

# Selective Destruction of Nerve Growth Factor Receptor-bearing Cells In Vitro Using a Hybrid Toxin Composed of Ricin A Chain and a Monoclonal Antibody Against the Nerve Growth Factor Receptor

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**ABSTRACT** A hybrid toxin composed of ricin A chain and a monoclonal antibody directed against the rat nerve growth factor (NGF) receptor (192-IgG) was prepared using the hetero-bifunctional cross-linking agent *N*-succinimidyl-3-(2-pyridyldithio)-propionate and purified by affinity chromatography. Characterization studies showed that the hybrid, 192-s-s-A, displaced bound <sup>125</sup>I-labeled 192-IgG from rat superior cervical ganglion (SCG) membranes with an IC<sub>50</sub> 3–5 times lower than that of unconjugated 192-IgG. When incubated with cultured rat SCG neurons, 192-s-s-A inhibited protein synthesis in a concentration-dependent fashion. The effect of 192-s-s-A on these neurons was reversed by coincubation with an excess of 192-IgG. The IC<sub>50</sub> of 192-s-s-A on protein synthesis in rat SCG neurons was 4 nM. Intact ricin and ricin A chain inhibited protein synthesis in these neurons with IC<sub>50</sub> values of 5 pM and 500 nM, respectively. The 192-s-s-A hybrid had no effect on mouse SCG neurons or a human melanoma cell line known to have NGF receptors. This is consistent with the finding that 192-IgG recognizes only the rat NGF receptor. Also, 192-s-s-A did not inhibit protein synthesis in primary cultures of rat skeletal muscle or Vero cells, which do not have cell surface receptors for NGF. 192-s-s-A was able to inhibit protein synthesis in PC12 cells but the potency was 10–100 times less in these cells compared to rat SCG neurons. Ricin and A chain were also 10–100 times less potent in PC12 cells than neurons.

Rat SCG neurons exposed to 192-s-s-A lost their refractile appearance under phase-contrast optics, showed granular degeneration of neurites, and died. Thus the decreased protein synthesis caused by the hybrid toxin correlated with the morphological destruction of the neurons.

192-s-s-A represents a potentially powerful tool by which to selectively destroy NGF receptor-bearing cells in vitro. The hybrid toxin may prove useful as an in vivo toxin.

A recent strategy used to deliver toxins to specific cell types has been to couple the toxic subunit of certain plant or bacterial toxins with a hormone or a monoclonal antibody directed against a cell surface component of a particular cell type (14). Ricin is one such toxin which is composed of a

binding (B chain) and a toxic (A chain) subunit linked by a disulfide bond. The B chain binds to cell surface galactose residues and facilitates the entry of the A chain. Once inside the cell, the A chain enzymatically inactivates ribosomes which ultimately leads to cell death through inhibition of

protein synthesis (13). The rationale for constructing hybrid toxins is to replace the B chain of ricin with an alternate carrier that will deliver the A chain to specific cell types and bring about their destruction. Such studies have been carried out using hormones; for example, human chorionic gonadotropin (hCG)<sup>1</sup> (12) and epidermal growth factor (EGF) (4) have both been coupled to A chain. In both instances the hormone was responsible for specifically directing toxicity to cells bearing hCG or EGF receptors. In a striking example of hybrid toxin strategy Krolick et al. (7) have linked ricin A chain to monoclonal antibodies directed against a murine B cell tumor and showed this hybrid to be effective both in vitro and in vivo against tumor cell proliferation. As with the hormone-A chain hybrids, the antibody was responsible for directing the toxin to the tumor cells.

In the present study we have synthesized and characterized a hybrid toxin composed of ricin A chain coupled to a monoclonal antibody (192-IgG) directed against the rat pheochromocytoma nerve growth factor (NGF) receptor (5). In a previous paper 192-IgG was shown to bind to NGF receptors and to be transported to the cell body of NGF receptor-bearing cells in vivo (17), thus, providing a potentially efficient delivery system of the A chain to NGF receptor-bearing cells. The hybrid toxin which we have synthesized in this report specifically destroys NGF receptor-bearing neurons in vitro and may be a useful tool to probe the biology of NGF. The results are discussed in terms of the possible in vivo applications of this hybrid molecule.

## MATERIALS AND METHODS

**Synthesis and Purification of 192-s-s-A Hybrids:** The monoclonal antibody, 192-IgG, was purified as previously described (5, 17). Ricin toxin and pure A chain were purified from castor beans (Hummert Seed Co., St. Louis, MO) by the method of Simmons and Russell (16). A chain was stored in 1 M mercaptoethanol to avoid oxidation to A chain dimers. Prior to use, A chain was desalted on a Sephadex G-25 molecular exclusion column. The heterobifunctional cross-linking agent *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pharmacia Fine Chemicals, Piscataway, NJ) was added in a 12-fold molar excess to 192-IgG in 20 mM phosphate-buffered saline (PBS), pH 7.4. The modification of 192-IgG was allowed to proceed for 1 h at 22°C. Modified 192-IgG was then dialyzed against PBS to eliminate unreacted SPDP. Concentrated, desalted ricin A chain was added to modified 192-IgG in a 3–5-fold molar excess at pH 8.0 for 2 h at 22°C. The 192-s-s-A hybrid was purified using sequential immunoaffinity columns. Briefly, affinity-purified polyclonal antibodies prepared against 192-IgG or ricin A chain were linked to sepharose 4B by the cyanogen bromide method (1). The 192-s-s-A reaction mixture was passed over the affinity columns at room temperature, washed with PBS, and eluted with 4 M MgCl<sub>2</sub> in sodium acetate buffer, pH 5.5. Fractions containing 192-s-s-A were dialyzed against PBS containing 0.5 M NaCl. Purity of the 192-s-s-A hybrid was confirmed by SDS PAGE (8). Proteins on the gel were stained with 0.1% Coomassie Blue and destained in 20% methanol and 10% acetic acid. 192-s-s-A was stored at 4°C and remained stable for at least 3 wk. 192-s-s-A was quantitated by measuring its ultraviolet absorption at 280 nm with an extinction coefficient of 1.2. This extinction coefficient is based on the molecular weight equivalents of 192-IgG and A chain, whose individual extinction coefficients were assumed to be 1.38 and 0.765, respectively.

**Preparation of Primary Dissociated Superior Cervical Ganglia (SCG) Cultures:** SCG were dissected from prenatal rats (E 21) under sterile conditions. Whole ganglia were washed twice in Hanks' balanced salt solution in bicarbonate-free HEPES buffer (Gibco Laboratories, Grand Island, NY), pH 7.2. Ganglia were then incubated with 1 mg/ml of collagenase (Worthington Biochemical Corp., Freehold, NJ) at 37°C for 20 min followed

by a 0.02% trypsin (Worthington Biochemical Corp.) incubation for 20 min. Ganglia were washed and triturated with a reduced-bore pipet. Dissociated neurons were plated out on collagen-coated 24-well Costar culture dishes (Cambridge, MA) at a density of 1/3–1/2 SCG per well. The culture medium consisted of 90% Eagle's minimal essential medium (Gibco Laboratories) with 10% fetal calf serum (Armor Biochemicals, Kankakee, IL), 100 U/ml of penicillin (Sigma Chemical Co., St. Louis, MO), 100 µg/ml of streptomycin (Sigma Chemical Co.), 2 mM glutamine, 20 µM fluorodeoxyuridine as an antimetabolic, and 50 ng/ml NGF. Cells were maintained in this medium in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air for 6 d before being subjected to quantitative bioassay of 192-s-s-A, ricin, and A chain. This was ample time for the fluorodeoxyuridine to eliminate nonneural, dividing cells (e.g., fibroblasts).

Mouse primary SCG cultures were prepared from 1-d-old mice as described above except that Gibco 309 fetal calf serum was used.

**PC12 Cells:** PC12 cells were maintained in 85% Dulbecco's modified Eagle's medium (Gibco Laboratories) with high glucose/low bicarbonate concentrations, 10% fetal calf serum (Hazleton Systems, Inc., Denver, PA), 5% heat inactivated horse serum (K. C. Biological Inc., Lenexa, KS) and 100 U/ml of penicillin and 100 µg/ml of streptomycin. Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. For protein synthesis assays PC12 cells were plated on type IV collagen-coated plates at a density of 1 × 10<sup>4</sup> cells per well.

**Vero Cells:** African green monkey kidney cells (Vero) were maintained in 89% Eagle's minimal essential medium, 10% fetal calf serum (Hazleton Systems, Inc.) and 1% non-essential amino acids in 5% CO<sub>2</sub> and 95% air. Vero cells were also plated in 24-well plates (1 × 10<sup>4</sup> cells per well) for bioassay of 192-s-s-A, ricin, and A chain.

Human melanoma cells (A875) were grown under the same conditions as with Vero cells.

**Primary Rat Muscle Cell Cultures:** Skeletal muscle cells from 19-d-old embryonic rats were cultured as previously described (9).

**Protein Synthesis Assay:** All toxins were dissolved in normal media for the respective cell types. Toxin incubations were carried out in 5% CO<sub>2</sub> and 95% air at 37°C. Neurons, PC12 cells, or Vero cells were incubated with 192-s-s-A, intact ricin, or ricin A chain for 45 min. The wells were washed, and normal medium was added back for 8 h. Each well then received 10 µCi of carrier-free [<sup>35</sup>S]methionine (New England Nuclear, Boston, MA) in normal medium for 16 h. In preliminary experiments it was found that [<sup>35</sup>S]methionine incorporation in neurons and other cells was linear for at least 24 h. Label was removed and cells were washed twice with ice cold Hank's/HEPES buffer, pH 7.2. 500 µl of 0.4 N NaOH were added to each well and the plates were gently shaken overnight. A 200-µl aliquot of the NaOH extract from each well was blotted to Whatman no. 3 papers (Whatman Inc., Clifton, NJ) and allowed to dry. The papers were boiled in 10% trichloroacetic acid (TCA) for 15 min and washed with cold 10% TCA. The TCA was extracted from the papers with two washes of 95% ethanol. The papers were dried and added to 6 ml of Budget Solv (Research Products International, Mount Prospect, IL) and counted using standard liquid scintillation spectrophotometry. Wells containing no cells were incubated with [<sup>35</sup>S]methionine and served as blanks. Blanks were subtracted from control or toxin-treated wells and all data are expressed as percent of control. Sample to blank ratios routinely were 15 to 1 for neurons and other cell types.

**Phase-Contrast Photomicrographs:** Dissociated rat SCG were incubated for 45 min with various concentrations of 192-s-s-A, ricin, 192-IgG, or A chain as described above. The cells were washed and placed back in their normal medium. Phase-contrast micrographs of the neurons were taken over the next 5 d using a Nikon Diaphot microscope with a 10x lens and a 20x phase-contrast objective. A Nikon F-2 35-mm camera was fitted to this scope and micrographs were taken using Kodak EPY 135 film (Eastman Kodak Co. Rochester, NY). In a related experiment, SCG cultures were constantly exposed to the various toxins for 5 d and photomicrographs of the neurons were taken daily as described above.

**Displacement of Specific <sup>125</sup>I-192-IgG Binding by 192-s-s-A:** In previous reports it was shown that <sup>125</sup>I-192-IgG binds specifically to the rat NGF receptor at a site distinct from the NGF binding site (5, 17). To determine the relative affinities of 192-s-s-A and 192-IgG for the NGF receptor, we monitored the ability of the ligands to displace <sup>125</sup>I-192-IgG from adult rat SCG membranes. 192-IgG was iodinated by the lactoperoxidase method (10). Adult rat SCG membranes were prepared as described by Banerjee et al. (3). Membranes were resuspended in 0.1% bovine serum albumin (BSA) in PBS. Membranes were incubated in PBS, 0.1% BSA with 0.1 nM <sup>125</sup>I-192-IgG in the presence of various concentrations of 192-s-s-A or 192-IgG for 45 min at 22°C. Membranes containing bound <sup>125</sup>I-192-IgG were separated from free label by quickly diluting the reaction mixture with 2 ml of ice cold buffer and filtering

<sup>1</sup> *Abbreviations used in this paper:* EGF, epidermal growth factor; hCG, human chorionic gonadotropin; NGF, nerve growth factor; SCG, superior cervical ganglion (ganglia); SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; TCA, trichloroacetic acid.

over 0.45  $\mu\text{m}$  Millipore filters. Each filter was washed with an additional 2 ml of ice cold buffer. Filters were counted in a Beckman gamma counter. Data are expressed as percent of displacement of specifically bound  $^{125}\text{I}$ -192-IgG, determined as the difference in binding in the presence and absence of unlabeled 192-IgG (100-fold excess).

## RESULTS

### Gel Electrophoresis of 192-s-s-A

Fig. 1 shows a 7–20% linear gradient SDS PAGE of A chain (lane 1), SPDP-modified 192-IgG (lane 2), and the 192-IgG + A chain reaction mixture after 2 h (lane 3). Passing this reaction mixture over an anti-A chain–Sepharose 4B column and subsequent elution yielded a mixture of hybrids and unreacted A chain with removal of unreacted 192-IgG (lane 4). After further purification on an anti-192-IgG immunoaffinity column, 192-s-s-A hybrids were obtained (lane 5). Note that more than one hybrid species was obtained, presumably representing increased numbers of A chain molecules attached to the 192-IgG. When SDS PAGE was performed on purified 192-s-s-A under reducing conditions, the complex broke down to ricin A chain, and light and heavy chains of immunoglobulin (lane 6). In 10 hybrid preparations, the conversion of 192-IgG to 192-s-s-A was ~60% and the final yield ranged from 25 to 40%. Thus, the use of anti-ricin A chain and anti-192-IgG immunoaffinity columns proved to be effective in obtaining 192-s-s-A hybrids devoid of A chain or 192-IgG contamination.

### Displacement of $^{125}\text{I}$ -192-IgG Binding by 192-s-s-A

To determine the effect of A chain conjugation on the affinity of 192-IgG for the NGF receptor, the ability of 192-IgG and 192-s-s-A to displace tracer amounts of bound  $^{125}\text{I}$ -192-IgG from rat SCG membranes was determined. The binding of  $^{125}\text{I}$ -192-IgG to rat SCG membranes has been characterized and the  $K_D$  of 192-IgG was found to be 5–7 nM

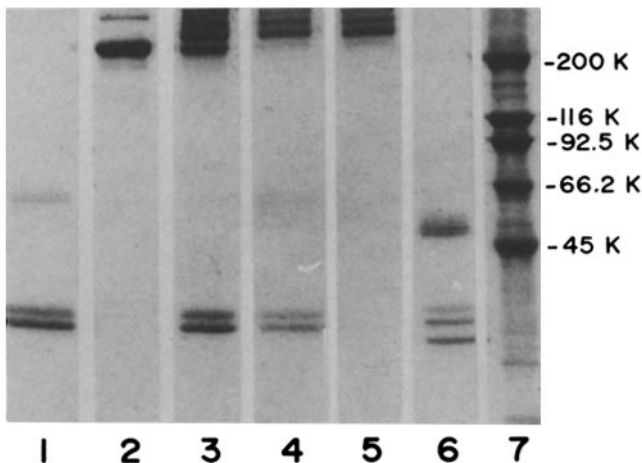


FIGURE 1 192-s-s-A synthesis and purification. 7–20% linear gradient SDS PAGE of ricin A chain (lane 1), SPDP-modified 192-IgG (lane 2), and the 192-IgG + A chain reaction mixture after 2 h (lane 3). Lane 4 shows partially purified hybrid after the anti-ricin A chain–Sepharose 4B affinity column step. The purified hybrid is shown in lane 5. Under reduced conditions the hybrid breaks down to ricin A chain, and light (22 kD) and heavy chains (55 kD) of IgG. Molecular mass standards ( $\times 10^3$ ) are shown in lane 7. Each lane was loaded with 15–40  $\mu\text{g}$  of protein and processed as in Materials and Methods.

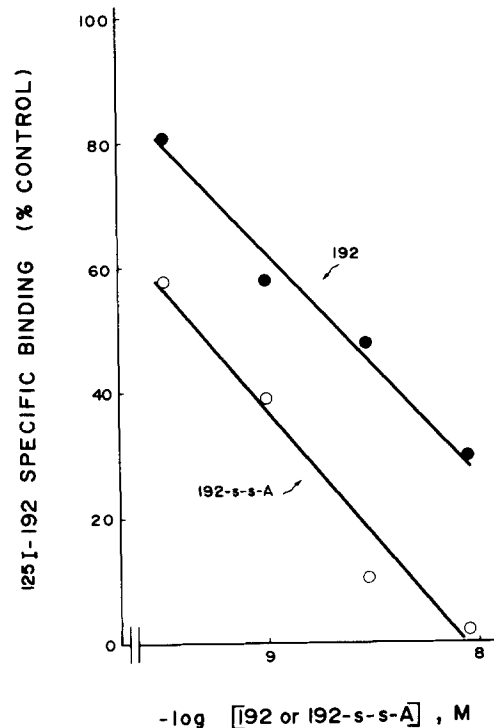


FIGURE 2 Displacement of  $^{125}\text{I}$ -192 binding by 192-s-s-A and 192-IgG. Plot of percent of control of specifically bound  $^{125}\text{I}$ -192-IgG (0.1 nM) vs. the negative log of unlabeled 192-s-s-A (O) or 192-IgG (●). The lines are from one experiment performed in duplicate. The experiment was performed three times (in duplicate) and the hybrid toxin was 3–5 times more potent a displacer than 192-IgG in each experiment. Specific binding was routinely 40–45%.

(Taniuchi, M., unpublished observation). When 192-s-s-A was compared with 192-IgG for its ability to block  $^{125}\text{I}$ -192-IgG binding to rat SCG membranes, it was found that the concentration of hybrid necessary to inhibit specific binding by 50% ( $\text{IC}_{50}$ ) was 0.5 nM, whereas that for 192-IgG itself was 2.2 nM (Fig. 2). This experiment was repeated 3 times and in each experiment 192-s-s-A was 3–5 times more potent at displacing  $^{125}\text{I}$ -192-IgG than was 192-IgG itself. These data demonstrate that 192-s-s-A is not only capable of displacing  $^{125}\text{I}$ -192-IgG bound to rat SCG membranes but that it is 3–5 times more potent a displacer than 192-IgG.

### Bioassay of 192-s-s-A on Cultured Cells

The efficacy and specificity of 192-s-s-A as a toxin was evaluated by determining its ability to inhibit protein synthesis in NGF receptor-bearing and nonbearing cell types. When incubated with primary cultures of dissociated rat SCG neurons at concentrations of 0.04–5  $\mu\text{g}/\text{ml}$  (0.2–27 nM) 192-s-s-A was effective in inhibiting [ $^{35}\text{S}$ ]methionine incorporation into TCA-precipitable proteins (Fig. 3). Over the course of eight preparations of 192-s-s-A, the mean  $\text{IC}_{50}$  of 192-s-s-A for this response was 4 nM. The variability was slight, as the  $\text{IC}_{50}$  value ranged between 1 and 8 nM. Intact ricin was 1,000 times more potent than 192-s-s-A ( $\text{IC}_{50}$  = 5  $\mu\text{M}$ ) whereas A chain alone was 100 times less potent than 192-s-s-A ( $\text{IC}_{50}$  = 500 nM). The inhibition of protein synthesis caused by 192-s-s-A was blocked by co-incubation with a 50–100-fold molar excess of 192-IgG (solid triangle in Fig. 3). This confirmed that the specificity of the 192-s-s-A hybrid was conferred by

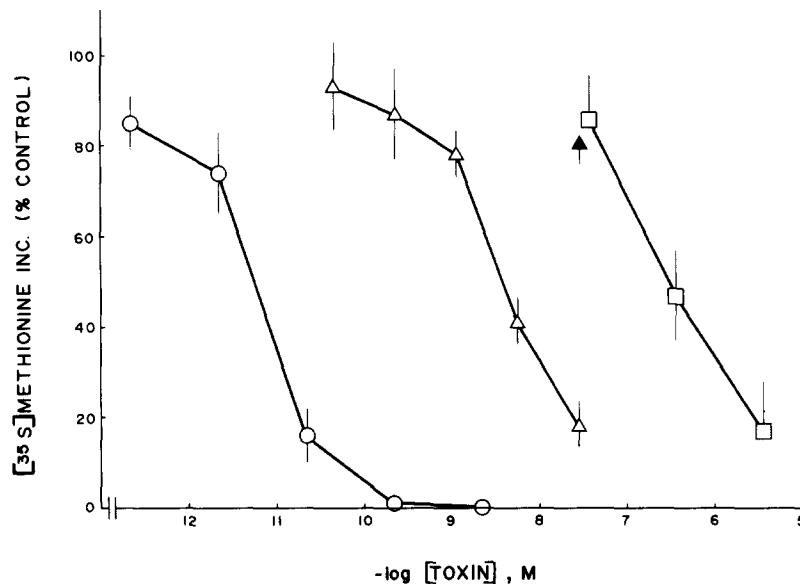


FIGURE 3 Inhibition of protein synthesis in cultured rat SCG neurons by 192-s-s-A, ricin and A chain. 1-wk-old cultures were incubated with toxins at various concentrations. Protein synthesis was measured as the TCA-precipitable counts after incubation of cells for 16 h with [<sup>35</sup>S]methionine. All data are expressed as percent of control (no treatment). O, ricin; Δ, 192-s-s-A; □, A chain. The solid triangle represents protein synthesis inhibition caused by 27 nM 192-s-s-A in the presence of an excess of 192-IgG. In a typical assay, control values were ~3,500–4,000 cpm and blanks were 200–300 cpm. Values represent mean ± SEM of 4–8 experiments performed in triplicate.

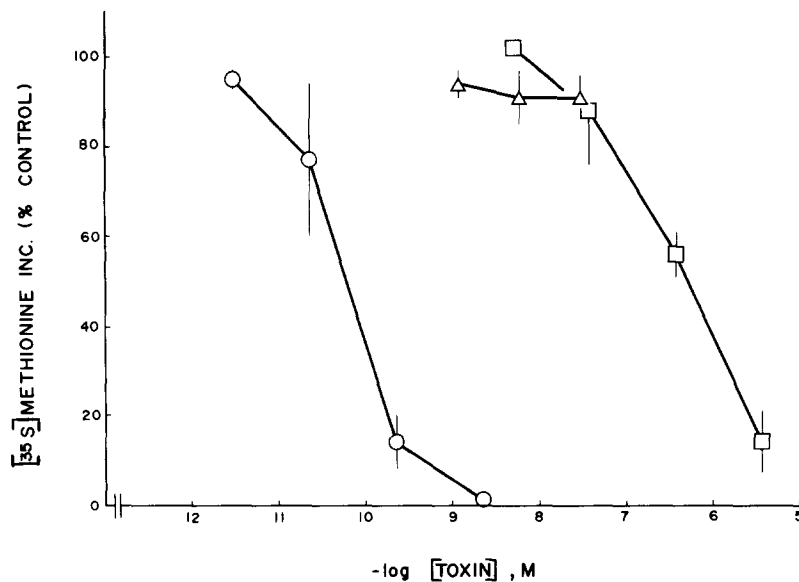


FIGURE 4 Protein synthesis assay of mouse SCG neurons exposed to toxins. Mouse neurons were prepared as in Materials and Methods and exposed to ricin (O), 192-s-s-A (Δ), or A chain (□) as in Fig. 3. Values are mean ± SEM of three independent experiments performed in triplicate.

the 192-IgG moiety of the molecule. Since 192-IgG does not appear to recognize the mouse NGF receptor (17), we predicted that 192-s-s-A would not be toxic to mouse SCG neurons. To test this, we incubated 192-s-s-A with cultured mouse SCG neurons as described above for rat neurons. Fig. 4 shows that 192-s-s-A had no significant effect on protein synthesis in cultured mouse neurons at concentrations which were effective on rat neurons. Four different 192-s-s-A preparations were tested on mouse SCG neurons. Ricin and A chain exerted slightly different toxicities to those observed in the rat. The IC<sub>50</sub> values for ricin and A chain on mouse SCG neurons were 20 pM and 400 nM, respectively. In a related

experiment, 192-s-s-A was incubated with A875 human melanoma cells, which have NGF binding sites on their cell surface (15). While ricin displayed potent protein synthesis inhibition in these cells, 192-s-s-A was without effect (Table I).

To show that 192-s-s-A was not toxic to rat tissue not containing NGF receptors, 192-s-s-A was tested on rat skeletal muscle. 192-s-s-A had no significant effect on protein synthesis in primary cultured rat skeletal muscle cells, whereas ricin and A chain displayed IC<sub>50</sub> values of 55 pM and 3 μM, respectively (Table I).

In a third test system, 192-s-s-A, ricin, and A chain were

TABLE I. Summary of Ricin, A Chain, and 192-s-s-A Toxicities on Various Cell Types

Cell type	Measurement of toxicity*			% Inhibition at 27 nM 192-s-s-A
	IC <sub>50</sub> ricin pM	IC <sub>50</sub> A chain nM	IC <sub>50</sub> 192-s-s-A nM	
Rat SCG	5	500	4	82 ± 5
Mouse SCG	20	400	NO*	9 ± 5
PC12 cells	400	>3,500	>27	28 ± 4
Rat muscle	55	3,000	NO	15 ± 4
Human melanoma cells (A875)	100	NO	NO	0
Vero cells	8	3,000	NO	11 ± 8

\* IC<sub>50</sub> values are given for protein synthesis by the various toxins on different cell types. Also, the inhibition of protein synthesis by 27 nM 192-s-s-A are given and expressed as a percentage of control (untreated) cells.

\* NO, not obtainable. Values of protein synthesis inhibition did not approach 50%.

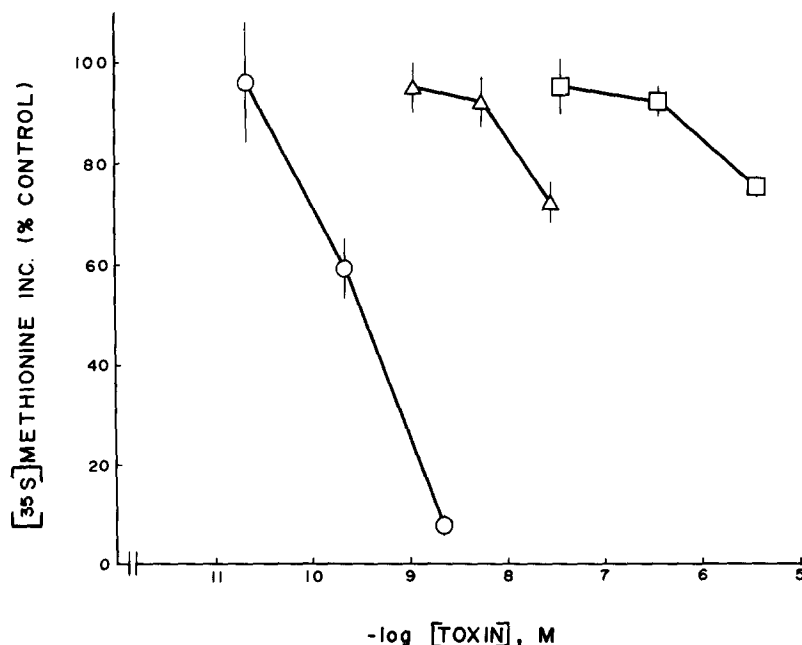


FIGURE 5 Inhibition of protein synthesis in PC12 cells by toxins. PC12 cells were exposed to toxins as with neuronal cultures. PC12 cells were 10–100 times more resistant than rat neurons to ricin (O), 192-s-s-A ( $\Delta$ ), and A chain ( $\square$ ). Control values ranged from 30,000–50,000 cpm and blanks were ~2,000–3,000 cpm. Values are mean  $\pm$  SEM of four experiments.

added to PC12 cells as previously described. It was found that all toxins were 50–100 times less potent on PC12 cells (Fig. 5) than on rat SCG neurons. This was observed with every 192-s-s-A preparation tested. The IC<sub>50</sub> value for ricin on PC12 cells was 400 pM; IC<sub>50</sub> values for 192-s-s-A and A chain were not obtainable, as protein synthesis inhibition did not reach 50% at the highest concentrations tested.

To show that 192-s-s-A was without effect on non-rat cells which contain no NGF receptors, the hybrid was incubated with Vero cells at concentrations similar to those used on neurons and PC12 cells. Vero cells were chosen due to their exquisite sensitivity to ricin. Table I shows that 192-s-s-A had no significant effect on protein synthesis in Vero cells, whereas ricin and A chain displayed their characteristic toxicities on these cells: the IC<sub>50</sub> values of ricin and A chain were 8 pM and 3  $\mu$ M, respectively. Taken together these experiments demonstrate that 192-s-s-A is capable of inhibiting protein synthesis in NGF receptor-bearing cells of rats in a concentration-dependent fashion. The effects are specific for the 192-IgG moiety of the hybrid, as the toxicity can be prevented with an excess of 192-IgG in the incubation mixture. The effect of 192-s-s-A is species specific (rat) and has no effect on cells which contain no NGF receptors.

### Visualization of Cell Death Caused by Toxins

In an attempt to correlate the biochemical toxicity (inhibition of protein synthesis) of 192-s-s-A with morphological changes indicative of cell death, rat SCG neurons were monitored in cells incubated with 192-s-s-A, ricin, A chain, and 192-IgG and monitored for a period of 5 d. This experiment was approached in two different ways. First, neurons were continuously exposed to 2 nM ricin (Fig. 6e) or 5 nM and 1 nM 192-s-s-A (Fig. 6, b and c) for a period of 5 d. After 2 d of incubation with ricin or 192-s-s-A the cell bodies and neurites took on a grainy appearance; the cell bodies lost their refractile appearance under phase-bright optics and began to lift off the collagen-coated wells after 5 d. When cells were constantly exposed to 60 nM A chain (Fig. 6f), they began to look like ricin- or 192-s-s-A-treated neurons. However, this effect was not as dramatic and required 1–2 d longer.

In a similar set of experiments, cultured rat SCG neurons were pulsed with 192-s-s-A, ricin, 192-IgG and A chain for 45 min, washed, and monitored for 5 d. It was found that neurons treated with 27 nM and 5 nM 192-s-s-A, or 2 nM ricin became grainy and nonrefractile as they did when they were constantly exposed to 192-s-s-A ricin. The only differ-

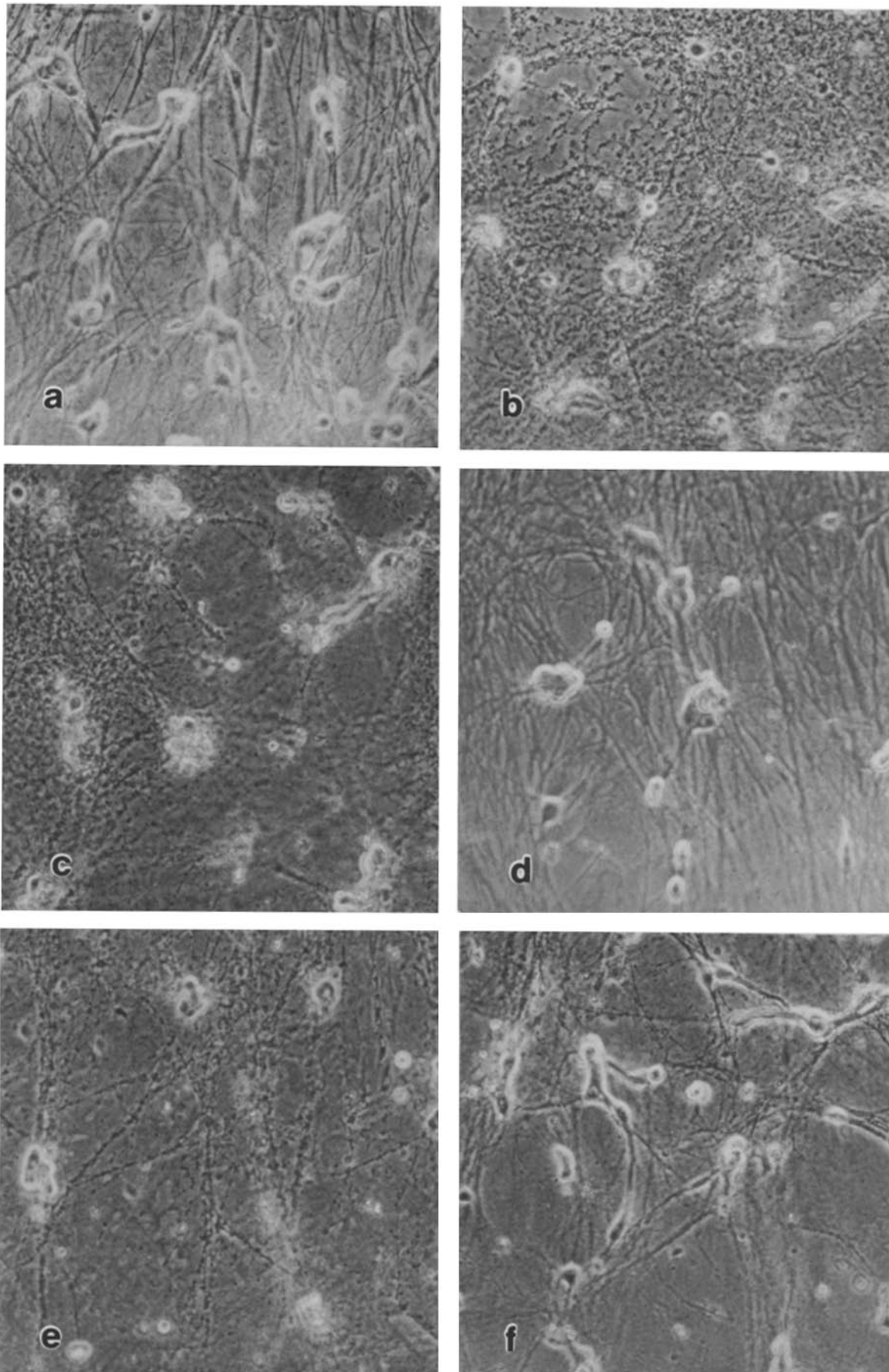


FIGURE 6 Visualization of neuronal death caused by incubation with toxins. Rat SCG neurons were constantly exposed to 192-s-s-A, ricin, A chain, or 192-IgG for a period of 5 d. Phase-contrast photomicrographs were taken after 3 d of exposure as described in Materials and Methods. (a) Control; (b) 5 nM 192-s-s-A; (c) 1 nM 192-s-s-A; (d) 25 nM 192-IgG; (e) 2 nM ricin toxin; (f) 60 nM A chain. All micrographs were taken after 2 or 3 d except A chain-exposed cells (f), which were taken at 4 d. In experiments where neurons were pulsed with the toxin, identical morphological changes occurred with a different time course (see Results).  $\times 200$ .

ence was that the onset of this morphologic degeneration was 1–2 d later with the pulsed neurons than with those neurons constantly exposed to the toxins (micrographs not shown). A pulse of 25 nM 192-IgG had no adverse effect on the neurons (Fig. 6*d*) and 300 nM A chain caused the cells to become grainy in appearance, but this response took 5 d.

To verify that the neurons constantly incubated or pulsed with the toxins exhibited decreased protein synthesis, 10  $\mu$ Ci of [<sup>35</sup>S]methionine was added to each well 5 d after the start of the experiment. The wells were then assayed for TCA-precipitable counts as described in Materials and Methods. Rat neurons constantly exposed to 192-s-s-A (0.2–5 nM) or ricin (2 nM) exhibited an almost complete inhibition of protein synthesis, whereas 60 nM A chain caused only a 50% reduction after 5 d (data not shown). Similar results were seen when cells were pulsed for 45 min with the toxins. 192-s-s-A and ricin inhibited protein synthesis by >95% and A chain decreased protein synthesis to ~50% of control. 192-IgG alone had little or no effect on protein synthesis at concentrations of 5 nM and 25 nM (data not shown). Thus, good agreement exists between the morphological deterioration of the rat neurons and the measured biochemical toxicity caused by 192-s-s-A.

## DISCUSSION

The use of cell-directed toxins has provided a means to ablate specific cell types both *in vitro* and *in vivo* (for reviews, see references 6 and 14). One of the most striking experimental applications is the use of hybrid toxins in experimental tumor therapy (7). We show here the potential of toxin-carrier strategy to study the hormone NGF. A hybrid toxin should satisfy a number of criteria. It should be demonstrated that its actions are receptor-mediated, saturable, reversible, and cell specific. Ideally, the hybrid toxin should specifically bind to a component on its target cell with an affinity comparable to that of the directing molecule. Our results show that 192-s-s-A effectively and specifically kills NGF receptor-bearing cells derived from the rat. The data indicate that the 192-IgG portion of the hybrid is responsible for directing the toxicity of the hybrid to the cultured rat SCG neurons. This is supported by the fact that excess 192-IgG blocks the toxic effect of 192-s-s-A and that A chain alone is 100 times less potent than 192-s-s-A (Fig. 3). In addition, the IC<sub>50</sub> of 192-s-s-A for inhibiting protein synthesis in the rat SCG neurons (4 nM) is very close to the binding constant of 192-s-s-A for <sup>125</sup>I-192-IgG binding sites on NGF receptors in these cells (Fig. 2). Several investigators have reported on the conjugation of hormones to the A chains of various plant or bacterial toxins. Cawley et al. (4) linked EGF to ricin A chain and showed that the potency of this hybrid on protein synthesis inhibition of mouse 3T3 fibroblast cells was similar to the binding constant of EGF for 3T3 EGF receptors (10<sup>-11</sup>–10<sup>-9</sup> M). The toxic effect of EGF-s-s-A was blocked by EGF, confirming that EGF directed the toxicity to the 3T3 cells. Bacha et al. (2) formed a thyrotropin releasing hormone (TRH)-diphtheria toxin hybrid which killed GH<sub>3</sub> rat pituitary cells with a high potency (3 nM) and this toxic effect was blocked by TRH.

Weaker protein synthesis inhibition was obtained with an hCG-ricin A chain hybrid on rat Leydig cells (12). In this study, the IC<sub>50</sub> of hCG-s-s-A was higher than the K<sub>D</sub> of hCG for its receptor on Leydig cells. Nonetheless, the effect of this

hybrid was blocked with an excess of hCG. Similarly an insulin-diphtheria toxin conjugate has also been synthesized by Miskimins and Shimizu (11) which has greatly diminished cytotoxic effects on mouse 3T3 cells than would be predicted by the potency of insulin binding to these cells. It is not apparent why certain hormones, lectins, or antibodies lose biological activity after conjugation with toxin subunits although there are numerous possibilities. One logical explanation is that chemical modification of these carriers with SPDP through lysine amino groups may greatly impair biological activity. Modification of two or more lysine groups on NGF, for example, results in dramatic loss of its biological activity. We have, in fact, prepared a hybrid toxin composed of NGF and ricin A chain and found it far less potent than 192-s-s-A in both binding and toxicity assays (DiStefano, P. S., J. B. Schweitzer, M. Taniuchi, and E. M. Johnson, Jr., unpublished observations).

That 192-s-s-A is specific for rat-derived tissues is not unexpected since the monoclonal antibody, 192-IgG, was raised against rat pheochromocytoma cells and previous characterization studies show that 192-IgG appears to bind exclusively to the rat NGF receptor (17). 192-s-s-A is ineffective on cultured mouse SCG neurons (Fig. 4) at concentrations which decreased protein synthesis by 80% in rat neurons. Similarly, 192-s-s-A was not effective in a human melanoma cell line known to have NGF receptors (Table I).

The monoclonal antibody, 192-IgG, was raised against solubilized membranes from the PC12 cell line; however, 192-s-s-A was 10–100 times less potent an inhibitor of protein synthesis in these cells compared to rat SCG neurons. Likewise, intact ricin and A chain were also much less potent on these cells as compared to rat neurons. Since the binding constant of 192-IgG is not different for PC12 cells and rat SCG membranes (5–7 nM), this probably reflects a difference in either internalization or intracellular processing of the hybrid. It is possible that internalized NGF receptor complexes are processed differently in neurons and PC12 cells. In neurons it is thought that when NGF binds to its receptor on nerve terminals it is internalized and transported to the cell body where it interacts specifically with some cytosolic component to exert its trophic effect on the neuron (for review see reference 18). PC12 cells have no such retrograde mechanism and the internalized NGF receptor complex may be channeled directly to lysosomes. The distinct differences in the potencies of both ricin and 192-s-s-A suggest that the PC12 cell may not be a faithful model of neuronal function, at least with respect to the processing of receptor-mediated internalization of certain ligands, including those bound to the NGF receptor.

The hybrid toxin 192-s-s-A offers a useful tool for probing the biology of NGF in a variety of ways. We propose to use the hybrid to selectively destroy NGF receptor-bearing cells *in vitro* and *in vivo*. By using strategic concentrations of 192-s-s-A, it may be possible to select for NGF receptorless mutants of PC12 cells and other cell populations containing NGF receptors. Mutant cells lacking NGF receptors would be useful in studying the interactions of NGF and its cell surface receptor. Another application for 192-s-s-A *in vitro* would be to eliminate NGF receptor-bearing cells in a mixed culture system.

*In vivo* it would be of interest to use 192-s-s-A to destroy NGF receptor-bearing cells not killed by anti-NGF treatment.

Certain cell types express NGF receptors through the course of development but do not appear to be dependent on NGF for survival. Adrenal medullary cells and postnatal peripheral sensory neurons represent two such cell types. In addition, NGF receptor-bearing tumors, such as pheochromocytoma, may also be destroyed by 192-s-s-A treatment. Another potential use of 192-s-s-A involves attempts to selectively denervate a specific end organ innervated by a particular sympathetic or sensory neuron. In a previous report it was shown that 192-IgG by itself is retrogradely transported from the rat iris to the SCG, much in the same manner as NGF (17). It is possible that injection of this hybrid toxin to an end organ would result in the specific transport of 192-s-s-A back to the cell body causing selective denervation of that end organ, leaving other NGF-responsive cells unaffected. This strategy could be used for experimental purposes or clinically to treat pain syndromes mediated by sensory neurons. Destruction of cell bodies after retrograde axonal transport of ricin has been obtained in the sciatic nerve (20), vagus nerve (19), and in SCG after intraocular injection (Schweitzer, J. B., and E. M. Johnson, Jr., unpublished observation). Though ricin is effectively retrogradely transported in sensory and sympathetic nerves, it offers little potential as a selective lesioning tool since it causes massive tissue destruction at the site of injection and is systemically extremely toxic. 192-s-s-A would affect only NGF receptor-bearing cells, making it a potentially specific neurotoxic agent associated with little or no tissue damage or systemic toxicity.

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