Intracranial Delivery of Recombinant Nerve Growth Factor: Release Kinetics and Protein Distribution for Three Delivery Systems

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Purpose. Three different polymeric delivery systems, composed of either poly(ethylene-co-vinyl acetate) (EVAc) or poly(lactide-co-glycolide) (PLGA), were used to administer recombinant human nerve growth factor (rhNGF) intracranially in rats.

Methods. The delivery systems were characterized with respect to release kinetics, both in the brain and in well-stirred buffer solutions.

Results. During incubation in buffered saline, the delivery systems released rhNGF in distinct patterns: sustained (EVAc), immediate ($PLGA_1$), and delayed ($PLGA_2$). One 10-mg delivery system was implanted in each rat and an ELISA technique was used to determine the amount of rhNGF in 1-mm coronal brain slices produced immediately after removal of the delivery system. High levels of rhNGF (as high as 60,000 ng in a brain slice of ~50 μL) were recovered from the brain tissue at 1, 2, and 4 weeks after implantation. With all three delivery systems, the amount of rhNGF in each brain slice decreased exponentially with distance from the implant site; the distance over which concentration decreased by 10-fold was 2–3 mm for all delivery systems. When rhNGF release was moderate (10 to 200 ng rhNGF/day), the total amount of rhNGF in the brain increased linearly with release rate, suggesting an overall rate of rhNGF elimination of 0.4 hr^{-1} or a half-life of 1.7 hr. With higher release rates (500 to 50,000 ng rhNGF/day), total amounts of rhNGF in the brain were considerably higher than anticipated based on this rate of elimination.

Conclusions. Polymeric controlled release can provide high, localized doses of rhNGF in the brain. All of the experimental data were consistent with penetration of rhNGF through the brain tissue with a diffusion coefficient $\sim 8 \times 10^{-7}$ cm^2/s, which is ~50% of the diffusion coefficient in water.

KEY WORDS: neurotrophin; controlled release; drug delivery; poly(lactide-co-glycolide); microspheres.

INTRODUCTION

Proteins and polypeptides, such as neurotrophins, may be useful in the treatment of central nervous system (CNS) disease (1). Most proteins have short half lives and penetrate slowly through tissue barriers (2), particularly the blood-brain barrier, making administration to the brain difficult. Therefore, new delivery methods are needed (3). Several delivery systems for direct administration of nerve growth factor (NGF) to the brain have been described, including poly(ethylene-co-vinyl acetate) (EVAc) disks (4–7), poly(lactide-co-glycolide) (PLGA) microspheres (8,9) and atelocollagen pellets (10). As a result, several alternate technologies for the long-term (i.e., >30 day) release of biologically active NGF from a localized depot are now available.

Studies of protein distribution after delivery in animals are essential for the characterization of intracranial protein delivery systems. Rates of polymer degradation and protein release may be different in tissue than in *in vitro* assay systems. The fate of protein drugs in the brain depends on multiple mechanisms (11); the relative importance of each mechanism is difficult to predict. In addition, the brain compartment is not well mixed (as are most *in vitro* measurement systems) and the composition of brain extracellular fluid may influence the rate of protein release (12). Previous studies have described the intracranial distribution of NGF after administration by intraventricular injection (13,14), infusion (14,15) and polymer delivery system implantation (6,7,16). NGF-releasing microspheres have been described (8,17), but the distribution of NGF in the brain after controlled delivery from microspheres has not been studied.

No previous studies have compared protein concentrations in the brain tissue after implantation of systems with different release characteristics. Therefore, in this study, the intracranial distribution of recombinant human NGF (rhNGF) delivered by biodegradable microsphere pellets was compared to the distribution obtained by implantation of non-degradable EVAc disks.

MATERIALS AND METHODS

Materials

Recombinant human nerve growth factor (rhNGF), rhNGF coating antibody and rhNGF antibody-horseradish peroxidase conjugate were provided by Genentech, Inc. (South San Francisco, CA). PLGA (75:25 w/w ratio, 85,900 M_w) was purchased from Birmingham Polymers Inc. (Alabama). EVAc was obtained from DuPont (Wilmington, DE) and prepared as described previously (18). Polyvinyl alcohol (PVA, 25,000 M_w, 88 mole % hydrolyzed) and carboxymethyldextran were purchased from Polysciences, Inc. (Warrington, PA). RPMI 1640 media, horse serum and fetal bovine serum were purchased from Gibco (Grand Island, NY). Methylene chloride was purchased from Aldrich Chemical Company (Milwaukee, WI). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

The anesthesia solution contained 14.2 ml of ethanol (95%), 2.5 ml of xylazine (100 mg/ml, Rompun, Mobay Company, Shawnee, KS), 25 ml of ketamine hydrochloride (100 mg/ml, Parke-Davis, Morris Plains, NJ) and 58ml sterile saline (0.9% NaCl). The solution was filter sterilized. The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Fabrication of Microspheres by Double Emulsion Solvent Evaporation Method

To make microspheres, 500 mg of PLGA was dissolved in 3-ml methylene chloride in a short glass test tube (5.8 cm

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long, diameter 1.2 cm). Carboxymethyl dextran or bovine serum albumin (BSA) was dissolved in less than 100 μL of deionized-distilled water. rhNGF was added to the dissolved BSA or dextran to create an 8:1 (protein/dextran: rhNGF) mixture. This mixture was added to the dissolved PLGA on ice and the solution was sonicated (Tekmar Sonic Disrupter model TM300, 40% duty cycle, microtip #4) for 10 sec to give a homogeneous milky mixture. One ml of aqueous 1% PVA was quickly added to this emulsion and vortexed for 10 sec at high speed. This double-emulsion was poured into a beaker with 100 ml aqueous 0.3% PVA stirring at high speed and the mixture was stirred for 3 hours to allow microsphere formation. The microspheres were collected by centrifuging the solution (including the foam) at 2000 rpm for 10 min and discarding the supernatant. The microspheres were rapidly frozen by swirling the centrifuge tubes in liquid nitrogen and then lyophilized for 16 to 24 hr. Microspheres with different loading were prepared similarly by increasing the amount of protein in the aqueous phase, while keeping the amount of polymer added constant. Particle size was determined by suspending a small quantity of microspheres in distilled water on a glass slide and measuring particle diameters by computerized image analysis. At least 140 spheres per sample were examined to estimate the size distribution; number-averaged quantities were determined. The morphology of microspheres was examined with scanning electron microscopy.

Fabrication of Microspheres by Spray Freeze-Drying Method

The encapsulation of recombinant human nerve growth factor (rhNGF) in poly (lactic co-glycolic acid) (PLGA) microspheres was performed using the Alkermes spray freeze-drying process (19). For preparation of the spray freeze-dried protein, a solution containing equal amounts of rhNGF and trehalose dihydrate was passed through an ultrasonic nozzle and atomized into liquid nitrogen. The frozen protein droplets were lyophilized to form dried, porous particles 2–5 μm in diameter and the dried powder contained 50% (w/w) rhNGF. For manufacturing of the microspheres, the spray freeze-dried powder, zinc carbonate salt, and trehalose dihydrate solid were homogenized into a solution of PLGA polymer (Boehringer Ingelheim, 12kDa, RG502H) in ethyl acetate to give a weight percentage of 10.1% rhNGF, 6.2% zinc carbonate, 13.8% trehalose, and 69.9% polymer. This emulsion was passed through an ultrasonic nozzle and atomized onto a frozen bed of ethanol contained in liquid nitrogen. The frozen ethanol was allowed to warm to −70°C for 24 hrs., where the resultant droplets (microspheres) were hardened by extraction of the ethyl acetate into ethanol. Further extraction of the organic solvent occurred for the next 48 hr with addition of an equal volume of −70°C ethanol. After 72 hr at −70 °C, the microspheres were collected and dried under a continuous flow of nitrogen at 5°C. The dried microspheres were then sieved through a 125 μm filter, placed in a vial, and stored at 5°C.

Fabrication of Microsphere Pellets

Microspheres were prepared by the double emulsion solvent evaporation method or the spray freeze-drying method described above. Approximately 10 mg of microspheres were placed in a mold and lightly compressed to form 3-mm diameter disks with a thickness ranging from 1 to 1.5 mm. The preservation of the spherical structures of the microspheres after mild compression was confirmed by light microscopy.

Fabrication of EVAc Disks

Five mg of rhNGF and 995 mg of BSA were dissolved in 15 ml of phosphate buffered saline (PBS) to form a 200:1 BSA:rhNGF mixture. The solution was quickly frozen in liquid nitrogen and then lyophilized for 26 hours. The lyophilized powder was ground and sieved to a particle size of <180 μm. EVAc was dissolved in methylene chloride to create a 10% (w/v) solution. The 200:1 BSA:rhNGF powder was added to the polymer solution at a concentration of 35% by weight, and the final mixture was vortexed for 10 seconds to form a homogeneous mixture. The polymer-protein mixture was then poured into a chilled mold. After the polymer solution had frozen, it was removed from the mold and the methylene chloride was evaporated for two days at −20°C, then for two days in a vacuum desiccator at room temperature. Small disks were punched from the EVAc matrix using a cork borer with an inner diameter of 3 mm.

Release of rhNGF from Delivery Systems into Buffered Saline

The rate of rhNGF release from the polymer delivery systems was determined by incubating EVAc disks (6.20 ± 0.5 mg), microsphere pellets prepared by w/o/w solvent evaporation method (5.3 mg), or microsphere pellets prepared by the spray freeze-drying method (12.0 ± 2.4 mg) in 4 ml of phosphate-buffered saline with 1 w/v% BSA and 0.02 wt% gentamicin sulfate in polypropylene centrifuge tubes. *In vitro* release profiles were obtained from freely suspended PLGA microspheres similarly. The centrifuge tubes were placed in a shaker-incubator at 37°C. Periodically, the buffer was replaced with fresh buffer; changes were performed frequently enough to minimize changes in pH. The concentration of rhNGF in the buffer was determined by rhNGF ELISA, using the protocol developed by Genentech, Inc.

Quantification of rhNGF by ELISA

Each well of a 96-well microtiter plate (Maxisorp™ Nunc-Immuno Plate II) (PGC, Frederick, MD) was coated with 100 μl of 1 μg/ml anti-rhNGF, incubated at 4°C overnight, and washed with 200 μl of wash buffer (phosphate buffered saline, PBS, with 0.05% Tween 20) for 5 min. After three washes, each well was blocked by adding 200 μl of assay diluent (PBS with 0.5% BSA, 0.05% Tween 20 and 0.01% Thimerosal) to each well and incubating for 90 min at room temperature. The plate was washed three times, blotted thoroughly, and 100 μl of sample (diluted to a range of 0.5–15 ng/ml) was added to each well. Standards with a concentration range of 0.097 ng/ml to 50 ng/ml were also prepared. The samples and standards were incubated for 2 hr at room temperature, the plate was washed three times with wash buffer, and 100 μl of anti-rhNGF antibody-horseradish peroxidase conjugate solution (82 ng/ml) was added to each well. The plate was then incubated for 2 hr at room temperature, washed three times, and 100 μl of a substrate solution (prepared by dissolving one 5 mg OPD tablet in 12.5 ml of 4 mM 30% w/w H_2O_2 in PBS) was added to

each well in the dark. The substrate solution was incubated in the dark at room temperature for 20 min, the reaction was stopped by adding 100 μl of 4.5 N sulfuric acid to each well, and the optical density of the solution in each well was measured (Molecular Devices Corporation, Menlo Park, CA), using two filters, 495 nm for absorbance and 405 nm for reference. The amount of rhNGF in the brain tissue and the blood was quantified similarly, except the rhNGF standards were diluted with blank brain homogenate and blood respectively to account for species capable of non-specific binding to rhNGF antibody.

Test of Biological Activity of Encapsulated rhNGF by PC12 Cell Assay

To demonstrate the biological activity of rhNGF after encapsulation, in vitro release samples (i.e., PBS supernatants that had been incubated with polymer delivery systems for various periods) from the microspheres were exposed to pheochromocytoma (PC12) cells (Paragon Biotech, Baltimore, MD) for 5 days. Six-well tissue culture polystyrene plates were coated with collagen at a concentration of 50 μg/well. PC12 cells were seeded at a density of 1×10^6 cells/well in 2 ml of RPMI 1640 medium supplemented with 10% horse serum and 5% fetal bovine serum. Two days after the PC12 cells were seeded, the medium was replaced with 2 ml of *in vitro* release samples diluted in fresh medium. The medium was replaced every two days over the course of 7 days, and neurite outgrowth was monitored.

Surgical Implantation of EVAc Disks and Microsphere Pellets

NGF-loaded microsphere pellets or EVAc disks were implanted to the brains of 6–7 week old male Fisher 344 rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN). EVAc disks weighed 6.0 ± 0.3 mg each (3.4 mm diameter, ~0.8–1 mm thick), while microsphere pellets ($PLGA_1$, 3 mm diameter, 1–1.5 mm thick) prepared by double emulsion solvent evaporation method weighed 6.8 ± 0.5 mg each, and microsphere pellets prepared by the spray freeze-drying method ($PLGA_2$) weighed 10.8 ± 1.3 mg each. EVAc disks and microsphere pellets containing only BSA, weighing approximately 8–9 mg each, were implanted in control rats. The rats were anesthetized with 0.7–0.8 ml of xylazine/ketamine solution intraperitoneally as above. A 3-cm incision was made to expose the bregma. A hole with a 5 mm diameter was drilled 5 mm posterior to the bregma and 3 mm lateral from the sagittal suture. Drilling was halted before piercing the dura. A 5 mm slit parallel to the midline was made with a scapel, followed by a 3 mm slit made in the brain tissue using the pointed end of the #10 scapel blade. The EVAc disk or microsphere pellet was inserted into this slit until the brain tissue covered it. The scalp was then closed with surgical staples. The rats were monitored closely for several hours following microinjection and were given ample food and water. All procedures were reviewed and approved by the Johns Hopkins Animal Care and Use Committee in accordance with NIH guidelines.

Preparation of Brain Tissue Samples

After 1, 2 or 4 weeks, the rats were anesthetized with xylazine/ketamine solution and were sacrificed by exsanguination in the absence of pedal reflex. Blood samples were collected by cardiac puncture. The brains were removed, quickly frozen in hexane at −70°C and weighed. The brains were stored at −70°C and subsequently sectioned into 15 ipsilateral and 15 contralateral coronal sections, each approximately 1 mm in thickness. Each section was weighed and placed in an extraction buffer containing 100 mM Tris HCl, 400 mM NaCl, 2 (w/v)% BSA, 0.05 (w/v)% sodium azide, 7 μg/ml aprotinin, 1 mM PMSF dissolved in dimethylsulfoxide and 4 mM EDTA at pH 7. Each brain section was homogenized for 30 to 40 seconds using a sonicator (Tekmar Sonic Disrupter model TM300, 40% duty cycle, microtip #4). The brain homogenate was centrifuged at 12000 rpm for 20 minutes at 4°C. The supernatant was diluted 1:1 with a solution of 20 mM $CaCl_2$ and 0.2 (w/v)% Triton® X-100. The final solution was stored at −20°C until the rhNGF ELISA was performed.

RESULTS

Characteristics of the Delivery Systems

Polymer discs, microspheres and microsphere pellets were produced from biocompatible materials: EVAc and PLGA. PLGA microspheres were prepared by two different techniques: double emulsion/solvent evaporation ($PLGA_1$) and spray freeze-drying ($PLGA_2$, provided by Genentech, Inc.). Ninety percent of the $PLGA_1$ microspheres were 4.8 to 59 μm in diameter with a number average diameter of 19 μm. Ninety percent of the $PLGA_2$ microspheres were 0.6 to 14 μm in diameter with a number average diameter of 6.3 μm. The majority of the microspheres were spherical, but the surfaces were somewhat irregular: pits were found on the surface of microspheres when observed by scanning electron microscopy (data not shown).

Active rhNGF was continuously released from EVAc disks and PLGA microspheres (Fig. 1); the three delivery systems released rhNGF into buffered saline at different rates, which varied with time. EVAc disks provided a sustained release of rhNGF for longer than 2 months (Fig. 1a): mass release was proportional to the square root of time. $PLGA_1$ microspheres gave an immediate burst (Fig. 1b): ~10% was released in the first hr, ~20% in the first day. The immediate release was followed by a slow, but steady, release of rhNGF. $PLGA_2$ microsphere pellets produced delayed release: the smallest initial burst (Fig. 1c: <1% in the first 24 hr) was followed by a second phase of rhNGF release, with the largest rate of release occurring 10 to 14 days after immersion in saline.

Mild compression to form implantable pellets did not alter the pattern of NGF release in either PLGA microsphere system (data not shown). The PLGA microsphere pellets were prepared to be similar in size to the EVAc disks, which contained 11,000 ng of rhNGF. The pellets were designed to contain 140,000 ng ($PLGA_1$) and 1,200,000 ng ($PLGA_2$), although it is possible that some of this material is lost during the encapsulation process, particularly during the solvent extraction step used to produce $PLGA_1$ spheres.

Release and Distribution in the Rat Brain

The three polymer delivery systems produced distinct patterns of rhNGF release *in vitro*: immediate release ($PLGA_1$), sustained release (EVAc), and delayed release ($PLGA_2$). To

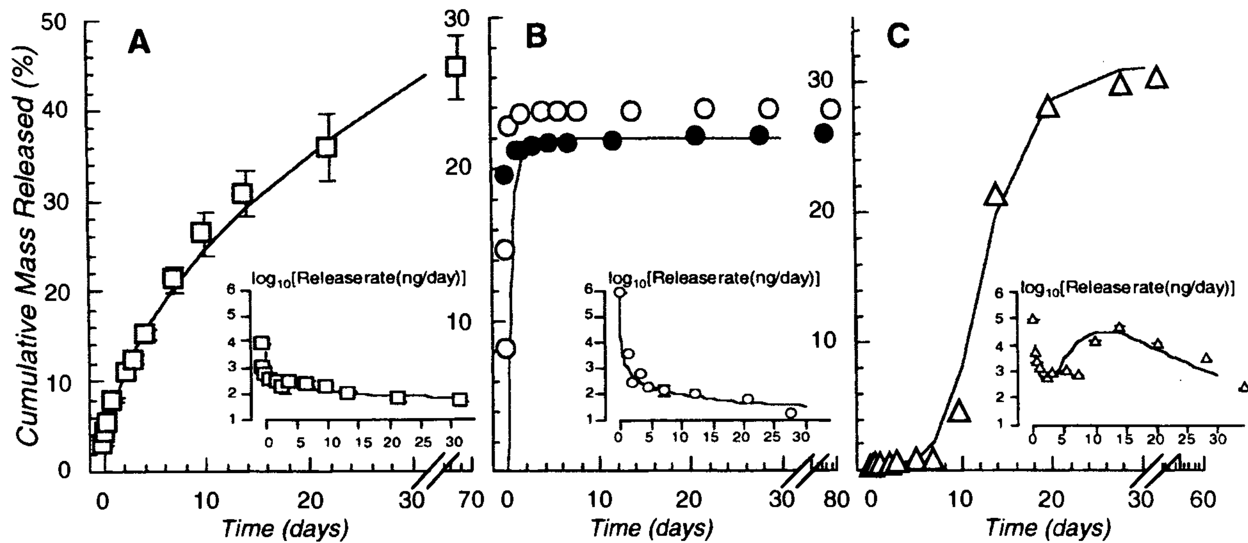

Fig. 1. Controlled release of rhNGF from polymer delivery systems into buffered saline. In all cases, the cumulative percent of rhNGF released was calculated based on the theoretical dose. A) The cumulative percent rhNGF released is plotted versus time for EVAc disks with 35% loading of 200:1 BSA:rhNGF powder (loading of rhNGF = 0.175%). The solid line shows that release follows the anticipated kinetics for diffusion from a slab: $\% \ released = 7\sqrt{t}$. B) Cumulative percent released from $PLGA_1$ microspheres (○) and pellets (●) with 23.9% loading of 8:1 carboxymethyldextran: rhNGF (loading of rhNGF = 3%). The solid line shows the function: $\% \ released = 20.82t^{0.018}$. C) Cumulative percent release from $PLGA_2$ microsphere pellets with 8.9% loading of pure rhNGF (▲). The solid line shows the function: $\% \ released = 0.482t^{0.131} - 1 + e^{0.0059t} + 30.4t^{5.0}/(288{,}000 + t^{4.96})$. The insets on each panel compare *in vitro* rates of rhNGF release ($\dot{R} = dM/dt$ where M is the cumulative amount of rhNGF released from the implant) from EVAc disks (□), $PLGA_1$ microsphere pellet (○) and $PLGA_2$ microsphere pellet (Δ). The solid lines indicate the release rate calculated from the derivative of the expressions provided above.

examine the impact of pattern of release on rhNGF delivery to the brain tissue, EVAc disks and pellets formed from PLGA microspheres were implanted into the brains of healthy rats. All rats survived to the scheduled time of sacrifice and no animals exhibited any unusual behavior during treatment. The polymers were completely removed from the rats implanted with EVAc and $PLGA_1$ pellets before the brain tissues were sectioned and analyzed. The $PLGA_2$ microsphere pellets frequently crumbled during removal: this problem occurred in one of the three rats sacrificed 1 week after implantation and all of the rats sacrificed 2 and 4 weeks after implantation.

After removal of the drug delivery system, total rhNGF content was measured in 1mm-thick sections from the hemisphere in which the delivery system was implanted (Figs. 2–4) and the opposite hemisphere. In all cases, the rhNGF content in the ipsilateral hemisphere was highest in sections closest to the implantation site and decreased with distance from the implantation site (Figs. 2–4). Over the course of the 28 day experiment, the mass of rhNGF in the sections closest to the polymer decreased 8-fold for rats implanted with EVAc disks, 11-fold for those with $PLGA_2$ microsphere pellets and 100-fold for those with $PLGA_1$ microsphere pellets. $PLGA_1$ microsphere pellets released extremely low levels of rhNGF towards the end of 28 days such that no rhNGF was found in one of the animals. Barely detectable levels of rhNGF (0.1 to 1 ng) were present in sections from the contralateral hemisphere immediately adjacent to EVAc disks and $PLGA_1$ microsphere pellets implant (data not shown). But substantial levels of rhNGF (1 to 1000 ng) were measured in almost every section from the contralateral hemisphere of rats implanted with $PLGA_2$ microsphere pellet.

Concentrations of rhNGF in the brain were normalized by dividing the amount of rhNGF in each section by the amount of rhNGF in the section with the highest value. One week after the implantation, the normalized distribution of rhNGF released from the three polymer implants was similar and consistent with the exponential drop with distance from the source expected for diffusion/elimination (Fig. 5). The penetration distance of rhNGF in brain tissue, which was defined as the distance at which the rhNGF concentration dropped to 10% of the value at the surface of the implant, was ~2–3 mm rostrally and caudally from the polymer implant (Table 1).

Except for $PLGA_2$ microsphere pellets, which could not be completely recovered from the rats 2 and 4 weeks after implantation, the amount of rhNGF remaining in the implant after removal from the brain was estimated either by placing the polymer in buffer for in vitro release or by sonication (Table 1). This rhNGF was biologically active as determined by stimulation of neurite outgrowth in PC12 cells.

Correlations Between *In Vitro* and *In Vivo* Release Rate

$PLGA_1$ microspheres gave the largest initial burst followed by a sharp decrease in release rate, whereas the release rate of rhNGF from EVAc discs decreased more slowly with time (Fig. 1). In both systems, the gradual decrease in release rate produced a decrease in peak rhNGF content in the brain sections closest to the polymer: peak concentrations dropped by 55% for EVAc disks and 95% for $PLGA_1$ microsphere pellets from week 1 to week 2 (Figs. 2 and 3). The relationship between release and rhNGF level in the brain was not as obvious for $PLGA_2$ microspheres: the peak rhNGF content in brain sections closest to

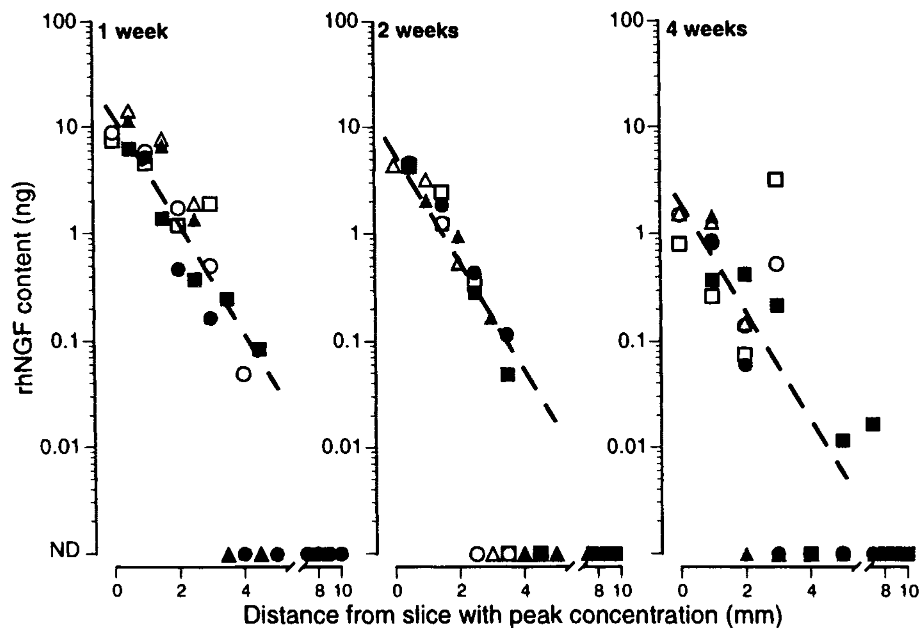

Fig. 2. Distribution of rhNGF in the ipsilateral hemisphere following delivery by 35% loading EVAc disks. Each symbol represents the mass of rhNGF in one 1 mm-thick brain sections of one rat. The dashed line represents the solution to the diffusion/elimination equation (equation 1), with the single parameter $\sqrt{k/D}$ = 1.2 mm^{-1}.

the $PLGA_2$ microsphere implant dropped by 53% from week 1 to week 2, despite the second burst of rhNGF release observed *in vitro* (Fig. 1c). Moderate release rates, between 10 and 200 ng/day, were achieved using either EVAc or $PLGA_1$ pellets; for these systems, total NGF in the brain was linearly related to release rate measured *in vitro* (Fig. 6a).

Significant quantities of rhNGF were detected in the blood of rats implanted with EVAc or PLGA systems (Fig. 6b). Although the rate of release, and therefore the total amount of rhNGF, was up to 1000 times higher for $PLGA_2$ implants, the concentration of rhNGF in the blood was only about 10 times higher in animals receiving $PLGA_2$ implants (Fig. 6b). We did,

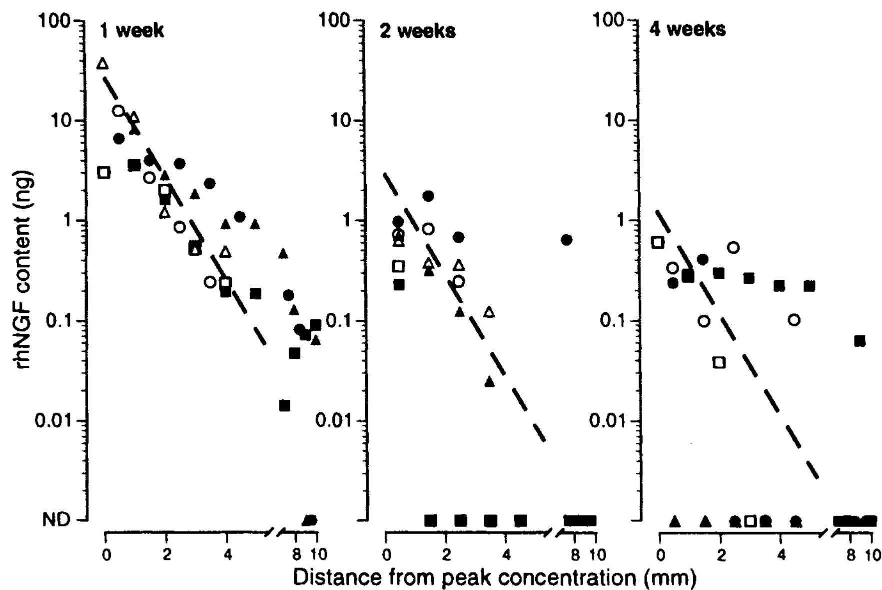

Fig. 3. Distribution of rhNGF in the ipsilateral hemisphere following implantation of $PLGA_1$ microsphere pellets. Each symbol represents the mass of rhNGF in each 1 mm-thick brain section of one rat. The dashed line represents the solution to the diffusion/elimination equation (equation 1), with the single parameter $\sqrt{k/D}$ = 1.2 mm^{-1}.

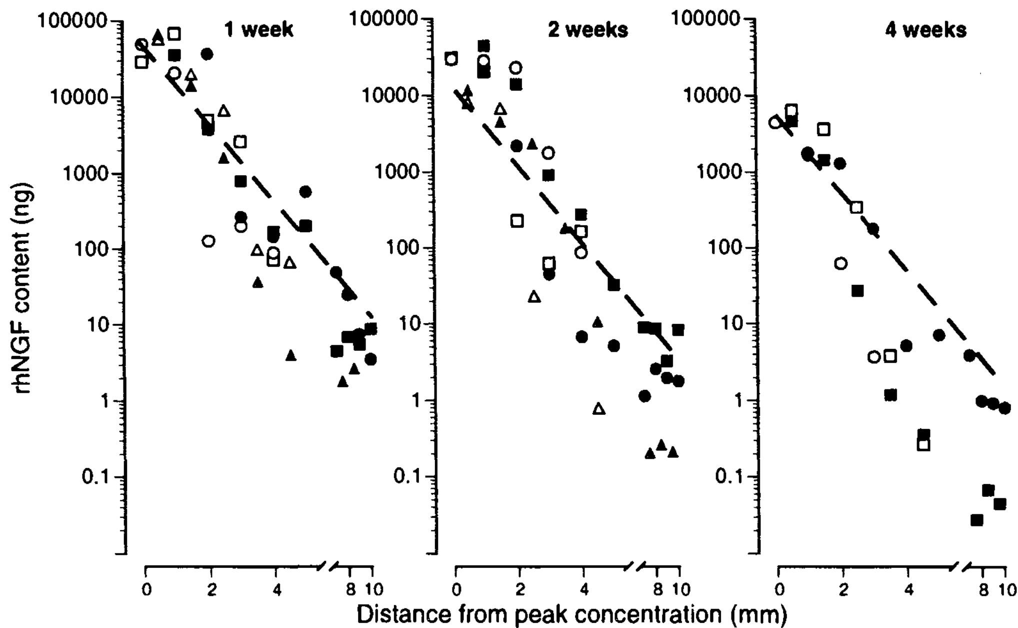

Fig. 4. Distribution of rhNGF in the ipsilateral hemisphere following delivery by $PLGA_2$ microsphere pellets. Each symbol represents the mass of rhNGF in each 1 mm-thick brain section of one rat. The dashed line represents the solution to the diffusion/elimination equation (equation 1), with the single parameter $\sqrt{k/D} = 1.2\ mm^{-1}$.

however, observe redness of the paws, tails and ears in all of the animals with $PLGA_2$ microsphere pellets on day 13 after implantation. No other changes were observed in any of the animals; the redness gradually subsided and completely disappeared by 28 days after implantation.

DISCUSSION

In previous studies, using EVAc delivery systems and identical methods of analysis, roughly 5 ng of rhNGF was measured after 1 week in the 1-mm thick tissue section nearest the intracranial implant: this bulk measurement reflected a local concentration of ~10,000 ng/mL in the tissue immediately adjacent to the delivery device (6). In this study, the EVAc delivery system provided similar levels of rhNGF in tissue near the implant; the $PLGA_2$ delivery system provided even higher levels, up to 60,000 ng of rhNGF in the 1-mm section nearest the implant. Assuming standard relationships between local point-wise concentration and bulk concentration in the tissue (as described in more detail elsewhere (20)), rhNGF concentrations of 10–100 mg/mL—near the expected solubility of the protein—may be possible at the $PLGA_2$ implant surface.

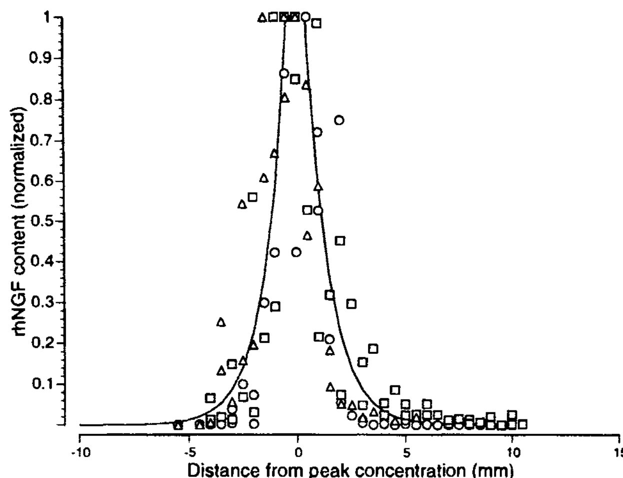

Fig. 5. Comparison of normalized concentration profiles of rhNGF in the ipsilateral hemisphere 1 week after implantation of EVAc disks, $PLGA_1$ microsphere pellet, or $PLGA_2$ microsphere pellet. The implant material was removed before the content of rhNGF was measured in animals receiving EVAc disks (□), $PLGA_1$ microsphere pellet (○) and $PLGA_2$ microsphere pellet (△).

For each delivery device, concentrations of rhNGF were highest in the brain tissue near the implant, and were substantially lower in tissue sections obtained rostral or caudal to the implant site. Within 2–3 sections (1-mm thick) of each implant, concentrations of rhNGF dropped to 10% of peak concentrations. This data is consistent with concentration profiles predicted by a simple steady-state mathematical model based on diffusion and first-order elimination (6,7):

$$C = C_0 \exp\left[-x\sqrt{\frac{k}{D}}\right] \qquad (1)$$

where, C_0 is the concentration at the implant site, k is the rate constant for elimination (assuming first-order elimination), and D is the diffusion coefficient for rhNGF in the brain tissue. The dashed lines in Figs. 2, 3, and 4 represent equation 1 with the parameter $\sqrt{k/D}$ set equal to $1.2\ mm^{-1}$, a value comparable to values obtained in other studies (6,7,16).

The mechanisms of rhNGF elimination from the brain are not known, but previous studies suggest a half-life of ~1 hr (6). Assuming quasi-steady-state, which would require that the

Table 1. Penetration Distance of rhNGF in the Ipsilateral Hemisphere After Implantation of EVAc Disks or Microsphere Pellets, and Percentage of rhNGF Recovered from Explanted Material

	EVAc disk	$PLGA_1$ pellet	$PLGA_2$ microsphere pellet
Penetration distance(mm)			
1 week			
R	2.2 ± 0.6	2.7 ± 1.3	2.2 ± 0.5
C	2.3 ± 0.5	2.3 ± 1.2	1.7 ± 1.0
2 weeks			
R	2.3 ± 0.6	1.7 ± 0.2	1.7 ± 0.2
C	2.0 ± 0.4	2.7 ± 1.9	2.5 ± 0.0
4 weeks			
R	2.0 ± 0.7	2.3 ± 2.3	2.0
C	2.0 ± 0.0	—	1.5
Residual rhNGF in explant (%)			
1 week	34 ± 10	0.20 ± 0.07	37 ± 22
2 weeks	13 ± 0.3	0.18 ± 0.01	—
4 weeks	8 ± 4	0.15 ± 0.04	—
Total rhNGF in brain tissue (%)			
1 week	0.31 ± 0.08	0.024 ± 0.013	17 ± 2
2 weeks	0.12 ± 0.008	0.002 ± 0.001	8.6 ± 3.3
4 weeks	0.05 ± 0.02	0.0005 ± 0.0004	1.2 ± 0.3

Note: Penetration was defined as the distance at which the rhNGF content was 10% of the maximum at that time point. Each distance was the average value ± standard deviation obtained from three rats, except for the penetration distances of rhNGF released from microsphere pellets 4 weeks after implantation (average obtained from 2 rats). If the rhNGF content dropped to 10% between the sections obtained 2 and 3 mm from the polymer, the penetration distance was designated as 2.5 mm. R = rostral to implant; C = caudal to implant. Residual rhNGF in polymer implants, total rhNGF in the brain, and rhNGF concentrations in blood are the average ± standard deviation from three animals, unless otherwise noted.

overall rate of release into the brain is equal to the rate of elimination from the brain, yields a simple relationship between release rate, $\dot{R}$, and k:

$$\dot{R} = k\overline{C} \tag{2}$$

where $\overline{C}$ is the total concentration of rhNGF in the brain. The data are consistent with a rate constant k of 0.4 hr^{-1}, or a half-life of 1.7 hr (Fig. 6). The parameter $\sqrt{k/D}$, which was estimated from the pattern of distribution (equation 1), and the elimination rate constant, which was estimated from the relationship between release rate and total concentration in the brain (equation 2), can be combined to yield an estimate of the diffusion coefficient in the brain, D, of ~8×10^{-7} cm^2/sec. This diffusion coefficient is quite reasonable, in that it is slightly less than the diffusion coefficient of rhNGF in water, 13×10^{-7} cm^2/sec.

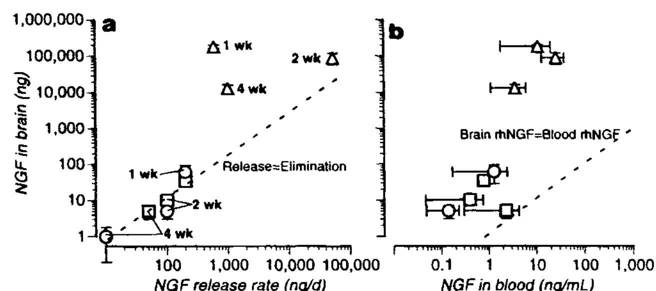

Fig. 6. Total amount of rhNGF in the brain compared to A) release rate from the polymer and B) concentration of rhNGF in the blood. Symbols represent data obtained with EVAc disks (□), $PLGA_1$ microsphere pellet (○) and $PLGA_2$ microsphere pellet (△). In panel A, the dashed line represents linear correlation (i.e. line of slope 1 on log-log plot) between release rate and total rhNGF in the brain. In panel B, the dashed line represents equal concentration in the brain and blood with the assumption that total volume of a hemisphere is 0.6 mL.

NGF can cross the blood-brain barrier, although very slowly (21,22): the permeability-surface area product (PS) for blood-to-brain transport is 2×10^{-6} ml/g-s. In our experiments, moderate levels of rhNGF were detected in the blood of rats (Table 1 and Fig. 6b). The observed blood levels were less than the concentration in the brain (dashed line Fig. 6b, assuming a total hemisphere volume of 0.6 mL) and higher than predicted by assuming that the rate of brain-to-blood permeation (with PS = 2×10^{-6} mL/g-s) is exactly balanced by the rate of rhNGF elimination from the blood (using an expression analogous to equation 2, but with $k = \ln(2)/t_{1/2,\text{plasma}}$). This finding suggests that mechanisms other than BBB permeation may be important in moving rhNGF from the brain interstitium into the blood. Previous investigators have measured substantial rates of protein transport from the brain into cervical lymphatics and, subsequently, blood (23,24); these mechanisms may be important for rhNGF clearance after delivery by polymeric implants, as well. A high dose of NGF, represented here by the $PLGA_2$ microspheres which give a burst of rhNGF between day 10 and day 13 of release, may lead to systemic activity. The highest systemic rhNGF concentrations were observed in animals receiving $PLGA_2$ implants; these animals had noticeable side effects (i.e., reddening of the ears and paws) at two weeks after implantation of $PLGA_2$ microsphere pellets, corresponding to the time of the large burst of NGF (Fig. 1c) and the highest blood levels

(Fig. 6b). These side effects are consistent with previous studies: NGF is known to influence several aspects of the acute inflammatory response in rats (25) and intravenous administration of rhNGF in humans caused myalgias whose duration and severity varied in a dose-dependent manner (26). Importantly, direct intracranial delivery of rhNGF by implanted polymer materials produced extremely high concentrations in the CNS, with only slight side effects at the highest doses.

In summary, our experimental data, obtained with three different polymeric delivery systems, is consistent with the following pharmacokinetic analysis (Fig. 7). Molecules of rhNGF were released from the implant into the interstitial tissue of the brain; release rates correlated with local rhNGF concentrations measured in tissue sections (Fig. 2–4) and bulk concentrations in the brain hemisphere (Fig. 6). Local distribution of rhNGF occured by diffusion through the tissue interstitium ($D \sim 8 \times 10^{-7}$ cm^2/sec) and first-order elimination ($t_{1/2}$ = 1.7 hr). Elimination may be a saturable process: higher than anticipated quantities of rhNGF accumulated in the brain (Fig. 6a), and lower than anticipated quantities were detected in the blood (Fig. 6b), during periods of high release rate provided by the PLGA2 delivery system. For the PLGA1 and EVAc systems, blood concentrations correlated with the total amount of rhNGF in the brain (Fig. 6b), although concentrations were near the detection limit, so estimation of the rate of brain-to-blood transport was not possible. NGF molecules reach the brain tissue in the hemisphere opposite of the implant. In most cases, these low concentrations probably represent the small quantity of rhNGF that was present in the vasculature during extraction of rhNGF. In cases where rhNGF concentration is high, as in the animals examined two weeks after implantation of $PLGA_2$ systems, rhNGF may enter the contralateral hemisphere by interstitial diffusion or transport of rhNGF molecules that entered the blood or CSF in the region of high rhNGF near the implant, as we have observed with high-dose chemotherapy using similar delivery systems (20).

The polymeric devices described in this study produced predictable, continuous, and sustained rhNGF levels in brain tissue. Generally, the rates of rhNGF release were greater than

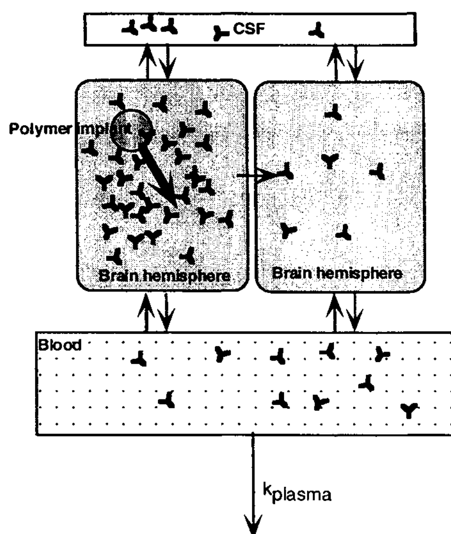

Fig. 7. Schematic diagram of the pattern of rhNGF distribution in the body after implantation of a polymeric delivery system.

100 ng/day, and occasionally as high as 100,000 ng/day. Doses within this range of rhNGF have some efficacy in treating animal models of Alzheimer's disease (27–29): for example, intraventricular administration of 100 ng/day of rhNGF produced 50% recovery of basal forebrain cholinergic neurons in primates, whereas higher doses (100,000 to 1,000,000 ng/day) led to complete recovery (30). In a recent preliminary clinical study, topical application of NGF (10 μg/eye, 6 to 10× daily) led to healing of corneal neurotrophic ulcers (31). Polymeric mircospheres or matrices, such as the ones described here, may provide sufficient doses for long-term beneficial effects, even after a single administration.

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