Neurochemical Research, Vol. 12, No. 4, 1987, pp. 393–397

Nerve Growth Factor Levels in Mouse Serum: Variations Due to Stress

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(Accepted October 24, 1986)

The presence of nerve growth factor (NGF) in the serum of adult male mice was assayed using the chick embryo dorsal root ganglion (DRG) bioassay technique in a serum free N1 supplemented medium. Wide variations in the serum-induced nerve fiber outgrowth response were observed when serum was obtained from animals maintained four per cage. Of 64 mice tested, sera of 7 animals induced a profound nerve fiber outgrowth response while the sera of 57 mice failed to show a similar response. In animals kept in isolation for 7 days prior to the start of the experiment, aggression provoked a marked increase in serum NGF levels. In contrast to the sera of aggression-unprovoked mice, the sera of all aggression-provoked mice stimulated a dense nerve fiber outgrowth. The sera of both groups of mice stimulated an intense proliferation and migration of nonneuronal cells. The neurite outgrowth responses elicited by sera from aggression-provoked and unprovoked mice were completely inhibited by the rabbit anti-NGF antiserum. In conclusion, both crowded housing and aggression in mice may provoke an elevation in the serum NGF levels that can be confirmed by the ganglion bioassay technique.

KEY WORDS: Nerve growth factor; aggressive behavior; ganglion bioassay; stress; submandibular gland; neurite formation.

INTRODUCTION

Nerve growth factor (NGF) is a protein that plays a key role in the survival and differentiation of sympathetic and sensory neurons of the peripheral nervous systems (1, 2). Deprivation of circulating NGF both by anti-NGF antibody administration (3), and by the experimental autoimmune approach (4), elicits a near-total destruction of target neurons. Despite this well-documented in-vivo biological role of NGF, its presence in tissues and physiological body fluids remain uncertain. Conflicting values have been reported for serum NGF levels as measured both by the chick embryo dorsal root ganglion bioassay (3, 5–7) and by various radioimmunoassay systems (7, 8–10). Part of the variations reported using the radioimmunoassay systems have been attributed to interference of serum binding proteins (7, 11). However, the discrepancies found using the chick embryo dorsal root ganglion bioassay system still remain unexplained. In view of this confusion, I have reinvestigated the evidence for the presence of NGF in serum using the ganglion bioassay technique in a serum-free environment. In this study, I have used aggression in mice as a model for stress, since intermale spontaneous aggression is a common phenomenon in this species. The results indicate that adult male mouse serum may contain NGF more than the bioassayable amounts (>10 ng/ml) if the animals have been stressed prior to blood sampling. In aggression-provoked mice, serum NGF levels were consistently higher than in aggression-unprovoked animals. The

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0364-3190/87/0400-0393$05.00/0

data strongly indicate that confrontation between adult male mice may increase serum NGF levels several fold higher than the basal levels.

EXPERIMENTAL PROCEDURE

Animals. Adult male Swiss-Webster mice (33–40 gm) were purchased from Simonsen Laboratory, Gilroy, California. On arrival, they were housed 4 per cage (17 × 27 cm). They were maintained in an air conditioned room with lights that were turned on from 0600 to 1800 hr. Tap water and a regular laboratory mouse chow were available ad libitum.

For studies involving aggression, a total of 36 mice were individually housed for at least 7 days prior to the start of experiment. In half of the animals, aggression was provoked by grouping 6 previously isolated mice in one cage for 20 minutes. Most of the time these animals adopted an upright position on their hind legs, struck at, wrestled with and bit each other. Bleeding was also observed.

All animals were sacrificed by CO_2 narcosis which ensured death in less than one minute. The abdomen was opened widely with a scissor and blood was collected from the inferior vena cava at the level of the renal veins. It was immediately transferred to polypropylene microfuge tubes. Blood was allowed to clot at room temperature for 30 minutes and then in an ice-water bath for a further 30 minutes. The tubes were centrifuged at 9000 *g* in a Beckman Microfuge B for 2 minutes at 4°. The samples were returned to the ice-water bath and the serum was transferred to fresh microfuge tubes and recentrifuged for 1 minute to remove any visible red cell contamination or fibrin clots. The clear serum samples were removed and stored in microfuge tubes at 70°C. Individual serum samples were used for the NGF bioassay after filter sterilization.

Dorsal Root Ganglion (DRG) Bioassay. White Leghorn chick embryos incubated for 8 days were used for this study. Lumbar ganglia were dissected out from the embryos for explantation to 24-well cluster culture dishes (Costar) precoated with collagen. Ganglia explants were cultured in a serum-free N1 supplemented medium of Bottenstein et al (12). Each well contained 4–5 ganglia in 250 μl of culture medium. Two wells were used for each test solution. In order to prevent the evaporation of the medium, adjacent wells were filled with medium. When serum samples were tested, they were substituted for 10–50 μl of culture medium. Cultures were incubated in a humidified atmosphere of 5% CO_2—95% air at 37°C. Ganglia were inspected at the end of 24 or 48 hours.

Nerve growth factor (NGF) used as a standard was purified from adult mouse submandibular glands by the method of Mobley et al. (13) as modified by Chapman et al. (14). Fresh NGF solutions were made on the day of the experiment. Dilutions of NGF (10–12 ng/ml) which gave optimal neurite outgrowth were designated as containing one biological unit per milliliter (IBU/ml). Ganglia were viewed using low power phase contrast microscopy at a magnification of 100 ×. The neurite outgrowth response was scored according to Varon et al. (15).

Anti-NGF Antiserum. NGF antiserum was raised in rabbits by repeated, multiple intradermal injections of purified mouse SMG—NGF. The antiserum obtained after a third booster showed a 50% binding of ^{125}I-βNGF in a final dilution of 1:250,000. In the present study the antiserum was used after 1:20 dilution in N1 medium. Control sera from non-immunized rabbits were used in the same concentrations as the antisera.

RESULTS

I. Bioassay of Mouse Serum NGF In Animals Housed Four Per Cage.

Figure 1 shows the two different kinds of bioassay responses observed with sera of animals housed 4 per cage. Ganglion explants cultured in the absence of added serum (Figure 1A) showed no fiber outgrowth at the end of a 24 hour culture period. Of three independent experiments consisting a total of

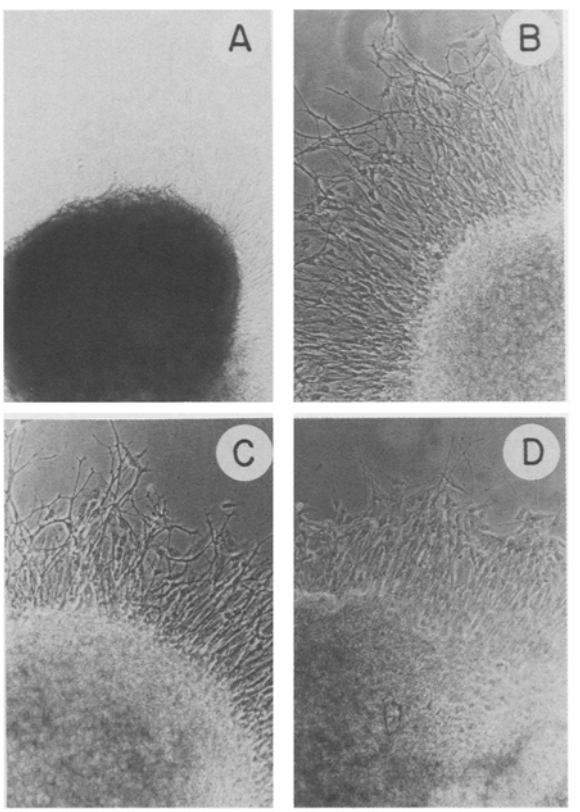

Fig. 1. Phase-contrast photo micrographs of 8-day old chick embryo dorsal root sensory ganglia cultured in N1 medium in the absence and presence of adult male mouse serum. Sera prepared ffrom groups of mice housed 4 per cage were tested for NGF in the ganglion bioassay. Animals were sacrificed three days after their arrival to vivarium. A = Ganglion in the absence of any added serum. B and C = Representative of dense nerve fiber growth seen in sera of 7 mice when tested at two concentrations (B = 50 μl/well, C = 10 μl/well). D = representative of poor neurite outgrowth response seen in sera of 57 animals when tested at 50 μl/well. Note the marked proliferation of non-neuronal cells in all ganglion explants treated with serum (B, C, D). Ganglia were photographed at 24 hrs. of incubation. (× 100).

64 mice (Exp. I = 28; Exp. II = 20 and Exp. III = 16 mice), sera of seven animals (4 in Exp. I, 2 in Exp. II and 1 in Exp. III) elicited a dense fiber outgrowth response when tested at a concentration of 50 μl/well (Figure 1B). Five-fold dilution did not diminish the nerve fiber outgrowth response (Figure 1C). In contrast, the majority of serum samples (57 out of 64) did not show any profound fiber outgrowth response (Figure 1D). Very few nerve fibers were seen to extend beyond the zone of non-neuronal cells.

II. Bioassay of Mouse Serum-NGF in Animals Provoked With Aggression. Individual serum samples of aggression-unprovoked (mice housed individually for 7 days prior to sacrifice) and aggression-provoked mice (mice involved in vigorous fighting for 20 minutes prior to sacrifice) were tested for NGF in the ganglion bioassay. The results are shown in Figure 2. DRG explants cultured in the absence of any added serum samples (Figure 2A) were different from the explants maintained in the presence of various mice sera (Figure 2B–I). Sera of aggression-unprovoked mice elicited few nerve fiber outgrowths (Figure 2B) and very often the presence of nerve fibers was masked by the intense proliferation of nonneuronal cells (Figure 2C). Sera of all aggression-provoked male mice induced a profound nerve fiber outgrowth response (Figure 2D and 2E). Serial dilution studies in four different sera of aggression-provoked animals indicated NGF concentrations in the range of 14–20 BU/ml. In addition, these sera enhanced the proliferation and migration of non-neuronal cells. The overall growth and migration of non-neuronal cells were more extensive with sera of aggression-provoked than unprovoked mice. Dense fiber outgrowth also extended beyond the zone of non-neuronal cells.

Sera of aggression-unprovoked mice did not inhibit either the nerve fiber outgrowth or the proliferation of non-neuronal cells when added simultaneously with the sera of aggression-provoked mice (Figure 2F).

Non-immune rabbit serum did not prevent the nerve fiber outgrowth produced by the sera of aggression-provoked mice (Figure 2G). Anti-NGF-antiserum completely blocked the neurite outgrowth but did not affect the proliferation of non-neuronal cells (Figure 2H). Similarly, anti-NGF-antiserum did not prevent the enhanced proliferation of non-neuronal cells seen in explants treated with sera of aggression-unprovoked mice (Figure 2I).

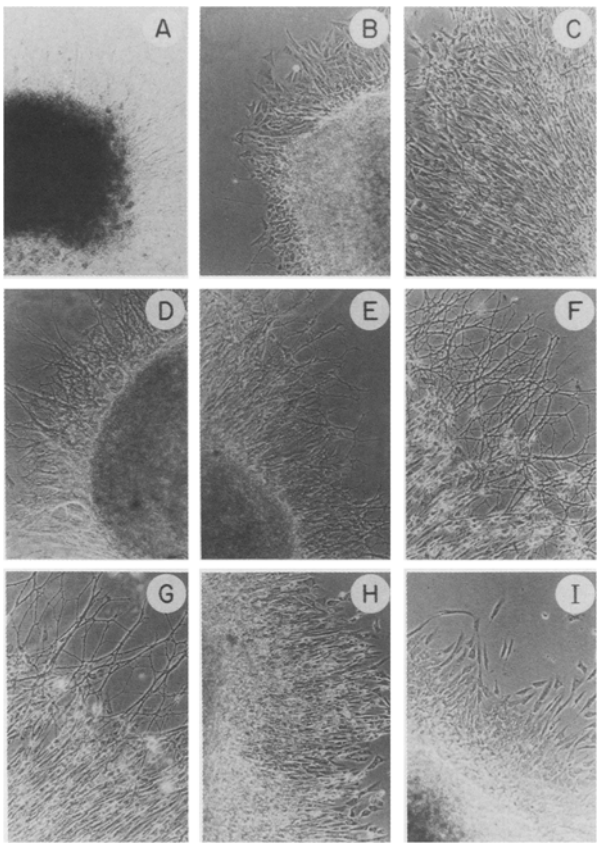

Fig. 2. Phase-contrast photomicrographs of 8-day old chick embryo dorsal root sensory ganglia cultured in N1 medium with the sera of aggression-unprovoked and aggression-provoked adult male mice. A = Ganglion in the absence of added serum; B and C = Ganglion with sera of aggression-unprovoked mice (20 and 40 μl); D and E = Ganglion with sera of aggression-provoked mice (20 and 40 μl); F = Ganglion with sera of aggression-unprovoked and aggression-provoked mice (20 μl each); G = Ganglion with sera of aggression-provoked mice (40 μl) and non-immunized rabbit serum (50 μl); H = Ganglion with sera of aggression-provoked mice (20 μl) and NGF-immunized rabbit serum (50 μl of 1:20); I = Ganglion with sera of aggression-unprovoked mice (20 μl) and NGF-immunized rabbit serum (50 μl of 1:20). All ganglia were photographed at 48 hr of incubation. (×100). See text for other details.

DISCUSSION

In the present study the ganglion explants were cultured in N1 medium to assess the presence of NGF in adult male mouse serum. Ganglion explants cultured in the absence of added serum did not show any significant nerve fiber outgrowth or proliferation of non-neuronal cells. When mice were housed 4 per cage, sera from 7 of these animals showed a dense fiber outgrowth with neat serum (10–50 μl/well). However, sera of most animals (57 out of 64)

did not show any pronounced neurite outgrowth. These observations were not surprising in light of the findings by Cohen (3), who was able to record the presence of NGF by the plasma clot-assay in one of four attempts. However, this author did not specify the number of animals used in each attempt. The poor neurite outgrowth response observed using sera from a large number of animals was in contrast to the findings of Levi-Montalcini and Booker (5), who were able to detect NGF in serum even after 1:10 dilution. Based on their observations in 15 male mice, these investigators concluded that bioassayable amounts of NGF are present in the general circulation. However, Suda et al. (7), using 10 male mice, were not able to confirm the findings of Levi-Montalcini and Booker (5). In the present study I have used sera of aggression-provoked mice to assess the stress-associated changes in serum NGF levels.

All sera of aggression-provoked mice showed a profound neurite outgrowth response. Similar responses were not seen with sera of aggression-unprovoked mice. The sera of mice in both groups elicited an overgrowth of ganglionic non-neuronal cells. Anti-NGF-antiserum totally neutralized the neurite outgrowth response while the proliferation of non-neuronal cells was unaffected. These results indicate that NGF is not the factor that is responsible for the overgrowth of non-neuronal cells. Significant numbers of non-neuronal cells were also noticed around the ganglion explants cultured in the presence of serum of individually housed animals (aggression-unprovoked animals). However, more extensive proliferation and migration of non-neuronal cells was observed with the sera of aggression-provoked mice. This points out that the responsible mitogen is a normal constituent and its level is greatly augmented in aggressive mice. Preliminary radioimmunoassay (RIA) results indicate the presence of extremely high concentrations of epidermal growth factor (EGF) in serum of male aggressive animals. Whether an increased serum EGF level was responsible for eliciting the proliferation of non-neuronal cells is presently under investigation.

Ganglion explants cultured in the presence of sera of aggression-unprovoked animals were consistently different from the explants cultured in the absence of added serum. Short neurites (shorter than the size of the ganglion explant) were seen overlying non-neuronal cells. The overgrowth of supporting cells causes some difficulty in scoring of nerve fiber outgrowth with confidence. The appearance of short neurites can be completely blocked by anti-NGF antiserum (Figure 2I). The minimal NGF concentration required to elicit the growth of neurites longer than the size of the ganglion is in the range of 2 ng/ml. Concentrations below this level provoke the protrusion of short neurites. These observations indicate that serum NGF levels in mice that are kept in isolation may not exceed 2 ng/ml. However, it should be cautioned that animals kept in isolation for 7 days prior to sacrifice need not reflect the true basal levels of NGF. It has not been completely resolved whether individual housing in mice constitutes a "stress-free" condition (16). However, isolation per se is considered as less "stressful" than group housing (16).

The data presented here indicate that stress due to crowded housing and aggression between animals may increase serum NGF levels far greater than the bioassayable amounts (>10 ng/ml). Presumably, this increase is due to release from the submandibular glands, since preliminary RIA results indicate that aggression does not increase serum NGF and EGF levels in sialoadenectomized adult male mice. Thus, different NGF levels reported by various investigators may have arisen from variable amounts of stress associated with animals prior to sacrifice (5, 6, 8, 9). The present study also suggests that fractionation of serum is not essential as reported by others (6) for assessing serum NGF levels in adult male mice. In conclusion, the results suggest that precautions should be taken in housing animals, particularly for experiments involving the analysis of NGF levels in physiological body fluids, such as blood. Stress appears to be a potential source of error in determining the basal serum NGF levels in adult male mice. Whether factors other than aggression—such as transport, odor, sound, handling of animals, changing cages, and food and water deprivation—will provoke an increase in serum NGF levels, needs to by systematically studied.

ACKNOWLEDGMENT

I thank Drs. R. Smith, C. Manes and C. Landel and Mr. G. Koshkarian for their critical review of the manuscript. Thanks are due to Ms. D. Finch, Ms. L. Jambrosic and Mrs. P. DeLeon for assistance in the preparation of the manuscript. This work was supported by NIH grant NS 174314.

Due to a clerical error in the editorial office the revised form of this manuscript, which was submitted on November 22, 1985, was accepted for publication on October 24, 1986.

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