies on the effect of CNI-1493 on LPS-mediated TNF release into the circulation in normal mice, full recovery in their sensitivity to LPS challenge was noted 4 h after i.p. treatment with 5 mg/kg CNI-1493 (data not shown), and pharmacologic studies have shown that the elimination half-life of CNI-1493 in the circulation is 5 h (33). Taken together, our results suggest that therapeutic intervention during this CNI-1493-sensitive window has profound clinical effects. Understanding and elucidating the pathogenic mechanisms affected in this therapeutic window will help in targeting more successful and specific treatments for conditions in which the activation of macrophages plays a key role in disease pathogenesis.

EAE can be successfully treated by a variety of immunotherapies directed at proinflammatory cytokine production. These include procedures that result in the down-regulation of proinflammatory cytokines TNF, lymphotoxin, and IL-1, as well as those that inhibit macrophage migration and activation (16–18, 20–23). The most extensive and successful strategies have examined the role of the TNF family of proteins. In initial studies, a central role for TNF was demonstrated by the ameliorating effect of administering Abs to TNF or the soluble TNF receptor (21, 23). These promising results then prompted further investigations of pharmacologic TNF inhibitors. Several of these have shown efficacy in EAE, including matrix metalloproteinase inhibitors that block processing of TNF; Rolipram, a selective inhibitor of phosphodiesterase type IV; other phosphodiesterase type IV inhibitors such as pentoxifylline, which inhibit TNF production by activated macrophages; thalidomide, which inhibits the stability of TNF mRNA without affecting the stability of mRNA for other cytokines, and phosphatidylserine, a phospholipid of the cell membrane, which has been found to inhibit LPS-induced TNF production in vitro (reviewed in Ref. 34). Pathologic analyses of the results obtained with these various anti-TNF treatment modalities suggest that these procedures principally interfere with the homing of inflammatory cells to the CNS and affect the development of EAE at a step subsequent to the generation of autoimmune cells, a situation relevant to MS where the putative autoimmune reactive cells have been generated before clinical presentation. However, although extremely effective in animal models, these specific immunotherapies are less likely to work in human disease in which the pathogenic mechanisms involved are expected to be more complex and to vary at different stages of the disease process.

The precise mechanism involved in suppression of EAE by CNI-1493 is not known. Results presented here demonstrate a lack of inhibitory activity of CNI-1493 on T cell proliferation in response to the CNS-specific Ags PLP and MBP. A more extensive study of the effects of CNI-1493 on T cells and macrophages has recently been published by Bjork et al. (35). They quantitated proinflammatory cytokine synthesis at the single cell level (using computerized image analysis) following different routes of cell activation. Their results clearly demonstrated that the production of IL-2, IFN-γ, TNF-α, and TNF-β by activated T cells (via CD3 and

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**FIGURE 5.** Effect of CNI-1493 on T cell proliferative responses. A, anti-CD3-induced T cell proliferative response. The results represent mean ± SD of triplicate wells from a representative experiment. Open bars represent 0.5 × 10^6 cells/ml, hatched bars represent 1.0 × 10^6 cells/ml, cross-hatched bars represent 5.0 × 10^6 cells/ml. B, T cell proliferation in response to Ag, C, to superantigen. The results represent mean ± SD of triplicate wells from a representative experiment. D, Culture supernatant TNF levels for experiment 5b.
CD28 ligation or PMA/ionomycin treatment) was not affected by CNI-1493 treatment (35). On the other hand, similar treatment resulted in a profound inhibition of LPS-induced TNF-α, IL-1α, IL-1β, IL-6, and IL-8 production by macrophages, independently of IFN-γ priming. These results correspond well with previous published reports in which CNI-1493 has been shown to inhibit the release from activated macrophages of the cytokines TNF and IL-1, the chemokines MIP-1α and MIP-1β, as well as NO production via the inducible form of nitric oxide synthase (iNOS) (30). Each of these factors has been implicated in the pathogenesis of EAE (11). Elevated levels of TNF, IL-1, MIP-1α, MIP-1β and iNOS have been clearly demonstrated in affected CNS tissues, with levels correlating with the kinetics and severity of disease expression. Inhibition of TNF, IL-1, MIP-1α, and iNOS have been found to block disease expression. Peroxynitrates formed as a major consequence of NO production via iNOS have been implicated in the pathogenesis of EAE (36). Thus, CNI-1493 provides a potentially wider range of inhibitory activities against these inflammatory mediators than the specific inhibitors described above.

Since CNI-1493 does not affect T cell proliferation or proinflammatory cytokine production, but profoundly inhibits the production of potentially pathogenic macrophage cytokines, we hypothesize that the mechanism of suppression of EAE by this drug is likely to involve macrophage deactivation. We found a decrease in the expression of the proinflammatory cytokines IL-6, IFN-γ, IL-1, and IL-2 at the lesion site. We interpreted the decrease of T cell-derived cytokines (IL-2 and IFN-γ) as an indirect effect due to macrophage deactivation and subsequent generalized decrease in the proinflammatory status of the lesion area, which is supported by the histologic data. Of particular note, however, is the observation that treatment of activated macrophages with CNI-1493 has not been shown to affect the production of TGF-β, a cytokine with known regulatory activity. Natural recovery from the acute clinical episode of EAE has been associated with the production of TGF-β by spleen cells, and administration of this cytokine to animals sensitized to develop EAE blocks progression of disease (34). Furthermore, enhanced TGF-β secretion has been proposed to mediate the immunosuppression observed following the induction of oral tolerance (37).

In addition to its effect in the acute clinical episode, treatment with CNI-1493 was found to significantly ameliorate the chronic phase of the disease. Although it is widely assumed that the mechanisms involved in the induction of a relapse resemble those found during the acute phase of the disease, this has by no means been formally tested (38, 39). Most of the studies that have addressed mechanisms of disease pathogenesis, as well as those that have tested various therapeutic regimens, have focused principally on the acute clinical episode in the mouse model or have used the monophasic disease that develops in the Lewis rat. The striking ability of CNI-1493 to inhibit chronic disease, even after the cessation of treatment, is in contrast to data obtained with either Abs to TNF or integrins, or with the soluble TNF receptor, where EAE usually develops rapidly following cessation of treatment (21, 22, 40). Since the in vivo anti-inflammatory effects of CNI-1493 are relatively short lived, these data suggest that the long-term beneficial effects of CNI-1493 result from the lack of effect on TGF-β that could result in a shift in the cytokine profile of the autoreactive cells from one dominated by proinflammatory cytokines to one in which a Th2 or Th3 profile dominates. If this is indeed the case, then CNI-1493 may, like other drugs such as retinoids and phosphodiesterase inhibitors, exert part of its effect by altering the balance between Th1 and Th2 type responses and further studies are planned to specifically address this issue.

In conclusion, we have documented the efficacy of a new type of pharmacologic agent, a multivalent guanylylhydrazone compound, in an inflammatory disease of the CNS. This compound has many potential benefits for diseases of a chronic inflammatory nature; it has broad inhibitory activity for proinflammatory factors released from activated macrophages while leaving immunoregulatory cytokines unaffected; the anti-inflammatory activities of CNI-1493 are short lived and thus should not result in long-term immunosuppression leaving the patient vulnerable to intercurrent infections and, like other successful pharmacologic agents, has a lower cost, higher specificity, and known biophysical characteristics compared with biologic immunotherapies. Finally, this compound provides a useful tool to study the effects of simultaneously removing multiple cytokines from an inflammatory disease model. In summary, these studies identify a prototype compound that should lead to the development of novel macrophage-modulating anti-inflammatory therapies that could have significant benefits for many diseases of a chronic inflammatory nature.

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References


