Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3739–3743, April 1994
Neurobiology

Mast cells synthesize, store, and release nerve growth factor

(plasticity/inflammation/hyperalgesia/neuroimmune interactions)

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ABSTRACT **Mast cells and nerve growth factor (NGF) have both been reported to be involved in neuroimmune interactions and tissue inflammation. In many peripheral tissues, mast cells interact with the innervating fibers. Changes in the behaviors of both of these elements occur after tissue injury/inflammation. As such conditions are typically associated with rapid mast cell activation and NGF accumulation in inflammatory exudates, we hypothesized that mast cells may be capable of producing NGF. Here we report that (*i*) NGF mRNA is expressed in adult rat peritoneal mast cells; (*ii*) anti-NGF antibodies clearly stain vesicular compartments of purified mast cells and mast cells in histological sections of adult rodent mesenchymal tissues; and (*iii*) medium conditioned by peritoneal mast cells contains biologically active NGF. Mast cells thus represent a newly recognized source of NGF. The known actions of NGF on peripheral nerve fibers and immune cells suggest that mast cell-derived NGF may control adaptive/reactive responses of the nervous and immune systems toward noxious tissue perturbations. Conversely, alterations in normal mast cell behaviors may provoke maladaptive neuroimmune tissue responses whose consequences could have profound implications in inflammatory disease states, including those of an autoimmune nature.**

Mast cells are involved in inflammatory and hypersensitivity reactions (1) and occur in many peripheral tissues, in perivascular regions in close apposition to innervating sensory or autonomic nerve fibers (2), and also within the peripheral and central nervous systems (3). Nervous and immunological mediators such as neuropeptides or IgE can affect the state of mast cell activation (4). Secretory products of activated mast cells can stimulate or facilitate axon reflexes, thereby inducing positive feedback loops (5). Activated mast cells also secrete a wide array of pluripotent cytokines and other inflammatory mediators (6) and may thus act as bidirectional carriers of information between the nervous and immune systems, suggesting profound implications for tissue homeostatic mechanisms.

Neurotrophic and/or tropic cues could be produced directly by mast cells. Nerve growth factor (NGF) reportedly accumulates in inflammatory sites or exudates (7, 8) caused by various noxious stimuli, including those of autoimmune origin (9, 10). NGF is the prototype of target-derived neurotrophic factors critical for development and maintenance of specific peripheral and central neuronal populations (11, 12). Evidence also points to an action for NGF on cells of the immune system (13, 14), including mast cells (15). NGF may thus play a key role not only within the nervous system but, more importantly, in cross-talk between cells of the nervous and the immune systems (16).

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NGF accumulation in acute inflammatory exudates has been attributed to cellular infiltrates or to the induction of tissue NGF expression. However, the rapid appearance (within 2 h) and degree of NGF accumulation in rat skin blister fluid (8) is more compatible with release of NGF from resident tissue cells containing the stored protein. Rapid induction of preprotachykinin mRNA expression, which is a NGF-sensitive process, in dorsal root ganglia (DRG) of rats with adjuvant monoarthritis (17) supports this hypothesis. In addition, the remodeling of intestinal mucosal nerve fibers during intestinal inflammation is correlated with changes in mast cell density (18). Mast cells and sympathetic neurons in culture form contacts (19), suggesting a mast cell–nerve ending chemotactic NGF-like gradient effect.

Given the potential involvement of mast cells and NGF in neuroimmune interactions and the close microanatomical associations between mast cells and sensory or autonomic fibers in several tissues (20, 21) mast cells may, in fact, produce NGF. This study investigates the capability of purified rat peritoneal mast cells to synthesize and release biologically active NGF.

MATERIALS AND METHODS

Immunocytology and Histochemistry. Rat peritoneal mast cells (RPMCs) were prepared from male Wistar rats (150–200 g) (Charles River Breeding Laboratories) as described (22). Over 90% cell purity was indicated by toluidine blue or safranin staining (23). Immunocytochemistry for NGF was performed with an affinity-purified rabbit polyclonal antibody to mouse NGF (1–0.1 μg/ml) and a peroxidase-conjugated goat anti-rabbit polyclonal antibody (Vectastain, Vector Laboratories). Cryostat sections (15 μm) from adult rat ear pinna were similarly processed. To control for staining specificity, primary antibody was preincubated with excess NGF, substituted with nonspecific rabbit IgG (2 μg/ml; Sigma), or omitted.

Biological Assays and Quantitation of NGF Activity. NGF activity in RPMC conditioned medium was tested on dissociated cultures of chicken embryonic day 8 (E8) DRG and E10 sympathetic neurons (11). Conditioned medium was obtained by incubating 10^6 RPMCs per ml in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS) for 24 h, at which time cells were still viable by microscopic appearance. Sympathetic and DRG neurons were cultured (24) for 24 and 48 h, respectively, without or with various amounts of the conditioned medium. The percentage of cells bearing neurites of >4 somal diameters was determined in random fields, and NGF was quantitated as described (24). Biospecificity was evaluated by adding a goat anti-mouse NGF polyclonal antibody (10 μg/ml) or a mouse

Abbreviations: NGF, nerve growth factor; DRG, dorsal root ganglia; RPMC, rat peritoneal mast cell; E8, embryonic day 8; BDNF, brain-derived neurotrophic factor.

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monoclonal anti-NGF antibody (1 μg/ml; clone 27/21; Boehringer Mannheim). A two-site ELISA was used to quantitate NGF in appropriate dilutions of mast cell conditioned media versus mouse 2.5S NGF as described (25). Blanks consisted of samples added to microwells coated with mouse myeloma IgG (Calbiochem) instead of anti-NGF antibody.

RNA Extraction and PCR. Total RNA was extracted as described (26) from RPMCs, rat basophilic leukemia (RBL-2H3) cells (P. Ghezzi, Mario Negri Institute, Milan), mouse neuroblastoma neuro-2a cells (ATCC-CCL 131), or male mouse submaxillary glands. The RNA was then precipitated with 4 M sodium acetate (4°C overnight) and separated by centrifugation (8000 × *g* for 15 min). PCR primers and an internal hybridization probe for rat NGF were from Severn Biotech (Kidderminster, U.K.). Their sequences were as follows: 5′ primer, 5′-TCA TCC ACC CAC CCA GTC TTC-3′, 5′ corresponding to residue 654; 3′ primer, 5′-GGC AGC CTG TTT GTC GTC TGT-3′, 3′ corresponding to residue 946; internal probe, 5′-CGC CTT GAC AAA GGT GTG AGT CGT-3′, corresponding to residues 896–919 of the NGF gene sequence (27). Primer sequence identity with other members of the NGF gene family was <50% and PCR of a brain-derived neurotrophic factor (BDNF) cDNA clone with the chosen primers gave negative results. First-strand cDNA synthesis was performed with the 3′ antisense NGF primer and Moloney murine leukemia virus reverse transcriptase (United States Biochemical). The volume was then increased 5-fold with water and 25 μl of the reverse transcriptase products were used for PCR in a final vol of 100 μl containing 4 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 μM primers, and 10 units of *Taq* DNA polymerase Stoffel fragment (Perkin–Elmer/Cetus). The reaction cycle consisted of 1 min each at 94°C, 50°C, and 72°C. Mast cell, neuro-2a, and submaxillary gland cDNAs were amplified for 23 cycles, while 35 cycles were used for RBL-2H3 cDNA. Samples were then subjected to electrophoresis and a 4% agarose gel and transferred to a nylon filter. The internal probe was 3′-end-labeled with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) (New England Nuclear) using terminal deoxynucleotidyl transferase (United States Biochemical). Hybridization was carried out in standard solutions

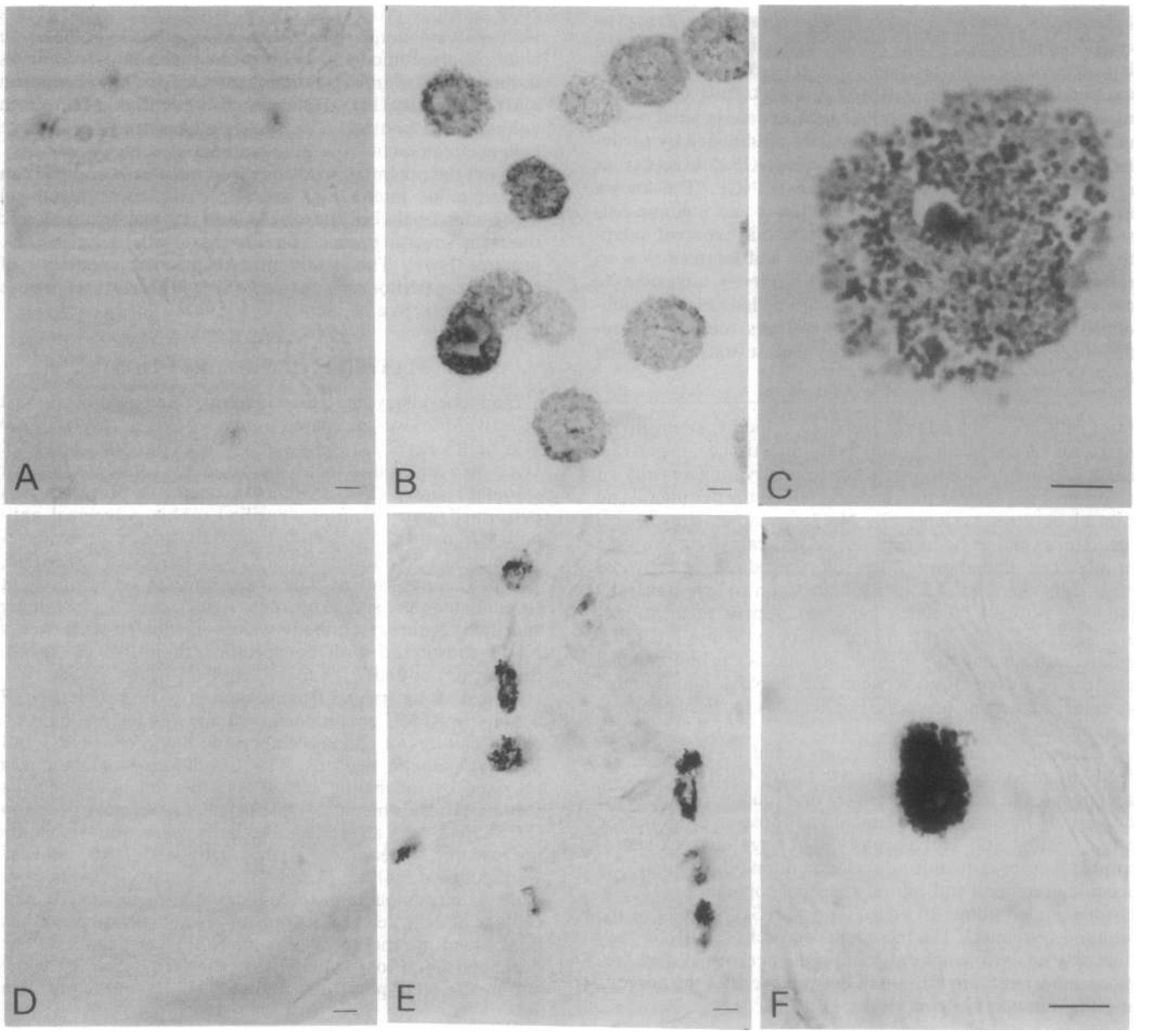

FIG. 1. NGF-like immunoreactivity in purified RPMCs (*A–C*) and rat ear pinna sections (*D–F*). (*A* and *D*) Secondary antibody only. (*B* and *E*) Polyclonal anti-NGF antibody. Higher magnifications of an anti-NGF immunostained isolated mast cell (*C*) or from a tissue section (*F*) are also shown. (Bars = 10 μm.)

(28) with 10% formamide (25°C overnight) and filters were washed in 1× standard saline citrate at room temperature before autoradiography (Hyperfilm-MP; Amersham).

RESULTS

NGF-Like Immunoreactivity in RPMCs. All mast cells, identified by safranin or toluidine blue staining, were clearly labeled by affinity-purified anti-NGF antibodies (Fig. 1*B*); highest levels of immunoreactivity appeared to be localized within granules (Fig. 1*C*). Omission of primary antibody or absorption with excess NGF yielded no or barely visible staining (Fig. 1*A*). Another primary rabbit anti-NGF polyclonal antibody (Sigma) gives positive labeling. No staining was seen in the non-mast cell resident cell populations present in the peritoneal cavity washes before mast cell purification.

NGF immunoreactivity was also observed in the mast cell line RBL-2H3 but with a lower intensity of staining (data not shown). Specific NGF-like immunoperoxidase staining was detected in mast cells of histological sections from rat ear pinna (Fig. 1 *D–F*).

RPMCs Contain NGF mRNA. The presence of NGF mRNA in RPMCs was examined by PCR, as no NGF hybridizing band was observed by Northern blot analysis. PCR amplification of mast cell reverse-transcribed total RNA (1 μg) with NGF-specific primers yielded a 292-bp band, which hybridized with a NGF-specific internal probe (Fig. 2), indicating the expression of the NGF gene in RPMCs. Mouse submaxillary gland total RNA served as a positive control (Fig. 2). No hybridizing band was found in PCR-processed mast cell RNA without the reverse transcription step. Although only a very minor proportion of non-mast cells was present in the purified RPMC preparation, PCR amplification could have resulted in a contribution to the NGF signal by the non-RPMCs. When total RNA (1 μg) from the rat mast cell line RBL-2H3 was reverse transcriptase PCR amplified, a NGF hybridizing band was clearly detected (Fig. 2), albeit at higher amplification values. No NGF hybridizing signal was evident in the PCR amplification products of mouse neuroblastoma neuro-2a cells (Fig. 2).

Biological Activity of Spontaneously Released NGF from Mast Cells. Chicken embryo DRG and sympathetic neurons in *in vitro* culture were used to measure NGF in medium conditioned for 24 h with RPMCs (Fig. 3). Mast cell viability was assessed by quantifying the histamine content of randomly selected conditioned medium using an enzyme immunoassay kit (Immunotech, Marseilles, France). Mast cells released 1.65 ± 0.2 μg of histamine per 10^6 cells (mean ± SD; $n = 4$) over the same 24-h period, being equivalent to 9.6% ± 1.2% of total histamine content and only slightly above routinely determined levels of basal histamine release (22).

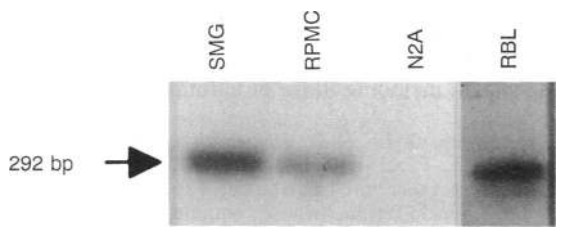

FIG. 2. NGF oligonucleotide probe hybridization of PCR amplified products from male mouse submaxillary glands (SMG), RPMCs, mouse neuro-2a cells (N_2A), and rat basophil leukemia cells (RBL). Autoradiograms of hybridization bands of SMG (positive control), N_2A (negative control), and RPMCs were obtained after 23 cycles of amplification. The RBL cDNA was amplified 35 cycles. No NGF hybridization band was observed in N_2A cDNA amplification products even after 35 cycles.

Sympathetic (E10) and DRG (E8) neurons were cultured 24 and 48 h, respectively, with conditioned medium (3–50 μl in 100-μl final vol). Maximal neurite-promoting activity for DRG neurons was obtained with 12 μl (Fig. 3*B*), the quantitation of which was biologically equivalent to ≈2 ng of NGF per ml per 10^6 mast cells over 24 h. Preincubation of RPMC conditioned medium with a goat polyclonal anti-NGF antibody (Fig. 3*B*) largely reduced this biological activity, excluding a significant contribution by factors other than those of the NGF neurotrophin family. RPMC conditioned medium was also active for sympathetic neurons (Fig. 3*A*), which respond neither to BDNF nor to neurotrophin 4 (29). Preincubation of conditioned medium with a monoclonal anti-NGF antibody (clone 27/21), which does not cross-react with other neurotrophins (30), significantly diminished biological activity for sympathetic neurons (Fig. 3*A*). Higher amounts of RPMC conditioned medium (≥25 μl) showed some cytotoxicity.

In agreement with the bioassay data, significant levels of β-NGF protein were detected in RPMC conditioned medium (2.56 ± 0.65 ng of β-NGF per 10^6 cells per ml per 24 h; mean ± SEM; $n = 10$), as measured by a specific two-site ELISA.

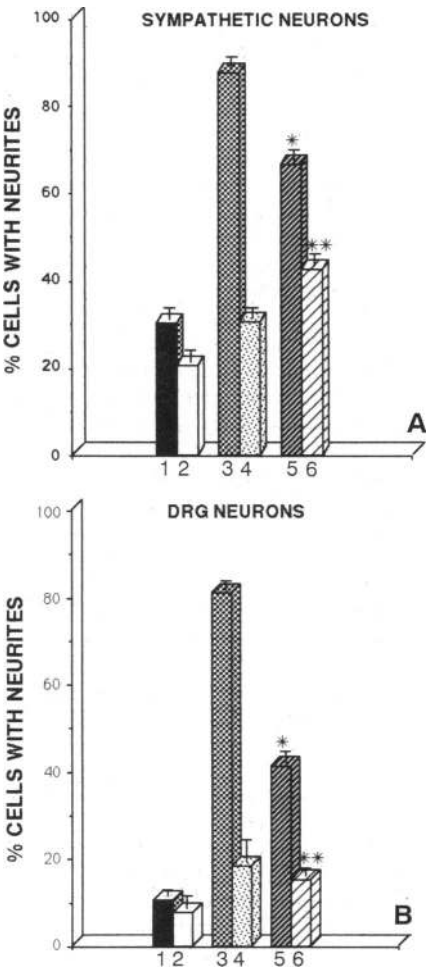

FIG. 3. Quantitation of RPMC-released NGF trophic activity. Histograms represent mean ± SEM of the percentage of neurite-bearing cells (*A*, $n = 6$; *B*, $n = 10$). Bars: 1, control culture medium; 2, anti-NGF antibody; 3, NGF (1 ng/ml); 4, NGF (1 ng/ml) and anti-NGF antibody; 5, RPMC conditioned medium; 6, RPMC conditioned medium and anti-NGF antibody. (*A*) Monoclonal anti-NGF antibody 27/21. (*B*) Polyclonal anti-NGF antibodies. Statistical analysis was conducted by Student's–Newman–Keuls *t* test for multiple comparisons. *, $P < 0.001$, control vs. mast cell conditioned medium; **, $P < 0.001$, mast cell conditioned medium alone vs. addition of anti-NGF antibody.

DISCUSSION

The present study shows that RPMCs express the gene transcript for NGF and display a NGF-like immunoreactivity largely associated with granule-containing compartments. The RBL-2H3 cell line behaved similarly, indicating that cells of the mastocytic–basophilic lineage are NGF-producing cells. Furthermore, a two-site ELISA detected abundant β-NGF protein levels in RPMC conditioned medium. NGF-like neurotrophic activity was also observed when the same conditioned medium was added to cultured avian embryonic sensory and sympathetic neurons, the latter of which respond to neither BDNF nor neurotrophin 4 (29). A monoclonal antibody recognizing NGF but not BDNF or neurotrophin 3 (30) effectively blocked mast cell-derived trophic bioactivity. Together with the occurrence of NGF-like immunopositive mast cells in histological sections of adult rat ear pinna, these data show that rodent connective tissue-type mast cells synthesize, store, and release biologically active NGF. The possibility that other mast cell types or basophils have similar properties, or that mast cells produce neurotrophic factors other than NGF, remains to be investigated.

Numerous cell types synthesize NGF including other hematopoietic cells (31). NGF expression and/or production has been observed *in vivo* either during development (32, 33) or during tissue injury (34) and *in vitro* following stimulation (e.g., with inflammatory agents) (31, 34, 35). Despite evidence of NGF in peripheral adult tissues such as skin, the cellular element(s) contributing to NGF production in normal or pathological conditions remains to be clearly identified (36, 37). Our results indicate NGF-like immunoreactivity in mast cells of mesenchymal adult rodent tissues or RPMCs. Although some cross-reactivity of the polyclonal antibody toward other neurotrophins cannot be excluded (38), the expression of NGF mRNA favors this immunostaining to reflect reactivity with one or more differently processed forms of the NGF protein. In addition, the vesicular localization of anti-NGF immunoreactivity indicates that connective tissue-type mast cells may store, in analogy to tumor necrosis factor (39), preformed β-NGF and/or specialized precursor storage forms. Independent of the intracellular NGF form(s), these experiments show that RPMCs release relatively large amounts of authentic biologically active β-NGF in the absence of any overt cellular stimulation. Preliminary studies with two-site ELISA indicate that β-NGF also occurs within unstimulated RPMCs (unpublished data). Given their defensive role, mast cells may represent a continuous source of NGF for tissue homeostatic functions and/or a prompt NGF supply for their reequilibration after tissue inflammatory insults; other cell types may contribute to tissue NGF levels at later stages of the inflammation process (7, 8). Mast cells could, by releasing cytokines such as tumor necrosis factor, also indirectly upregulate NGF production in surrounding cells (35).

Inflammatory insults to a variety of tissues, including peripheral nerves, are often accompanied by mast cell activation, hyperalgesia (40), and extracellular NGF accumulation (7, 8). Mast cells are frequently found closely apposed to small caliber or unmyelinated fibers (20), which may contain substance P and/or other neuropeptides (21). Small peptidergic fibers are generally assumed to be nociceptive sensory fibers, whose dependence on NGF for survival and differentiation is developmentally restricted. However, in the adult these neurons continue to express high-affinity NGF receptors and to respond to NGF with enhanced neuropeptide expression (12), while anti-NGF antibodies modify their morphology (41). Induction of neuropeptide synthesis in sensory neurons is also found in many models of tissue inflammation. In some instances, neuropeptide induction occurs within hours of initiation of inflammation (17, 40) and is abolished by anti-NGF antibodies (42). The increased levels of neuropeptides that accompany tissue inflammation may, upon primary afferent stimulation, result in their increased release from the sensory terminals in the spinal cord (43) and likely underlie centrally mediated prolonged behavioral hyperalgesia (40). Interestingly, a single high systemic dose of NGF in adult rats caused long-lasting thermal and mechanical hyperalgesia (44). These data, together with ours, support the notion that activated mast cells may, by releasing NGF, directly control plastic modifications in the central processing of sensory nociceptive inputs after tissue injury and inflammation. At the same time, NGF-induced neuropeptide upregulation may cause increased neuropeptide release at peripheral nociceptor terminals, and proteolytic products of NGF in inflamed tissues may indirectly modulate their excitation (45). Given the potential NGF effects on collateral sprouting or growth of sensory and sympathetic fibers (11, 36), mast cells, via release of NGF, could control peripheral short- and long-term modifications in the sensitivity and/or amplitude of sensory terminal fields toward persistent or subsequent, even subthreshold, noxious tissue stimuli. The degree of mast cell activation may thus contribute significantly to the hyperalgesic and pathological pain states frequently accompanying tissue inflammation, especially in peripheral nerves.

Mast cell progenitors migrate from the bloodstream to those tissues where they undergo terminal phenotypic differentiation, proposing that these cells represent potential mobile quanta for replenishment of tissue NGF. NGF itself can increase tissue mast cell number *in vivo* (15, 46) and affect mast cell survival, differentiation, and mediator release *in vitro* (47–50). RPMCs express the functional NGF receptor tyrosine kinase (51), indicating possible autocrine and paracrine actions of NGF on mast cells. Given that NGF can affect not only neurons but also hematopoietic cells, mast cells may, through the release of NGF, convey information to the nervous system as well as modulate their own behavior and the reactivity of tissue-infiltrating cells.

The capacity of at least connective tissue-type mast cells to produce NGF points to their having a pathophysiological potential far beyond that currently recognized. The ability of mast cells to synthesize and release NGF may represent a well-integrated tissue defense mechanism for maintaining and/or restoring homeostatic functions after noxious perturbations (16). On the other hand, mast cell hyerplasia and nerve remodeling are found in some chronic inflammatory states (18, 52–54). Such tissue modifications may be triggered and/or sustained by mast cell-derived NGF and lead to long-lasting changes in tissue reactivity and behavioral responses toward persistent or recurrent inflammatory stimuli. Accordingly, alterations in mast cell properties—e.g., in the entity and duration of their activation—could play a critical role in the progression and/or secondary complications of inflammatory tissue responses. A more complete understanding of their local stimulatory and inhibitory regulation (55) might open avenues to the management of inflammatory disease states, including those of autoimmune origin.

The authors thank Dr. S. D. Skaper for useful discussions and comments, Dr. L. Facci for the histamine measurements, and Ms. P. Lentola for expert secretarial assistance. A special note of thanks to the late Prof. Angelo Burlina, whose continuous effort and initiative in biomedical research helped to make this work possible.

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