

## Ciliary neurotrophic factor is an endogenous pyrogen

(fever/cytokines/thermoregulation)

LELAND SHAPIRO\*, XI-XIAN ZHANG\*, RANDALL G. RUPP†, SHELDON M. WOLFF\*,  
AND CHARLES A. DINARELLO\*‡

\*Department of Medicine, Tufts University School of Medicine and New England Medical Center, Boston, MA 02111; and †Regeneron, Inc., Tarrytown, NY 10591

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**ABSTRACT** Fever is initiated by the action of polypeptide cytokines called endogenous pyrogens, which are produced by the host during inflammation, trauma, or infection and which elevate the thermoregulatory set point in the hypothalamus. Ciliary neurotrophic factor (CNTF) supports the differentiation and survival of central and peripheral neurons. We describe the activity of CNTF as intrinsically pyrogenic in the rabbit. CNTF induced a monophasic fever which rose rapidly (within the first 12 min) following intravenous injection; CNTF fever was blocked by pretreatment with indomethacin. The fever induced by CNTF was not due to contaminating endotoxins. Increasing doses of CNTF resulted in prolongation of the fever, suggesting the subsequent induction of additional endogenous pyrogenic activity. After passive transfer of plasma obtained during CNTF-induced fever, endogenous pyrogen activity was not present in the circulation; CNTF also did not induce the endogenous pyrogens interleukin 1, tumor necrosis factor, or interleukin 6 *in vitro*. Nevertheless, a second endogenous pyrogen may originate within the central nervous system following the systemic injection of CNTF. Of the four endogenous pyrogens described to date (interleukin 1, tumor necrosis factor, interferon, and interleukin 6), CNTF, like interleukin 6, utilizes the cell-surface gp130 signal-transduction apparatus.

Endogenous pyrogens are polypeptide mediators of fever which are produced by the host in response to injury, inflammation, or infection (1, 2). When injected intravenously, endogenous pyrogens usually induce a rapid rise (within 12 min) in core temperature; this rapid rise in core temperature is preceded by a rise in brain prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels (3, 4). A fundamental scheme for the production of fever is that injury, inflammation, or infection stimulates the synthesis of endogenous pyrogens in the periphery which, in turn, affect the thermoregulatory center leading to fever.

The interaction of endogenous pyrogens with the cerebrovascular periventricular organs is likely to be a critical step in initiating the febrile response from systemically derived endogenous pyrogens, since it is unlikely that endogenous pyrogens by themselves cross the normal blood-brain barrier (5, 6). The ability of an endogenous pyrogen to produce fever appears to be mediated by a common pathway involving the generation of PGE<sub>2</sub> (reviewed in ref. 7). Cerebrospinal fluid and hypothalamic levels of PGE<sub>2</sub> correlate with the onset, magnitude, and duration of fever (3, 4). The cytokines interleukin 1 (IL-1), tumor necrosis factor (TNF), interferon (IFN), and IL-6 are endogenous, intrinsically pyrogenic cytokines (1, 2).

The first and most studied endogenous pyrogen is IL-1 (reviewed in ref. 8). In experimental animals (9) or humans (10), the intravenous injection of recombinant IL-1 at 10–100

ng/kg induces fever. IL-6 is also an endogenous pyrogen (11, 12) and elevated circulating levels of IL-6 in humans with infection or following experimental endotoxin fever are 100- to 1000-fold greater than those for IL-1 (13, 14). IL-6 belongs to a family of cytokine growth factors which use the gp130 transmembrane molecule for signal transduction (15). Ciliary neurotrophic factor (CNTF) also uses gp130 for initiating signal transduction in target cells as part of its receptor complex (16). The common pathway of IL-6 and CNTF using gp130 for signal transduction suggests the possibility that triggering this receptor leads to a pyrogenic response and that like IL-6, CNTF possesses endogenous pyrogenic activity.

### MATERIALS AND METHODS

**Cytokines and Neurotrophic Factor.** Recombinant human CNTF purified from *Escherichia coli* was used throughout these studies. The EC<sub>50</sub> of CNTF in the embryonic day 8 chicken ciliary ganglion neuronal survival bioassay was 0.24 ng/ml (17). Recombinant human IL-1β (specific activity, 10<sup>7</sup> units/mg) had an N terminus at position 117 (alanine) of the IL-1β precursor molecule (9). The recombinant proteins used in these studies did not contain measurable endotoxin (<100 pg/mg) as determined by the *Limulus* amoebocyte lysate assay with a sensitivity of 20 pg/ml (Associates of Cape Cod, Woods Hole, MA) and completely lost their pyrogenicity after heating at 70°C for 30 min. Lipopolysaccharide (B055; LPS) was purchased from Sigma. Polymyxin B (PmxB) for injection (Pfizer) was dissolved in sterile water.

**Rabbit Pyrogen Test.** Female New Zealand albino rabbits with an average weight of 3 kg were used. Measurement of rectal temperatures with a Digistrip II apparatus (Kaye Instruments, Bedford, MA) was as described (18). Rabbits were trained in metal restrainers for 4 days with indwelling rectal probes. On the day of the experiment, rectal temperatures were monitored every 6 min for 1 hr (8:30 to 9:30 am), after which intravenous injections took place. Mean temperature elevations were calculated from baseline temperature (mean of three consecutive recordings 18 min prior to injection). Rabbits receiving an intravenous injection of pyrogen-free saline had a slight drop (0.1–0.2°C) in rectal temperature within the first hour. With the exception of studies on pyrogenic tolerance and dose-response, rabbits were used once for pyrogen testing. Injections were 1.0-ml/kg volumes into a lateral ear vein.

**Passive Pyrogen Transfer Experiment.** Three donor rabbits were injected with CNTF at a dose of 330 μg/kg. After 190 min, blood was drawn from the central ear artery into heparinized polypropylene syringes and immediately centrifuged. The plasmas were combined and injected intrave-

Abbreviations: CNTF, ciliary neurotrophic factor; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; PmxB, polymyxin B; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; TNF, tumor necrosis factor.

‡To whom reprint requests should be addressed.

nously into each of three recipient rabbits at 6 ml/kg and temperatures were recorded.

**Peripheral Blood Mononuclear Cells (PBMCs).** Human blood was drawn from healthy adult male volunteers into heparinized (10 units/ml) syringes. PBMCs were separated by centrifugation on Ficoll/Hypaque gradients (9). The cells were washed with pyrogen-free saline and resuspended in RPMI 1640 medium (Sigma) which had been subjected to ultrafiltration to eliminate endotoxins (19). PBMCs were counted and suspended at  $5 \times 10^6$  cells per ml in RPMI with 2% (vol/vol) heat-inactivated human AB serum. Samples (500  $\mu$ l) of the cell suspension were added to 5-ml polypropylene tubes. An equal volume of either RPMI or materials diluted in RPMI was added. After incubation for 24 hr at 37°C in 5% CO<sub>2</sub>, the cultures were frozen (-70°C) and thawed (37°C) for three cycles prior to radioimmunoassay (RIA) for cytokines.

**RIA.** RIA for IL-1 $\alpha$ , TNF $\alpha$ , IL-6, and IL-8 were as described (20–23). For assay of PGE<sub>2</sub>, supernatant culture medium was removed after 24 hr of incubation as described previously (12).

**Statistics.** Means of groups were compared by two-tailed unpaired *t* test or ANOVA. A *P* value of <0.05 is considered significant.

## RESULTS

**Pyrogenicity of CNTF.** Following intravenous injection of CNTF at 1  $\mu$ g/kg into conscious rabbits, there was no significant change in body temperature; however, CNTF at 5  $\mu$ g/kg produced a monophasic fever with a brisk increase following the injection. Fever reached a peak elevation of  $0.35 \pm 0.03^\circ\text{C}$  ( $n = 6$ ) after 36 min. At 10  $\mu$ g/kg, there was a significant elevation ( $>0.15^\circ\text{C}$ ,  $P < 0.05$ ) above baseline 18 min after the injection. At this dose, CNTF produced a peak elevation of  $0.45 \pm 0.11^\circ\text{C}$  after 30 min (Fig. 1A). For comparison, rabbits injected with human IL-1 $\beta$  had peak fevers of  $0.78 \pm 0.07^\circ\text{C}$  with a latent period of 10 min at a dose of 100 ng/kg (100-fold less than CNTF at 10  $\mu$ g/kg) (Fig. 1B). With higher doses of CNTF (50 or 150  $\mu$ g/kg), a higher peak of fever was observed and fever was prolonged (data not shown). Fig. 2 depicts the dose-response of CNTF pyrogenicity. Significant fever occurred at a dose of 5  $\mu$ g/kg, with increasing maximal temperature elevations at higher concentrations until 150  $\mu$ g/kg. Increasing the dose to 250 or 330  $\mu$ g/kg (data not shown) did not result in a further increase in the maximal febrile response, although the fever persisted beyond 3–4 hr. Pretreatment with indomethacin prevented CNTF-induced fever, suggesting that, as with IL-6 and other endogenous pyrogens, inhibition of cyclooxygenase prevents fever (12).

**Passive Transfer of Possible Circulating Pyrogen.** Because of the prolonged nature of CNTF-induced fevers at the higher CNTF doses, we considered the generation of a second endogenous pyrogen, as is the case with IL-1 or TNF (9, 24). Three rabbits were infused with CNTF at 330  $\mu$ g/kg, which produced fever until the time of plasma collection (190 min). After removal of plasma from these rabbits, intravenous bolus infusion of the pooled plasma into recipient rabbits did not result in elevation of body temperature (Fig. 3).

**Ruling Out Endotoxin Contamination.** Higher doses of CNTF (150  $\mu$ g/kg) produced more prolonged fevers than the monophasic fevers observed at lower doses (5–50  $\mu$ g/kg). Since the duration of fevers induced by endotoxins also exhibits dose dependency, experiments were designed to exclude the possibility that CNTF was pyrogenic due to the presence of contaminating endotoxins. Four daily injections of CNTF (150  $\mu$ g/kg) into six rabbits did not result in progressively decreased pyrogenicity, also termed pyrogenic tolerance (25) (data not shown). Since PmxB neutralizes the

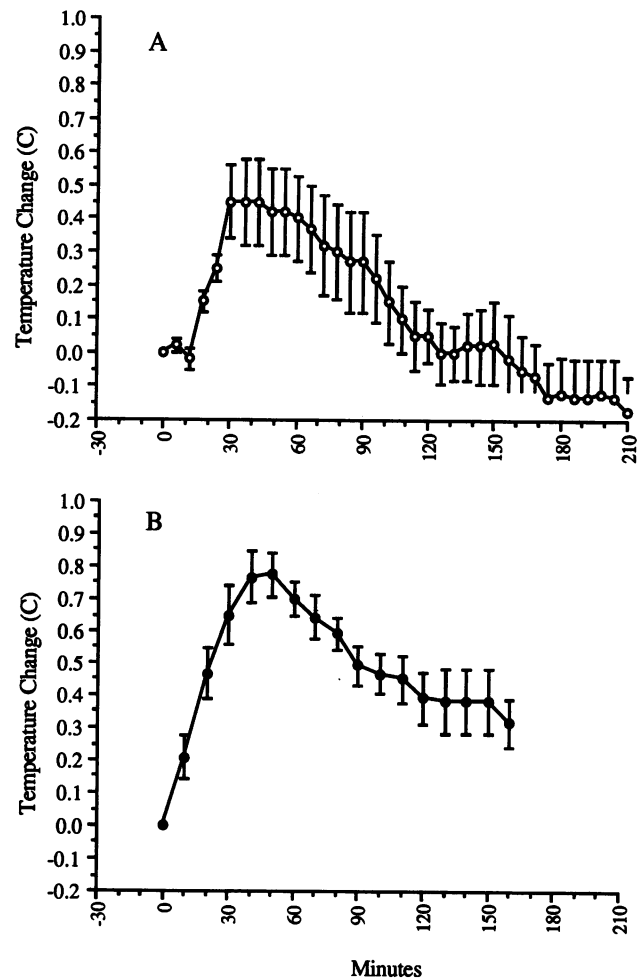


FIG. 1. Comparison of pyrogenicity of CNTF and IL-1 $\beta$ . (A) Change in temperature over baseline in six rabbits (mean  $\pm$  SEM) following injection of CNTF at 10  $\mu$ g/kg. CNTF-induced fever was significantly elevated above baseline from 18 to 102 min ( $P < 0.05$ , by ANOVA). (B) Change in temperature in six rabbits following injection of IL-1 $\beta$  at 100 ng/kg. IL-1 $\beta$ -induced fever was significantly elevated over baseline from 10 to 160 min ( $P < 0.05$ , by ANOVA).

pyrogenicity of LPS (26), we evaluated the ability of PmxB to affect CNTF-induced fever. Preincubation of CNTF with PmxB showed no significant alteration in the febrile response compared with the fever induced by CNTF preincubated with saline (Fig. 4A). For comparison, the pyrogenicity of LPS was blocked by PmxB (Fig. 4B).

**PBMC Incubations.** To further exclude the possibility of presence of endotoxin in recombinant CNTF, serial 1:2 dilutions of CNTF from 12.5 to 1.56  $\mu$ g/ml were incubated overnight with PBMCs. CNTF did not induce IL-1 $\alpha$  production, whereas LPS at 1 ng/ml induced IL-1 $\alpha$  production of  $>20$  ng/ml (Fig. 5). Furthermore, production of TNF $\alpha$ , IL-6, or IL-8 was not induced by CNTF. The viability of PBMCs was confirmed by trypan blue exclusion. In addition, IL-1 $\alpha$ , TNF $\alpha$ , or IL-8 production in response to LPS at 1 ng/ml was unaffected by the presence of the same concentrations of CNTF (data not shown).

## DISCUSSION

These data demonstrate that CNTF is intrinsically a pyrogen. The brisk rise of the fever, blockade by indomethacin, and lack of evidence of the presence of exogenous pyrogens support a role of CNTF as an endogenous pyrogen. CNTF is the fifth molecularly defined cytokine found to be intrinsi-

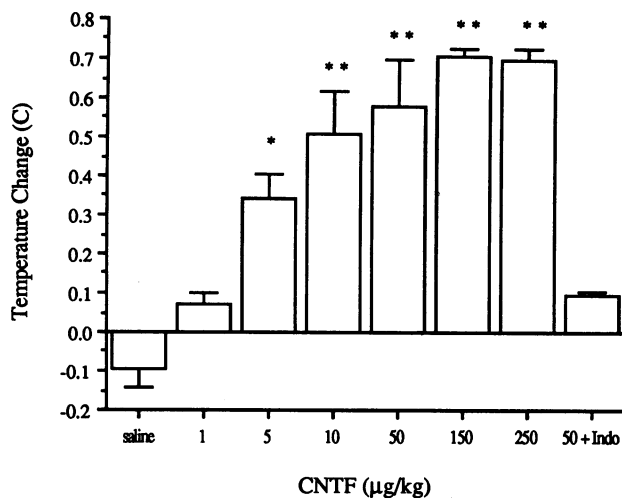


FIG. 2. Dose-response of CNTF-induced fever. Maximum fever peak over baseline occurring within 1 hr after injection as a function of CNTF dose is shown. The effect of indomethacin (Indo, 2.5 mg/kg) injected intravenously 12 min prior to injection of CNTF (50  $\mu$ g/kg) is shown in the last bar. Depicted are mean  $\pm$  SEM for six rabbits in each group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with saline-injected controls by unpaired  $t$  test.

cally pyrogenic, following the descriptions of IL-1, TNF, IFN, and IL-6, four cytokines that are pyrogenic in rabbits and in humans.

Low doses of CNTF produced a monophasic fever, whereas higher doses produced prolonged fever, suggesting the induction of a second endogenous pyrogen. The half-life of CNTF in the circulation is several minutes (27). This supports the concept that the prolongation is not due to residual CNTF. Similar to CNTF, increasing the dose of IL-1 or TNF from 100 ng/kg to 10  $\mu$ g/kg also results in the appearance of a prolonged fever peak (9, 24). During the second peak due to high doses of IL-1 or TNF there is a

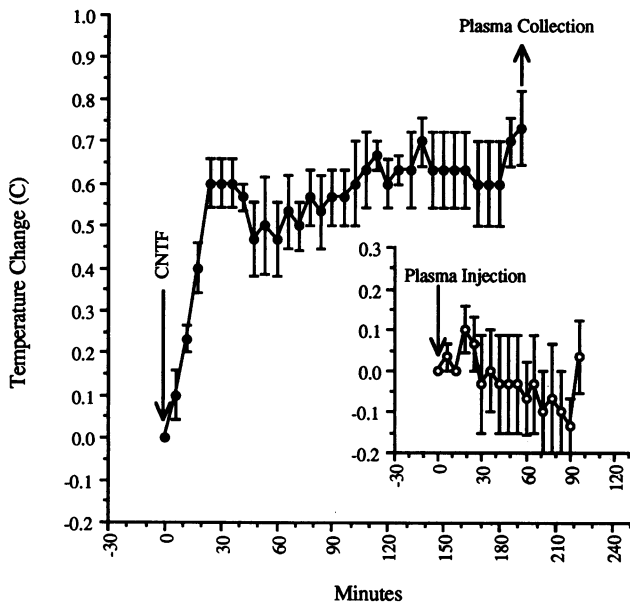


FIG. 3. Passive transfer during CNTF-induced fever. Three donor rabbits were infused with CNTF (330  $\mu$ g/kg). Temperature elevation was significant from 12 to 190 min ( $P < 0.05$ , ANOVA). Plasmas were collected at 190 min and combined. The pooled plasma was infused (6 ml/kg) into three recipient rabbits (*Inset*). Data are expressed as mean  $\pm$  SEM. The axes of the *Inset* are the same as those of the main figure. Injection of plasma did not result in statistically significant temperature elevation.

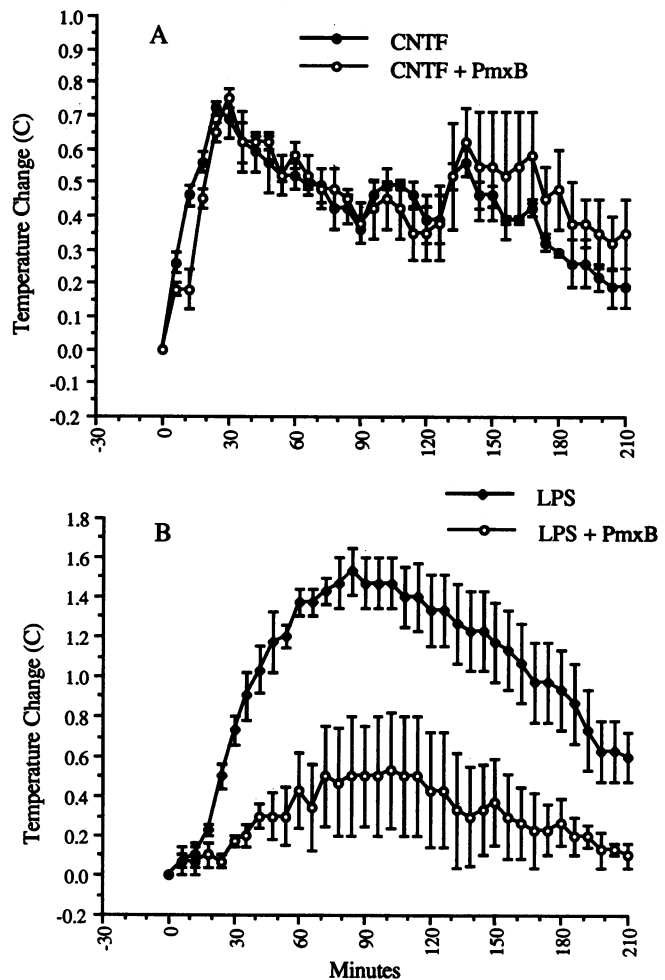


FIG. 4. Effect of PmxB on CNTF- or LPS-induced fever. (A) CNTF (250  $\mu$ g/ml) was incubated with PmxB (2 mg/ml) for 30 min at 37°C and then injected into three rabbits (CNTF, 250  $\mu$ g/kg). Control rabbits ( $n = 3$ ) received CNTF (250  $\mu$ g/kg) that had been incubated with saline. CNTF-induced fever was significant from 12 to 210 min ( $P < 0.05$ , by ANOVA); there were no significant differences between CNTF-induced fever and fever induced by CNTF after incubation with PmxB. (B) LPS (300 ng/ml) was incubated with PmxB (100  $\mu$ g/ml) and injected into three rabbits (LPS, 100 ng/kg). Control rabbits ( $n = 3$ ) received LPS (100 ng/kg) that had been incubated with saline. There was a statistically significant difference between fever induced by LPS and fever induced by LPS which had been incubated with PmxB ( $P < 0.05$ , 24–210 min, by ANOVA). Data are expressed as mean  $\pm$  SEM for each condition.

circulating endogenous pyrogen. However, passive transfer experiments showed that the prolonged fever following a high dose of CNTF is not due to the appearance of circulating endogenous pyrogen activity.

IL-1 and TNF induce the production of each other as well as the production of IL-6 *in vitro* (9, 23, 24, 28). The same cascade of induction of IL-1, TNF, and IL-6 also takes place *in vivo* (1, 9, 24, 28), which most likely accounts for the circulating endogenous pyrogen activity during the prolonged fever induced by higher doses of IL-1 or TNF. However, using passive transfer of plasma obtained from febrile rabbits injected with high dose CNTF, we excluded the induction of a peripheral (non-central nervous system) second pyrogen. Additional studies demonstrated the lack of *in vitro* induction of IL-1, TNF, or IL-6 production in PBMCs exposed to CNTF, further supporting the concept that the prolongation of CNTF fever is not due to the intermediate production of a peripheral endogenous pyrogen. On the other hand, we

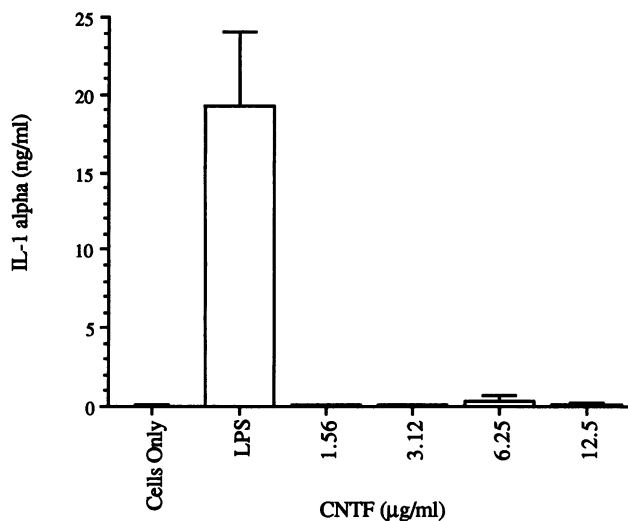


FIG. 5. Production of IL-1 $\alpha$  by PBMCs. PBMCs were incubated with RPMI 1640. (control), LPS at 1 ng/ml, or the indicated concentrations of CNTF. Data depict mean  $\pm$  SEM for PBMCs from three donors. LPS-induced IL-1 $\alpha$  production was significant compared with unstimulated cells ( $P < 0.05$ , by paired  $t$  test). There was no significant production of IL-1 $\alpha$  at any concentration of CNTF compared with unstimulated cells ( $P > 0.05$ , by ANOVA). Similar data were observed with PBMCs from another six donors.

speculate that there may be an induction of an endogenous pyrogen(s) originating within the central nervous system following the peripheral injection of CNTF. This concept has some basis, as peripherally injected IL-1 induces IL-6 in the cerebrospinal fluid of the third cerebral ventricle (29–32). This phenomenon of prolonged elevation in temperature and the appearance of a second peak of fever has been observed following the injection of exogenous pyrogens such as bacterial LPS (33, 34); however, in the present studies, we have excluded LPS as a cause of the prolonged fever due to CNTF.

CNTF and IL-6 share the gp130 signal-transduction apparatus, which may account for some of the functional similarities between these molecules (15). Both are endogenous pyrogens and both induce acute-phase proteins from hepatic cell lines *in vitro* (35). CNTF signals cellular events by combining with a trimeric cell-surface receptor complex consisting of a specific CNTF receptor protein (CNTFR $\alpha$ ), leukemia inhibitory factor  $\beta$  receptor, and gp130 (16). CNTFR $\alpha$  has a limited tissue distribution and has been found only in neural, muscular, and hepatic tissues (35, 36).

The gp130 molecule is responsible for signal transduction via tyrosine phosphorylations (16). Signal transduction does not result in the induction of PGE $_2$  by CNTF in PBMCs (data not shown) as has been observed with IL-6 (12). Others have reported that IL-6 does not induce PGE $_2$  in peripheral tissues (37–39). Nevertheless, the pyrogenicity of CNTF was blocked by indomethacin, suggesting that CNTF—like IL-1, TNF, IFN, and IL-6—causes fever by inducing the synthesis of prostaglandins. However, similar to IL-6 (12), CNTF likely triggers prostaglandin synthesis in the brain by a mechanism which utilizes constitutive rather than inducible cyclooxygenase (40).

The known physiologic roles for CNTF include support of differentiation and survival in both central and peripheral neurons. These properties have led to the initiation of clinical trials for the treatment of degenerative neurologic diseases. Based on the association of gp130-mediated events and the intrinsic pyrogenicity of IL-6 and CNTF, it is anticipated that IL-11, oncostatin M, and leukemia inhibitory factor—other cytokines which use gp130 to transmit a signal—may also function as endogenous pyrogens.

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