Nerve growth factor (NGF) promotes mast cell survival in vitro (Horigome, K., Bullock, E. D., and Johnson, E. M., Jr. (1994) J. Biol. Chem. 269, 3695–2702). NGF survival promotion is cell density-dependent, and conditioned medium experiments have shown that NGF increases the production of an autocrine mast cell survival activity. Cytokines are potential candidates for autocrine survival factors. In rat peritoneal mast cells (RPMC), NGF caused an increase in the messenger RNAs for interleukin (IL)-3, IL-4, IL-10, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor. This induction was NGF dose-dependent, was blocked by NGF-neutralizing antibodies, and was not observed in the non-mast peritoneal cell population. The immunosuppressive agent, cyclosporin A, blocked both cytokine induction and NGF-activated survival promotion but not survival promotion activated by IL-3 or stem cell factor, suggesting that NGF enhanced RPMC survival by increasing cytokine production. We also examine the effects of NGF on the expression levels of some members of the bcl-2 family and the interleukin-1β-converting enzyme-like cysteine protease families. NGF markedly increased bcl-2 expression but had little or no effect on the other genes studied. The induction of bcl-2 mRNA by NGF was not blocked by cyclosporin A. These data suggest that induced cytokine gene expression but not increased expression of bcl-2 mediates NGF-survival promotion in RPMC.

Nerve growth factor (NGF) is the best characterized member of a family of related proteins known as neurotrophins (2). NGF is absolutely required for survival during development and functional maintenance into adulthood of sympathetic neurons and dorsal root ganglion neurons derived from the neural crest. NGF also supports the survival of cholinergic neurons of the cranial and dorsal root ganglion neurons derived from the neural crest. NGF also supports the survival of cholinergic neurons of the cranial and dorsal root ganglion neurons derived from the neural crest. NGF supports the survival of cholinergic neurons of the cranial and dorsal root ganglion neurons derived from the neural crest. NGF also supports the survival of cholinergic neurons of the cranial and dorsal root ganglion neurons derived from the neural crest. NGF also promotes the survival of cholinergic neurons of the cranial and dorsal root ganglion neurons derived from the neural crest. NGF also promotes the survival of cholinergic neurons of the cranial and dorsal root ganglion neurons derived from the neural crest. NGF also supports the survival of cholinergic neurons of the cranial and dorsal root ganglion neurons derived from the neural crest. NGF promotes the survival of cholinergic neurons of the cranial and dorsal root ganglion neurons derived from the neural crest.
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NGF survival is cell density-dependent, and NGF treatment of RPMC induces an increase in the production and/or secretion of autocrine survival factor(s) into the medium (1).

These findings led us to analyze further the effects of NGF on RPMC survival promotion and cytokine gene induction. Here we demonstrate that NGF is able to up-regulate transiently mRNA encoding the cytokines TNF-α, IL-3, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-4. Cytokine induction was NGF dose-dependent, blocked by NGF-neutralizing antibodies, and did not occur in the non-mast peritoneal cell population. The immunosuppressive agent cyclosporin A (CsA) blocked both NGF cytokine gene induction and survival promotion but did not block survival activated by IL-3, or stem cell factor (SCF), two other well characterized mast cell growth and survival-promoting factors (26, 27).

We also examined the effects of NGF on expression of genes thought to be involved in regulation of cell death including members of the bcl-2 and ICE/ced-3 families (28). NGF increased RPMC expression of bcl-2 mRNA but had little or no effect on the other genes examined. The increased expression of bcl-2 was not blocked by CsA treatment, suggesting that NGF directly up-regulated bcl-2 expression by a pathway that was not inhibited by CsA and that up-regulation of bcl-2 was not sufficient for NGF-mediated RPMC survival.

**EXPERIMENTAL PROCEDURES**

**MATERIALS**—Mouse NGF (2.5S) was purified from male mouse submaxillary glands by the method of Bocchini and Angeletti (29). Recombinant SCF was a generous gift from Dr. Qiao Yan (Amgen Inc., Thousand Oaks, CA). Rat interleukin-3 was obtained from supernatant of D44CHO cells, a gift from Dr. Jeffrey Milbrandt (Washington University, St. Louis, MO), transfected with pIRL1 (30) (pSV2-neo with the dIII fragment carrying the entire rat IL-3 gene), a generous gift from Dr. Ian Young (Australian National University, Canberra, Australia). IL-3 activity was determined by serial 2-fold dilution and measurement of [3H]thymidine incorporation in 3-day cultures of rat bone marrow cells (30). Unit of IL-3 is defined as the dilution that is three standard deviations above the titration end point (31). Adult female Sprague-Dawley rats (200 g; Harlan, Indianapolis IN) were used in this study. All reagents were purchased from Sigma unless otherwise indicated.

**Mast Cell Preparation**—The method used for RPMC isolation was a slight modification of that previously described (32). Briefly, cells from a peritoneal lavage with mast cell medium (150 mM NaCl, 3.7 mM KCl, 3 mM dibasic sodium phosphate, 3.5 mM monobasic potassium phosphate, 0.9 mM CaCl₂, 0.1% glucose, 0.1% bovine serum albumin, 10 units/ml heparin, pH 6.8) were washed and separated by centrifugation (1500 × g) on a single step gradient of 23% metrizamide. Mast cells of greater than 95% purity were routinely obtained in the cell pellet. The purity of the mast cell population was determined by staining in toluidine blue (0.05% toluidine blue in 150 mM NaCl, 1% acetic acid, 10% formaldehyde, and 50% ethanol). At least 98% of cells were viable as estimated by trypan blue exclusion. For the RT-PCR experiments, the mast cells were separated from contaminating cells by running an additional metrizamide (23%) step gradient, resulting in a 98% pure population of mast cells. Non-mast peritoneal cells were prepared by running two consecutive step gradients and harvesting the cells on top of the metrizamide cushion. This cell population was confirmed to be free from mast cells by staining with toluidine blue.

**Cell Culture**—Primary cultures of mast cells were maintained in complete medium consisting of 90% RPMI (Life Technologies Inc.), 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan UT), 2 mM glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin. The cultures were incubated at 37 °C in 95% air, 5% CO₂. Depending on the experiment, cells were maintained in 4-well (Nunclon), 24-well (Costar), or 96-well (Costar) plates. For determination of cell number, cells were detached from the substrate by gentle trituration in phosphate-buffered saline containing 0.02% EDTA and then centrifuged (500 × g, 5 min.). The cell pellet was resuspended in a solution containing the vital dye, trypan blue, to determine the number of viable mast cells.

**cDNA Preparation**—Immediately following isolation, RPMC cultures (about 3 × 10⁶ mast cells/well in 4-well plates) were maintained for 3 h in complete medium alone to allow cells to recover from the isolation procedure. Total RNA was isolated from these cultures after the appropriate treatment by using the single-step RNA-isolating reagent, Tri-solv (Biotex Laboratories, Houston, TX). From total RNA, Poly(A)+ RNA was isolated by using an oligo(dT)-cellulose mRNA purification kit (QuickPrep Micro kit; Pharmacia Biotech Inc.). The mRNA was reverse-transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase (Superscript II; Life Technologies Inc.) and random hexamers (16 µl) as primers. The 30-µl reaction contained 50 mM Tris (pH 8.3), 75 mM KCl, 6 mM MgCl₂, 10 mM dithiothreitole (DTT), dCTP, dGTP, and dTTP, and 10 units of RNasin (Promega Corp., Madison WI). After 5 min at 20 °C, the reactions were incubated for 1 h at 42 °C; the reactions were terminated by the addition of 170 µl of TE buffer (10 mM Tris (pH 8.0) and 1 mM EDTA) and then heating to 95 °C for 5 min.

**RT-PCR Analysis**—The method used for semiquantitative PCR analysis was adopted from that of Estus and Freeman (33, 34). For PCR amplification of specific cDNAs, stock reactions of 50 µl, prepared on ice, contained 10 mM Tris (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 1 µM each primer, 50 µM dCTP, 100 µM each dATP, dGTP, and dTTP, 15 µCi of [α-³²P]dCTP (3000 Ci/mmol), 1 unit of Tq DNA polymerase, and approximately 3000 cells worth of reverse transcribed material or 1% of the cDNA synthesized in the reverse transcription reaction. The primer sequences used for amplification of the various gene products analyzed in these experiments are presented in Table I. All primers were designed based on the rat sequence of the analyzed gene product; amplified products were verified by sequencing. The stock reaction solutions for each test sample being analyzed were separated into three equal aliquots, covered with 1 drop of mineral oil, and then subjected to the polymerase chain reaction for different cycle numbers to determine the minimum number of cycles necessary to detect amplified product for each primer set. The typical reaction conditions were 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C; with few exceptions (noted under “Results”), the results presented represent 20–25 cycles. Because of the differences in efficiency of reverse transcription that result from a varying degree of secondary structure present in different mRNAs and the differences in amplification efficiency among unique primer pairs, comparing the amount of amplified product for two different gene products is only a rough indicator of the absolute amount of mRNA. However, the efficiency of RT-PCR is quite consistent for a given message and primer pair, and the yield of amplified product is a reliable indicator of changes in the abundance of mRNA between samples. Amplified PCR product yields are linear with respect to input cDNA template over the range of PCR cycles used in these experiments (data not shown). After amplification, the cDNAs were separated by electrophoresis on 10% polyacrylamide gels, which were subsequently dried, visualized, and then quantified with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

**RESULTS**

NGF Activates Cytokine Expression in Mast Cells—NGF-mediated survival is cell density-dependent (1). Experiments demonstrated that mast cell-conditioned medium, conditioned in the presence of NGF, with subsequent depletion of NGF activity by the addition of excess neutralizing antibody, is able to support mast cell survival as well as fresh medium supplemented with NGF (1). Taken together, these results suggested that NGF survival promotion was indirect and mediated through increased expression of autocrine factor(s). Cytokines are good potential candidates for autocrine survival-promoting factors.

To characterize the pattern of NGF cytokine gene induction, equal numbers of freshly isolated RPMC were plated in complete RPMI medium and allowed to equilibrate at 37 °C. Cells were then treated with L-15 medium or L-15 with 200 ng/ml NGF incubated for the indicated times and processed for RNA purification. The samples for time 0 were isolated from cells maintained in vitro for 3 h and represent the state of mast cells at the time of factor addition. To determine a base line of gene expression, general cellular markers including β-actin (18 cycles) and cyclophilin (18 cycles), both constitutively expressed at high levels in most cell types, and the mast cell/basophil-specific FceRI-α subunit were examined. Fig. 1A shows the expression pattern profile for β-actin. Cyclophilin and the FceRI subunit displayed a similar pattern (data not shown). No sig-
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TABLE I

| Gene      | Accession number | Forward Primer sequence | Reverse Primer sequence | Product size (base pairs) |
|-----------|------------------|-------------------------|-------------------------|--------------------------|
| IL-1a     | D00403           | GACAAGCTTGGTTGCTGAGG    | ATCCGAGTGAAGTGGAGG      | 93                       |
| IL-1β     | M98820           | AGCTGGCTTCTGAGCTGG      | ATCCGAGTGAACCTCGAGG     | 117                      |
| IL-2      | M22899           | GAGATGAGCTTGGACCTGCG    | CAGAGTTGCTGACCATCTGG    | 167                      |
| IL-3      | X03914, X03846   | TCCTGACTGCTCTACCC       | TCGAGCTGACAGATACACCTG   | 302                      |
| IL-4      | X53085, X53089   | TATTGAGCTGCTACCCCC      | GAGTCCCTTCTTCTGAGCTGG   | 140                      |
| IL-5      | X55419           | GGTGAAAGACAGGCTTTACACG  | CGCAACACTTCTTTCTGAGG    | 252                      |
| IL-6      | M26774           | AAGAGCCTTCCAAGCTGGGCC   | GTCCCACCTCTTTCTGAGG     | 151                      |
| IL-10     | X60675           | TTAGAGTGAAGCTCGACCC     | TACCTTCCACGCTCCACCTG    | 159                      |
| GM-CSF    | U06620           | TGGCTGGAGACGAAAGACG     | ATTAGGTTGGGATGGTGGCCC   | 132                      |
| TNF-α     | D00475           | TTGCACTCTCATTACAGGAGAA  | TCCAGAAAGATGCTCCCC      | 226                      |
| TGF-β1    | X52498           | AACTGATATACCTGAGTGG     | GAAGCAAGATTGTTACACCC    | 290                      |
| CSF-1     | M84361           | GCCTCTGTTCTCAACAGTGG    | CAGAAAGATTGCTGGAGG      | 218                      |
| β-Actin   | J00691           | TATGGAGAAAGTGGACAC     | CTGCAGCAAGACAGCTGGCAT   | 300                      |
| Cyclophilin | M19533          | ATGGCTACCAACCAAGTGGT       | CTGGTGAAGTACACCCCT       | 220                      |
| Fce R1α   | J03906           | TGAAAGATCAATGATGACAGG   | GTGCTTGTTAAGTACACCTGG   | 185                      |
| bel-2 (mouse) | M16506       | CTTTGGTGGAGCTTTGGAGAGG  | ACAGCTGACATCTGTAGGTACATC | 251                      |
| bel-α (human) | J03906       | GCCTCTGTTCTCAACAGTGG    | AGCTGGTGGAGATGGTGA      | 150 (short), 337 (long)  |
| bax (mouse) | L24472        | GSGAATCTGAGCTCAGGATGATT | GGGGCTCAGTTTGGCACTG    | 96                       |
| ICE       | S79676           | TGAGGGCAAAGAGGAAGCG     | GTAAATACCTGCGGCACTG     | 204                      |
| cpp-32    | U34885           | GATTTTGTGATGTGATACGGAGG | AGAGAAAGATGCTGGAGG      | 218                      |
| ich-1/weed-2 (human) | U13021    | GTGGAGTGAAGAACCTGCT     | CGCAGCATCCTCCACAA       | 250 (short), 200 (long)   |

Significant differences were evident at the early times; at later times (15 h), only a slight increase of approximately 2-fold was apparent for RPMC maintained in NGF. The small increase observed in β-actin mRNA in NGF-treated RPMC is consistent with the reported activation of new transcription for β-actin in the pheochromocytoma cell line, PC12, within 1 h after treatment with NGF (35).

Cytokine gene induction in mast cells, similar to other immune cell types, is characterized by a rapid increase in the steady-state message levels, usually peaking within 1–2 h and then returning to base-line levels within 4–6 h (36). NGF activated such a response in the message levels for the cytokines IL-3, IL-4 (27 cycles), IL-10, GM-CSF, and TNF-α (Fig. 1, A and B). The induction response was maximal at 1 h, and mRNA levels returned to near base line by 6 h after NGF treatment.

IL-1α, IL-1β, and IL-6 had a high expression level at the time of treatment initiation; message levels decreased with increasing time in culture (Fig. 1C). NGF seemed to slow the rate at which message levels decreased as compared with vehicle-only-treated cells (Fig. 1C), but NGF treatment did not increase steady-state message levels significantly above that observed at time 0. The elevated mRNA levels for these cytokines at the time of treatment initiation may have resulted from the stress endured during the mast-cell isolation procedure. To address the possibility of resistance to NGF activation resulting from a refractory period after RPMC isolation, cells were maintained for 15 h in complete medium alone and then treated with 200 ng/ml NGF. RNA samples taken 1 h after the addition of NGF demonstrated no change in the levels of mRNA coding for IL-1α, IL-1β, and IL-6, whereas TNF-α was induced by NGF treatment (Fig. 1D).

In contrast to mRNA for other cytokines, expression of the mRNA for TGF-β1 was not affected by NGF and resembled the expression pattern observed for β-actin, cyclophilin, and the Fce-α subunit (data not shown). Expression of IL-2 or IL-5 was not detected in RPMC preparations except at high cycle numbers (>30 cycles), suggesting either very low levels of expression in mast cells or expression in some subset of mast cells or the small number (approximately 2%) of contaminating cells.

The time course for initial characterization of the effects of NGF on RPMC mRNA expression relied on treatment with 200 ng/ml of NGF. This NGF concentration is supramaximal for both activation of mast cell degranulation (25) and survival promotion (1). Such a high concentration was chosen for initial experiments to ensure a strong readout for any changes in mRNA activated by NGF. RPMC exposed for 1 h to incremental concentrations of NGF show a strong dependence for cytokine mRNA expression on NGF concentration (Fig. 2, A and B). The NGF concentration eliciting a half-maximal response is approximately 0.5–1.0 nM (~12–24 ng/ml) (Fig. 2B); this result is consistent with the dose response characteristics of NGF-activated mast cell mediator release (25) and survival promotion (1).

The high sensitivity of RT-PCR and the potential for non-mast peritoneal cell contamination of RPMC cultures requires that adequate controls be conducted to ensure that observed changes in mRNA levels are occurring in mast cells and not in some contaminating cell type. RT-PCR analysis of non-mast peritoneal cells revealed no detectable expression of the known receptors for NGF (1), and NGF treatment showed no increase for the mRNA encoding TNF-α, IL-3, IL-10, or GM-CSF (Fig. 3A). In RPMC cultures, the addition of 100 ng/ml of NGF that had been preincubated for 12 h with an excess of neutralizing antiserum was unable to increase mRNA levels of TNF-α, IL-3, IL-10, or GM-CSF (Fig. 3B). This result is consistent with the cytokine induction response being dependent on NGF and not the result of endotoxin contamination of the NGF preparation.

Cyclosporin A Blocks NGF- but Not IL-3- or SCF-mediated Survival—The induction of some cytokine genes in mast cells can be abrogated by the immunosuppressive agents CsA (37) and the glucocorticoid analog dexamethasone (38). The addition of either of these immunosuppressive agents to cultured RPMC in conjunction with NGF blocked survival promotion (Fig. 4, A and B). After 3 days in the presence of NGF, only 45% of the input cells survived compared with 5% survival observed when both NGF and CsA (1 μM) were added together (Fig. 4A). Similar results were observed when dexamethasone was used.
However, the same concentration of either of these immunosuppressants had no effect when added to RPMC maintained in IL-3 (Fig. 4A and B); CsA did not prevent the survival promotion or activation of proliferation by SCF (Fig. 4A). Thus, SCF and IL-3, in contrast to NGF, supported mast cell survival in the presence of CsA. We then tested the ability of CsA to block the NGF-activated increase in mRNA encoding IL-3 and IL-10, two potential autocrine-acting cytokines (Fig. 5). NGF induction of both messages was blocked by the addition of CsA (1 μM). These results are consistent with previous data indicating that NGF mediates survival promotion of RPMC by up-regulation of autocrine factors (1) and support the hypothesis that NGF maintains mast cell survival indirectly through increased expression of cytokines.

Survival-promoting Factors Increase RPMC Expression of bcl-2—bcl-2 and homologous genes that comprise the bcl-2 family have been implicated in the regulation of cell survival and programmed cell death in a variety of cell types (28). We determined whether NGF had any effect on the steady-state level of mRNA expression for bcl-2 (Fig. 6). RPMC maintained in the presence of NGF for 2 h expressed a 5-fold greater amount of bcl-2 than control cells (Fig. 6A). Unlike the cytokine mRNAs, bcl-2 message remained elevated for at least 15 h. This pattern of sustained increase in mRNA was, thus, different from the pattern observed for cytokines but was similar, although greater in magnitude, to changes in mRNA observed for β-actin, cyclophilin, and FcεRIα subunit. The observed increase of bcl-2 mRNA was delayed with respect to cytokine induction (Fig. 6B), suggesting that NGF may act through first up-regulating expression of cytokines that can feed back and activate bcl-2 expression.

Further characterization of the levels of mRNA encoding potential cell death-regulatory genes of the bcl-2 and ICE/ced-3 families was done by determining the relative expression levels at 15 h after treatment with the RPMC survival-promoting factor NGF, IL-3, SCF, or NGF plus IL-3. Three independent template sets were generated and normalized to the amount of product observed after PCR amplification of cyclophilin. Representative results after electrophoresis and autoradiography of bcl-2, bax, and cyclophilin are shown (Fig. 6C). Expression of
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DISCUSSION

NGF Increases Cytokine Expression in RPMC—We found that NGF induced the mRNAs for IL-3, GM-CSF, IL-4, IL-10, and TNF-α. Various mast cell lines and bone marrow-derived mast cell cultures respond to activation by increasing production of a broad range of different cytokines and lymphokines (36, 39–43). However, except in the case for TNF-α, little information exists regarding cytokine production by freshly isolated mast cells, which have many phenotypic differences from in vitro bone marrow-derived mast cells and mast cell lines (36). Our results are the first showing NGF induction of cytokine mRNAs in freshly isolated rat mast cells.

NGF exerts influences on several different immune cell types. NGF enhances the production of certain immunoglobulin isotypes (44) and supports survival and function of human neutrophils (45), and both circulating monocytes (46) and activated CD4+ Th2 T-cell clones (8) express functional Trk A receptor. Likewise, many different cell types can serve as a potential NGF source for mast cells in vivo. For example, fibroblasts, which are typically found in contact with connective tissue mast cells, respond to TNF-α (5) or IL-1 (4) by greatly increased production of NGF. In mast cell/fibroblast co-culture experiments, fibroblast-derived NGF was demonstrated to direct a phenotypic change in the mast cells (23), and single cell PCR experiments revealed an increase in IL-3 mRNA present in mast cells that are in contact with fibroblasts (48). Furthermore, NGF concentration increases measurably at the site of an inflammatory response (49) and in the serum of male mice after fighting (12).
Induction in mast cells of mRNA encoding several different cytokines suggests a role for NGF in the modulation of inflammation and other immunologic responses and the potential for involvement in certain pathological processes (9). For example, mast cell production of TNF-α results in local infiltration of leukocytes and enhances their function (50). GM-CSF enhances the growth and functional activity of mature cells derived from the myeloid lineage (51) and promotes the accumulation of dendritic cells (52). IL-3 specifically stimulates the proliferation of mast cell precursors (51) and enhances the in vitro survival but does not activate the proliferation of peritoneal mast cells (26). Both GM-CSF and IL-3 are essential factors in bone marrow hemopoiesis (51); and, although local production by mast cells is unlikely to influence bone marrow cells, a large systemic increase in NGF concentration, such as after fighting of male mice (12) or therapeutic administration of NGF, may activate a large enough number of mast cells to increase circulating levels of these two factors and thereby affect hemopoiesis. These factors have numerous actions in inflammation, clotting, angiogenesis, wound healing, and tissue repair, and their increased expression in NGF-activated mast cells supports the involvement of both NGF and mast cells in these normal healing processes.

Pathological processes such as chronic inflammation may be mediated by NGF and mast cells. One scenario easily envisioned might involve activated fibroblasts producing increased amounts of NGF, leading to activation of mast cell TNF-α production. Mast cell-derived TNF-α can activate fibroblast proliferation, increase collagen deposition, and induce greater NGF production. Such a feed forward mechanism is consistent with the participation of mast cells and fibroblasts in fibrosis.

![Fig. 4. The effect of immunosuppressive agents on RPMC survival promotion.](image)

![Fig. 5. Cyclosporin A inhibits the induction by NGF of IL-3 and IL-10 in RPMC.](image)
and chronic inflammation (53).

The RT-PCR methodology used here is a very reliable measure of changes in relative levels of specific mRNAs (54); however, it is not able to address the potential differences in absolute amounts of mRNA species in individual cells. Mast cells are a very heterogeneous population capable of expressing a broad range of phenotypic characteristics (14). RPMC are considered to express the connective tissue mast cell phenotype, but within this population, distinct subsets of cells may exist. T-helper cells can be classified as either Th1 or Th2 type based on differences in the pattern of cytokines produced after activation (55). Mast cells may be similarly restricted in the pattern of cytokines they are able to produce. Characterization by in situ hybridization with probes for the different cytokine genes after NGF activation would provide further insight into the degree of heterogeneity present in rat peritoneal mast cells.

Mast Cell Expression of Gene Products Potentially Involved in Regulation of Cell Death—Precisely how different trophic factors act to support mast cell survival is unknown, but survival for many other cell types is thought to be under genetic control. During development of the nematode Caenorhabditis elegans, cell death is controlled by the genes ced-3 and ced-4; expression of ced-9 blocks such deaths (56). The mammalian gene bcl-2, the first characterized member of a growing family of genes, is homologous to ced-9; its overexpression slows the rate of cell death activated by a number of different stimuli (57, 58). The cysteine protease ICE is the first well characterized member of a growing family of cysteine proteases (59) that are homologous to ced-3 (60). Although targeted gene disruption of ICE has no apparent effect on trophic factor deprivation-induced cell death (61), the role other members of this family may have in controlling cell death is currently being characterized.

RPMC maintained in NGF show a sustained increase in expression of bcl-2 compared with cells maintained in the absence of any trophic factor. Examination of different trophic

![Fig. 6. Assessment of RPMC expression levels for messenger RNA encoding bcl-2. A, representative autoradiograph for the PCR-amplified product generated with primers specific for bcl-2. B, the same data presented graphically after quantification. The PCR product demonstrating the greatest incorporation of [32P]dCTP was assigned a value of 100%; all other samples were normalized accordingly. NGF causes an increase in expression of bcl-2 mRNA levels that is delayed (in comparison with cytokine induction) and sustained for at least 15 h. These data are representative of two different time courses. C, representative autoradiographs showing expression levels of bcl-2 and bax after 15 h in the presence of 200 ng/ml NGF, 200 ng/ml SCF, 10 units/ml IL-3, or NGF plus IL-3. Poly(A)+ RNA was isolated after 15 h of treatment in three separate experiments. Template sets were generated by reverse transcription into cDNA and analyzed by PCR. D, quantification of PCR-amplified products for the indicated genes. Data represent the average ± S.D. of the amount of amplified product for the three different template sets after normalization within each template set.](image-url)
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Fig. 7. Effect of cyclosporin A on NGF induction of bcl-2 mRNA. Quantification of PCR-amplified products for bcl-2 in RPMC maintained in RPMI or in RPMI supplemented with NGF (100 ng/ml), CsA (1 μg/ml), or both for 15 h. After quantification, the values obtained for each template set were normalized. Data represent the average ± S.D. of the amount of amplified product for the three independent template sets. cDNA templates were first normalized to one another based on equivalent levels of cyclophilin expression.

factors demonstrated that bcl-2 expression was elevated, compared with untreated controls, when cells were treated with NGF, IL-3, or NGF plus IL-3; cells maintained in SCF demonstrated the greatest increase (approximately 20-fold) in bcl-2 message expression. Expression levels of the other messages, cpp-32, ICE, bcl-x long, bcl-x short, bax, ich-1 long, and ich-1 short, were approximately the same under all tested conditions. The absence of change in mRNA levels for the ICEced-3 family members does not indicate a lack of involvement for these cell death-associated gene products in the execution of mast cell death. The ICEced-3 family of proteases are made in an inactive, or less active, pro form that becomes activated after proteolytic processing. Such post-translational activation could theoretically serve as a trigger for a cascade of proteases responsible for execution of apoptosis (60).

Insight into the Potential Mechanism of NGF Survival Promotion of RPMC—Consistent with the hypothesis that some component of NGF survival promotion is acting indirectly through increasing cytokine production, the transient rise in cytokine expression may explain the transient nature of NGF-activated survival promotion (1). TNF-α at concentrations as high as 1000 units did not promote the survival of RPMC (data not shown). However, IL-3 supports the survival of rat peritoneal mast cells (26), and IL-10 significantly enhances the rate of proliferation observed for bone marrow-derived murine mast cells after activation by IL-3 (62). In conjunction with IL-3, IL-4 can enhance murine bone marrow-derived mast cell colony formation and activates proliferation of mouse peritoneal mast cells (63, 64). Therefore, several of the cytokines induced by NGF possibly contribute to the survival-promoting effects of the factor. Concentrations of CsA that blocked NGF survival promotion of RPMC (but not survival activated by IL-3 or SCF) also blocked induction of IL-3 and IL-10, supporting the hypothesis that NGF activates survival indirectly through increased cytokine expression. The inability of CsA to block IL-3- or SCF-mediated survival signals indicates direct action by these factors. Similarly, CsA has no effect on NGF survival promotion of rat sympathetic neurons.2 NGF was able to activate bcl-2 expression in RPMC in either the presence or the absence of CsA (Fig. 7). These data suggest that NGF signal transduction in RPMC has both CsA-sensitive and -insensitive pathways.

The ability of NGF to act directly, up-regulating bcl-2 mRNA in the presence of CsA, suggests that the increase in bcl-2 mRNA is insufficient to effect survival or that the levels of bcl-2 are sufficient and CsA somehow acts downstream of bcl-2 and blocks its survival-promoting activity. We are not aware of any evidence supporting the hypothesis that CsA acts downstream inhibiting bcl-2 function. Typically, investigators testing the effects of increased levels of bcl-2 drive overexpression by using strong promoter/enhancer constructs, resulting in very high expression (57). Careful titration of the degree of inhibition of cell death with the magnitude of bcl-2 overexpression would be required to better understand what effects, if any, a 5–7-fold increase in bcl-2 expression might have on RPMC survival. Another possibility is that RPMC treated with CsA die through a mechanism that is resistant to inhibition by bcl-2 expression. CsA activates certain WEHI-231 cell lines to undergo programmed cell death that is blocked by bcl-x but not bcl-2 overexpression, implying the existence of a bcl-2-resistant cell death pathway (65). If such an alternative bcl-2-resistant apoptotic pathway is utilized by RPMC, then an increase in bcl-2 will have no impact on cell survival.

NGF is currently being considered for use in clinical trials for treatment of both peripheral neuropathy and Alzheimer’s disease (47). Long term elevation of NGF may result in repeated mast cell activation and induction of mast cell cytokine expression, and it may be proinflammatory and promote fibrosis and other autoimmune disorders. The potential for chronic and repeated mast cell activation and other immunomodulatory effects of NGF should be studied in more detail when considering possible adverse effects of using NGF clinically by systemic or intracerebroventricular administration.

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REFERENCES
1. Horigome, K., Bullock, E. D., and Johnson, E. M., Jr. (1994) J. Biol. Chem. 269, 2685–2702
2. Snider, W. D., and Johnson, E. M., Jr. (1989) Ann. Neurol. 26, 489–506
3. Hefti, F. (1986) J. Neurosci. 6, 2155–2162
4. Lindholm, D., Heumann, R., Hengerer, B., and Thoenen, H. (1988) J. Biol. Chem. 263, 16348–16351
5. Hattori, A., Tanaka, R., Murase, K., Ishida, N., Chatani, Y., Tsujimoto, M., Hayashi, K., Kohno, M. (1993) J. Biol. Chem. 268, 2577–2582
6. Goodwin, D., and Tattile, J. B. (1991) Hypertension 18, 730–741
7. DiPersio, E., Marchisio, P. C., Francis, J. P., Cancedda, R., and De Luca, M. (1991) J. Biol. Chem. 266, 21718–21722
8. Ehrhard, P. B., Erb, P., Graumann, U., and Otten, U. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10964–10968
9. Levi-Montalcini, R., Tsoe, R. D., Valle, F., Skaper, S. D., and Leon, A. (1995) J. Neurosci. Sci. 130, 119–127
10. Scully, J. L., and Otten, U. (1985) Cell Biol. Int. 19, 459–469
11. Li, A. K. C., Koroly, M. J., Schattenkerk, M. E., Malt, R. A., and Young, M., Jr. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6757–6761
12. Aloe, L., Alleva, E., Bohn, A., and Levi-Montalcini, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6184–6187
13. Aloe, L., and Levi-Montalcini, R. (1977) Brain Res. 133, 358–366
14. Galli, S. J. (1990) Lab. Invest. 62, 5–33
15. Galli, S. J. (1990) J. Clin. Invest. 86, 386–400
16. Galli, S. J. (1990) J. Exp. Med. 167, 7–14
17. Shahanian, F., Denburg, J. A., Fox, J., Bienenstock, J., and Befus, D. (1985) J. Immunol. 135, 1351–1357
18. Ansel, J. C., Brown, J. R., Payan, D. G., and Brown, M. A. (1993) J. Immunol. 150, 4478–4485
19. Bruni, A., Bigon, E., Boarato, E., Mietto, L., Leon, A., and Toffano, G. (1982) J. Immunol. 128, 533–542
20. Otten, U., Baumann, J. B., and Girard, J. (1984) Eur. J. Pharmaco. 106, 199–201
21. van, Y., Sato, E., and Wilkins, M. R. (1991) Clin. Sci. 80, 565–569
22. Pearson, F. L., and Thompson, H. L. (1986) J. Physiol. 372, 379–393
23. Matsuda, H., Kannan, Y., Ushio, H., Kiso, Y., Kanemoto, T., Suzuki, H., and Kitamura, Y. (1991) J. Exp. Med. 174, 7–14
24. Getchell, M. L., Kulkarni-Narla, A., Takami, S., An, T. J., and Getchell, T. V. (1992) Neuroreport 6, 2577–2582
25. Horigome, K., Bullock, E. D., and Johnson, E. M., Jr. (1993) J. Immunol. 150, 1351–1357

2 T. Deckwerth, personal communication.
