4. M. J. Berridge and R. F Irvine, *Nature* **312**, 315 (1984); *ibid.* **341**, 187 (1989).
5. A. H. Tashjian, Jr., J. P. Heslop, M. J. Berridge, *Biochem. J.* **243**, 305 (1987); A. K. Saluja, R. E. Powers, M. L. Steer, *Biochem. Biophys. Res. Commun.* **164**, 8 (1989); P. Mollard, B. Dufy, P. Vacher, J. L. Barker, W. Schlegel, *Biochem. J.* **268**, 345 (1990); H. Koshiyama and A. H. Tashjian, Jr., *Endocrinology* **128**, 2715 (1991).
6. T. Chiba *et al.*, *Biochem. Biophys. Res. Commun.* **145**, 263 (1987).
7. P. J. Dargie, M. C. Agre, H. C. Lee, *Cell Regul.* **1**, 279 (1990).
8. H. Koshiyama, H. C. Lee, A. H. Tashjian, Jr., *J. Biol. Chem.* **266**, 16985 (1991).
9. The pancreases of male Wistar rats (240 to 280 g) that had been fed ad libitum were removed. Two thousand islets were isolated (*18*) and then homogenized with a Pellet mixer (Treff, Degersheim, Switzerland) in 200 μl of acetate intracellular medium (AcIM) (*10*) composed of 250 mM potassium acetate, 250 mM *N*-methyl-glucamine, 1 mM $MgCl_2$, and 20 mM Hepes supplemented with 0.5 mM adenosine triphosphate (ATP), 4 mM phosphocreatine, creatine phosphokinase (2 U/ml), 2.5 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride. After the homogenates had been centrifuged for 45 s (13,000*g*), microsomes were prepared by Percoll density gradient centrifugation [D. L. Clapper and H. C. Lee, *J. Biol. Chem.* **260**, 13947 (1985)]. For cerebellum microsomes, rat cerebellum was homogenized with a Dounce-type glass tissue homogenizer and microsomes were then prepared as described above. Release of Ca^{2+} was monitored in 3 ml of intracellular medium (GluIM) (*10*) composed of 250 mM potassium gluconate, 250 mM *N*-methyl-glucamine, 1 mM $MgCl_2$, 20 mM Hepes (pH 7.2) containing 1 mM ATP, 4 mM phosphocreatine, creatine phosphokinase (2 U/ml), 2.5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 3 μM fluo 3, a fluorescent Ca^{2+} indicator [J. P. Y. Kao, A. T. Harootunian, R. Y. Tsien, *J. Biol. Chem.* **264**, 8179 (1989)], with the addition of 150 μl of islet microsome fraction (11 μg of protein) or cerebellum microsome fraction (88 μg of protein). Fluorescence was measured at 490-nm excitation and 535-nm emission (*7*) at 37°C with a circulating water bath.
10. D. L. Clapper, T. F. Walseth, P. J. Dargie, H. C Lee, *J. Biol. Chem.* **262**, 9561 (1987).
11. Cyclic ADP–ribose, prepared enzymatically from NAD^+ with ADP–ribosyl cyclase purified (*24*) from ovotestes of *Aplysia kurodai*, a species common around the Japanese coast, was purified by high-performance liquid chromatography (HPLC) (*25*) and characterized by ^1H nuclear magnetic resonance and fast atom bombardment mass spectrometry [H. C. Lee, T. F. Walseth, G. T. Bratt, R. N. Hayes, D. L. Clapper, *J. Biol. Chem.* **264**, 1608 (1989)]. Cyclic ADP–ribose, thus obtained, showed a single peak on HPLC and a peak at a mass-to-charge ratio (*m/z*) 540.2 corresponding to molecular ion of $(M-H)^-$ on negative ion fast atom bombardment mass spectrometry.
12. P. F. Worley, J. M. Baraban, S. Supattapone, V. S. Wilson, S. H. Snyder, *J. Biol. Chem.* **262**, 12132 (1987).
13. S. Takasawa, K. Nata, H. Yonekura, H. Okamoto, unpublished data.
14. A. Galione, H. C. Lee, W. B. Busa, *Science* **253**, 1143 (1991); A. Galione, *Trends Pharmacol. Sci.* **13**, 304 (1992).
15. Md. S. Islam, P. Rorsman, P.-O. Berggren, *FEBS Lett.* **296**, 287 (1991).
16. Islets were permeabilized by digitonin treatment [J. R. Colca, B. A. Wolf, P. G. Comens, M. L. McDaniel, *Biochem. J.* **228**, 529 (1985)]. In brief, freshly isolated islets were incubated in a modified Hepes-Krebs buffer (25 mM Hepes, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% bovine serum albumin, pH 6.8) at 37°C with digitonin (20 μg/ml) (Merck) for 20 min. Thirty islets were randomly selected, washed three times in tris buffer (50 mM tris, 100 mM KCl, 5 mM $MgCl_2$, 0.1% bovine serum albumin, pH 6.8), and incubated at 24°C for 15 min in 100 μl of tris-Pipes buffer (55 mM tris, 11 mM KCl, 5 mM $MgCl_2$, 1.1 mM EGTA, 1 mM ATP, 10 mM phosphocreatine, creatine kinase (10 units/ml), 0.1% bovine serum albumin, pH 7.5). Islets were treated with O_2:CO_2 (19:1). At the end of the incubation period, the medium was withdrawn and a radioimmunoassay kit (Novo Nordisk Biolabs, Baysvaerd, Denmark) was used to quantify the released insulin with rat insulin used as the standard.
17. For preparing the glucose-treated islet extract, 500 islets were isolated from the pancreases of male Wistar rats (240 to 280 g) that had fasted for 48 hours; these islets were incubated with 2.8 mM or 20 mM glucose in 100 μl of Krebs-Ringer's bicarbonate medium (*2, 18*) for 40 min at 37°C, washed three times with cold AcIM, and homogenized in 50 μl of AcIM. The homogenates were centrifuged at 4100*g* for 15 min in a Sorvall HB-4 rotor, and the supernatant was used as the glucose-treated islet extract. No changes in fluorescence were observed when islet extracts were added to a GluIM-fluo 3 system without microsomes.
18. H. Okamoto, *Mol. Cell. Biochem.* **37**, 43 (1981).
19. H. Yamamoto, Y. Uchigata, H Okamoto, *Nature* **294**, 284 (1981)
20. Y. Uchigata, H Yamamoto, A. Kawamura, H. Okamoto, *J. Biol. Chem.* **257**, 6084 (1982); H. Okamoto, *BioEssays* **2**, 15 (1985); in *Molecular Biology of the Islets of Langerhans*, H. Okamoto, Ed. (Cambridge Univ. Press, Cambridge, U.K., 1990), pp. 209–231.
21. P. Masiello, T. L. Cubeddu, G. Frosina, E. Bergamini, *Diabetologia* **28**, 683 (1985).
22. K. Shima, M. Hirota, M. Sato, S. Numoto, I. Oshima, *Diabetes Res. Clin. Practice* **3**, 135 (1987).
23. Five hundred islets were preincubated at 37°C for 15 min in 100 μl of Krebs-Ringer's bicarbonate medium containing 2.8 mM glucose in the presence or absence of a poly(ADP-ribose) synthetase inhibitor (3 mM nicotinamide or 0.4 mM 3-aminobenzamide), then incubated for 20 min with or without the addition of 2 mM streptozotocin. After preincubation, islets were further incubated for 40 min with an additional 100 μl of medium containing 37.2 mM glucose. The final concentration of glucose was 20 mM.
24. M. R. Hellmich and F. Strumwasser, *Cell Regul.* **2**, 193 (1991).
25 H. C. Lee and R. Aarhus, *ibid.*, p. 203.
26. T. F. Walseth, R. Aarhus, R. J. Zeleznikar, Jr., H. C. Lee, *Biochim. Biophys. Acta* **1094**, 113 (1991).
27. M. Prentki *et al.*, *Nature* **309**, 562 (1984).
28. T. J. Biden, M. Prentki, R. F. Irvine, M. J. Berridge, C. B. Wollheim, *Biochem. J.* **223**, 467 (1984); S. K. Joseph, R. J. Williams, B. E. Corkey, F. M. Matschinsky, J. R. Williamson, *J. Biol. Chem.* **259**, 12952 (1984); M. Prentki, B. E. Corkey, F. M. Matschinsky, *ibid.* **260**, 9185 (1985); C. B. Wollheim and T. J. Biden, *ibid.* **261**, 8314 (1986).
29. B. A. Wolf, P. G. Comens, K. E. Ackermann, W. R. Sherman, M. L. McDaniel, *Biochem. J.* **227**, 965 (1985).

30 We thank K. Shikama and T. Numakunai for useful suggestions and for preparing *Aplysia* ovotestes; R. Namba, K. Komatsu, S. Yoshida, M. Tajima, and M. Yanagi for fast atom bombardment mass spectrometry and proton nuclear magnetic resonance analyses; and B. Bell for valuable assistance in preparing the manuscript for publication.

4 September 1992; accepted 18 November 1992

Blood-Brain Barrier Penetration and in Vivo Activity of an NGF Conjugate

Phillip M. Friden,* Lee R. Walus, Patricia Watson, Susan R. Doctrow, John W. Kozarich, Cristina Bäckman, Hanna Bergman, Barry Hoffer, Floyd Bloom, Ann-Charlotte Granholm

Nerve growth factor (NGF) is essential for the survival of both peripheral ganglion cells and central cholinergic neurons of the basal forebrain. The accelerated loss of central cholinergic neurons during Alzheimer's disease may be a determinant of dementia in these patients and may therefore suggest a therapeutic role for NGF. However, NGF does not significantly penetrate the blood-brain barrier, which makes its clinical utility dependent on invasive neurosurgical procedures. When conjugated to an antibody to the transferrin receptor, however, NGF crossed the blood-brain barrier after peripheral injection. This conjugated NGF increased the survival of both cholinergic and noncholinergic neurons of the medial septal nucleus that had been transplanted into the anterior chamber of the rat eye. This approach may prove useful for the treatment of Alzheimer's disease and other neurological disorders that are amenable to treatment by proteins that do not readily cross the blood-brain barrier.

The degeneration of specific neuronal cell types during age-related neurological disorders such as Alzheimer's disease presents opportunities for neurotrophic therapies. For example, the degeneration of certain cholinergic neurons in patients dying with Alzheimer's disease has been linked to their memory impairment (*1*). If the loss of cholinergic neurons were responsible at least in part for the clinical symptoms of Alzheimer's disease, a treatment that limits

P. M. Friden, L. R. Walus, P. Watson, S. R. Doctrow, J. W. Kozarich, Alkermes, Inc., 64 Sidney Street, Cambridge, MA 02139.
C. Bäckman and A.-C. Granholm, Department of Basic Sciences, University of Colorado School of Dentistry, Denver, CO 80262.
H. Bergman, Department of Cell Biology, University of Linköping Health Science Center, Linkoping, Sweden.
B. Hoffer, Department of Pharmacology, University of Colorado School of Medicine, Denver, CO 80262.
F. Bloom, Department of Neuropharmacology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

*To whom correspondence should be addressed.

their loss would be desirable (*2*). Cholinergic neurons of the basal forebrain, which degenerate in the brains of patients with Alzheimer's disease, depend for their survival on the receipt of NGF through retrograde axonal transport from their synaptic target areas in the hippocampal formation and the cerebral cortex (*3*, *4*). Exogenous administration of NGF in animals increases cholinergic markers in the central nervous system (CNS), rescues degenerating cholinergic neurons in the forebrain, and also leads to functional recovery after lesions of the cholinergic pathways to the hippocampal formation in rats (*4*, *5*). Cholinergic degeneration and impaired maze performance in aged rats are also ameliorated by intracerebral administration of NGF (*6*).

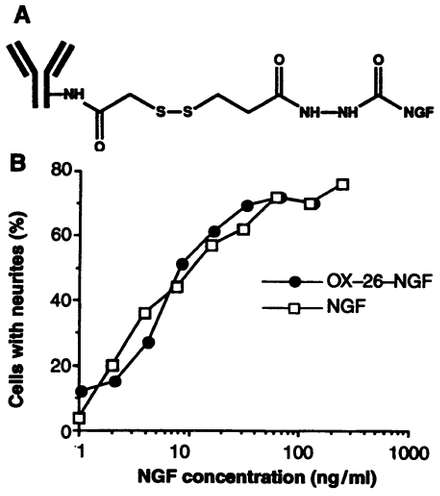

Fig. 1. Conjugation of biologically active NGF to the antibody to the transferrin receptor OX-26. (**A**) PDP-hydrazide was used to attach a thiol reactive group to NGF by means of carboxyl groups that had been activated with EDC. SATA was reacted with lysine amines on the antibody to introduce a protected sulfhydryl group. The sulfhydryl group on the antibody exchanged with the 2-pyridyl-sulfide group on NGF, forming a cleavable disulfide bond between the two proteins (*12*). (**B**) The neurite outgrowth response of PC-12 cells treated with either unmodified NGF or OX-26–NGF conjugate was examined over a range of doses (*14*). The response is expressed as percent of cells extending neurites as a function of NGF dose. Closed circles, OX-26–NGF; open squares, unmodified NGF.

Currently, one of the primary obstacles to clinical testing of NGF for neurodegenerative disorders is the lack of an efficient, noninvasive means to deliver the peptide across the blood-brain barrier (BBB) to the target cells (*2*). The BBB, which is composed principally of specialized capillary endothelial cells joined by highly restrictive tight junctions, prevents the passage from the bloodstream to the CNS of proteins with therapeutic potential (*7*). After intravenous (i.v.) administration, antibodies to the transferrin receptor preferentially bind to brain capillary endothelial cells (*8*). These antibodies, as well as drugs conjugated to these antibodies, can cross the BBB as a result of their interaction with the transferrin receptor, which suggests that such conjugates may be of value in the delivery of blood-borne therapeutic agents to the brain (*9*). We now demonstrate the feasibility of this approach with NGF. NGF can be linked in a biologically active form to an antibody (OX-26) against the rat transferrin receptor and can be delivered across the BBB to medial forebrain tissue that has been transplanted to the anterior chamber of the rat eye. These transplants are responsive to NGF administered into the ocular fluid (*10*) but, because they retain normal BBB properties, would not be expected to respond to NGF administered peripherally. However, we now report that NGF delivered by means of the antibody carrier was able to stimulate the survival and growth of these brain tissue transplants.

We synthesized OX-26–NGF conjugates by a strategy that has been used extensively in the synthesis of antibody-protein conjugates, primarily in the area of immunotoxins (*11*). This process involved the introduction of a protected sulfhydryl group onto the antibody to the transferrin receptor through lysine ϵ-amines and the introduction of a heterobifunctional cross-linker that contained a thiol reactive group onto NGF through carboxyl groups (Fig. 1A) (*12*). The advantage of using a heterobifunctional cross-linking agent is that the coupling is performed in a stepwise manner, thus avoiding the formation of homoprotein polymers.

To ascertain the effects of both protein derivatization and conjugation on the biological activity of NGF, we used the PC-12 cell neurite outgrowth assay. In response to stimulation with NGF, these cells undergo a reversible differentiation in which the cells adopt a neuronal phenotype and extend neurites. This morphological response to exogenous NGF can be used as the basis for a semiquantitative assay for NGF biological activity (*13*). A comparison of the OX-26–NGF conjugate with unmodified NGF in the PC-12 cell neurite outgrowth

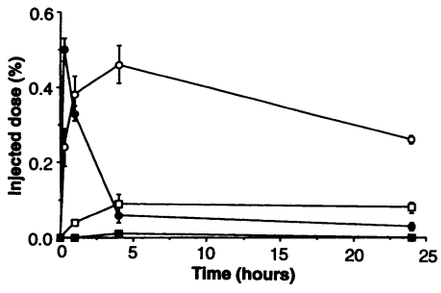

Fig. 2. Enhanced delivery of conjugated NGF across the BBB. Radiolabeled NGF, either as an OX-26–NGF conjugate or as free protein, was injected into rats intravenously. Capillary depletion was performed on brains taken from the animals at various times after injection (*9*, *16*, *17*). Results are expressed as the percent of the injected dose per brain in either the parenchyma or capillary fraction and shown as means ± SEM (n = three animals per time). Open circles, OX-26–NGF in brain parenchyma; closed circles, OX-26–NGF in brain capillaries; open squares, NGF in brain parenchyma; closed squares, NGF in brain capillaries.

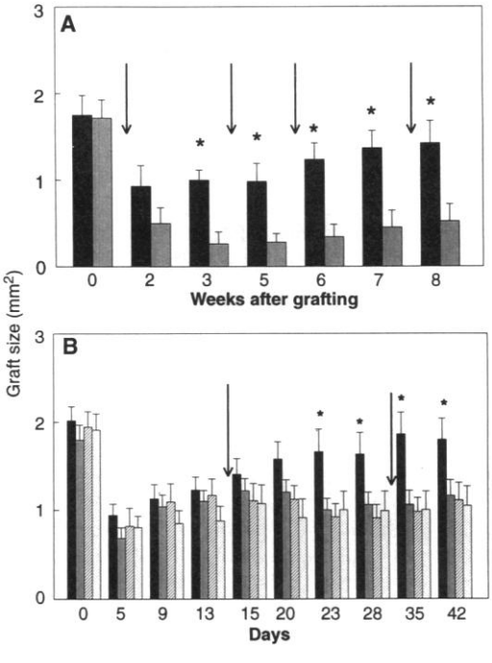

Fig. 3. The intraocular growth of medial forebrain transplants from the time of transplantation (t = 0). (**A**) The sizes of the grafts in the first experimental series. (**B**) The sizes of the grafts in the second series. In both series, OX-26–NGF conjugate treatment is shown by the solid bars, and OX-26 administration alone is shown by the bars with the narrow cross-hatching. In (B), peripheral injection of an equivalent dose of NGF and peripheral injection of an equivalent volume of saline are shown by the bars with wide cross-hatching and by the stippled bars, respectively (*20*). Asterisk, $P < 0.05$. In both (A) and (B), vertical arrows indicate day of treatment.

assay demonstrated that there was full retention of NGF's specific biological activity after its conjugation to the antibody (Fig. 1B) (*14*).

The antibody to the rat transferrin receptor OX-26 can be detected immunohistochemically in brain vasculature after i.v. administration (*9*). We performed similar studies with the OX-26–NGF conjugate to demonstrate the ability of the carrier antibody to localize NGF to the brain vasculature of rats after conjugation. By using brain sections prepared from rats killed 1 hour after the injection of the conjugate, we were able to detect immunohistochemically a pattern of localization of the carrier antibody in the brain vasculature that was indistinguishable from that seen with antibody alone (*15*). This result indicates that the attachment of NGF did not appreciably alter the targeting of the antibody to brain capillary endothelial cells after i.v. injection. In addition, when these sections were probed with an antibody to NGF, staining of the vasculature was also observed, which demonstrates that NGF was present in the brain capillaries and localized with the carrier antibody.

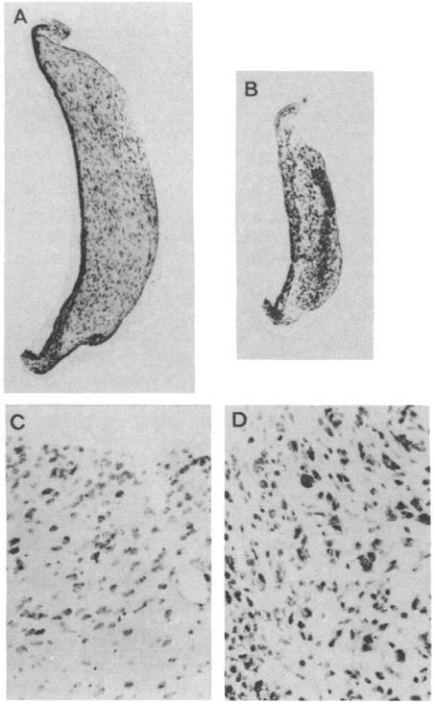

Fig. 4. Toluidine blue staining of sections of intraocular septal transplants in a rat host injected with OX-26–NGF (**A** and **C**) and in a control host injected with only OX-26 (**B** and **D**). Note that there is a significant difference in size between the two transplants in (A) and (B), whereas the density of cells within the grafts appears to be the same in (C) and (D). A cross section of the host iris can be seen to the left in each transplant in (A) and (B).

To ascertain whether the OX-26–NGF conjugate crossed the BBB, we performed capillary depletion experiments in which neural tissue was separated from the brain vasculature and the amount of NGF in each fraction was determined (*16*). Experiments such as these have shown that unconjugated antibody to the transferrin receptor and antibody-methotrexate conjugates can cross the BBB (*9*). The conjugated NGF initially accumulated in the capillary fraction of the brain, which is consistent with its binding to, and internalization by, brain capillary endothelial cells (Fig. 2) (*17*). The amount of radiolabeled NGF in the capillaries then decreased while the amount in the brain parenchyma increased. These results, in which the temporal pattern of the tracer's distribution indicates that transport across the BBB occurs, are in accord with results obtained previously with unconjugated antibody (*9*). In contrast, after i.v. injection unconjugated NGF did not accumulate in the capillaries, although some radioactivity accumulated to a small degree in the brain parenchyma (Fig. 2) (*17*).

We tested the feasibility of using the antibody carrier for the delivery to the brain of therapeutic amounts of NGF by using intraocular transplants of septal tissue in rats (*18*). At the time of vascularization of the transplanted tissue (*19*), vessels from the iris connect with capillary primordia in the brain grafts. The grafts develop the endothelial and astrocytic barrier mechanisms characteristic of brain parenchyma (*18*). That the BBB formed in such transplants within 2 weeks of transplantation was confirmed by dye exclusion experiments (*19*).

Direct injection of NGF into the anterior chamber of the rat eye enhances the growth of septal transplants (*10*). Because the growth of these transplants can be followed noninvasively by repeated direct measurements through the cornea, such grafts provided an excellent model system for evaluation of the OX-26 conjugate. The growth curve of septal transplants, with or without i.v. injections with the OX-26–NGF conjugate, is shown in Fig. 3 (*20*). In one experimental series, four injections of the conjugate, administered once every 2 weeks during the development of the septal grafts, induced a significantly greater growth of these transplants as compared to transplants in a control group that received only the carrier antibody (Fig. 3A) (*20*). In a second series, the results of peripheral injections of OX-26–NGF conjugate were compared with the results of injections of NGF alone, OX-26 alone, or saline. Again, the conjugate-treated grafts showed a highly significant increase in growth, which was not seen in the three control groups (Fig. 3B) (*20, 21*). We intentionally chose a fetal donor stage in rats, embryonic day 18, that would lead to a less than optimal growth of the control group [figure 1 of (*10*)] to facilitate determination of the impact of NGF on the survival and maturation of neurons in the grafts.

Despite the marked differences in overall graft sizes, histological examination of the septal transplants showed no obvious differences in the density of neural or glial structures within the two groups of grafts in the first experimental series (Fig. 4). Specifically, the density of cells and the vascularization of grafts in the two groups appeared to be the same. To specifically investigate the survival of septal cholinergic neurons in these transplants, we used immunohistochemistry with a monoclonal antibody directed against the enzyme choline acetyl-

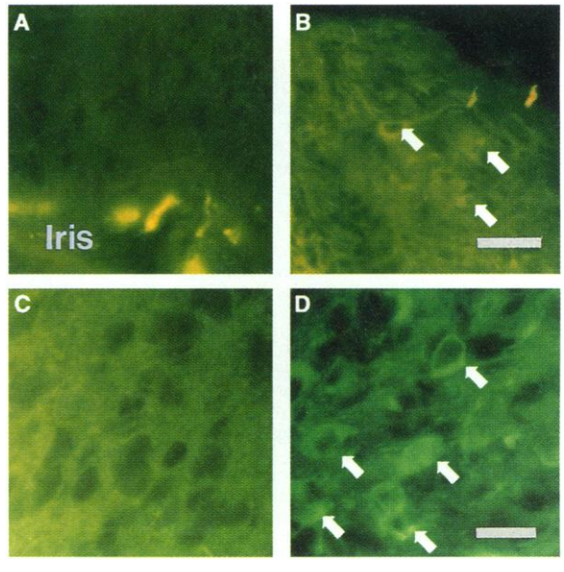

Fig. 5. Photomicrographs of transplant tissue sections incubated with rat antibody to ChAT and antibody to rat IgG conjugated with fluorescein. (**A**) and (**C**) demonstrate the lack of ChAT-immunoreactive neurons in control (OX-26) transplants, whereas (**B**) and (**D**) show the abundance of CAT-positive neurons in two separate conjugate-treated grafts. Bars in (A) and (B), 100 μm; bars in (C) and (D), 50 μm. The host iris, containing ChAT-positive parasympathetic fibers, can be seen in the control graft shown in (A). Arrows in (B) and (D) demarcate ChAT-immunoreactive neurons.

transferase (ChAT) (*22*). This antibody is reliable for the visualization of cholinergic structures in nervous tissue (*23*). In comparison to the control group, three and a half times more cholinergic neurons were found in the group of transplants injected with OX-26–NGF (Fig. 5). Similar changes were seen in the second experimental series. Because the transplants in the NGF conjugate groups were significantly larger, the total numbers of both cholinergic and noncholinergic neurons surviving were larger than in the control groups, a finding in keeping with previous studies that used intraocular injections of NGF (*10*). These results further support the concept that NGF passed through the BBB into the grafts and enhanced the survival of cholinergic neurons in the groups injected with the NGF conjugate (*24*).

In summary, we have taken a biological approach to drug delivery by exploiting a cellular mechanism, the transport of iron across brain capillary endothelial cells, to enable the large polypeptide growth factor NGF to reach its site of action within the brain and enhance the survival of cholinergic neurons. The ability to deliver proteins to the brain will facilitate a critical examination of the efficacy of neurotrophic factors on the amelioration of neurodegenerative disorders.

REFERENCES AND NOTES

1. P. J. Whitehouse *et al.*, *Science* **215**, 1237 (1982); D. L. Price, *Annu. Rev. Neurosci.* **9**, 489 (1986); D A. Drachman and J. Leavitt, *Arch. Neurol.* **30**, 113 (1974); R. T. Bartus, R. L Dean III, B. Beer, A. S. Lippa, *Science* **217**, 408 (1982); H. E. Haigler *et al.*, *Brain Res.* **362**, 157 (1985).
2. L. Olson *et al.*, *Arch. Neurol.* **48**, 373 (1991); L. Olson *et al.*, *J. Neural Transm.* **4**, 79 (1992).
3. L. A. Greene and E. M. Shooter, *Annu. Rev. Neurosci.* **3**, 353 (1980); B. H. Gahwiler *et al.*, *Neurosci. Lett.* **75**, 6 (1987); J. Hartikka and F. Hefti, *J. Neurosci.* **8**, 2967 (1988); L. F. Kromer, *Science* **235**, 214 (1987); W. C. Mobley *et al.*, *Mol. Brain Res.* **1**, 53 (1986); S. R. Whittemore and A. Seiger, *Brain Res. Rev.* **12**, 439 (1987); M. E. Schwab *et al.*, *Brain Res.* **168**, 473 (1979).
4. F. Hefti, *J. Neurosci.* **6**, 2155 (1986).
5. T. Hagg *et al.*, *Exp. Neurol.* **101**, 303 (1988); L. R. Williams *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9231 (1986); E. O. Junard *et al.*, *Exp. Neurol.* **110**, 25 (1990); T. Hagg *et al.*, *J. Neurosci.* **10**, 3087 (1990); D. Hoffman *et al.*, *Exp. Neurol.* **110**, 39 (1990); B. Will and F. Hefti, *Behav. Brain Res* **17**, 17 (1985).
6. W. Fischer *et al.*, *Nature* **329**, 65 (1987).
7. G. W. Goldstein and A. L. Betz, *Sci. Am.* **255**, 74 (September 1986); M. W. Brightman, *Exp. Eye Res.* **25**, 1 (1977); T. S. Reese and M. J. Karnovsky, *J. Cell Biol.* **34**, 207 (1967).
8. W. A. Jefferies *et al.*, *Nature* **312**, 162 (1984).
9. P. M. Friden *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4771 (1991); W. M. Pardridge *et al.*, *J. Pharmacol. Exp. Ther.* **259**, 66 (1991).
10. M. Eriksdotter-Nilsson *et al.*, *Exp. Brain Res* **74**, 89 (1989); M. Eriksdotter-Nilsson *et al.*, *Neuroscience* **30**, 755 (1989).
11. E. J. Wawrzynczak and P. E. Thorpe, in *Immunoconjugates: Antibody Conjugates in Radioimaging and Therapy of Cancer*, C.-W. Vogel, Ed. (Oxford Univ. Press, Oxford, 1987), pp. 28–55; A. H. Blair and T. I. Ghose, *J. Immunol. Methods* **59**, 129 (1983).
12. The strategy for conjugating NGF to OX-26 is as follows. We used PDP (pyridyldithiopropionate)-hydrazide {a derivative of SPDP [*N*-succinimidyl 3-(2-pyridyldithio)propionate] [J. Carlsson *et al.*, *Biochem. J.* **173**, 723 (1978)] in which the *N*-hydroxy-succinimide ester has been replaced by a hydrazide group} to attach a thiol reactive group to NGF by means of the carboxyl groups. We achieved this by first activating the carboxylates with EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) to make them susceptible to attack by the hydrazide group. Correspondingly, SATA (*N*-succinimidyl-*S*-acetylthioacetate) was reacted with lysine amines on the antibody to introduce a protected sulfhydryl group. Quantitative assays were used to determine the number of sulfhydryl and pyridyldithio groups that were introduced onto OX-26 and NGF, respectively. By limiting the number of modifications on each protein, we kept the likelihood of forming large protein multimers to a minimum. In general, approximately 1 mol of the thiol reactive group was added per NGF dimer, and approximately two to five thiols were added to each antibody molecule. We prepared OX-26–NGF conjugates by reacting carboxyl-modified NGF with the derivatized antibody at a 7.5:1 molar ratio. The free sulfhydryl group on the antibody exchanged with the 2-pyridyl-sulfide group on NGF, forming a disulfide linkage between the two proteins and releasing pyridine-2-thione We purified the OX-26–NGF conjugate by passing the crude conjugate over a protein A–Sepharose (Pharmacia) column and then passing the eluted material over an affinity column prepared by linking a monoclonal antibody to NGF (1G3) [B N Saffran *et al.*, *Brain Res* **492**, 245 (1989)] to Sepharose beads with cyanogen bromide–activated Sepharose beads. We monitored the conjugation and purification processes by analyzing samples taken at various steps using SDS–polyacrylamide gel electrophoresis [U. K. Laemmli, *Nature* **227**, 680 (1970)]. Immunoblots prepared from gels run under nonreducing conditions, in which the disulfide linkage between the antibody and NGF was not cleaved, were used to show that NGF (26,000 daltons) was now migrating at a molecular mass corresponding to ~180,000 daltons, the size of the expected conjugate. In addition, we also used a two-site enzyme-linked immunosorbent assay, formated such that only conjugate consisting of NGF and antibody would give rise to a positive signal, to demonstrate the composition of the purified conjugate [E. Harlow and D. Lane, Eds., *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988)].
13. S. Buxser *et al.*, *J. Neurochem* **56**, 1012 (1991).
14. In this assay, PC-12 cells were plated onto bovine type IV collagen–coated 96-well microtiter plates at a density of ~1 × 10^3 cells per well. Six hours after plating, the cells were exposed to native NGF or OX-26–NGF conjugate To generate a dose-response curve, we diluted the samples in duplicate in growth medium (serially, in twofold increments) to cover a wide range of concentrations. After 5 days of their exposure to the samples, we scored the plates by counting the total number of cells and the number of cells with neurites longer than two cell diameters in length in two to three representative fields of view Intact conjugate was recovered from the incubation medium at the end of the assay, which suggests that the observed activity was a result of NGF linked to the antibody. These results are representative of other experiments that have been done.
15. L. R. Walus and P M Friden, unpublished data.
16. D. Triguero *et al.*, *J. Neurochem.* **54**, 1882 (1990).
17. For the capillary depletion experiments, OX-26–NGF conjugate was prepared with NGF that was radiolabeled with ^{3}H to a high specific activity [U. Kummer, *Methods Enzymol.* **121**, 670 (1986)]. The radiolabeled conjugate was injected into rats intravenously by means of the tail vein, and the animals were killed at various times after injection and their brains removed. Homogenization of the brain followed by density gradient centrifugation through 12% dextran separated the vascular elements from the remaining brain tissue. Although precipitable by trichloroacetic acid, the very small amount of ^{3}H detected in the brain parenchyma after administration of radiolabeled NGF may represent a partially degraded form of the protein, as intact NGF is not thought to cross the BBB.
18. Intraocular allogenic transplantation was performed in Sprague-Dawley rats according to the protocol of Olson and co-workers [L. Olson *et al.*, *Brain Res.* **119**, 87 (1977)]. Fetal basal forebrain was dissected from rat fetuses of embryonic day 18 (*10*) and injected into the anterior chamber of the eye of chloral hydrate–anesthetized [300 mg per kilogram of body weight, intraperitoneally (i.p.)] adult rat hosts through an incision in the cornea [A.-Ch. Granholm, *Methods Neurosci.* **7**, 327 (1991)]. To control for small variations in the fetal age of the donors, which would influence posttransplantation growth, we evenly distributed among all treatment groups brain tissue from any given donor dam. The growth and vascularization of transplants in hosts lightly anesthetized with halothane was evaluated routinely with an ocular micrometer in a stereomicroscope. The surface area of each graft was calculated repeatedly, and growth curves were generated. We previously demonstrated a high correlation between graft linear dimensions and wet weight [H. Björklund *et al.*, *Environ. Res.* **22**, 229 (1990)].
19. Intraocular transplants in rats form an intact BBB. Vessels growing into the graft from the host iris make connections with vascular elements in the grafted nervous tissue [R. L. Knobler *et al.*, *J. Neuropathol. Exp. Neurol.* **51**, 36 (1992)]. To determine if the BBB had formed specifically in our transplants at the time of the first injection, 2 weeks after grafting we injected Evan's blue (20 mg/kg; i.v.) into two rats with transplants in both eyes. After 30 s, the rats were killed, and the transplants were sectioned. This experiment demonstrated that a large quantity of stain collected in iris vessels, with iris tissue strongly stained and with some staining in vessels in transplants but no leakage into the brain tissue in grafts. Therefore, we started injections of OX-26–NGF at this time after grafting. In mature grafts, immunocytochemical studies showed that blood vessels accumulate the OX-26 antibody after i.v. injection with a density and intensity similar to intact brain vasculature. No vascular staining was seen after i.v. injection of control antibody [mouse immunoglobulin G2a (IgG2a)].
20. The dose of NGF administered, either as free protein or a conjugate with OX-26, was 6.2 μg per injection. We calculated this dose by estimating an optimal concentration of 5 × 10^{-10} M of NGF at the graft neuronal receptor site. This represents a 50-fold excess over the dissociation constant value of 10^{-11} M for the high-affinity NGF receptor in order to achieve a tissue level of NGF on the plateau of the dose-response curve. In the first series, five animals with transplants in both eyes received the OX-26–NGF conjugate, and four animals received OX-26 alone. Injections were given in the tail vein (0.5 ml per rat) at 13, 27, 41, and 55 days after transplantation. In the second series, 11 animals were injected in each group with OX-26–NGF, OX-26 alone, NGF alone, or saline at 14, 29, and 43 days after grafting. A one-way analysis of variance (ANOVA) was used to assess overall statistical significance of differences in growth among groups with $P < 0.05$. The Bonferroni post hoc test was used to compare means from individual groups.
21. The growth and survival of target-deprived basal forebrain cholinergic neurons in the anterior chamber of the rat eye is promoted by NGF administered into the intraocular fluid (*10*), and intraocular administration of NGF can increase the number of ChAT-positive cells in rat basal forebrain tissue grafts by approximately 80% over that observed in untreated control grafts (*10*).
22. Host rats were anesthetized with chloral hydrate (300 mg/kg i.p.), and graft tissue was fixed with a transcardial perfusion of paraformaldehyde (2%)

and picric acid (3%) in phosphate buffer. Transplants were dissected and sectioned on a cryostat. Sections were washed in phosphate-buffered saline (PBS) and incubated with antibody to rat IgG for 1 hour, washed, and incubated again with a monoclonal antibody against ChAT (Boeringer Mannheim; 1:10 in PBS with 0.2% Triton X-100) for 48 hours. They were washed and incubated with antibody to rat IgG conjugated with fluorescein (1:10 in PBS with 0.2% Triton X-100). Sections were washed and mounted in glycerin-PBS (9:1) and studied with an epi-illumination Nikon fluorescence microscope. With the use of a nonparametric measure of cholinergic neuron density (0 to 4) and ratings by a blinded observer, cholinergic neuron density in the conjugate group was determined to be 3.65 ± 0.71. There were no significant differences in the control groups; the average cholinergic neuron density was 1.14 ± 0.22.

23. B. H. Wainer *et al.*, *Neurochem. Int.* **6**, 163 (1984); J. Rossier, *ibid.*, p. 183.
24. Consideration of the role of transport of NGF from the circulation to the graft via the vasculature of the ciliary epithelium, with subsequent diffusion through the aqueous humor, is very relevant because the ciliary epithelium has been shown to possess several properties of brain blood vessels [S. I. Harik *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4261 (1990); G. Raviola, *Exp. Eye Res.* (suppl.) **25**, 27 (1977); G. Raviola and J. Butler, *Invest. Opthalmol. Visual Sci.* **25**, 827 (1984); M. Kupersmith and M. Shakib, in *Implications of the Blood-Brain Barrier and its Manipulation*, E. A. Neuwelt, Ed. (Plenum, New York, 1989), pp. 369–390], although the existence of transferrin receptors on ciliary vessels has not been examined. Several considerations suggest this mechanism is less likely than direct delivery through the brain graft vasculature. First, as shown by direct measurement [M. Kottler *et al.*, *Invest. Ophthalmol.* **9**, 758 (1970); F. J. Macri and J. O'Rourke, *Arch. Ophthalmol.* **83**, 741 (1970)], the aqueous humor turns over rapidly, with a half-life of substantially less than 1 hour. Thus, this route would be an inefficient means to deliver materials to the brain transplant, considering the rapid loss of the conjugate from the bloodstream. Second, as shown by direct histological examination, the transplants also develop a pia mater and glia limitans surface covering like intact brain. These membranes would further constitute diffusional barriers from the aqueous humor to the transplant. Third, in order to achieve trophic effects on the survival of medial septal brain transplants (*10*) direct intraocular administration of approximately 1 μM doses of NGF were required. In our experiments, the NGF conjugate concentration in the blood was in the range of only 10 to 100 nM, and our direct measurements of NGF brain levels after systemic administration of the conjugate only approximate a 20 to 40 pM level. Thus, greater than 1000 times more NGF was required for comparable effects when placed directly into the anterior chamber, which further supports the conclusion that within the anterior chamber a diffusional barrier exists for large molecules to pass into the transplant and that specific transport of the NGF conjugate across the BBB via the transferrin receptor is a much more efficient process. Still higher doses of NGF were required for medial septal neuron rescue in vivo after intracerebroventricular administration (*4, 5*).
25. We thank S. Putney, R. Pops, and R. Starzyk for helpful discussions and critical reading of this manuscript. Supported in part by a Small Business Innovation Research (SBIR) grant from NIH (NS29601-01).

24 August 1992; accepted 10 November 1992

Retinal Degeneration in Choroideremia: Deficiency of Rab Geranylgeranyl Transferase

Miguel C. Seabra, Michael S. Brown, Joseph L. Goldstein

Rab geranylgeranyl transferase (GG transferase) is a two-component enzyme that attaches 20-carbon isoprenoid groups to cysteine residues in Rab proteins, a family of guanosine triphosphate–binding proteins that regulate vesicular traffic. The mutant gene in human choroideremia, an X-linked form of retinal degeneration, encodes a protein that resembles component A of rat Rab GG transferase. Lymphoblasts from choroideremia subjects showed a marked deficiency in the activity of component A, but not component B, of Rab GG transferase. The deficiency was more pronounced when the substrate was Rab3A, a synaptic vesicle protein, than it was when the substrate was Rab1A, a protein of the endoplasmic reticulum. The data imply the existence of multiple component A proteins, one of which is missing in choroideremia.

Choroideremia (CHM) is an X-linked form of retinal degeneration that is subsumed under the broad classification of retinitis pigmentosa (*1*). Affected males experience night blindness in their teens, which usually progresses to tunnel vision or complete blindness by middle age. Histologically, there is degeneration of the retinal pigment epithelium and its two adjacent cell layers, the choroid which contains the blood vessels, and the retinal photoreceptor cells. Which of these three layers is the primary site of the disease is not known. Carrier females are generally asymptomatic, but they have patchy pigmentation and degeneration of the pigment epithelium and choroid consistent with the presence of clonal areas of disease attributable to random X-inactivation.

The defective gene in CHM was localized initially by linkage analysis and identified by positional cloning on the basis of information on deletions and translocations surrounding the locus at the chromosome Xq21 band (*2, 3*). Analysis of a partial cDNA encoded by the CHM gene isolated from a retinal cDNA library (*2, 3*) revealed that the CHM mRNA is expressed in cell types other than those of the retina, including Epstein-Barr virus–immortalized B lymphoblasts. The CHM 5.4-kb mRNA encodes a protein of more than 395 amino acids (*2, 3*) whose function has heretofore been unknown. The sequence of the CHM protein from the human and the mouse resembles that of the bovine protein designated *smg*p25/Rab3A GDI (*4, 5*), which was identified and purified (*6*) on the basis of its ability to inhibit the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) bound to Rab3A, a low molecular weight guanine nucleotide-binding protein present in synaptic vesicles (*7*).

The Rab family includes more than 20 sequence-related proteins (~25 kD) that are bound to the cytoplasmic surfaces of specific membranous organelles in all eukaryotic cells (*7*). By analogy to other guanine nucleotide-binding proteins, the Rab proteins are considered inactive in their GDP-bound form (*7*). When activated, the GDP is exchanged for GTP, and the Rab proteins then participate in the process by which membranous vesicles fuse with each other in a highly selective and unidirectional fashion. The Rab proteins become inactivated when they hydrolyze the bound GTP to GDP, allowing the cycle to be repeated (*7*).

In order for Rab proteins to bind to membranes, they must contain a covalently bound hydrophobic prenyl group, generally the 20-carbon isoprenoid geranylgeranyl (GG) (*8, 9*). Shortly after Rab proteins are translated, one or more GG groups are attached in thioether linkage to cysteine residues at or near the COOH-terminus (*8*). Rab3A, which terminates in Cys-Ala-Cys, contains GG groups on each of these cysteines (*8*). Rab1A, a protein of the endoplasmic reticulum, contains at least one GG group on its COOH-terminal Cys-Cys sequence (*8*).

Prenylation of Rab3A and Rab1A is catalyzed by a two-component enzyme called Rab geranylgeranyl transferase (GG transferase) that has been purified from rat brain (*10, 11*). Component B of this enzyme consists of two (60 and 38 kD) (*10*) tightly associated polypeptides whose amino acid sequences resemble those of the α and β subunits, respectively, of $p21^{ras}$ farnesyltransferase, another prenyltransferase (*12*). Component A of Rab GG transferase, which has no counterpart in the farnesyltransferase, is a 95-kD polypeptide (*11*). Component B has little, if any GG transferase activity in the absence of component A (*10, 11*).

Although the sequences of six peptides

Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235.