Ciliary Neurotrophic Factor Inhibits Brain and Peripheral Tumor Necrosis Factor Production and, When Coadministered with Its Soluble Receptor, Protects Mice From Lipopolysaccharide Toxicity

Fabio Benigni,* Pia Villa,*† Maria Teresa Demitri,* Silvano Sacco,* Jean D. Sipe,‡ Laura Lagunowich,§ Nikos Panayotatos,§ and Pietro Ghezzi*

*"Mario Negri" Institute for Pharmacological Research, Milan, Italy
†CNR, Center of Cytopharmacology, Department of Pharmacology, Milan, Italy
‡Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts, U.S.A.
§Regeneron Pharmaceuticals Inc., Tarrytown, New York, U.S.A.

ABSTRACT

Background: The receptor of ciliary neurotrophic factor (CNTF) contains the signal transduction protein gp130, which is also a component of the receptors of cytokines such as interleukin (IL)-6, leukemia-inhibitory factor (LIF), IL-11, and oncostatin M. This suggests that these cytokines might share common signaling pathways. We previously reported that CNTF augments the levels of corticosterone (CS) and of IL-6 induced by IL-1 and induces the production of the acute-phase protein serum amyloid A (SAA). Since the elevation of serum CS is an important feedback mechanism to limit the synthesis of proinflammatory cytokines, particularly tumor necrosis factor (TNF), we have investigated the effect of CNTF on both TNF production and lipopolysaccharide (LPS) toxicity.

Materials and Methods: To induce serum TNF levels, LPS was administered to mice at 30 mg/kg i.p. and CNTF was administered as a single dose of 10 μg/mouse i.v., either alone or in combination with its soluble receptor sCNTFRα at 20 μg/mouse. Serum TNF levels were then measured by cytotoxicity on L929 cells. In order to measure the effects of CNTF on LPS-induced TNF production in the brain, mice were injected intracerebroventricularly (i.c.v.) with 2.5 μg/kg LPS. Mouse spleen cells cultured for 4 hr with 1 μg LPS/ml, with or without 10 μg CNTF/ml, were also analyzed for TNF production.

Results: CNTF, administered either alone or in combination with its soluble receptor, inhibited the induction of serum TNF levels by LPS. This inhibition was also observed in the brain when CNTF and LPS were administered centrally. In vitro, CNTF only marginally affected TNF production by LPS-stimulated mouse splenocytes, but it acted synergistically with dexamethasone (DEX) in inhibiting TNF production. Most importantly, CNTF administered together with sCNTFRα protected mice against LPS-induced mortality.

Conclusions: These data suggest that CNTF might act as a protective cytokine against TNF-mediated pathologies both in the brain and in the periphery.

INTRODUCTION

Proinflammatory cytokines, particularly tumor necrosis factor (TNF) and interleukin (IL)-1, play important roles in infectious, inflammatory and autoimmune diseases. In particular, TNF appears to mediate the toxicity induced by lipopolysaccharide (LPS) and to act in some diseases of the central nervous system, such as allergic encephalomyelitis, bacterial meningitis, and cerebral malaria (1).

Address correspondence and reprint requests to: Pietro Ghezzi, Mario Negri Institute, Via Eritrea 62, 20157 Milano, Italy.

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The pathogenic effects of proinflammatory cytokines result from their action on several cellular targets, some of which, in turn, cause the triggering of "anti-inflammatory" feed-back responses. These include the induction of hepatic acute-phase proteins (such as proteinase inhibitors and antioxidants), and the activation of the hypothalamus-pituitary-adrenal axis (HPAA), which ultimately increases serum levels of corticosterone (CS), a potent inhibitor of cytokine synthesis (2).

Induction of at least some acute-phase proteins by either IL-1 or TNF is mediated by IL-6 (3). IL-6 also synergizes with IL-1 to activate the HPAA (4), and thus appears to play a central role in mediating anti-inflammatory responses. It should be noted that, while administration of TNF or IL-1 induces LPS shock-like symptoms and toxicity, administration of IL-6 does not induce toxicity but only some metabolic changes associated with the acute-phase response, namely, APP synthesis and fever (5). This absence of toxicity further illustrates the anti-inflammatory role of IL-6.

It was recently established that the gp130 subunit of the IL-6 receptor is an accessory component of the receptors of many other cytokines, such as leukemia inhibitory factor (LIF), IL-11, oncostatin M, ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (6,7). Interestingly, some of these "gp130-user cytokines" have common pharmacological properties, including a protective action in animal models of inflammation. LIF protects against endotoxic shock (8,9) and pulmonary inflammation induced by intratracheal injection of LPS (10); IL-6 suppresses de-myelination in a viral model of multiple sclerosis (11) and inhibits TNF production both in vivo and in vitro (9,12). All gp130-user cytokines tested (LIF, IL-6, oncostatin M, and CNTF) act as hepatocyte-stimulating factors, directly stimulating APP synthesis (13–15). Finally, both CNTF and IL-6 enhance the activation of the HPAA by IL-1 (15), which might further contribute to the protective action of gp130-user cytokines.

Because it targets primarily neuronal cells, CNTF was not expected to have a wide range of activities compared with the other gp130-user cytokines. In vitro experiments have however shown that CNTF, in combination with its soluble receptor sCNTFRα, inhibits the synthesis of proinflammatory cytokines in human peripheral blood mononuclear cells and human fibroblasts (16). This suggests that circulating sCNTFRα may render a wide range of non-neuronal cells responsive to CNTF. This is supported by the observation that recombinant sCNTFRα confers functional responsiveness to CNTF to cells that do not express the a subunit of the receptor complex on their surface, with the same relative affinity and specificity of the cell-surface form (17,18).

In order to evaluate the physiopathological relevance of these actions of CNTF, we have characterized the effects of administering CNTF (either alone or in combination with sCNTFRα) in an animal model of LPS toxicity. We also studied the effect of CNTF on TNF production in vivo, by measuring serum TNF levels after i.p. administration of LPS, and in vitro in mouse splenocytes, and its ability to act in synergy with the synthetic glucocorticoid dexamethasone (DEX).

Since CNTF is a neurotrophic factor, we also studied its effect on TNF production in the brain. For this purpose, mice were injected with LPS intracerebroventricularly (i.c.v.), as peripheral injection of LPS does not produce detectable levels of TNF in the brain (19).

MATERIALS AND METHODS

Materials

Recombinant human CNTF, purified from *Escherichia coli*, was obtained from Regeneron Pharmaceuticals Inc. (Tarrytown, NY, U.S.A.). EC50 of CNTF in the E8 chicken ciliary ganglion neuronal survival assay was 0.24 ng/ml (20). Recombinant human soluble CNTF receptor a (sCNTFRα) was prepared from *E. coli* as previously described (18). LPS (phenol-extracted preparation from *E. coli* O55:B5) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals and Treatments

Male CD-1 mice (30 g body weight, Charles River, Calco, Como, Italy) were used. Procedures involving animals and their care was conducted in conformity with the institutional guidelines, in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana No. 40, February 18, 1992; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

CNTF was administered as a single dose of 10 μg/mouse i.v. (15) and LPS at 30 mg/kg i.p. Blood was taken 1.5 hr later from the retroorbital plexus under ether anaesthesia and serum prepared. When indicated, LPS (2.5 μg/mouse in a final volume of 20 μl) was injected intracerebroventricularly (i.c.v.) via a 28-gauge needle into ether anesthetized mice (21,22).

Splenocyte Cultures

BALB/c mice were sacrificed and spleens were removed and disaggregated by washing with RPMI 1640 medium using a 2.5-ml syringe. Splenocytes were counted and plated at 10×10^6/ml in 96-well culture plates (100 μl/well). Cells were cultured in RPMI 1640 medium with 10% FCS in the presence of 1 μg/ml LPS, with and without CNTF, sCNTFRα, or DEX, at the indicated concentrations in a final volume of 200 μl/well. Four hours later, supernatants were harvested and assayed for TNF levels.

The IC_{50} values for DEX were established by simultaneous nonlinear curve fitting according to the logistic equation reported by De Lean et al. (23), using the "Allfit" program.

Miscellaneous Assays

TNF activity was measured by cytotoxicity on L929 cells as previously described (24), using mouse recombinant TNFα as a standard (Genzyme, Cambridge, MA, U.S.A.). CS was measured by radioimmunoassay, using an antiserum obtained from Sigma (C-8784) per manufacturer's instructions. ^{3}H-corticosterone was purchased from Amersham (Amersham, United Kingdom). SAA was measured in serum samples by ELISA, as previously described (25).

LPS Lethality Studies

Male, adult (22–24 g) BALB/c mice were given LPS at a single dose of 30 mg/kg i.p., with or without a single dose of 10 μg/mouse of CNTF, and survival was assessed daily. When indicated sCNTFRα was administered i.v. in combination with CNTF (CNTF, 10 μg/mouse + sCNTFRα, 20 μg/mouse). Animals were followed up to 7 days, then sacrificed.

RESULTS

CNTF Inhibits Serum TNF Induction in Vivo and, When Coadministered with sCNTFRα, Protects from LPS Lethality

Figure 1 shows the survival curves of mice treated with a lethal dose of LPS (30 mg/kg i.p.). As a positive control, pretreatment with DEX (30 mg/kg i.p. 30 min before LPS) conferred 100% protection. Also, pretreatment with a monoclonal anti-TNF antibody 30 min before LPS administration completely protected against LPS toxicity (26), indicating that this is an experimental model of TNF-mediated LPS toxicity.

A statistically significant protection was achieved only when CNTF was administered in combination with its soluble receptor (CNTF, 10 μg/mouse + sCNTFRα, 20 μg/mouse). The same dose of CNTF alone did not significantly protect against LPS toxicity.

As shown in Fig. 2, CNTF (10 μg/mouse i.v.) inhibited the increase in TNF serum levels induced by a lethal dose of LPS (30 mg/kg i.p.), both at the time when peak levels occurred (1.5 hr) and at a later time point (3 hr). Concomitant administration of sCNTFRα (20 μg/mouse) did not further augment this effect of CNTF.

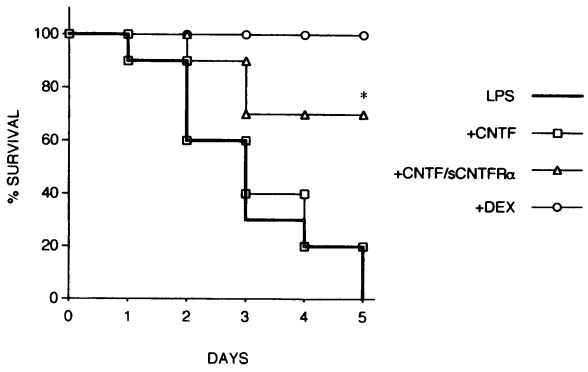

FIG. 1. Effect of CNTF on LPS toxicity

Mice were treated with LPS (30 mg/kg), either with or without CNTF (10 μg/mouse) and sCNTFRα (20 μg/mouse) administered 30 min before LPS, as described in Materials and Methods. Survival was assessed daily and expressed as a percentage (data from 10 mice per group). Animals were followed for up to 7 days; further deaths did not occur over this period and surviving mice did not appear sick.
*$p < 0.05$ versus LPS alone by Fisher's exact test.

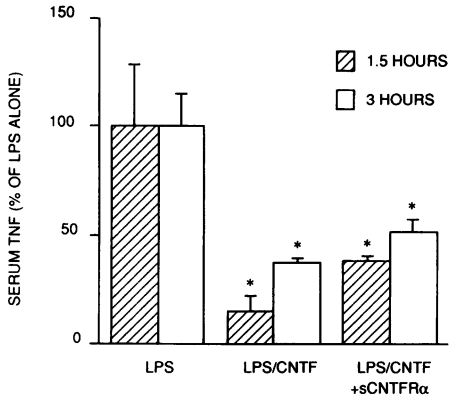

FIG. 2. CNTF inhibits serum TNF induction by a lethal dose of LPS

Mice were treated with LPS (30 mg/kg i.p.) with or without CNTF and sCNTFRα (10 μg/mouse and 20 μg/mouse, respectively, i.v. 30 min before LPS). Serum TNF levels were measured 1.5 and 3 hr later. Data (mean ± SE, $n = 5$) from two experiments are reported and TNF levels are expressed as percentage of LPS alone (100% was 4.0 ± 2.0 ng/ml at 1.5 hr and 0.27 ± 0.08 ng/ml at 3 hr). No TNF was detected in mice treated with saline or CNTF alone (not shown).
*$p < 0.05$ versus respective control (LPS alone) by Dunnett's test.

CNTF Inhibits Brain TNF Induced by Central LPS Administration

When mice were injected i.c.v. with 2.5 μg/mouse of LPS, high TNF levels were detected in brain homogenates; TNF was not detectable in the brain following either i.p. or i.v. injections with the same dose of LPS (data not shown). Coadministration of CNTF i.c.v. (10 μg/mouse) with LPS significantly prevented the induction of brain TNF levels (Fig. 3).

Effect of CNTF on LPS-Induced CS and SAA

As shown in Table 1, CNTF administered simultaneously with LPS slightly but significantly increased the peak levels of serum CS. CNTF alone had no effect serum CS levels. As previously reported (15), CNTF alone induced a significant elevation of serum SAA levels (Table 1); although SAA levels induced by CNTF were 5-fold lower than the SAA levels induced by LPS. When CNTF was given in combination with LPS, no additive effect was observed. Coadministration of sCNTFRα did not enhance the effects of CNTF on serum CS and SAA levels.

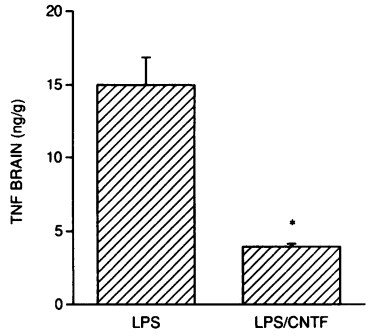

FIG. 3. CNTF inhibits TNF production in the brain

Mice were injected i.c.v. with LPS (2.5 μg/mouse) with or without CNTF (10 μg/mouse). TNF was measured in brain homogenates 1.5 hr later, and expressed as ng/g tissue (mean ± SE, $n = 5$).
*$p < 0.01$ versus LPS alone by Dunnett's test.

Effect of CNTF on TNF Production in Vitro by Splenocytes

In order to show a direct effect of CNTF on LPS-induced TNF synthesis, mouse spleen cells were used. Spleen cells were cultured for 4 hr with 1 μg LPS/ml, since, in preliminary experiments these were found to be the optimal conditions for TNF production. In some experiments, sCNTFRα was also included, as previously described (16). A trend towards an inhibition of TNF production by CNTF was observed in three experiments, where 10 μg/ml CNTF inhibited TNF production by 27%, 35%, and 11% (data

TABLE 1. Effect of CNTF on LPS-induced CS and SAA

Treatment	CS (ng/ml)	SAA (μg/ml)
Saline	42 ± 38	<0.06
CNTF	46 ± 11	0.6 ± 0.1
LPS	269 ± 36	3.0 ± 1.0
CNTF + LPS	315 ± 32	2.7 ± 0.8
CNTF + sCNTFRα + LPS	347 ± 56[a]	4.5 ± 0.9

Mice were treated with LPS (80 μg/kg, i.v.), sCNTFRα (20 μg/mouse i.v., along with CNTF) and CNTF (10 μg/mouse i.v., simultaneously with LPS). CS was measured 2 hr and SAA 24 hr after treatment. Data are mean ± SD (five mice/group) from a representative experiment.
[a]$p < 0.01$ versus LPS alone by Duncan's test.

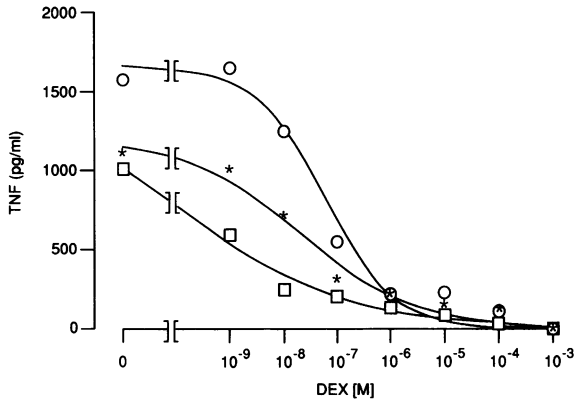

FIG. 4. CNTF potentiates DEX inhibition of TNF production in vitro

Mouse spleen cells were cultured 4 hr as described in Materials and Methods with 1 μg/ml LPS, 10 μg/ml CNTF, and 20 μg/ml sCNTFRα. No TNF was detected in the absence of LPS (data not shown). DEX was added at various concentrations (0 or 10^{-3} to 10^{-9} M) in the presence and absence of CNTF (10 μg/ml). Curve fitting was performed by the Allfit program (23). (○), LPS alone; (□), LPS + CNTF; (*), LPS + CNTF + sCNTFRα. TNF production in the absence of DEX was: 1570 ± 479 for LPS alone; 1007 ± 308 pg/ml (mean ± SD; $n = 3$) for LPS with CNTF.

not shown). However, the effect was not statistically significant. In other experiments, CNTF was tested at various concentrations (from 0.2 to 20 μg/ml) without any significant effect on TNF production. Addition of sCNTFRα (20 μg/ml) did not increase the effect of CNTF (data not shown).

CNTF, however, augmented the inhibitory effect of DEX, shifting its IC_{50} from 5.8×10^{-8} ($\pm 1.4 \times 10^{-8}$) to 2.1×10^{-9} ($\pm 1.3 \times 10^{-9}$) M (Fig. 4). The difference between the two IC_{50} was statistically significant ($p < 0.05$ using the Allfit program). Once again, exogenously added sCNTFRα (20 μg/ml) did not enhance this effect of CNTF.

DISCUSSION

The present report shows that CNTF is an inhibitor of LPS-induced serum TNF levels and protects against LPS toxicity when combined with its soluble receptor. In our previous work, CNTF was shown to increase the levels of serum CS, SAA, and IL-6, induced by IL-1 (15). Production of endogenous corticosteroids represents an important feedback mechanism against proinflammatory cytokines, as demonstrated by the observation that adrenalectomy sensitizes mice to the lethal effect of LPS, TNF, and IL-1 (27), and increases TNF production in vivo (28). The activation of the HPAA response by CNTF might therefore have a role in the observed inhibition of TNF production.

A similar protective role has been demonstrated for at least some acute-phase proteins. Mice treated with α-acid glycoprotein are protected against TNF-induced lethality (29), and transgenic mice expressing rabbit C-reactive protein are protected from complement-mediated lung injury (30). SAA was also reported to inhibit IL-1- and TNF-induced fever and PGE_2 production (31). It is possible that these mechanisms are involved both in the protective effect of CNTF reported in this study, and in the anti-LPS actions of LIF and IL-6 previously reported (8–10,12).

CNTF was, however, found to have only a minor effect on the levels of serum CS induced by LPS and no effect on SAA levels, most likely because LPS induces maximal levels of these proteins. It is therefore unlikely that the inhibition of TNF production and of LPS toxicity by CNTF is due only to the activation of feedback regulatory mechanisms, but suggests that other, direct effects of CNTF are involved.

The data obtained in vitro, using cultured mouse spleen cells, indicate that CNTF, even at high concentrations, only slightly inhibits TNF production. Once again this can hardly explain the marked inhibition of serum (and brain) TNF levels observed in LPS-treated mice. On the other hand, the finding that CNTF enhances the inhibitory effect of a glucocorticoid, dexamethasone, decreasing its IC_{50} by about 25-fold, suggests that CNTF not only affects the activation of the HPAA but also the inhibitory effect of glucocorticoids on TNF production. Inhibition of TNF production may therefore result from a combination of effects of CNTF: (1) direct inhibition of TNF production; (2) activation of the HPAA; and (3) increasing the inhibition of TNF production by glucocorticoids.

As far as the protective effect on LPS toxicity is concerned, inhibition of serum TNF levels does not entirely account for the protective action of CNTF. The inhibition of TNF production (both at peak levels and at 3 hr) did not differ whether CNTF was administered alone or together with sCNTFRα; similarly, serum CS and SAA were not significantly elevated when sCNTFRα was added.

sCNTFRα, however, significantly enhanced the protection against a lethal dose of LPS. In addition, it should be noted that, for this experimental model, CNTF (with or without its soluble receptor) was not able to completely block TNF production, raising the possibility that CNTF acts both on TNF production and on its toxicity.

The requirement for sCNTFRα to gain a maximal protective effect might be explained by the following hypotheses: (1) injection of soluble receptors might confer responsiveness to cells that normally do not respond to CNTF because they display gp130 and LIF receptors, but not the specific receptor subunit; or (2) the soluble receptor might change the pharmaco-kinetics and tissue distribution of CNTF, as soluble cytokine receptors have been shown to prolong the pharmaco-kinetic half-life of recombinant cytokines. Further studies are necessary to investigate these possibilities.

In conclusion, our data are consistent with the idea that cytokines like CNTF, LIF, or IL-6 (gp130-users) have an anti-inflammatory action rather than a pathogenetic role in septic shock. It is noteworthy that the only report of protection against LPS toxicity by anti-IL-6 antibodies was associated with increased, rather than inhibited, serum IL-6 levels (32), probably due to a "chaperone" effect of the antibody. In fact, IL-6 knockout mice have higher serum TNF levels after LPS injection than normal mice (33), supporting the hypothesis that IL-6 inhibits TNF production.

The present study has also shown that CNTF inhibits TNF production in the central nervous system. This observation might be important to TNF-mediated pathologies of the central nervous system; in particular, in experimental allergic encephalomyelitis susceptibility to the disease is related to endogenous corticosteroids levels and to the efficiency of the HPAA feedback response (34,35).

It was recently reported that TNF induces cell death in oligodendrocytes and that this effect can be prevented by CNTF (36). It is therefore possible that, under certain conditions, CNTF might counterbalance the effects of TNF. Further studies involving measurements of CNTF levels after LPS treatment or neutralization of CNTF with antibodies, will be required to determine whether CNTF represents yet another feedback regulator of TNF production.

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