Isolated Hepatic Granulomas from Mice Infected with *Schistosoma mansoni* Contain Nerve Growth Factor

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Nerve growth factor (NGF) is a neurotrophic factor essential for the development and maintenance of specific neuronal cell populations. In addition, NGF has biological effects on inflammatory cells. The aim of this study was to determine whether NGF is present in chronic inflammation, using isolated hepatic granulomas from mice infected with *Schistosoma mansoni* as the model. The schistosome granuloma is a complex T-cell-mediated immune response to the egg. Intact granulomas were isolated from the livers of infected mice and examined for the presence of NGF. In homogenized granuloma samples, radioimmunoassay and immunoblotting analyses detected an immunoreactive NGF that had the same molecular mass as that of purified murine NGF (13 kDa). Isolated granulomas cultured in vitro released soluble factor(s) with NGF-like neurite-promoting activity in a rat pheochromocytoma (PC12) bioassay. This activity was partially inhibited by a blocking anti-NGF antibody. There were two potential sources of this NGF-like neurite-promoting activity, either the schistosome egg or the host inflammatory response. Since neither isolated eggs nor soluble egg antigen had neurite-promoting activity, the inflammation was the source of this activity. The inability of the anti-NGF antibody to inhibit completely the granuloma-induced neurite outgrowth in the bioassay signifies that NGF is not the only neurotrophic factor present in these granulomas. The presence of NGF within the granulomas may indicate that NGF has a role in the granulomatous response.

Nerve growth factor (NGF) is a neurotrophic factor that is essential for the development and maintenance of specific neuronal cell types, including sympathetic and sensory neurons in the peripheral nervous system (12). In addition to its effects on neurons, NGF has biological effects on inflammatory cells, such as the induction of mast cell degranulation (5), promotion of the proliferation of splenic lymphocytes (28), and stimulation of the expression of interleukin-2 (IL-2) receptors on human lymphocytes (29). These effects on immune cells support the hypothesis that NGF modulates immune responses.

In an inflammatory response, the presence of NGF at physiologically relevant concentrations would also support the hypothesis that NGF is a mediator of immune responses. NGF is present in some inflammatory processes. Bioassays have detected NGF activity in carageenan-induced granuloma explants (11). These explants were harvested from skin at 72 h, and the cells were predominantly fibroblasts, with a small number of macrophages. Another study showed that NGF exhibits a transient increase in the fluid of heat-induced blisters and is detected in carageenan-induced pleural exudates (36). In these instances, NGF was identified during the early stages of inflammation. The source of NGF was not determined. NGF may arise either from the injured tissues or from the host inflammatory response.

The aim of this study was to determine whether NGF is present in the granulomatous inflammation of mice infected with *Schistosoma mansoni*. Schistosome granulomas are chronic, focally destructive lesions. They are complex T-cell-mediated immune responses to the schistosome eggs. The cell types within these lesions are predominantly eosinophils, macrophages, and fibroblasts, with other cell types such as lymphocytes and mast cells less abundant (27).

These granulomas can be isolated from the livers of infected mice for in vitro experiments (21).

In this study, radioimmunoassay (RIA) and immunoblotting detected NGF in isolated schistosome granulomas. Granulomas may produce other neurotrophic factors as well, since the granulomas cultured in vitro had neurotrophic activity that was not inhibited by anti-NGF antibody. The presence of NGF in schistosome granulomas supports the hypothesis that NGF participates in the inflammatory injury and repair process.

MATERIALS AND METHODS

Materials. NGF was isolated from mouse submandibular glands either by initial purification of 7S-NGF (32) followed by isolation of the β -subunit (24) or by the method of Bocchini and Angeletti (3). Polyclonal antiserum was raised in rabbits against purified β -NGF (18). The immunoglobulin G fraction was isolated, and the specificity of the antibody was determined by four criteria. (i) Ouchterlony plates revealed that the antibody reacted with pure NGF (18). (ii) The antibody specifically immunoprecipitated ¹²⁵I-labelled NGF (18). (iii) RIA using the antibody detected NGF on an isoelectric focusing gel (18). (iv) The antibody detected NGF in immunoblotting experiments (19). Schistosome soluble egg antigen was obtained from the World Health Organization. Recombinant human IL-6 was obtained from R & D Systems (Minneapolis, Minn.).

Animals and infection. Female 8-week-old CBA/J mice (Jackson Laboratory, Bar Harbor, Maine) were infected by subcutaneous injection of 35 cercariae of the Puerto Rican strain of *S. mansoni* (22). The animals were sacrificed by cervical dislocation after 8 weeks of infection. The livers were removed aseptically.

Isolation of granulomas. Granulomas were isolated from the liver tissue by homogenization of the livers in a Waring blender at low speed followed by repeated washes and

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sedimentation in RPMI 1640 medium. The samples were centrifuged at $350 \times g$ for 5 min at 4°C (21). The resulting pellet contained intact granulomas with no residual liver tissue, as described previously (35). Samples of granulomas were sectioned and stained with Wright's stain, and it was determined that there was no liver tissue contamination.

Granuloma homogenization. For the RIA, 1 ml of phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) was added for each gram (wet weight) of granulomas. The granulomas were homogenized with a Ten Broeck tissue grinder for 5 min. The grinder was rinsed with an additional 1 ml of the above solution, and the rinse solution was added to the homogenate. The homogenate was dialyzed against 0.01 M ammonium acetate (pH 7.0), using dialysis membranes with an 8,000-molecular-weight cutoff. These samples were then centrifuged for 25 min at $10,000 \times g$ to remove particulate matter. The resulting supernatant was freeze-dried and stored at -70°C. For Western blot (immunoblot) analysis, the same homogenization procedure was followed, except that 0.01 M ammonium acetate (pH 7.0) was used for the first step in place of PBS containing 0.1% Triton X-100.

Granuloma cultures and conditioned medium. The isolated granulomas were cultured in a defined medium, N2 (4), for 48 to 72 h at 37°C in an atmosphere containing 5% CO₂ and with a relative humidity of 100%. The resulting conditioned medium was dialyzed and freeze-dried as described above.

Pheochromocytoma (PC12) cell bioassay. The neurite-promoting activity of granulomas was determined by PC12 cell bioassay. In the presence of NGF or NGF-like agents, these cells will produce neurites which are evident at 48 h and which become extensive by 5 to 7 days. The PC12 cells were obtained from the American Type Culture Collection (Rockville, Md.). Stocks of PC12 cells were grown in RPMI 1640 medium supplemented with 10% horse serum (HyClone Laboratories, Ogden, Utah) and 5% fetal calf serum (HyClone). The cells were incubated at 37°C in an atmosphere containing 5% CO₂ and with a relative humidity of 100%.

The first step in the assay is to plate PC12 cells in 24-well tissue culture plates (25,000 cells per well) in the RPMI medium described above. To promote adherence, the wells were precoated with laminin (10 µg of laminin per ml of RPMI medium) for a minimum of 3 h at room temperature and washed with RPMI medium. After cell attachment (minimum of 3-h incubation), the plating medium was removed and replaced with test samples. The test samples (300 to 500 µl) consisted of intact granulomas, isolated eggs, or soluble egg antigen (100 pg to 1 µg/ml) suspended in N2 medium, or freeze-dried samples of conditioned medium reconstituted to a 1/4 dilution with N2. Other wells received either NGF (20 ng/ml) in N2 as the positive control or N2 alone as the negative control. Since liver tissue was not present in the granuloma preparations, no liver tissue controls were utilized. In several experiments, various dilutions of anti-NGF antibody (1/25, 1/50, and 1/100) were added to block the effects of NGF in the samples. Some conditioned medium wells received additional NGF (20 ng/ml) along with the antibody to assure that sufficient antibody was present to block all of the potential granuloma NGF. The bioassays were incubated at 37°C for 7 days. The cells were examined for neurite outgrowth and photographed with an inverted phase-contrast microscope. The outgrowth was quantified as described in the legend to Fig. 3.

In separate experiments, IL-6 (1 to 100 ng/ml) was tested in the PC12 bioassay to determine whether IL-6 stimulates neurite outgrowth.

NGF RIA. The presence of immunoreactive NGF was determined by a competitive RIA (18), using purified murine submandibular gland NGF as the standard. NGF was iodinated by the chloramine-T method (9). Standard curves and NGF concentrations were calculated (logit-log) (23), using the Packard MPIO software (Packard Instrument Company, Inc., Downers Grove, Ill.).

Western blot analysis. Immunoblotting was used to confirm the presence of NGF in granuloma homogenates (19). The freeze-dried granuloma homogenates were concentrated fivefold by reconstituting the lyophilized powder in a reduced volume of sodium dodecyl sulfate (SDS) sample buffer (5% 2-mercaptoethanol, 10% glycerol, 4% [wt/vol] SDS, 0.015% [wt/vol] bromophenol blue, 124 mM Tris). The samples were heated in a boiling water bath for 3 min and loaded into the wells of a 7.5 to 15% gradient polyacrylamide-SDS gel (10). Colored standards (Bethesda Research Laboratories, Gaithersburg, Md.) were used as molecular weight markers. Purified NGF (100 ng), which was similarly reconstituted with SDS sample buffer and heated for 3 min in a boiling water bath, was the positive control. After the electrophoresis was completed, the proteins were transferred to a nitrocellulose membrane electrophoretically (30). The membrane was washed in blot buffer (PBS containing 0.25% nonfat powdered milk) for 30 min to reduce nonspecific binding. The membrane was then placed in a solution containing rabbit anti-NGF antibodies (1:300 dilution in blot buffer) and shaken on a rocker at 4°C overnight. The membrane was washed three times with blot buffer over a period of 30 min. This washing step was repeated between each of the following steps. A method using avidin-biotin complexes (Vector Laboratories, Burlingame, Calif.) was used to localize NGF. The membrane was initially incubated in biotinylated goat anti-rabbit immunoglobulin G (40 µl in 10 ml of blot buffer) for 4 h at room temperature. The membrane was washed and then incubated in avidin-horse-radish peroxidase complex (40 µl in 10 ml of blot buffer) for 90 min at room temperature. The membrane was washed and finally incubated in peroxidase substrate solution (PBS containing 0.07% 3,3'-diaminobenzidine and 0.002% H₂O₂) for 5 to 9 min. The reaction was stopped by repeated washes with blot buffer. The membrane was photographed immediately.

RESULTS

Pheochromocytoma (PC12) cell bioassay. In this study, the PC12 cell bioassay was used to determine whether schistosome granulomas have NGF-like neurite-promoting activity in culture. Initially, isolated granulomas were cocultured with the PC12 cells. At 48 h, there was evidence of neurite outgrowth from PC12 cells near the granulomas (Fig. 1A). The outgrowth was similar to that stimulated by purified NGF (Fig. 1B). The granuloma coculture experiments were limited to a 72-h observation period, because granuloma cells migrate from the granulomas, which impairs morphological identification of PC12 cells. Neither isolated schistosome eggs nor soluble egg antigen stimulated neurite outgrowth.

To further study the neurite-promoting effect of granulomas, granulomas were cultured in N2 medium for 72 h and the resulting conditioned medium was removed from the

FIG. 1. PC12 bioassay of isolated granulomas. Isolated hepatic granulomas were cocultured with PC12 cells (A). Neurite outgrowths (indicated by arrows) from PC12 cells were observed in the vicinity of the granulomas (G) by 48 h. This outgrowth was similar to NGF (100 ng/ml)-stimulated neurite outgrowth (B). Magnification, $\times 250$.

cultures and examined in the PC12 bioassay. Since high levels of cyclic AMP can stimulate neurite outgrowth from PC12 cells, the conditioned medium was dialyzed against 0.01 M ammonium acetate, using dialysis membranes with an 8,000-molecular-weight cutoff to remove cyclic AMP. These samples were freeze-dried for storage and reconstituted to a 1/4 dilution with N2 for testing. The granuloma-conditioned medium stimulated neurite outgrowth from PC12 cells (Fig. 2A). This outgrowth was first evident at 48 h and became extensive by 5 to 7 days. The neurite outgrowth was similar to that produced by purified NGF (Fig. 2B). Anti-NGF antibody was added to the test samples in dilutions of 1/25, 1/50, and 1/100. These dilutions partially inhibited outgrowth stimulated by the conditioned medium to the same extent (only the results with the 1/50 dilution shown in Fig. 2C), whereas all three dilutions completely inhibited the outgrowth stimulated by NGF (20 ng/ml) (only the results with the 1/50 dilution shown in Fig. 2D). The degree of inhibition was quantified and is presented in Fig. 3. Since the 1/25, 1/50, and 1/100 dilutions of antibody inhibited outgrowth to the same extent, only the 1/50 dilution results are presented. Quantification confirmed that the anti-NGF antibody partially inhibited neurite outgrowth stimulated by the conditioned medium. In contrast, neurite outgrowth induced by pure NGF (20 ng/ml) was completely inhibited. Medium alone or with antibody did not stimulate neurite outgrowth.

Since several antibody dilutions were tested and only a

partial inhibition was seen, it was our hypothesis that all of the granuloma NGF was blocked and the remaining neurite outgrowth was not due to NGF. One concern was that insufficient antibody was added to block all of the NGF in the conditioned medium. Purified NGF (20 ng/ml) was added to a conditioned medium sample along with the blocking antibody (1/50 dilution) to determine whether there was still antibody available to bind the added NGF. This added NGF did not overcome the partial inhibition of the antibody. This result demonstrates that sufficient antibody was added to the conditioned medium samples to block all of the granuloma NGF as well as additional NGF. Therefore, the unblocked neurite outgrowth was not induced by NGF but by some other unidentified neurotrophic factor(s).

Since IL-6 is a cytokine that modulates immune function and has neurotrophic effects, it could have been responsible for the neurite outgrowth that was not inhibited by the anti-NGF antibody. However, IL-6 (1 to 100 ng/ml) did not stimulate neurite outgrowth in the PC12 bioassay.

NGF RIA. An NGF RIA was used to determine whether an immunoreactive NGF was present in granuloma homogenates and conditioned medium. Granuloma homogenates were serially diluted and assayed by RIA. These serial dilutions produced an RIA displacement curve (dashed line in Fig. 4) which paralleled the standard curve generated with pure NGF. Since the two lines were parallel, this indicates that the granuloma immunoreactive NGF was indistinguish-

FIG. 2. PC12 bioassay of granuloma-conditioned medium. Granulomas were cultured in N2 medium for 48 h, and the conditioned medium was collected, dialyzed, freeze-dried, and reconstituted to a 1/4 dilution in N2. The PC12 cells were cultured in this conditioned medium and photographed at 5 days. The granuloma-conditioned medium stimulated neurite outgrowth from PC12 cells (A), which was similar to the neurite outgrowth produced by NGF (20 ng/ml) (B). This outgrowth was partially inhibited by anti-NGF antibody (1/50 dilution) (C). This same antibody dilution completely inhibited outgrowth produced by NGF (20 ng/ml) (D). Magnification, $\times 250$.

able from pure murine NGF by RIA. The RIA detected 3.11 fmol (standard error = 0.47; $n = 5$) or 57 ng of immunoreactive NGF per g (wet weight) of granuloma tissue. In granuloma-conditioned medium, low levels of immunoreactive NGF (<5 ng/ml) were detected by RIA. Since these levels were not on the linear portion of the standard curve, an accurate measurement was not possible.

Western blot analysis. Western blot analysis was per-

formed to provide additional evidence of NGF in granuloma homogenates and to determine whether the granuloma NGF had a molecular mass similar to that of purified NGF. NGF is a noncovalently bound homodimer of 26 kDa which under denaturing conditions (SDS) has a molecular mass of 13 kDa. As shown in Fig. 5, granuloma NGF (lane A) had a molecular mass of 13 kDa, which is identical to that of purified NGF (lane B).

FIG. 3. Quantification of PC12 bioassays. The PC12 bioassays were quantified by determining the percentages of cells bearing neurites. Random fields were scored, and a minimum of 200 cells were counted per test well. Cells were considered positive for neurite outgrowth if they had a process extending greater than one cell diameter. Each bar represents six samples tested in duplicate and is expressed as the mean $\pm$ standard error of the mean. The degree of outgrowth stimulated by conditioned medium (C.M.) (1/4 dilution) was less than that stimulated by purified NGF (20 ng/ml) ($\square$). The antibody (1/50 dilution) partially inhibited neurite outgrowth stimulated by conditioned medium ($P < 0.0001$, Student *t* test), whereas it completely inhibited NGF (20 ng/ml) ($\square$). The addition of NGF (20 ng/ml) to a conditioned medium sample along with antibody did not overcome this partial inhibition ($\square$). Medium alone or with antibody did not induce neurite outgrowth.

DISCUSSION

This study showed that schistosome granulomas contain NGF, as determined by RIA and immunoblotting. The molecular mass of the granuloma NGF was 13 kDa in denaturing buffers, which is identical to that of NGF purified from mouse submandibular glands. In addition, intact granulomas and conditioned medium from granuloma cultures stimulated neurite outgrowth from PC12 cells. This neurite outgrowth was partially inhibited by anti-NGF antibody. This suggests that granulomas release multiple neurite-promoting factors, one of which is NGF. The antibody blocks the NGF, but other neurite-promoting factors are still active, resulting in reduced neurite outgrowth. The nature of the other neurite-promoting factors in granulomas are not known. Several molecules have neurite-promoting activity (25). Of these, fibroblast growth factors (acidic and basic) stimulate neurite outgrowth from PC12 cells (26, 33) and have been described for granulomatous inflammation (37). The inflammatory mediator, IL-6, has neurotrophic effects on spinal and sensory neurons by promoting their survival (8). However, as reported here, IL-6 does not stimulate

FIG. 4. NGF RIA of granuloma homogenates. Serial dilutions of granuloma homogenates produced an RIA displacement curve (Unknown) that paralleled pure murine NGF (Standard). The graph is a logit-log plot. The y axis is the logit of B/B_0 (where B is labeled NGF bound in the presence of the unknown factor, and B_0 is labeled NGF bound in absence of the unknown factor). The x axis is the log of the NGF concentration (in nanograms per milliliter).

neurite outgrowth in the PC12 bioassay. Whether the known neurotrophic factors or other as yet uncharacterized factors are responsible for the unblocked neurite outgrowth requires further study.

Schistosome granulomas are an excellent model for the study of NGF or other neurotrophic factors in inflammation. These lesions are chronic, focally destructive inflammatory reactions. They begin as an initial acute response to the schistosome eggs and evolve into a complex T-cell-mediated inflammatory mass. In this study, the granulomas varied in

FIG. 5. Western blot analysis of granuloma homogenates. Mouse NGF in denaturing buffers (SDS) has a molecular mass of 13 kDa. In granuloma homogenates, the anti-NGF antibody detected an immunoreactive NGF at approximately 13 kDa (lane A), a molecular mass identical to that of pure mouse NGF (lane B).

age but were no older than 3 weeks. At these ages, the predominate cell types are eosinophils, macrophages, and fibroblasts, with other cell types such as lymphocytes and mast cells less abundant (27). Since neither schistosome eggs nor soluble egg antigen stimulated neurite outgrowth in the PC12 bioassay, the source of NGF as well as of the unblocked neurite-promoting activity is the inflammation. The granuloma cells responsible for NGF synthesis are not known. Several cell types can synthesize NGF, including fibroblasts (17–19, 38), epithelial cells (6), striated muscle cells (16), and astrocytes and Schwann cells (1). In addition, the macrophagelike amoeboid microglial cells synthesize NGF (13). Thus, in granulomas, fibroblasts and macrophages are likely sources of NGF. Studies are in progress to determine the cell type(s) responsible for NGF synthesis.

The function of NGF in granulomatous inflammation is unknown. Perhaps NGF participates in the modulation of inflammation or is necessary for the repair of injured nerves. NGF has biological effects on inflammatory cells. For instance, NGF stimulates mast cell degranulation (5, 20) and increases the expression of IL-2 receptors on cultured human lymphocytes (29). NGF stimulates T-cell-dependent, human basophil differentiation (14) and the proliferation of rat splenic lymphocytes (28). Whether these NGF effects play a role in the regulation of the granulomatous inflammation is not known.

Alternatively, NGF could function to promote nerve repair or modulate neural function in the vicinity of the injury. This modulation could take the form of affecting neurotransmitter release or altering the density and distribution of specific types of nerves or neurotransmitters. It is interesting that morphological changes in innervation in chronic inflammations have been described. In adjuvant-induced arthritis, substance P- and calcitonin gene-related peptide-containing nerves stain more intensely and neurite outgrowth is evident in inflamed joints, whereas neuropeptide Y-containing nerves are not affected (34). Morphological changes in innervation are also observed in chronic liver diseases. In chronic active hepatitis, the number of nerves stained for neuron-specific enolase, S-100, or neuropeptide Y are increased in portal areas. In cirrhotic livers, the number of these nerve fibers are increased in the fibrous septa (15).

Similarly, changes in the enteric nerves have been identified in granulomatous diseases involving the gastrointestinal tract. In Crohn's disease, there is an increase in the number of enteric ganglion cells (7) and vasoactive intestinal polypeptide-containing nerves are larger and stain more intensely in inflamed bowel segments. These latter changes are more pronounced in the vicinity of granulomas (2). Similar morphological changes of the vasoactive intestinal polypeptide-containing enteric nerves are found in mice infected with *S. mansoni*. In the chronic stage of this infection (>18 weeks), nerve cell bodies in the vicinity of schistosome granulomas stain with greater intensity for vasoactive intestinal polypeptide than normal controls or uninvolved segments (31). Whether neurotrophic factors released by inflammation produce these morphological changes in the nerves is unknown. Further investigation is required to delineate the etiology and significance of these nerve changes in relation to organ and immune function.

In summary, this initial report links NGF to granulomatous inflammation and demonstrates that granulomas have neurite-promoting activity in culture. The functional roles of NGF and other neurotrophic factors in the granulomatous response remain to be determined.

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