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(57) Abstract

Methods are provided for the production and purification of recombinant ciliary neurotrophic factor. The purification of C-terminal truncated forms of ciliary neurotrophic factor are also described. Methods are provided for preventing and treating peripheral nerve damage. The method comprises administering to patients in need thereof a therapeutically effective amount of CNTF. A preferred method for producing CNTF is by recombinant DNA technology.

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PURIFICATION OF RECOMBINANT CILIARY NEUROTROPHIC FACTOR AND C-TERMINAL TRUNCATED CILIARY NEUROTROPHIC FACTOR AND METHODS FOR TREATING PERIPHERAL NERVE DAMAGE

BACKGROUND OF THE INVENTION

The present invention relates to neurotrophic factors and ciliary neurotrophic factor (CNTF) in particular, as well as methods of purifying CNTF and producing recombinant CNTF.

Severe mental and physical disabilities result from the death of nerve or glial cells in the nervous system. The death of nerve or glial cells can be caused by neurodegenerative diseases such as Alzheimer's and Parkinson's diseases and multiple sclerosis, by the ischemia resulting from stroke, by a traumatic injury, or by the natural aging process.

Neurotrophic factors are a class of molecules that promote the survival and functional activity of nerve or glial cells. Evidence exists to suggest that neurotrophic factors will be useful as treatments to prevent nerve or glial cell death or malfunction resulting from the conditions enumerated above. Appel, 1981, Ann. Neurology 10:499.

The best characterized of such neurotrophic factors is nerve growth factor (NGF). NGF has been demonstrated to be a neurotrophic factor for the forebrain cholinergic nerve cells that die during Alzeheimer's disease and with increasing age. The loss of these nerve cells is generally considered responsible for many of the cognitive deficits associated with Alzheimer's disease and with advanced age.

Experiments in animals demonstrate that NGF prevents the death of forebrain cholinergic nerve cells after traumatic injury and that NGF can reverse cognitive losses that occur with aging. Hefti and Weiner, 1986, Ann. Neurology 20:275; Fisher et al., 1987, Nature, 329:65. These results suggest the

potential clinical utility in humans of this neurotrophic factor in the treatment of cognitive losses resulting from the death of forebrain cholinergic nerve cells through disease, injury or aging.

A complication of the use of neurotrophic factors is their specificity for only those subpopulations of nerve cells which possess the correct membrane receptors. Most nerve cells in the body lack NGF receptors and are apparently unresponsive to this neurotrophic factor. It is, therefore, of critical importance to discover new neurotrophic factors that can support the survival of different types of nerve or glial cells than does NGF.

New neurotrophic factors have been searched for by their ability to support the survival in culture of nerve cells that are not responsive to NGF. One widely used screening assay is designed to discover factors that promote the survival of ciliary ganglionic motor neurons that innervate skeletal and smooth muscle. These ciliary ganglionic nerve cells belong to the parasympathetic nervous system and their survival is not supported by NGF.

The presence of factors which promote the survival of ciliary ganglionic nerve cells have been reported from a variety of tissues and species. Many of these ciliary ganglionic neurotrophic activities have the following similar chemical and biological properties: (1) the activity is present in high concentration in sciatic nerves; (2) the neurotrophic activity survives exposure to the ionic detergent sodium dodecyl sulfate (SDS) and to the reducing agents beta-mecaptoethanol (BME) or dithiothreitol (DTT) during electrophoresis on SDS polyacrylamide reducing gels; and (3) on such gels the activity migrates with an apparent molecular weight between 24-28 kd.

Collins, 1985, Developmental Biology, 109:255-258, Manthorpe, et al., 1986, Brain Research, 367:282-286.

Based on these similar properties, it had been proposed that the same or closely related molecules, typically referred to as "ciliary neurotrophic factor" or "CNTF", are responsible for the ciliary ganglionic neurotrophic activities. Thus, the term CNTF was used as an operational definition referring to agents with the above properties that promote the survival of ciliary ganglionic nerve cells in culture.

Despite widespread scientific interest in CNTF the difficulty in purifying substantial amounts from natural sources and the unavailability of human CNTF hampered attempts to demonstrate its value in sustaining the viability of nerve cells during disease or after injury. Prior attempts to purify a rat CNTF had resulted in an 800-fold enrichment over crude nerve extract in terms of specific activity. Manthorpe et al., 1986, Brain Research 367:282-286.

However, an eight hundred-fold increase in specific activity was insufficient to produce a single protein species. Therefore, the product showing increased activity obtained from the method described by Manthorpe et al. was insufficient as it included multiple protein species. It was desirable to achieve a purification of CNTF such that a single protein species is obtained with the appropriate biological activity.

In a published PCT application (PCT/US90/00022, Int'l Publication No. WO 90/07341) Collins et al. have described the purification of CNTF from rabbit sciatic nerve extract, and have set forth the nucleic acid sequences encoding rabbit and human CNTF and the amino acid sequences of rabbit and human CNTF. The Collins' reference also describes the

recombinant production of CNTF in both animal and bacterial expression systems. This published application is specifically incorporated herein by this reference.

The present invention further describes methods for the prevention and treatment of a variety of diseases and medical conditions. The common element of the diseases and medical conditions that are suitable for prevention or treatment according to the methods described herein is damage to the peripheral nervous system.

The peripheral nervous system consists of those nerve cells that extend axonal processes outside the spinal cord and brain. The principle nerve cell types in the peripheral nervous system are primary motor neurons innervating skeletal muscle and controlling movement, autonomic neurons (both sympathetic and parasympathetic) innervating the cardiovascular system and other internal organs and regulating their function, and sensory neurons innervating sensory receptors throughout the body and conveying sensations including pain and proprioception.

Conditions that compromise the survival and proper function of one or more of these types of peripheral nerve cells cause peripheral nerve damage. Such damage may occur through physical injury, which causes the degeneration of the axonal processes of peripheral nerve cells that pass through or near the site of injury. Such damage may also occur because of intentional or accidental exposure to neurotoxins, such as the cancer and AIDS chemotherapeutic agents cisplatinum and dideoxycytidine (ddC), respectively. Such damage may also occur because of chronic metabolic diseases, such as diabetes or renal dysfunction. Such damage may also occur because of neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS),

which causes the degeneration of primary motor neurons and consequently motor dysfunction.

As stated above, the defining characteristic of such peripheral nerve damage is compromised function and/or survival of peripheral nerve cells and their axonal processes. This invention describes treatments that can support peripheral nerve cells: that is, promote the normal function and survival of peripheral nerve cells against the effects of conditions that typically lead to peripheral nerve damage, or to reverse or minimize the effects of peripheral nerve damage.

The methods described herein for treating peripheral nerve damage involve the administration of the human protein ciliary neurotrophic factor (CNTF). It has been shown that CNTF supports the survival of embryonic rodent or embryonic chicken peripheral sympathetic and parasympathetic autonomic neurons and peripheral sensory neurons in cell culture (Manthorpe et al. (1989) Ciliary Neurotrophic Factors, Nerve Growth Factors, R.A. Rush ed., John Wiley & Sons, Ltd.). The single experiment on animal peripheral nerve cells suggests that CNTF was able to rescue cranial motor neurons after damage to their axonal processes in newborn rats (Sendtner et al. 1990 Nature **345**:440). No work has been reported demonstrating that CNTF can support autonomic or sensory nerve cells in animals. In addition, prior to this invention there was no evidence that CNTF can support any type of peripheral nerve cells in adult animals.

Obviously, little or no useful information is known regarding the proper doses and routes of administration for the prevention or treatment of peripheral nerve damage with CNTF. In the one experiment reported in animals, a single dose of CNTF was directly applied to the severed end of the

experimentally-damaged nerve (Sendtner et al. 1990 Nature 345:440). Most peripheral nerve damage does not involve individual severed nerves, but is widespread and involves nerve cells and their processes throughout the body. To treat these systemic conditions, CNTF will need to be administered systemically or regionally and not simply to the severed end of a single nerve. The information on dosing and route of administration contained in Sendtner, therefore, is of very limited practical use. To date, no methods have been reported for systemically or regionally delivering CNTF in doses that are effective in preventing or reversing peripheral nerve damage.

In the present invention, methods are provided for systemic and regional dosing with CNTF and it is demonstrated, for the first time, that CNTF used according to these methods can be effective in preventing or reversing peripheral nerve damage in adult animals. The appropriate route of administration and the appropriate dosing of CNTF needed to treat different forms of peripheral nerve damage are also disclosed. These methods differ among different diseases primarily in the way CNTF is administered and the dosing that is used.

SUMMARY OF THE INVENTION

An object of this invention is to provide recombinant human ciliary neurotrophic factor protein having an amino acid sequence as in Figure 1 where the amino acids at the C-terminal end are cleaved. Preferred truncated forms of CNTF are truncated by two or six amino acids.

The invention also provides for an expression vector comprising a DNA sequence encoding for C-terminal truncated forms for CNTF and their expression in bacterial and preferably E. coli expression systems.

In addition, this invention provides for recombinant human CNTF substantially free of truncated forms of CNTF.

Furthermore, the present invention provides a method for preparing a substantially purified CNTF comprising: (a) applying cell lysate containing soluble CNTF protein to an anion exchange column which reversibly binds CNTF; (b) collecting fractions comprising CNTF by eluting the CNTF protein bound to the anion exchange column with salt; (c) applying the fractions containing CNTF protein to a cation exchange column; (d) collecting fractions comprising CNTF by eluting the CNTF protein with a pH gradient of from about 7 to about 8.5; (e) applying the fractions containing CNTF protein to an anion exchange column; and (f) eluting the substantially purified CNTF protein with a salt gradient.

The present invention also includes a method for preventing or treating peripheral nerve damage which comprises administering to a patient in need thereof a therapeutically effective amount of CNTF; the use of a therapeutically effective amount of CNTF for the manufacture of medicament suitable for preventing or treating peripheral nerve damage; and an agent for preventing or treating peripheral nerve damage which

comprises a therapeutically effective amount of CNTF. In particular, the invention provides methods for administering therapeutically effective amounts of CNTF by therapeutically effective routes of administration in order to prevent and reverse peripheral nerve damage. The invention also demonstrates the adequacy of these methods to prevent or reverse peripheral nerve damage from a variety of insults.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the DNA and inferred amino acid sequence of human CNTF. The human CNTF coding sequence is interrupted by a single ca. 1.3-kb intron located between amino acids 38 and 39. The splice acceptor/donor sequences at this site are: [GTAAGT...1.3kb...TTTCCTGTATCCTCGGCCAG]. The internal HindIII and NheI sites used in construction of the expression vector are underlined as are the oligonucleotides used for cloning.

Figure 2 shows the inferred amino acid sequences of human, rabbit and rat CNTF. The amino acid sequences are presented in single letter code. Numbers to the right indicate position in the human sequence. Regions in which the sequence is identical in all three species are shaded. Since the inferred rabbit protein is one amino acid shorter than either human or rat CNTF, a gap (indicated by a dash) has been introduced into the rabbit sequence to maximize alignment.

Figure 3 shows the SDS-Page analysis of selected fractions eluted from initial Q-Sepharose ion-exchange chromatography column (Step 3). Cell extract

was chromatographed on a column of Q-Sepharose (1.5 X 20 cm). The chromatogram was developed at 2 ml/min and 2 ml fractions were collected. Selected fractions were subjected to SDS-PAGE and the gels stained with CBB. For electrophoresis, samples (15 μl, lanes 1 and 2 or 30 μl, lanes 3-28) were diluted in SDS-sample buffer (final concentrations: 10% glycerol, 1% DTT, 0.5% SDS, 0.002% bromophenol blue and 25 Mm Tris-HCl, pH 6.8) and boiled for 2 min. Key to gel lanes: 1 & 2 = crude extract before and after PEI treatment, respectively; 3-28 = even-numbered fractions from 12 to 62. Numbers in the left-hand margin indicate M_r values (X 10^{-3}) of protein standards electrophoresed simultaneously. CNTF co-migrates with the trypsinogen standard (M_r = 24,000) and the CNTF pool represents fractions 18-50.

Figure 4 shows the Q-Sepharose ion-exchange chromatography (Step 4). The CNTF pool from the first Q-Sepharose column was dialyzed and chromatographed on a second Q-Sepharose column (1.5 X 15 cm). The chomatogram was developed at 2 ml/min and 2 ml fractions were collected. Selected fractions were prepared for electrophoresis, subjected to SDS-PAGE and the gels silver-stained. The inset shows the protein content of electrophoresed fractions of the CNTF pool (fractions 114-130). Key to lanes: 1-9 5 μl of fractions, 114, 116, 118, 120, 122, 124, and 10 μl of fractions 126, 128 and 130, respectively. Numbers in the left-hand margin indicate M_r values (X 10^{-3}) of protein standards electrophoresed simultaneously.

Figure 5 shows the S-Sepharose ion-exchange chromatography (Step 5). The CNTF pool from Step 4 was dialyzed and chromatographed on an S-Sepharose column (1 X 10 cm). The chromatogram was developed at 2 ml/min and 4 ml fractions were collected. Selected fractions were prepared for electrophoresis, subjected to SDS-PAGE and the gels silver-stained. The inset

shows the protein content of electrophoresed fractions of the CNTF pool (fractions 25-29). Key to lanes: 1-5 = 15 µl of fractions 25, 26, 27, 28 and 29, respectively. Numbers in the left-hand margin indicate the M_r values (X 10^{-3}) of protein standards electrophoresed simultaneously.

Figure 6 shows the Zn^{2+}-affinity chromatography (Step 6). The CNTF pool from Step 5 was dialyzed and chromatrographed on a Zn^{2+}-IDA-agarose column (1 X 10 cm). The chromatogram was developed at 1.5 ml/min and 3 ml fractions were collected. Selected fractions were prepared for electrophoresis, subjected to SDS-PAGE and the gels silver-stained. The inset shows the protein content of electrophoresed fractions of the CNTF pool (fractions 30-38). Key to lanes: 1-9 = 25 µl of fractions 30, 31, 32, 33, 35, 36, 37 and 38, respectively.

Figure 7 shows the RP-HPLC analysis of purified recombinant human CNTF. CNTF, 5 µg (A) and 50 µg (B) were applied to SynChrom RP-8 reverse phase HPLC column (250 X 4.6 mm) equilibrated with 0.1% TFA. Protein was eluted with a linear gradient of acetonitrile containing 0.1% TFA (1% acetonitrile/min; flow rate, 1 ml/min).

Figure 8 shows the multiple forms of CNTF. Purified human recombinant CNTF (12 µg) was subjected to SDS-PAGE with (A) or without (B) prior heating in SDS sample buffer to 100°C for 2 min. The protein was transblotted onto a nitrocellulose membrane and treated with primary antibody (rabbit anti-CNTF) and secondary antibody (goat anti-rabbit IgG-alkaline phosphatase).

Figure 9 shows the u.v.-absorption spectrum of recombinant human CNTF. The u.v. absorption spectrum was recorded on a Beckman DU-50 spectrophotometer and the concentration of CNTF (1.28 mg/ml) determined by amino acid analysis.

Figure 10 illustrates the rate of recovery of cutaneous sensation after sciatic nerve crush (on day 0) in adult rats treated with vehicle only or with vehicle containing 0.25mg/kg human recombinant CNTF delivered in daily subcutaneous injections on days -2 to +11.

Figure 11 illustrates the rate of recovery of motor function (measured by recovery of the ability to spread toes 1-5) after sciatic nerve crush (on day 0) in adult rats treated with vehicle only or with vehicle containing 0.25mg/kg human recombinant CNTF delivered in daily subcutaneous injections on days -2 to +11.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the following examples, serve to explain the principles of the invention.

This application includes recombinant methods of production of human ciliary neurotrophic factor (CNTF). Also included in this invention are various human C-terminal truncated CNTFs. In the preferred embodiments of the invention, the C-terminal truncated CNTFs are identical to full length human CNTF as shown in figure 1 but are truncated at the C-terminus by either two or six amino acid residues. The C-terminal truncated CNTFs of the present invention preferably are produced during the bacterial expression of human CNTF by the expression of vectors containing the gene coding for CNTF. Such C-terminal truncated CNTFs may also be produced by the expression of vectors containing the gene coding for the C-terminal truncated CNTFs. This invention also includes purification processes for obtaining substantially purified CNTF obtained from recombinant production systems.

The purification of CNTF from rabbit sciatic

nerve is described in PCT application WO 90/07341 of Collins et al. (PCT/US90/00022). The Collins application includes a description of the rabbit and human genes coding for CNTF and the production of recombinant human CNTF from mammalian and bacterial expression systems. The WO 90/07341 application is specifically incorporated herein, in its entirety, by this reference, including without limitation all definitions and experimental procedures.

A novel process for the production and purification of recombinant human CNTF is given below in Example 1. Further included in this example is a six step process for the purification of recombinant human CNTF comprising:

1. the preparation of cell free extracts;
2. the removal of nucleic acids from the extract;
3. Q-sepharose ion-exchange chromatography;
4. a second Q-sepharose ion-exchange chromatography;
5. S-sepharose ion-exchange chromatography; and
6. Zn^{2+} - affinity chromatography.

According to these procedures, a CNTF composition is prepared that contains less than 0.1% non-CNTF proteins.

A preferred method of purification of human recombinant CNTF as taught in Example 2 below comprises: (a) applying cell lysate containing soluble CNTF protein to an anion exchange column which reversibly binds CNTF; (b) collecting fractions comprising CNTF by eluting the CNTF protein bound to the anion exchange column with salt; (c) applying the fractions containing CNTF protein to a cation exchange column; (d) collecting fractions comprising CNTF by eluting the CNTF protein with a pH gradient of from

about 7 to about 8.5; (e) applying the fractions containing CNTF protein to an anion exchange column; and (f) eluting the substantially purified CNTF protein with a salt gradient.

An alternate purification of CNTF is described in Example 2 below, wherein C-terminal truncated CNTFs are isolated and identified. The C-terminal truncated CNTFs are isolated from a bacterial expression of a vector containing the gene coding for full length human CNTF. The C-terminal truncated CNTFs so isolated are identical to human CNTF -- as shown in Figure 1 -- but are truncated at the c-terminus by either 2 or 6 amino acid residues. Also described are procedures for isolating CNTF from C-terminal truncated CNTFs and substantially purified CNTF which is substantially free from C-terminal truncated CNTFs. Included within the scope of this application are all C-terminal truncated CNTFs that retain any of the biological activity associated with CNTF.

It has also been found that recombinant CNTF produced as described in Example 2 can be further purified by using an additional chromatography step. As described in Example 3 below, columns that have been found effective in lowering the amount of non-CNTF proteins from purified CNTF solution include hydroxy-apatite resin, butyl HIC (hydrophobic interaction chromatography) resin and Zn-IMAC (immobilized metal affinity chromatography) resin.

As noted above, the present invention further relates to methods for preventing and treating peripheral nerve damage in patients suffering therefrom. These methods comprise the route of administration of a therapeutically effective amount of a ciliary neurotrophic factor (CNTF) to a patient suffering from peripheral nerve damage or to a patient at risk of suffering peripheral nerve damage.

A disease or medical indication is to be considered to be peripheral nerve damage if the survival or function of peripheral nerve cells and/or their axonal processes is compromised. In a preferred embodiment, a patient is at risk of suffering peripheral nerve damage or actually has peripheral nerve damage as the result of one of the following conditions: 1) Physical injury, which causes the degeneration of the axonal processes of peripheral nerve cells that pass through or near the site of injury; 2) Exposure to neurotoxins, such as the cancer and AIDS chemotherapeutic agents cisplatinum and dideoxycytidine (ddC), respectively; 3) Chronic metabolic diseases, such as diabetes or renal dysfunction; and, 4) Neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), which causes the degeneration of primary motor neurons and consequently motor dysfunction. A non-exclusive list of conditions involving peripheral nerve damage includes Amyotrophic Lateral Sclerosis, Diabetic Peripheral Polyneuropathy, Toxic Peripheral Neuropathy caused by the cancer chemotherapeutic agents taxol or cisplatin or vincristine, Toxic Peripheral Neuropathy caused by the AIDS chemotherapeutic agents ddI or ddC, and physical damage to peripheral nerves such as that caused by crush or cut injuries to the arm and hand.

The treatment of peripheral nerve damage includes the ability to reverse permanent peripheral nerve damage and the ability to enhance naturally occurring recovery processes by either speeding up such processes or by effecting a more complete recovery from the peripheral nerve damage. The prevention of peripheral nerve damage includes the ability to totally prevent nerve damage against the effects of conditions that typically lead to peripheral nerve damage, as well as the ability to lessen the extent of peripheral nerve

damage associated with such conditions.

In one embodiment, preferred CNTFs are naturally occurring proteins. The naturally-occurring proteins are preferred in part because they pose a comparatively low risk of producing unforeseen and undesirable physiological side effects in patients treated therewith. Human CNTFs are preferred for use in this invention. However, to the extent that non-human CNTFs are substantially equivalent to human CNTFs and possess equivalent biological activity, they are considered to be within the scope of this invention.

For purposes of the specification and claims, a protein is deemed to be "naturally-occurring" if it or a substantially equivalent protein can be found to exist normally in healthy humans. "Naturally-occurring" proteins specifically includes forms of proteins found to exist in healthy humans that are partially truncated at the amino or carboxyl terminus of such proteins or that have amino acids that are deamidated or otherwise chemically modified. "Naturally-occurring" proteins may be obtained by recombinant DNA methods as well as by isolation from cells which ordinarily produce them. "Naturally-occurring" also encompasses proteins that contain or lack an NH_2-terminal methionyl group as a consequence of expression in E. coli.

"Substantially equivalent" as used throughout the specification and claims is defined to mean possessing a very high degree of amino acid residue homology (See generally M. Dayhoff, Atlas of Protein Sequence and Structure, vol. 5, p. 124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated herein by reference) as well as possessing comparable biological activity.

Particularly preferred CNTFs of the present invention are the naturally-occurring proteins that

have previously been described in PCT application WO 90/07341 of Collins, et al. entitled "Purified Ciliary Neurotrophic Factor."

The nucleic acid sequences of the genes encoding human and animal CNTFs and the amino acid sequences of such proteins are given in the Collins et al. application. The present invention encompasses non-glycosylated forms of CNTF as well as truncated forms of the naturally-occurring and recombinant CNTF proteins as described in the Collins et al. application. In a further embodiment, CNTF is modified by attachment of one or more polyethylene glycol (PEG) or other repeating polymeric moieties.

Methods for producing the naturally occurring or modified CNTFs are also disclosed herein and in the above-mentioned application. One disclosed method consists of isolating CNTF from various sources, such as peripheral nerve tissues. A second disclosed method involves isolating the genes responsible for coding CNTF, cloning the gene in suitable vectors and cell types, and expressing the gene in order to produce the CNTF. The latter method, which is exemplary of recombinant DNA methods in general, is a preferred method of the present invention. Recombinant DNA methods are preferred in part because they are capable of achieving comparatively higher amounts at greater purity.

Preferably, the above described CNTFs are produced by the aforementioned method in "substantially pure" form. By "substantially pure" it is meant that CNTF, in an unmodified form, has a comparatively high specific activity. It is to be recognized, however, that derivatives of CNTF may have different specific activities. In a preferred embodiment of the present invention, a therapeutic composition comprising CNTF is administered in an effective amount to patients

suffering from peripheral nerve damage.

Because it is possible that the neurotrophic function of the preferred CNTFs is imparted by one or more discrete and separable portions of the CNTF protein, it is also envisioned that the method of the present invention could be practiced by administering a therapeutic composition whose active ingredient consists of that portion (or those portions) of CNTF which controls (or control) CNTF neurotrophic function.

The therapeutic composition of the present invention is preferably administered parenterally by injection or intrathecally by continuous infusion from an implanted pump. Also, other effective administration forms, such as parenteral slow-release formulations, inhalant mists, orally active formulations, or suppositories, are also envisioned. One preferred carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one preferred embodiment it is envisioned that the carrier and the CNTF constitute a physiologically-compatible, slow-release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the CNTF. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dose or multi-dose form or for intrathecal delivery by continuous or periodic infusion from an implanted pump or

intrathecally by periodic injection.

Once the therapeutic composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The preferred storage of such formulations is at temperatures at least as low as 4°C and preferably at -70°C. It is also preferred that such formulations containing CNTF are stored and administered at or near physiological pH. It is presently believed that storage and administration in a formulation at a pH below approximately pH 5.5 and above approximately pH 8.0 is undesirable.

Preferably, the manner of parenterally administering the formulations containing CNTF is via a subcutaneous or intramuscular route. To achieve the desired dose of CNTF, repeated daily or less frequent subcutaneous or intramuscular injections may be administered. It is believed that the administration of CNTF in daily doses below approximately 0.001mg/kg may not be effective, while the administration of daily doses of greater than 1mg/kg have undesirable side effects.

A preferred dosage range for the parenteral treatment of peripheral nerve damage is between about 0.01 and 0.25 mg per kg of patient body weight per 24 hours administered in a single dose per 24 hours. In a preferred mode for the prevention or minimization of peripheral nerve damage, the administration of CNTF will begin up to one week before the condition or initiation of events that typically leads to peripheral nerve damage. For example, in a preferred embodiment to prevent toxic neuropathy due to cancer chemotherapeutic agents, administration of CNTF, will

begin up to 1 week before the initiation of treatment with the chemotherapeutic agent and will continue during the period of exposure to the agent. The frequency of dosing will depend on pharmacokinetic parameters of CNTF in the formulation used and will be readily ascertained by one skilled in the art.

To achieve the desired dose of CNTF to motor and other damaged nerve cells whose cell bodies are within the spinal cord, CNTF may be administered intrathecally into the subarachnoid space of the spinal cord. Administration may be continuous or periodic and may be accomplished by a constant- or programmable-flow implantable pump or by periodic injections.

It is also contemplated that certain formulations containing CNTF are to be administered orally. Preferably, CNTF which is administered in this fashion is encapsulated. The encapsulated CNTF may be formulated with or without those carriers customarily used in the compounding of solid dosage forms. Preferably, the capsule is designed so that the active portion of the formulation is released at that point in the gastro-intestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional excipients may be included to facilitate absorption of CNTF. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

Regardless of the manner of administration, the specific dose is calculated according to the approximate body weight or surface area of the patient. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by

them without undue experimentation, especially in light of the dosage information and assays disclosed herein. These dosages may be ascertained through use of the established assays for determining dosages utilized in conjunction with appropriate dose-response data.

It should be noted that the CNTF formulations described herein may be used for veterinary as well as human applications and that the term "patient" should not be construed in a limiting manner. In the case of veterinary applications, the dosage ranges should be the same as specified above.

It is understood that the application of teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of representative uses of the present invention appear in the following examples.

Example 4 below describes the application of the present invention to peripheral nerve damage from physical injury to a peripheral nerve, as described herein. The differences, if any, between this treatment and the treatment of patients suffering from other forms of peripheral nerve damage would be readily and routinely identified by one of ordinary skill in the art. The ability to accelerate the recovery of sensory and motor function after physical injury to peripheral nerves by administering CNTF as shown in the following example, shows that the administration of CNTF may be equally effective in preventing and treating other forms of peripheral nerve damage, as defined herein.

EXAMPLE 1: PURIFICATION OF RECOMBINANT CILIARY NEUROTROPHIC FACTOR

Materials and Methods - The purification, cloning and expression of CNTF has been previously described and is incorporated by reference herein, Collins et al., WO 90/07341. (See also, Collins, et al., U.S. Patent 5,011,914 and Collins et al., U.S. Patent 4,997,929). Numbers in brackets refer to references listed below in the section entitled References for Example 1.

Cloning the Human CNTF Gene - Fully degenerate oligonucleotides were synthesized corresponding to the amino acid sequence of rabbit CNTF [7]. The sense orientation of each oligonucleotide is given starting with the 5′ end together with the corresponding rabbit protein sequence (N denotes A,C,G, or T) CNTF-1: TAT/C GTN AAA/G CAT/C CAA/G GG (Tyr-Val-Lys-His-Gln-Gly); CNTF-2: AAT/C AAA/G AAT/C ATT/C/A ATT/C C/TT (Asn-Lys-Asn-Ile-Asn-Leu); CNTF-3a: AAA/G TTA/G TGG GGN TTA/G AA; CNTF-3b: AAA/G TTA/G TGG GGN CTN AA; CNTF-3c: AAA/G CTN TGG GGN TTA/G AA; CNTF-3d: AAA/G CTN TGG GGN CTN AA (Lys-Leu-Trp-Gly-Leu-Lys). Oligonucleotides CNTF-3a to 3d were used in separate PCR reactions to reduce degeneracy.

Oligonucleotides 1 (sense) and 3a to 3d (anti-sense) were used as primers in PCR with human genomic DNA. Polymerase chain reactions were performed as previously described [1] except that each reaction contained 1.75 mM $MgCl_2$, 100 ng of each oligonucleotide, and 0.5 μg of human genomic DNA prepared from placenta [2]. To identify DNA bands amplified from the CNTF gene, DNA (Southern) blots of the PCR products were probed with ^{32}P-labeled oligonucleotide 2, which occurs just downstream of oligonucleotide 1 in the rabbit gene [7]. A single ca.

400-bp band of amplified DNA hybridized to this probe in Southern blots of the PCR products from human genomic DNA. This band was most intense in the reaction using CNTF-3d. This band was cloned and sequenced to give the DNA sequence of the human CNTF gene between oligonucleotides 1 and 3 in Fig. 1.

The ca. 400-bp fragment amplified from human genomic DNA was labeled with ^{32}P by random priming and used to screen a human genomic DNA library at high stringency. The human genomic DNA library was constructed by cloning genomic DNA [18], partially digested with Sau3AI, into the BamHI site of Charon 30 [3]. Out of $1x10^6$ clones, nine positive clones were isolated. Two of these clones were sequenced and the rest appeared related to these based on DNA (Southern) blot analysis. The sequenced clones contained an open reading frame (Fig. 1) that was 89% identical to the rabbit CNTF coding sequence [7]. In addition, each open reading frame contained a segment identical to the fragment amplified from human genomic DNA by PCR. Restriction endonuclease fragments from the human genomic DNA clones corresponded to those observed on DNA (Southern) blot analysis of human genomic DNA, indicating that the clones were representative of the organization of the CNTF gene in genomic DNA.

Preparation of DNA for the Expression of CNTF. A human genomic DNA clone for CNTF in phage Charon 30 was digested with the restriction enzymes SalI and HindIII and a 4.3 kb-fragment was subcloned into the Bluescript KS M13(-) plagemid vector (Stratagene). This fragment contains the CNTF coding sequences upstream of the HindIII site in the coding sequence (Fig. 1). This 4.3 kb-fragment also contains a single, approximately 1.3 kb intron (Fig. 1). To allow expression in bacterial cells, the intron was removed by site-directed mutagenesis in vitro [4] using

the synthetic oligonucleotide 5′- GATGTTCTTGTTCAGGCCCTGATGCTTCACATAGGATTCCGTAAGAGCAGT CAGGTCTGAACGAATCTTCC-3′ to produce phagemid 1.

The 5′ end of the CNTF coding sequence in phagemid 1 was reconstructed for cloning into the expression vector and to make changes found to increase the efficiency of expression in E. coli. Phagemid 1 contains a single NheI site at amino acids 22-23 in the human CNTF coding sequence (Fig. 1). Partially overlapping complementary oligonucleotides, (5′-GATCCGATCTTGGAGGATGATTAAATGGCTTTCACTGAACACTC TCCGCTGACCCCGCACCGTCGAGATCTGTGCAGCCGCTCTATCTGG - 3′/5′ - CTAGCCAGATAGAGCGGCTGCACAGATCTCGACGGTGCGGGGTCA GCGGAGAGTGTTCAGTGAAAGCCA TTTAATCATCCTCCAAGATCG - 3′) containing a 3′ NheI overhang were synthesized, annealed together, and ligated to NheI-cut phagemid 1 to produce phagemid 2. These oligonucleotides alter the human codon usage to that used preferentially by E. coli [5] without changing the amino acid sequence, and contain a 5′ BamHI overhang that creates a BamHI site in phagemid 2. Oligonucleotides 2 and 3 also contain a transnational coupler to promote effective translation in E. coli [6].

Phagemid 2 DNA was then digested with BamHI and HindIII to release the DNA fragment referred to as CNTF-Syn1 which contains DNA sequences suitable for expression in E. coli and encoding human CNTF upstream of the HindIII site (Fig. 1).

To prepare the 3′ end of the expression construct, a human genomic DNA clone for CNTF in phage Charon 30 was cut with the restriction enzyme HindIII and a 2.1 kb-fragment, containing the CNTF coding sequences downstream of the HindIII site (Fig. 1), was subcloned into HindIII-cut plasmid pEMBL8 [7]. A SpeI site was inserted into the 2.1 kb-insert DNA by oligonucleotide directed mutagenesis 13 base pairs

downstream of the stop codon ending the CNTF sequence using the synthetic oligonucleotide 4 (5′ - ATG TAG CAG TTA GTC ACT AGT CTC TTC CTT GCT - 3′). The mutated plasmid was cut with HindIII and SpeI to release the DNA referred to as CNTF-Syn2.

CNTF-Syn1 and CNTF-Syn2 were ligated at the HindIII overhangs to produce CNTF-Syn1/2, which was subcloned into the BamHI- and SpeI-cut phagemid expression vector pJU1003 [8] to produce pJU1003-huCNTF, which was transformed into E. coli strain BL21(DE3) [9]. This places expression of the CNTF insert under control of the T7 phage promoter upon induction with isopropyl β-D-thio-galactopyranoside (IPTG) [24]. One transformant, CNTF-A, producing CNTF after induction with IPTG was selected.

Expression of recombinant human CNTF - Overnight cultures of CNTF-A were prepared in Luria broth [10] supplemented with 10 μg/ml of tetracycline. These cultures were diluted (1 to 50) with the same medium and grown until the A_{600} reached 1.0 (3-4 h). Expression of CNTF was achieved by adding IPTG to a final concentration of 0.5 mM and incubating for 4 h. Cells were harvested by centrifugation (9,000 X g, 5 min), washed with 50 mM sodium phosphate, pH 8.0, and recentrifuged. Cell pastes were either used immediately or stored frozen at -80°C.

Purification of recombinant human CNTF - All purification steps were carried out on ice or at 4°C and fractions from the various chromatography columns were analyzed by SDS-PAGE.

Step 1. Preparation of Cell Free Extracts - A cell paste (4-5 g wet weight) was suspended in 3-4 volumes of buffer A (50 mM sodium phosphate, pH 8.0, containing 5 mM EGTA and 5 mM EDTA) and passed through a French pressure cell at 18,000 lb./in.^{2} The

resultant mixture was centrifuged at 48,000 X g for 20 min and the supernatant filtered through glass wool.

Step 2. Removal of Nucleic Acids - PEI was added to the supernatant to a final concentration 0.25% (v/v) to facilitate removal of nucleic acids [11]. Without this treatment, the nucleic acid contained in the supernatant would bind to the anion-exchange resin and decrease the number of times that the Q-Sepharose could be regenerated and reused. After incubating for 10 min, the mixture was centrifuged as above and the resultant supernatant filtered through the glass wool.

Step 3. Q-Sepharose Ion-exchange Chromatography - Cell extract was loaded onto a column (1.5 X 20 cm) of Q-Sepharose previously equilibrated with buffer A. After loading, the column was washed with buffer A until the A_{280} reached baseline. CNTF was detected in the column flow-through/wash. The CNTF pool was dialyzed twice against 10 volumes of buffer B (5 mM sodium phosphate, pH 8.0, containing 10 mM NaCl, 1 mM EGTA and 1 mM EDTA).

Step 4. Q-Sepharose Ion-exchange Chromatography - The above CNTF pool was loaded onto a column (1.5 X 15 cm) of Q-Sepharose previously equilibrated with buffer B. After loading, the column was washed with buffer B until the A_{280} reached baseline. Bound proteins were eluted with a gradient (150 ml) of 10 to 80 mM NaCl in buffer B. The CNTF pool was dialyzed twice against 10 volumes of buffer C (5 mM sodium phosphate,

pH 7.1, containing 0.1 mM EGTA and 0.1 mM EDTA).

Step 5 - S-Sepharose Ion-exchange Chromatography - The above CNTF pool was loaded onto a column (1 X 10 cm) of S-Sepharose previously equilibrated with buffer C. After loading, the column was washed with buffer C until the A_{280} reached baseline. Bound proteins were eluted with a gradient (60 ml) of 0 to 0.5 M NaCl in buffer C. The CNTF pool was dialyzed twice against 10 volumes of buffer D (10 mM Hepes, pH 7.5, containing 50 mM NaCl, 0.1 mM EGTA, and 0.1 mM EDTA).

Step 6. Zn^{2+}-affinity Chromatography - The CNTF pool was loaded onto a column (1 X 10 cm) of Zn^{2+}-IDA agarose previously equilibrated with buffer D without the metal ion chelators, EGTA and EDTA. After loading, the column was washed with the same buffer until the A_{280} reached baseline. Bound proteins were eluted with a gradient (50 ml) of 0 to 50 mM histidine in buffer D (without chelators). The final, purified CNTF pool was dialyzed twice against 10 volumes of 10 mM phosphate, pH 8.0, containing 50 mM NaCl, 0.1 mM EGTA and 0.1 mM EDTA and stored at -80°C.

RP-HPLC - TFA and acetonitrile were added to protein samples to final concentrations of 0.1% (v/v) and 5% (v/v), respectively, prior to injection. RP-HPLC was performed using a 250 X 4.6 mm SynChropak RP-8 column (SynChrom, Inc., Lafayette, IN) with 0.1% aqueous TFA as solvent A and 0.1% TFA in acetonitrile as solvent B.

Electrophoresis and Blotting Techniques - Electrophoresis was performed in 12.5% polyacrylamide slab gels (1.5 mm thick), with a 5% acrylamide stacking gel, in the presence of 0.1% (w/v) SDS at 40 mA, with the discontinuous buffer system of Laemmli [12]. Gels were stained with CBB as described previously [13] or silver-stained using a Rapid-Ag-Stain Kit (ICN Radiochemicals, Irvine, CA). Gels to be used to separate proteins prior to Western blotting and protein sequencing were pre-electrophoresed for 16 h at 15 mA in the presence of 25 mM thioglycolic acid and 10 mM DTT. This prevents blockage of amino-terminal amino acid groups during electrophoresis of protein samples [14]. Western blotting was preformed as previously described [15] using Immobilon-P (Millipore Corporation, Bedford, MA) or nitrocellulose (Schleier and Schuell, Inc., Keene, NH) membranes. Immobilon-P membranes were stained with CBB and the appropriate protein bands excised for sequencing. Nitrocellulose membranes were subjected to treatment with antibodies to CNTF, and subsequently, with goat anti-(rabbit IgG) conjugated to alkaline phosphatase (Cappel). The secondary antibody was detected using a kit with 5-bromo-4-chloro-indo-3-yl phosphate and nitroblue tetrazolium supplied by Promega (Madison, WI).

Preparation of Antibodies to CNTF - Highly-purified recombinant human CNTF in 10 mM sodium phosphate, pH 8.0 containing 50 mM NaCl, 0.1 mM EGTA and 0.1 mM EDTA was emulsified with 2 volumes of Freund's complete adjuvant and injected subcutaneously into multiple dorsal sites of two New Zealand rabbits (100 μg of CNTF per rabbit). Booster injections of 100 μg of CNTF emulsified in Freund's incomplete adjuvant were than given at 2 to 3-week intervals. Sera were prepared from blood collected 7 weeks after the initial injection, and again at 2-week intervals and stored at

-70°C. The titer of the antiserum was 3,000 as determined by ELISA [16].

Bioassays - Bioassays for CNTF activity were performed as described by Lin et al., [10]. Briefly, the in vitro assay for CNTF activity [17] measures the survival of chick embryo ciliary ganglion (E8), sympathetic chain (E11) or dorsal root ganglion (E10) neurons. Two thousand purified neurons were placed into each well of a 96-well dish and serial dilutions of samples to be assayed were added. After 20 h (ciliary ganglion neurons) or 44 h (sympathetic chain and dorsal root ganglion neurons), neuronal survival was estimated by the ability of live cells to reduce the vital dye MTT (3-4[,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium) (Sigma). The titer of bioactivity in trophic units (TU) per ml was defined as the dilution that gave 50% of the maximal neuronal survival in the MTT assay. For example, if a dilution of 1:1000 was required to give 50% survival, the titer was defined as 1,000 TU/ml.

Peptide Mapping and Protein Sequencing - Generation of C-terminal peptides of CNTF was achieved by first digesting the protein with CNBr overnight at room temperature in hexafluoro-acetone hydrate. Peptides were separated on a narrow-bore C8 RP-HPLC column (Brownlee, Inc., Santa Clara, CA), with 0.085% aqueous TFA as solvent A and 0.085% TFA in 80% acetonitrile as solvent B. The C-terminal peptide was then subdigested with endoproteinase ASP-N [7] and the peptides separated as above. Amino-acid analysis and protein sequencing were carried out as described by Armes and Forney [18].

Other Methods - Protein concentrations were determined by the BioRad microassay based on CBB dye-binding [19] or by amino-acid analysis. U.V.-absorption spectra were recorded in a Beckman D-50

spectrophotometer and densitometry performed using an LKB Ultrascan XL laser densitometer.

Materials - S- and Q-Sepharose were purchased from Pharmacia; IDA-agarose from Pierce Chemical Co., and IPTG, PEI, M_r markers and tetracycline from Sigma.

RESULTS and DISCUSSION

Human CNTF Gene - The genomic DNA sequence and inferred amino acid sequence encoding human CNTF are shown in Figure 1. The human DNA and protein sequences are 89% and 86% identical to the rabbit CNTF [7] and 85% and 83% identical to the rat CNTF [8] DNA and protein sequences, respectively. Alignment of the inferred amino acid sequences of human, rabbit and rat CNTF is shown in Figure 2. Only a single band that hybridized to CNTF-specific probes was observed in DNA (Southern) blots of human genomic DNA digested with various restriction endonucleases (not shown), consistent with only a single gene in human genomic DNA hybridizing at high stringency.

Purification of recombinant CNTF - Upon induction with IPTG, cultures of the bacterial transformant pJU1003-CNTF-A synthesized recombinant human CNTF. At the end of the culture period, CNTF accounted for approximately 13% of the soluble protein in cell extracts (25 mg/liter/A_{600} unit) as judged by laser densitometer analysis of CBB-stained gels (Fig. 3, lane 1).

When the soluble material from crude cell extract was subjected to anion-exchange chromatography on Q-Sepharose in a relatively high-ionic strength buffer at pH 8.0, CNTF was slightly retarded on the column and emerged in the flow-through and column wash (Fig. 3, lanes 6-22) just after the passage of cellular debris and other proteins (Fig. 3, lanes 3-5). The bulk of E. coli proteins were retained on the column

under these conditions. When the CNTF pool from the first column was dialyzed into a low ionic strength buffer at pH 8.0, CNTF now bound to a second column of Q-Sepharose and could be eluted by application of a salt gradient as a peak at 55-60 mM NaCl (Fig. 4).

The resultant CNTF pool (Fig. 4, inset) was dialyzed into a low ionic strength buffer at pH 7.1 and subjected to cation-exchange chromatography on S-Sepharose. CNTF bound to the resin was eluted by application of a salt gradient as a peak between 125-250 mM NaCl (Fig. 5). The CNTF pool (Fig. 5, inset) was then subjected to a final affinity chromatography step on a Zn^{2+}-IDA-agarose column. CNTF bound to the column, probably via an interaction between zinc and histidine residues, of which CNTF possesses ten per molecule (Table I). CNTF was eluted from the column by application of a histidine gradient at 30-35 mM histidine (Fig. 6).

A summary of the purification of recombinant human CNTF is shown in Table II. The average yield of CNTF was 19 $\pm$ 1.5% (n=4). The percent CNTF in protein pools collected after each chromatography step was determined by laser densitometer analysis of CBB stained gels. These percentages were used to calculate the total amount of CNTF in any given pool and, from this, the fold purification and yields (Table II). Silver-stained gels were not used for this purpose, since the intensity of staining with silver was not proportional to the amount of protein and this method, therefore, was not reliably quantitative. However, silver-stained gels were used to qualitatively assess the degree of purity of CNTF contained in the various pools (insets, Figs. 4, 5 and 6).

The pI of human CNTF, calculated from the amino acid composition, is 6.4. This is significantly higher than that calculated for rabbit (pI = 5.8) or

rat (pI = 5.7) CNTF [7,8]. This difference in calculated pI suggests that the above purification protocol, which relies heavily on ion exchange chromatography at carefully controlled pH and ionic strengths, might require modification in order to be used to purify recombinant rat or rabbit CNTFs from bacterial expression systems.

Purity of CNTF - The amino acid composition of human recombinant CNTF, purified as above, corresponded well to the amino acid composition predicted from the human coding sequence (Table I). In addition, peptide map and amino acid sequence analyses of the purified protein indicated only the presence of CNTF sequences. The amino acid sequence of recombinant human CNTF was that expected from the human coding sequence (Fig. 1), except for the failure to detect an amino-terminal methionine. Amino acid sequence analysis of three different CNTF preparations yielded less than 0.1% of the expected amount of methionine at the amino-terminal position. Removal of the amino-terminal methionine during expression in bacteria is not uncommon for proteins, such as CNTF, in which the amino-terminal methionine is following by an alanine residue [20, 21].

The purity of CNTF was further analyzed by RP-HPLC. Elution profiles obtained at 214 and 280 nm (Fig. 7a and 7b, respectively) revealed two symmetric protein peaks accounting for 95 $\pm$ 2% and 5 $\pm$ 2 % (n = 3) of the total protein eluted. The minor form did not appear to derive from the major form during chromatography, since some preparations of CNTF exhibited no detectable minor form on RP-HPLC. Peptide map and amino- and carboxyl- terminal amino acid sequence analyses of the protein contained in both fractions revealed only the presence of full-length CNTF. Therefore, the two peaks represent forms of CNTF

that differ in an unknown way. These CNTF forms may be a consequence of deamidation. A second possible explanation is proline isomerization, which has been reported for other proteins, including insulin [22, 23, 24].

When an amount of CNTF in excess of 100 μg was subject to RP-HPLC and monitored at 214 nm, a peak representing less than 0.1% of the detected protein was observed immediately prior to the two peaks discussed above. Since insufficient quantities of this protein could be obtained for sequence analysis by RP-HPLC, 100 μg amounts of purified CNTF were subjected to SDS-PAGE and Western blot analyses in an attempt to identify what protein species was present at approximately 0.1% of the total.

CBB staining of such gels revealed the presence of faint protein bands corresponding to M_r = 46,000, 21,000, 18,000 and 16,000, in addition to native CNTF (data now shown). These bands were, however, more easily visualized upon immunoblotting using antibodies to CNTF (Fig. 8, lane A). This observation in itself does not identify these bands as CNTF since the antisera was raised against this preparation of CNTF. The proteins of M_r = 18,000 and 16,000, appeared to be heat generated fragments of native CNTF, since these bands increased with time of heating at 100°C for at least 2 min prior to electrophoresis (compare lanes A and B, Fig. 8). The proteins of M_r = 46,000 and 21,000 were blotted from a heavily loaded gel onto immobilon-P and subjected to amino-terminal protein sequencing. The only sequencing detected were those of CNTF, which suggests that the 46,000 dalton species may be a dimer of CNTF (M_r = 23,000 daltons). It is unlikely that dimerization occurred by disulfide-bond formation, since the samples were reduced with DTT before electrophoresis. No CNTF

sequence was detected from a strip of immobilon-P excised from between the 46,000 and 23,000 dalton species. This suggests that the detection of any CNTF sequence at the 46,000 dalton level is not a consequence of streaking of native CNTF upon SDS-PAGE. The 21,000 dalton species may be a carboxyl-terminally truncated form of CNTF, since its amino terminus was intact (too little material was available for sequencing of the carboxyl-terminal peptide).

Based on later densitometry of CBB stained gels, the apparent dimer accounted for approximately 0.01% and the truncated CNTF for approximately 0.1% of the total protein. The 0.1% RP-HPLC peak, discussed above, was tentatively identified as a putative truncated form of CNTF. Loss of the highly charged carboxyl-terminus of CNTF (Fig. 1) would reasonably be expected to alter migration on RP-HPLC. These results indicate that contamination of purified CNTF by non-CNTF proteins was significantly below 0.1%.

Ultraviolet Absorption Spectrum of CNTF - The u.v.-absorption spectrum of the recombinant human CNTF is shown in Fig. 9. An absorption maximum was observed at 279 nm, with a shoulder at 290-295 nm, indicative of the presence of tryptophan. This is consistent with the amino acid composition (Table I) which reveals the presence of 4 mol of tryptophan per mol of CNTF. An absorption coefficient of the protein was calculated from the u.v.-absorption spectrum and the protein concentration (determined by amino acid analysis).

Bioactivity of recombinant human CNTF - There would appear to be no need to perform a refolding step in order to produce biologically active CNTF from the bacterial expression system. Since there is only one cysteine in human CNTF (amino acid 17 in Fig. 1), there can be no intramolecular disulfide bonds that would need to reform correctly. Also, since CNTF can regain

biological activity after exposure to SDS, acetonitrile, and TFA [10], CNTF appears able to spontaneously refold after some forms of denaturation. As anticipated, both crude bacterial lysate and purified recombinant CNTF exhibited CNTF biological activity.

Highly-purified recombinant CNTF promoted the survival in culture of chick embryo parasympathetic (ciliary), sympathetic chain, and sensory (dorsal root) neurons (Table III). The specific activities in Table III are equivalent within experimental error to those observed for recombinant human CNTF expressed in animal (COS-7) cells (ciliary neurons = 1.7± 1.2 TU/ng (N=5); sympathetic neurons = 7.7 ± 2.1 TU/ng (N=3)). This suggests that the purified CNTF from E. coli is fully biologically active.

Crude or partially purified extracts from various tissues have also been reported to promote the survival in culture of all three of the chick embryo neuronal populations used above [25, 26, 27]. The activity of these extracts has been ascribed to CNTF. Our results indeed demonstrate that a single molecule, CNTF, has these activities.

The primary structure of human CNTF exhibits no N-linked glycosylation sites (N-X-T/S) (Fig. 1). In addition, the biological activity of bacterially-expressed human CNTF indicates that other forms of glycosylation, even if they occur in vivo, are not essential for the biological activity of CNTF.

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TABLE I

Amino acid composition of recombinant human CNTF

Amino Acid		Calculated[a]	Expected[b]
Asp/Asn	(D/N)	18.7	18
Thr	(T)	11.0	12
Ser	(S)	13.6	13
Glu/Gln	(E/Q)	26.0	26
Gly	(G)	10.5	10
Ala	(A)	15.5	15
Val	(V)	7.3	8
Met	(M)	4.6	4[c]
Ile	(I)	11.9	12
Leu	(L)	25.7	26
Tyr	(Y)	5.0	5
Phe	(F)	6.9	7
Lys	(K)	8.6	9
His	(H)	n.d.	10
Arg	(R)	12.1	12
Cys	(C)	n.d.	1
Trp	(W)	n.d.	4
Pro	(P)	6.9	7
Total residues		176.2	200
M_r			22,931

(a) Amino acid analysis performed as described by Armes and Forney (1990).

(b) From sequence in Fig. 1.

n.d. = not determined

(c) Based on absence of the amino-terminal methionine.

TABLE II

Purification of recombinant human CNTF from E. coli

The percentage of CNTF in crude extract and subsequent pooled fractions was estimated by laser densitometer analysis of CBB, rather than silver stained gels. The percent values are the mean of at least three separate determinations.

	Step	Total Protein	% CNTF	CNTF-PURIFICATION-YIELD
1.	Crude extract	4061353	(1)	(100)
2.	PEI treatment	3701348	(1)	90
3.	Q-Sepharose	72.53626	2.8	49
4.	Q-Sepharose	265714.8	4.4	28
5.	S-Sepharose	147610.6	5.9	20
6.	Zn^{2+}-affinity	10	>99.910	19

TABLE III

Neuronal specificity of recombinant human CNTF

The apparent specific activity in trophic units/ng is reported for purified human CNTF. Biological activity was determined for the survival of chick embryo E8 ciliary ganglion (CG), E11 sympathetic chain (SC) and E10 dorsal root ganglion (DRG) neurons.

CNTF SPECIFIC ACTIVITY (TU/ng)		
CG NEURONS	SC NEURONS	DRG NEURONS
2.12 ± 0.2[a]	3.6 ± 1.6[a]	1.75[b]

(a) Average ± S.E. of five separate experiments with dose response curves performed in duplicate.

(b) Single experiment determinations with dose response curves performed in quadruplicate.

EXAMPLE 2: IDENTIFICATION OF C-terminal TRUNCATED FORMS OF CNTF

In an alternate process for the production and purification of CNTF, C-terminal truncated forms of CNTF were identified.

Fermentation

Strain description:

1) Alternatives to the lead Q-Sepharose (CNTF capture) column:

Q-Sepharose Bid Beads by Pharmacia was tested successfully. Fermentation run: The tank was inoculated with seed culture and the cells were induced with IPTG at 10 OD (600 nm). The cells were harvested by centrifugation at 55 OD. The cell sludge (50% solids) was either used immediately or the cells were frozen at --20°C. Typical production scales were 10 or 160 liters.

Cell Processing

The cells were thawed and water was added to obtain 20% cell solids and the pH was adjusted to 8.2 with 0.5 M Tris-base. Alternatively freshly harvested cells were used. The entire process from performed at 4-8°C.

The cells were lysed by a continuous homogenizer. The lysate was clarified by centrifugation and diluted with 10-15 volumes of cold water to a conductivity equal to that of the lead column equilibration buffer.

Purification

Step 1: (CNTF CAPTURE)

Q-SEPHAROSE FAST FLOW COLUMN STEP

The product was captured on an anion exchange column (2.5 cm diameter by 7.1 cm) with 35 mL bed volume of Q-Sepharose fast flow resin (Pharmacia), equilibrated with 10 mM Tris-HCl pH 8.1 and 1 mM EDTA. The clarified

lysate was pumped at 11 mL per minute through a 3 uM filter and then on the column. The column was washed at the same flowrate with column equilibration buffer until the OD (280 nm) returned to base line (approximately three bed volumes). CNTF was step eluted from the column at 3.7 mL per minute with 80 mM NaCl prepared in 10 mM Tris-HCl pH 8.2 and 1 mM EDTA. The entire peak was pooled and diluted two fold with water and the pH was adjusted to 7.2 with 0.1 N H_3PO_4.

Step 2: S-SEPHAROSE FAST FLOW COLUMN STEP (CNTF C-terminal TRUNCATED FORMS I, II AND III SEPARATION)

The pooled CNTF was diluted two-fold with cold water and loaded at 4.7 mL/minute onto the cation-exchange (S-Sepharose, Pharmacia) column (2.5 cm diameter and 2,500 mL bed volume) equilibrated with 25 mM NaPO pH 7.1, 25 mM NaCl, and 0.1 mM EDTA. The column was washed at the same flowrate with equilibration buffer until the OD (280 nm) returned to baseline. The CNTF was eluted with a pH gradient going from 7.1 to 8.1. This was accomplished by a 200 mL gradient made up with two buffers. The low pH buffer was 25 mM NaPi pH 7.1, 25 mM NaCl, and 0.1 mM EDTA. The high pH buffer was made up of 25 mM Tris-HCL, pH 8.1, 25 mM NaCl, and 0.1 mM EDTA.

The CNTF was gradient eluted at a flowrate of 1.2 mL/minute. Of the three peaks, the last peak (pH 7.8-8.1) was pooled from OD (280 nm) 0.2 to 0.2.

The last peak contains substantially pure CNTF Form III, which contains the full amino acid sequence as set forth in Figure 1. The second peak contains C-terminal truncated CNTF Form II, wherein the last two amino acids of the C-terminal end of the amino sequence as set forth in Figure 1 are cleaved. The first peak contains C-terminal truncated CNTF Form I, wherein the

last six amino acids of the C-terminal end of the amino acid sequence as set forth in Figure 1 are cleaved.

Alternatives to CNTF C-terminal Truncated Forms I, II, III Separation column:

Cation-exchange resin from Toso Haas (TSK SP-650) and S-Sepharose HP from Pharmacia were tested successfully.

The CNTF C-terminal truncated forms I, II, and III were also separated using NaCl gradients at pH 7.1 using 50 mM Phosphate buffer.

Step 3: Q-SEPHAROSE FAST-FLOW COLUMN STEP

The pH of the S-Sepharose pool was adjusted to 8.0 with 0.1 N HCl or NaOH. The dimension of the Q-Sepharose (Pharmacia) column was 2.5 cm in diameter with a bed volume of 20 mL. The resin was equilibrated with 10 mM Tris pH 8.0, 50 mM NaCl. The column was loaded at a flowrate of 3.4 mL/minute and washed with equilibration buffer until the OD (280 nm) returns to baseline. The CNTF was eluted at 1.1 mL/minute with a 200 mL salt gradient composed of 2 buffers. The low salt buffer was 10 mM Tris-HCl pH 8.0 and 50 mM NaCl. The high salt buffer was 10 mM Tris-HCl pH 8.0 and 200 mM NaCl. The pooling of the fractions took place between an OD (280 nm) of 0.3 and 0.9.

Step 4: BULK STORAGE

The pH of the Q column eluate was adjusted to 7.1-7.2 using 0.1 N H_3PO_4. Alternatively the CNTF concentration was increased to approximately 8 mg/ml by ultrafiltration using an Amicon YM10 membrane. Finally the material was frozen at -70°C.

EXAMPLE 3: FURTHER CNTF PURIFICATION PROTOCOLS

The present chromatography train is Q----S----Q. The CNTF at this stage is purified to equal to or greater than 99.9% purity with respect to ECP (E. coli protein). Furthermore it did pass DNA and endotoxin specifications. These are less than 100 pg DNA per dose and less than 5 E.U. per kg body weight per day, respectively. The amounts of CNTF forms were typically 97, 3 and 0.1% of CNTF C-terminal truncated forms III, II, and I, respectively. Note that III is the full size CNTF. The amount of ECP in the final product was between 50-200 ppm as judged by ELISA to ECP. To further lower the amount of ECP in a range below 25 ppm an additional column was needed.

The locations for this fourth column in the process flow diagram can be either in between the 2nd ('S') and the third column ('Q') or after the third column. In the following paragraphs examples are given of the various column resins tried that produced material that had a lower ECP content.

Example A. Hydroxy Apatite Resin

The column with ceramic Hydroxy Apatite (HA) resin (AIC) was equilibrated with 5 mM NaPi pH 7.0. The pH of the load (the S column) was adjusted to 7.0 with 0.1 N HCl and water was added to the load until its conductivity was equal to the conductivity of the HA column equilibration buffer. The column was washed with equilibration buffer until the OD (280 nm) returned to baseline and the CNTF was eluted with a 10 bed volume phosphate gradient. The low phosphate buffer was 5 mM NaPi pH 7.0. The high phosphate buffer was 150 mM phosphate pH 7.0. Fractions were pooled between an OD (280 nm) of 0.3 and 1.0 at the leading and at the

trailing edge of the peak, respectively. The ECP loan decreased to less than 25 ppm.

In addition to the ceramic HA a spheroidal HA from BDH was also successful in removing ECP but it required a lower phosphate concentration throughout all steps. The HA column can also be used after the third column in which case there is no phosphate present in the final bulk product.

Example B. Butyl HIC resin.

Butyl Toyopearl 650 M (Toso-Haas) is a resin used in hydrophobic interaction chromatography (HIC). The butyl column (2.5 diameter by 10.2 cm) with 50 mL resin was equilibrated with 200 mL of 300 mM NaCl in 20 mM NaPi pH 7.5. The NaCl and phosphate concentrations in the load of 200 mg CNTF were adjusted to 300 mM and 20 mM pH 7.5, respectively. The column was loaded at 9.6 mL per minute with CNTF and washed with column equilibration buffer. The CNTF was step eluted with 175 mL of a 20 mM imidazole buffer pH 7.5. Alternatively, the CNTF could be eluted with 20 mM Tris pH 7.5 of 50% (v/v) ethylene glycol in 20 mM phosphate buffer pH 7.5 or water or 20% ethanol or 10% glycerol in 20 mM imidazole pH 7.5. The resin was regenerated with a 6 M urea followed by washing with water and requilibration. Butyl resins with bead sizes of 650 M or 650 S are expected to yield similar results.

Example C. Zn-IMAC (immobilized metal affinity chromatography) resin.

The column had a diameter of 1 cm and was filled with 4 mL chelating Sepharose Fast-Flow from Pharmacia. The column was equilibrated with 10 mM Hepes pH 7.5 and 50 mM NaCl followed by a charging step using a solution of 1 mg $ZnCl_2$/mL prepared in water. The CNTF

was loaded to a capacity of 5 mg/mL resin at 3 mL/minute followed by a wash in column equilibration buffer. The CNTF was eluted at 1 mL/minute with a histidine gradient of 80 mL. The gradient was 0 to 75 mM histidine prepared in column equilibration buffer. The column was regenerated with a solution containing 5 mM EDTA in 10 mM Hepes and 1 M NaCl at a pH of 7.5, followed by a 1 hour soak in 1 M NaOH. Then the column was washed in water and requilibrated followed by a recharge with zinc. The location of this zinc column was tried both after the S column as well as after the Q column. Alternative charging metals are copper, cobalt, and nickel.

EXAMPLE 4: DEMONSTRATION OF THE ABILITY OF CNTF TO ACCELERATE THE RECOVERY OF SENSORY AND MOTOR FUNCTION AFTER PERIPHERAL NERVE DAMAGE.

A. Protocol for creating peripheral nerve damage.

Female Sprague Dawley rats weighing 120-140 g were used. The surgical procedure for producing damage to the sciatic nerve was performed on rats anesthetized with methoxyflurane. Induction was in a bell chamber. Anesthesia was maintained by nose cone.

The fur on the left hind limb was clipped from the thigh and hip regions. The clipped area was cleaned with betadine soap and rinsed with ethanol. Using sterile technique throughout the procedure, a 15 mm skin incision was made in the proximal half of the line between trochanter major and knee joint. The vastus lateralis and biceps femoris muscles were separated by blunt dissection and the sciatic nerve exposed where it emerges from under the gluteus maximus and runs over the semi-membranous and semitendinosus muscles. The nerve was elevated and Crile hemostatic forceps placed around

the nerve 5 mm distal to the trochanter major. The Crile forceps were closed maximally for 30 sec. The muscles were not reopposed. The skin was closed with wound clips. A single intramuscular injection of penicillin G procaine and penicillin G benzathine in an aqueous suspension was given.

The rats are ambulatory 10 to 15 min after the surgery. Because the femoral nerve is intact, the rats are able to bear weight on the lesioned limb.

B. Methods for assessing recovery of sensory and motor function after peripheral nerve injury.

i. Sensory function: Recovery of cutaneous sensation to the plantar surface of the footsole of the lesioned leg was performed by eliciting a withdrawal reflex after applying an electrical stimulus to the footsole. If sensory nerves have regenerated, a reflex arc is completed which causes the muscles of the hind limb to contract. A graded series of electrical currents were applied to the footsole in 100 μA decrements in a range of 800 to 300 μA. The current was generated by a constant-current generator (53500 Precision Instrument, Stoelting, Wood Dale, IL) and transmitted to the skin by dual stimulating electrodes with poles that are 1/8" apart (Lafayette Instrument, Lafayette, IN). The current was applied to the plantar surface of the paw immediately distal to digit 5. The lowest current to which the rats responded by withdrawing that limb was determined. A percentage recovery was calculated based on the lowest detectable current. Rats responding to 300 μA were considered to be 100% recovered, since this was the smallest current level that caused withdrawal reproducibly in normal rats.

ii. Motor function: The sciatic nerve crush

results in denervation of the extensors of the digits in the hindlimb. The digits are hyperflexed and held abnormally close together. This loss of toe spreading was used as an index of motor function after sciatic nerve crush. Toe spreading was measured from footprints made by walking rats. Measurements were compared in CNTF-treated and untreated rats during the course of regeneration of the sciatic nerve.

The plantar surfaces of both hindlimbs were pressed against an ink pad that was soaked lightly with black ink. The rat was placed, hindlimbs first, at the entrance of a walkway. The walkway was composed of a three-sided cardboard tunnel with its ground surface removed. The tunnel, which served to direct the rat's movements, was placed on a strip of white butcher paper. The rat was allowed to move freely through the tunnel. The object was to obtain at least two paired footprints on the butcher paper while the rat was in a walking mode.

Two parameters, the footspread (FS) and the distance between the intermediary toes (ID), were measured from the footprints. FS is the linear distance to the nearest millimeter from the medial edge of digit 1 to the lateral edge of digit 5. ID is linear distance to the nearest millimeter between the medial edge of digit 2 and the lateral edge of digit 4. The mean distances for FS and ID were determined for each foot. For each parameter, a ratio was calculated from values determined on the lesioned left foot and non-lesioned right foot. Thus, as motor function recovered as a function of time after sciatic nerve crush, ratios of either FS or ID, which at day 7 after nerve crush were less than 50% of normal, began to increase approaching a normal ratio of 1.0. Ratios were calculated daily beginning at day 7

from values determined for the paired lesioned and non-lesioned feet and compared for each parameter between CNTF-treated and untreated groups.

C. Administration of CNTF.

Rats were injected subcutaneously with CNTF at the dorsal midline in the region of the scapulae. The CNTF administered was human recombinant CNTF, produced as described in the Collins et al. patent application described above. Injections were made with an insulin syringe with a built-in 28 gauge needle. Minimal physical restraint was required during the injections. The volume of injected CNTF was 1.0 ml per kg body weight. Control rats were injected with 1.0 ml per kg of buffer vehicle, using the same technique.

D. Experimental design.

Injections with CNTF or vehicle were begun two days before the sciatic nerve injury and continued for 11 days after the injury for a total of 14 days. The completeness of the sciatic nerve crush was tested by the footsole test on day 3. Regeneration of the sensory nerves was determined by the foot sole test on a daily basis beginning 11 days after nerve crush.

E. Effects of CNTF on recovery of function after peripheral nerve damage.

Administration of CNTF to rats at a dose of 0.1 and 0.25 mg per kg of body weight, as described above, accelerated the rate of recovery of sensory (Figure 10) and motor (Figure 11) function. The CNTF-treated rats recovered 50% of normal sensory function 2.5 days earlier than rats treated with vehicle alone (Figure 10). It should be noted that vehicle-treated or untreated rats recover sensory and motor function without difficulty after physical nerve injury. Acceleration of recovery,

in what is already considered to be a rapidly recovering system is, therefore, a significant finding.

F. Controls showing the non-toxic nature of CNTF alone.

Separate controls were included to assess the effects of CNTF alone. CNTF had no effect on mortality and no significant effect on body weight at the doses found to accelerate recovery after peripheral nerve damage. In animals receiving CNTF in the experiments described above, there were no abnormalities apparent in the motor or sensory function on the control, unlesioned side. This indicates that CNTF had no obvious effect on sensory or motor function in the absence of nerve injury.

G. Conclusions.

CNTF was effective in accelerating recovery from peripheral nerve damage after physical injury in rats when CNTF was administered by daily subcutaneous injection around the time of injury. These results demonstrate that subcutaneously-administered CNTF is able to modify in a positive way the response of peripheral sensory and motor nerve cells to injury. A similar therapeutic regimen can be readily accomplished in a patient suffering from peripheral nerve damage.

Although the present invention has been described in connection with preferred embodiments, it is understood that those skilled in the art are capable of making modifications and variations without departing from the scope or spirit of the present invention. Therefore, the foregoing description of preferred embodiments is not to be taken in a limiting sense, and the present invention is best defined by the following claims and their equivalents.

WHAT IS CLAIMED IS:

1. A recombinant human ciliary neurotrophic factor (CNTF) protein having an amino acid sequence as in Figure 1 wherein the two amino acids at the C-terminal end are cleaved.

2. A recombinant human ciliary neurotrophic factor (CNTF) protein having an amino acid sequence as in Figure 1 wherein the six amino acids at the C-terminal end are cleaved.

3. The recombinant CNTF protein according to Claim 1, obtained from the expression of a vector comprising a DNA sequence encoding for CNTF.

4. The recombinant CNTF protein according to Claim 1, obtained from the expression of a vector in E. coli comprising a DNA sequence encoding for CNTF.

5. The recombinant CNTF protein according to Claim 2, obtained from the expression of a vector comprising a DNA sequence encoding for CNTF.

6. The recombinant CNTF protein according to Claim 2, obtained from the expression of a vector in E. coli comprising a DNA sequence encoding for CNTF.

7. A recombinant human CNTF protein having an amino acid sequence as in Figure 1, substantially free of CNTF having C-terminal amino acids cleaved.

8. The recombinant human CNTF protein according to Claim 7 where the human CNTF protein is obtained from the expression of a vector in E. coli comprising a DNA sequence encoding for CNTF.

9. A method for preparing substantially purified CNTF comprising:

(a) applying a cell lysate containing soluble CNTF protein to an anion exchange column which reversibly binds CNTF;

(b) collecting fractions comprising CNTF by eluting the CNTF protein bound to the anion exchange column with salt;

(c) applying the fractions containing CNTF protein to a cation exchange column;

(d) collecting fractions comprising CNTF by eluting the CNTF protein with a pH gradient of from about 7 to about 8.5;

(e) applying the fractions containing CNTF protein to an anion exchange column; and

(f) eluting the substantially purified CNTF protein with a salt gradient.

10. A recombinant human CNTF protein made by the process according to Claim 9.

11. A method of preparing substantially purified CNTF comprising:

(a) growing cells containing a nucleic acid sequence encoding for CNTF;

(b) harvesting the cells;

(c) solubilizing the CNTF protein;

(d) applying a cell lysate containing soluble CNTF protein to an anion exchange column which reversibly binds CNTF;

(e) collecting fractions comprising CNTF by eluting the CNTF protein bound to the anion exchange column with salt;

(f) applying the fractions containing CNTF protein to a cation exchange column;

(g) collecting fractions comprising CNTF by eluting the CNTF protein with a pH gradient of from about 7 to about 8.5;

(h) applying the fractions containing CNTF protein to an anion exchange column;

(i) eluting the substantially purified CNTF protein with a salt gradient; and

(j) eluting the CNTF protein by a salt gradient.

12. A method for preventing or treating peripheral nerve damage which comprises administering to a patient in need thereof a therapeutically effective amount of CNTF.

13. The method of claim 12 wherein said CNTF is a protein.

14. The method of claim 13 wherein said CNTF is naturally occurring CNTF.

15. The method of claim 13 wherein said CNTF is produced by recombinant DNA methods.

16. The method of claim 12 wherein said CNTF is in substantially pure form.

17. The method of claim 12 wherein said CNTF is administered in a pharmaceutically acceptable carrier.

18. The method of claim 12 wherein said CNTF is administered in a liquid form.

19. The method of claim 12 wherein said CNTF is human recombinant CNTF.

20. The method of claim 12 wherein said peripheral nerve damage is caused by a condition selected from the group consisting of: physical injury; exposure to neurotoxins; chronic metabolic diseases; and neurodegenerative diseases.

21. The use of a therapeutically effective amount of CNTF for the manufacture of a medicament suitable for preventing or treating peripheral nerve damage.

22. An agent for preventing or treating peripheral nerve damage which comprises a therapeutically

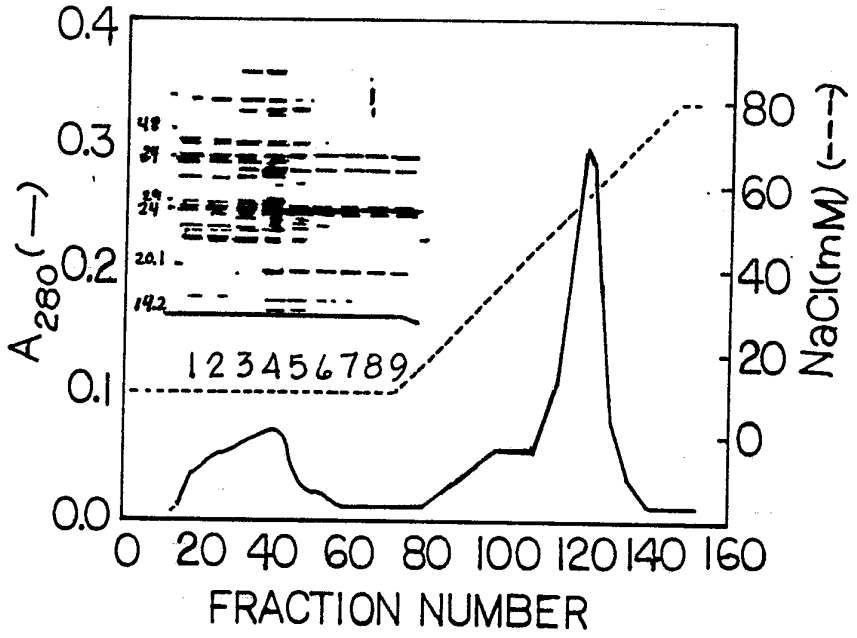

0.4
0.3
0.2
0.1
0.0
A280(—)
48
29
24
20.1
19.2
1 2 3 4 5 6 7 8 9
80
60
40
20
0
NaCl(mM) (---)
0 20 40 60 80 100 120 140 160
FRACTION NUMBER

FIG.4

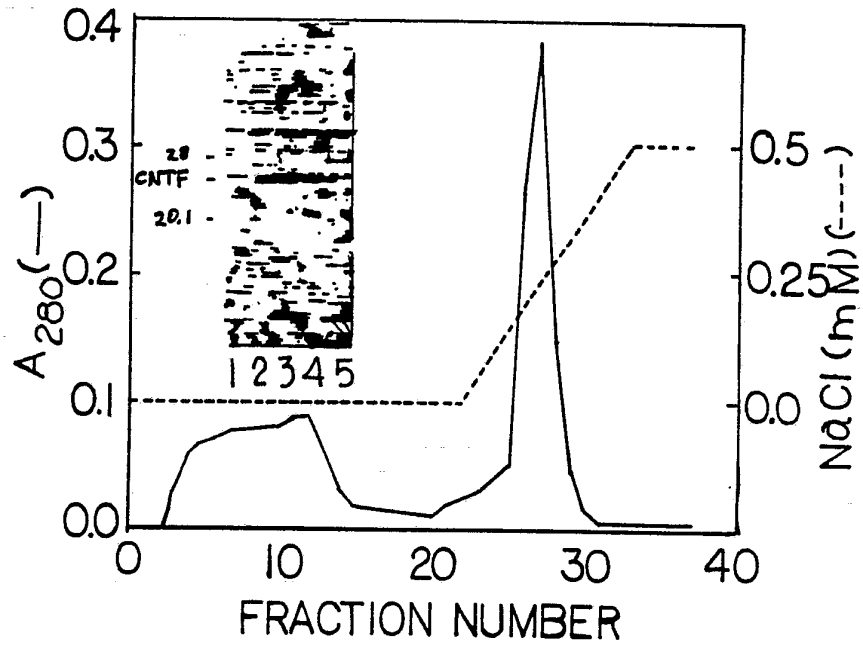

0.4
0.3
0.2
0.1
0.0
A280(—)
28
CNTF
20.1
1 2 3 4 5
0.5
0.25
0.0
NaCl (mM) (---)
0 10 20 30 40
FRACTION NUMBER

FIG.5

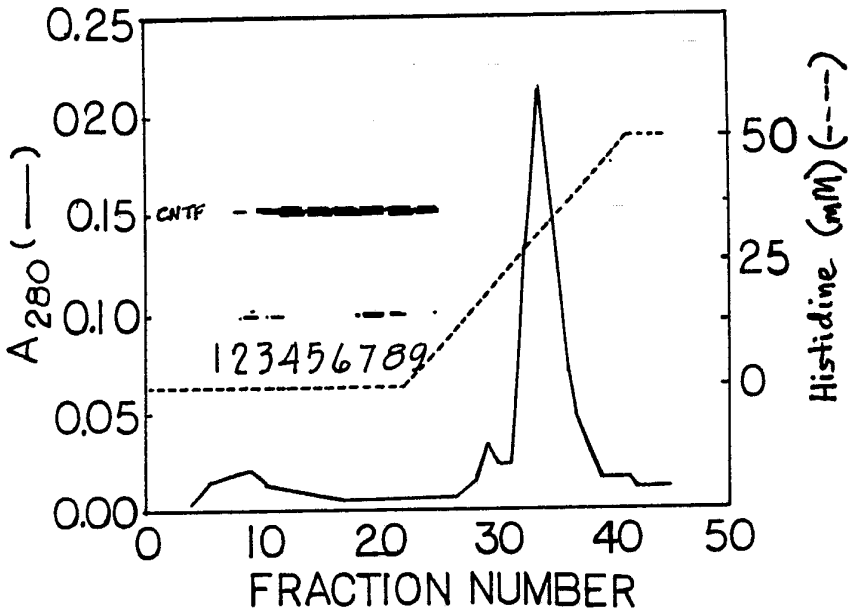
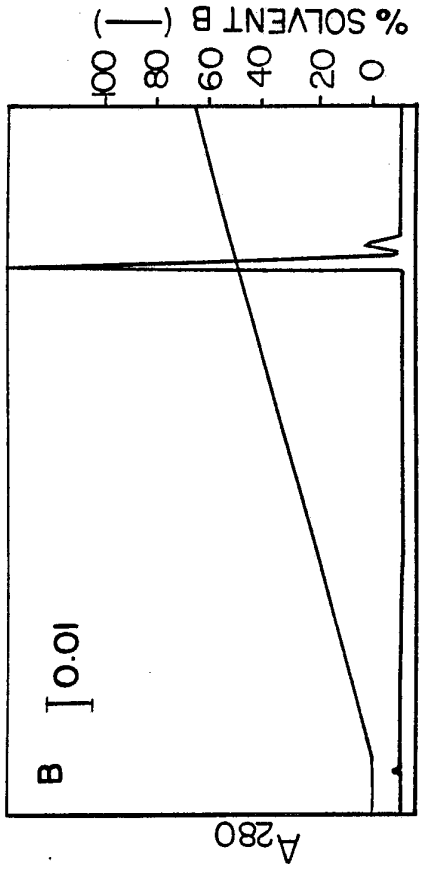

0.25
020
0.15
0.10
0.05
0.00
A280 (——)
CNTF
123456789
50
25
0
Histidine (mM) (- - -)
0
10
20
30
40
50
FRACTION NUMBER

FIG.6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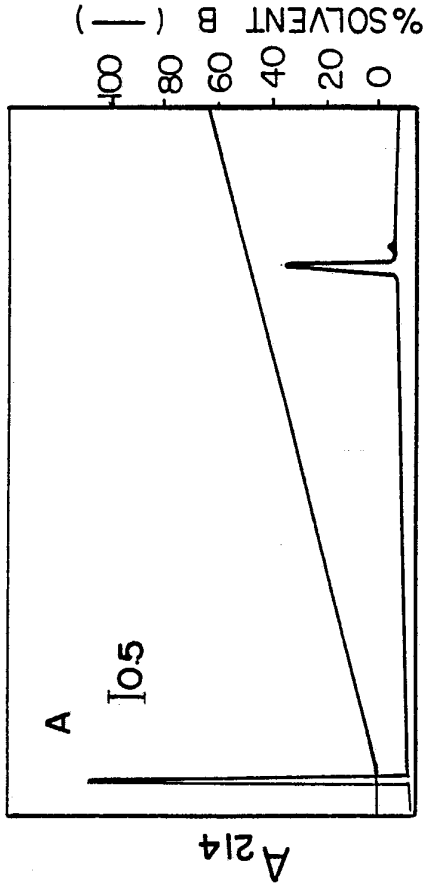
80
60
40
20
0
B
0.01
A280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80
60
40
20
0
A
0.5
A214

FIG. 7

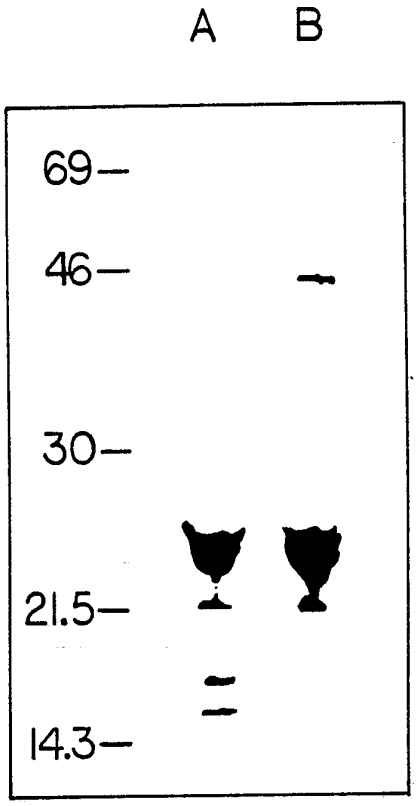

FIG. 8

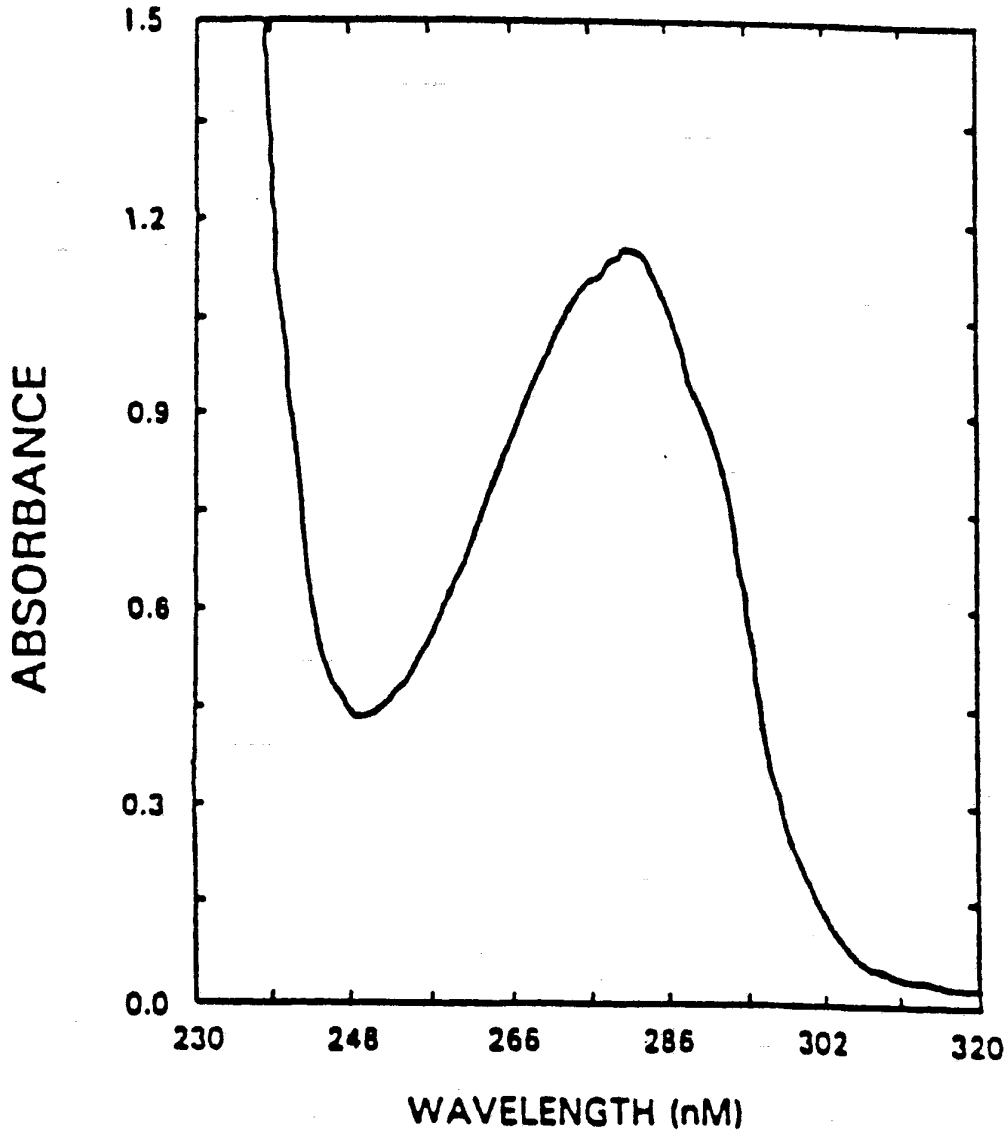
1.5
1.2
0.9
0.6
0.3
0.0
ABSORBANCE
230
248
266
286
302
320
WAVELENGTH (nM)

FIG. 9

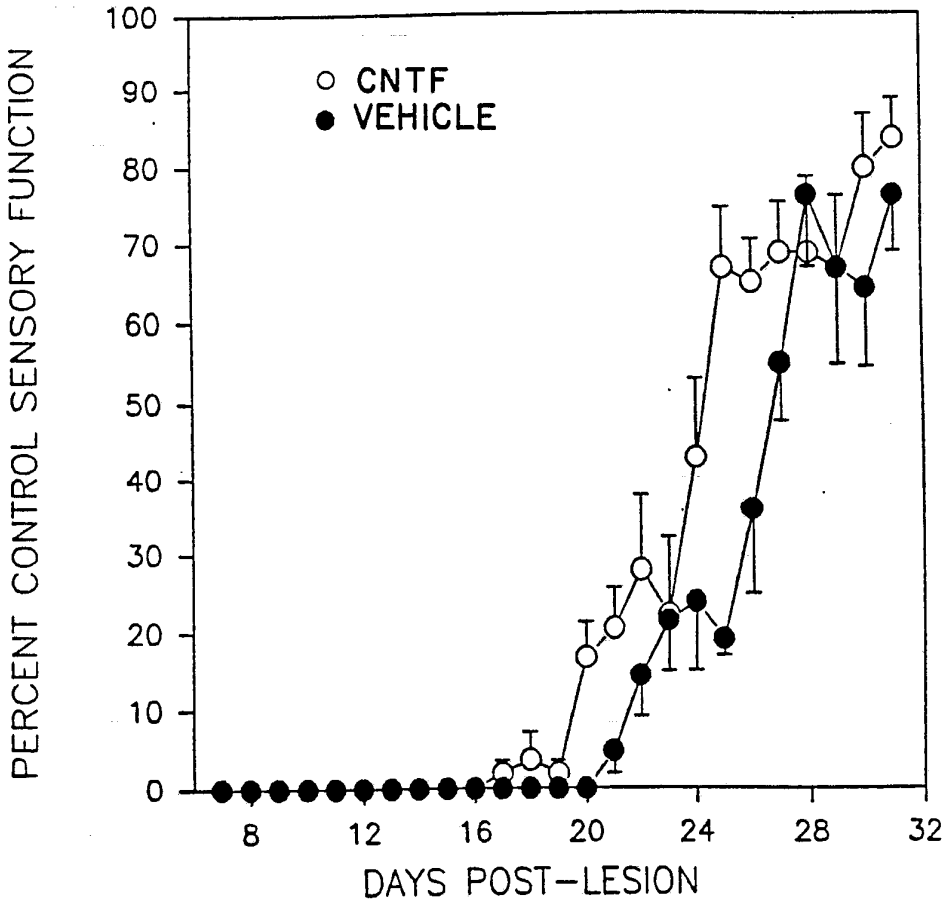
PERCENT CONTROL SENSORY FUNCTION
100
90
80
70
60
50
40
30
20
10
0
CNTF
VEHICLE
8
12
16
20
24
28
32
DAYS POST-LESION

FIG. 10

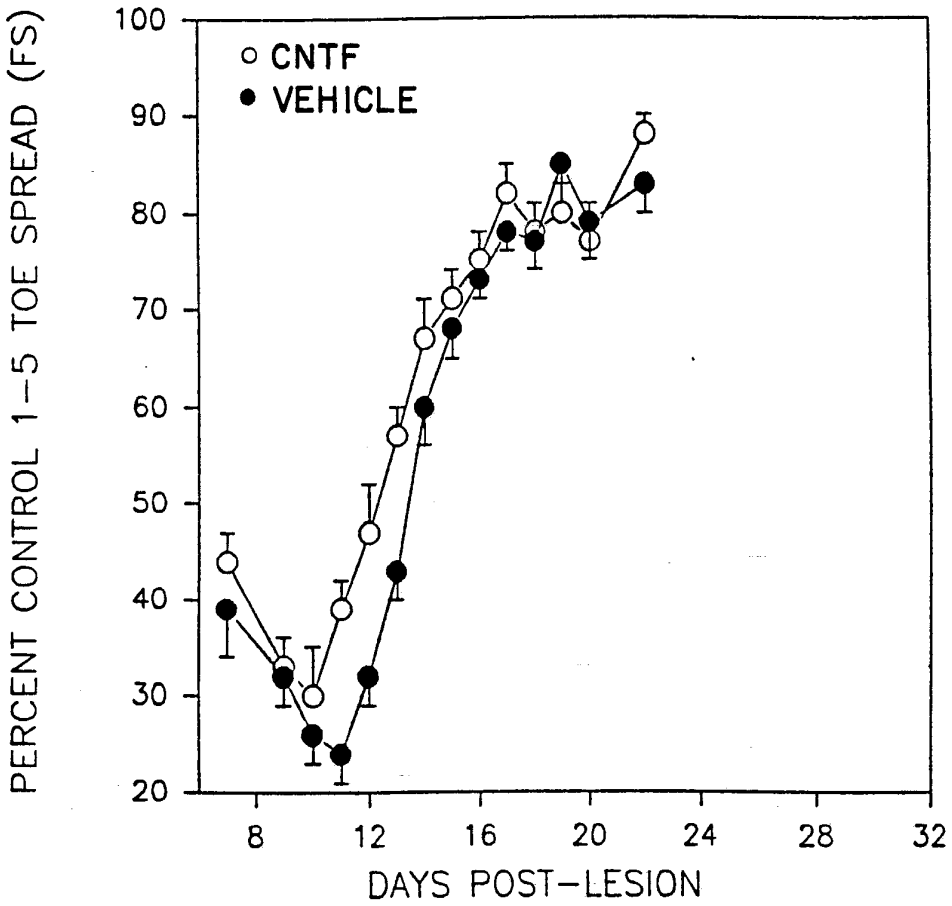
PERCENT CONTROL 1-5 TOE SPREAD (FS)
100
90
80
70
60
50
40
30
20
CNTF
VEHICLE
8
12
16
20
24
28
32
DAYS POST-LESION

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/06136

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C 12 P, 21/06; C 07 K, 3/00; A 01 N, 37/18.
US CL :514/2; 435/69.1, 69.4; 530/350, 399; 536/27.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2; 435/69.1, 69.4; 530/350, 399; 536/27.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS AND DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,011,914 (COLLINS ET AL.) 30 APRIL 1991, SEE THE ENTIRE DOCUMENT.	1-25
Y	WO, A 90/10647 (MANTHORPE ET AL.) 20 SEPTEMBER 1990, SEE THE ENTIRE DOCUMENT.	1-25
Y	WO, A 91/04316 (MASIAKOWSKI ET AL.) 04 APRIL 1991, SEE THE ENTIRE DOCUMENT.	1-25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 OCTOBER 1992	Date of mailing of the international search report 25 NOV 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT	Authorized officer GIAN WANG