Nerve Growth Factor Induces Development of Connective Tissue-type Mast Cells In Vitro from Murine Bone Marrow Cells

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Summary

The effect of nerve growth factor (NGF) on proliferation/differentiation of mast cells was investigated in vitro. Although NGF alone neither supported colony formation of bone marrow–derived cultured mast cells (BMCMC) nor induced development of mast cell colonies from nonadherent bone marrow cells (NBMC), addition of NGF to the suboptimal dose of interleukin 3 (IL-3) significantly increased the numbers of mast cell colonies produced by BMCMC or NBMC in methylcellulose. When stimulated by IL-3 alone, cells in mast cell colonies were not stained by berberine sulfate, a fluorescent dye. In contrast, mast cells developing in methylcellulose cultures obtaining both IL-3 and NGF were stained by berberine sulfate. The fluorescence was abolished by the treatment of heparinase but not of chondroitinase ABC, suggesting that mast cells stimulated by IL-3 and NGF produced and stored heparin proteoglycan. The histamine content of BMCMC maintained by IL-3 was also increased by addition of NGF. Since BMCMC showed mucosal mast cell–like phenotype, NGF appeared to induce the phenotypic change to connective tissue-type mast cells (CTMC). In the culture containing BMCMC, 3T3 fibroblasts, and IL-3, the phenotypic change of BMCMC to CTMC was observed as well. Since NGF was detected in this coculture and since addition of anti-NGF monoclonal antibody suppressed the phenotypic change, NGF produced by fibroblasts appeared to induce the phenotypic change. Neither BMCMC alone nor IL-3 alone increased the concentration of NGF. Therefore, there is a possibility that BMCMC stimulated by IL-3 may induce the production and/or release of NGF by fibroblasts.

Mast cell precursors that are derived from the multipotential hematopoietic stem cell circulate in the bloodstream, invade into tissues, and differentiate into two phenotypically distinct populations: connective tissue-type mast cells (CTMC)[1] and mucosal mast cells (MMC). CTMC and MMC are distinguishable by location, histochemical and biochemical characteristics, electron microscopic features, and T cell dependency (1–4). Murine bone marrow–derived cultured mast cells (BMCMC), which develop in the presence of T cell–derived growth factors (IL-3, IL-4, and IL-9) (5, 6), resemble MMC in their phenotype; both are stained with alcian blue but not berberine sulfate, contain little or no cytoplasmic electron-dense granules, and low levels of histamine (7–10). Coculture of BMCMC with 3T3 fibroblast cell lines induced a phenotypic change to CTMC-like cells (11), indicating that fibroblasts may influence the phenotype of mast cells. However, the exact role of fibroblasts has not been clarified.

Nerve growth factor (NGF) is a well-characterized neurotropic polypeptide for the survival, development, and function of basal forebrain cholinergic neurons in the central nervous system, as well as peripheral sympathetic and embryonic sensory neurons (12–15). Despite its name, NGF has been shown to elicit other biological effects on nonneuronal tissues. For example, NGF modulates both T and B cell–mediated immune responses (16, 17), induces shape changes in platelets (18), enhances the viability and functional properties of neutrophils (19), and accelerates wound healing (20).

Recently, we demonstrated that NGF promoted human

[1] *Abbreviations used in this paper:* BMCMC, bone marrow–derived cultured mast cell; CTMC, connective tissue-type mast cell; EGF, epidermal growth factor; GM, granulocyte/macrophage; MMC, mucosal mast cell; NBMC, nonadherent bone marrow cell; NGF, nerve growth factor; PMC, peritoneal mast cell.

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hematopoietic colony formation, and acted in a relatively selective fashion to induce the differentiation of eosinophils and histamine-containing cells (basophils or mast cells) (21, 22). Aloe and Levi-Montalcini (23) have shown that injections of NGF into neonatal rats result in the significant increase of mast cells in various tissues. Since NGF is secreted by fibroblasts (24), there is a possibility that development of CTMC-like mast cells in the coculture of BMCMC and fibroblasts is mediated by NGF released from fibroblasts. In the present study, we showed that NGF induced the phenotypic change from BMCMC to CTMC-like cells and that colonies composed of CTMC-like cells developed when murine bone marrow cells were cultured in methylcellulose in the presence of IL-3 and NGF.

Materials and Methods

Cell Suspensions. Male $WBB6F_1$-+/+ mice were used at 10–15 wk of age. These mice were raised by mating WB females with C57BL/6 males in our laboratory. The method to prepare cell suspensions has been described (25).

Cytokines and Other Reagents. 2.5S NGF purified from murine submaxillary glands was kindly provided by Drs. A. M. Stanisz and J. Bienenstock (McMaster University, Ontario, Canada) (21). Neurotropic biological activity of the preparations was measured in the dissociated cell assay, using neonatal mouse superior cervical ganglion neurons; half-maximal response was at 1 ng/ml. IL-3 purified from WEHI-3 supernatant was a kind gift from Dr. J. N. Ihle (National Cancer Institute, Frederick, MD) (26). Human rIL-1β was obtained from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Murine rIL-4, rIL-5, granulocyte/macrophage-CSF (GM-CSF), and TNF-α were obtained from Genzyme (Boston, MA). Murine epidermal growth factor (EGF) was provided by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Purified murine IgG mAb to 2.5S NGF (clone β1) was a kind gift from Dr. E. M. Shooter (Stanford University, Stanford, CA).

BMCMC. Bone marrow cells of $WBB6F_1$-+/+ mice were cultured at a density of 10^6 cells/ml in α-MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10^{-4} M 2-ME (Sigma Chemical Co., St. Louis, MO), 10% FCS (Hyclone Labs, Logan, UT), and 10% PWM-stimulated spleen cell–conditioned medium (PWM-SCM). The method to prepare PWM-SCM has been described (27), and PWM-SCM contains IL-3, IL-4, and probably IL-9 (6, 28). Culture flasks (Nunc, Roskilde, Denmark) were incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air. Half of the medium was replaced every 7 d. As described by Nakano et al. (10), >95% of cells were identified as immature mast cells 4 wk after the initiation of the culture; the cells were harvested, washed, and resuspended in α-MEM.

Clonal Cell Culture. Methylcellulose culture was carried out by using a modification of the technique described by Nakahata et al. (27). Bone marrow cells obtained from femurs and tibias of $WBB6F_1$-+/+ mice were suspended in MEM containing 10% FCS and allowed to adhere in plastic culture flask (Nunc) for 2 h at 37°C. The resulting nonadherent bone marrow cells (NBMC) were resuspended in α-MEM. 10^4 BMCMC or 5×10^4 NBMC were cultured in 1 ml of α-MEM containing 0.9% methylcellulose (Sigma Chemical Co.), 30% FCS, 10^{-4} M 2-ME, 1% BSA (Sigma Chemical Co.), 50 U/ml penicillin, and 50 mg/ml streptomycin in the presence of 50 U/ml IL-3 with or without other cytokines at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. As a control, 50 ng/ml cytochrome C (Sigma Chemical Co.) was added to methylcellulose culture instead of cytokines. 20 d after the initiation of the culture, mast cell colonies containing >20 cells were identified according to the criteria described by Nakahata et al. (27) and counted under an inverted microscope.

Staining. Individual colonies were picked up from methylcellulose cultures by a Pasteur pipette under direct microscopic visualization and placed onto glass slides. The preparations were fixed in Carnoy's fluid and stained with 0.025% berberine sulfate (Sigma Chemical Co.) at pH 2.5. Berberine sulfate is a fluorescent dye that specifically stains heparin (3, 9, 29). To confirm the specificity of staining, some specimens were treated with either heparinase (heparin lyase from *Flavobacterium heparium*; Seikagaku Chemical Industry Co. Ltd., Tokyo, Japan) or with chondroitinase ABC (chondroitin ABC lyase from *Proteus vulgaris*, Seikagaku Chemical Industry Co. Ltd.) before staining according to the method described by Nakano et al. (10). Specimens were fixed in chilled calcium acetate formalin at 4°C for 18 h and overlaid with heparinase or chondroitinase ABC dissolved in 0.1 M Tris-HCl buffer at the concentration of 0.5 IU/ml (pH 7.0) or 5.0 IU/ml (pH 8.0), respectively. After washing with Tris-buffer of the same pH, specimens were stained with berberine sulfate. Fluorescent intensity of cells in mast cell colonies was determined by using a systemphotometer (OSP-1; Olympus Japan Co. Ltd., Tokyo, Japan), which can detect the fluorescence of individual cells stained with berberine sulfate. After the measurement of fluorescent intensity, the same specimens were stained with alcian blue at pH 2.5.

Coculture of mast cells and fibroblasts (see below) was trypsinized, and the cells were washed with PBS and spun with a cytocentrifuge (Cytospin, Shandon Southern, Elliott, IL) at 600 rpm for 5 min. The cytospin preparations were stained with berberine sulfate and alcian blue as stated above.

Isolation of Peritoneal Mast Cells (PMC). Murine PMC were collected according to the method described by Yurt et al. (30). $WBB6F_1$-+/+ mice were intraperitoneally injected with 5 ml of Tyrode's buffer containing 0.1% gelatin (Sigma Chemical Co.). Peritoneal cells were collected from lavage of the peritoneal cavity. After washing with Tyrode's buffer containing 0.1% gelatin, 1 ml of the cell suspension was layered on 2 ml of 22.5% (wt/vol) metrizamide (Daiichi Pure Chemical Co. Ltd., Tokyo, Japan) and centrifuged at room temperature for 15 min at 400 *g*. The cell pellets were washed, resuspended in α-MEM containing 10% FCS, and spun in the cytocentrifuge.

Electron Microscopy. Some mast cell colonies were collected in microcentrifuge tubes containing 2.5% glutaraldehyde-2% paraformaldehyde solution and fixed at 4°C for 1 h. After washing with PBS, the specimens were postfixed at 4°C for 2 h in 1% osmium tetroxide, processed routinely, and then embedded in Epon 812. Thin sections (60 nm thick) were stained with uranyl acetate and lead citrate, and examined with an electron microscope (H-600; Hitachi).

Histamine Assay. 2 ml of a culture mixture containing 2×10^5 BMCMC, α-MEM, 10% FCS, antibiotics, and 100 U IL-3 with various amounts of NGF were layered onto 35-mm culture dishes (Corning Grass Works, Corning, NY) and incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. As a control, 100 ng cytochrome C was added into the culture instead of NGF. Half of the medium was replaced 1 wk after the initiation of the culture. 2 wk later, the cultured cells were harvested, washed, resuspended in 0.4 ml PBS, and then the number of mast cells was counted. After adding 0.1 ml of 12% perchloric acid, the cell suspensions were stored at −80°C. Histamine concentration was de-

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termined by a fluorometric assay described by Shore et al. (31), and the content of histamine per 10^5 mast cells was calculated.

Coculture of BMCMC with Fibroblasts. Coculture of BMCMC with 3T3-Swiss albino fibroblasts, obtained from the Japanese Cancer Research Resources Bank, was carried out by using a modification of the technique described by Levi-Schaffer et al. (11) and Fujita et al. (32). Briefly, 10^5 BMCMC suspended in 2 ml of α-MEM containing 10% FCS and antibiotics with or without 50 U/ml IL-3 were added onto a confluent monolayer of 3T3 fibroblasts in 35-mm culture dishes. The culture medium was collected and replaced with 2 ml of fresh culture medium every 2 d. NGF levels in the collected culture media were measured by an ELISA using anti-NGF mAb (Boehringer Mannheim Biochemicals, Mannheim, FRG) (33). 14 d after the initiation of the coculture, the fluorescent intensity of mast cells after staining with berberine sulfate was examined in cytocentrifuge preparations of trypsinized cultures.

In other experiments, 50 U/ml IL-3 and 10 μg/ml anti-NGF mAb (clone β1) were added into the coculture of BMCMC with 3T3 fibroblasts. Dishes were incubated for 2 wk at 37°C in a humidified atmosphere with 5% CO_2 in air. The culture medium was aspirated and replaced with 2 ml of fresh culture medium with IL-3 and anti-NGF mAb every 2 d.

Results

Effect of NGF on Mast Cell Colony Formation. 10^4 murine BMCMC were incubated in 1 ml of methylcellulose culture with various doses of IL-3. Without IL-3, no colonies developed for the incubation period of 20 d. Addition of IL-3 at doses of 0.5–100 U/ml resulted in formation of mast cell colonies, and the number of colonies was increased in a dose-dependent manner (Fig. 1).

Since NGF promotes differentiation of basophilic cells containing histamine from human peripheral blood (21) and cord blood (22), we examined its effect on formation of mast cell colonies from BMCMC and NBMC. NGF alone (50 ng/ml) did not support colony formation of BMCMC, but addition of NGF (50 or 500 ng/ml) to a suboptimal dose of IL-3 (50 U/ml) significantly augmented the number of colonies (Table 1). The specificity of the effect of NGF was examined by using mAb to 2.5S NGF. Addition of the anti-NGF mAb completely abolished the effect of NGF. Cytochrome C (50 ng/ml) did not affect development of mast cell colonies with or without IL-3.

In the next experiment, differentiation of mast cells from NBMC was examined. Addition of NGF (50 and 500 ng/ml) to the suboptimal dose of IL-3 increased the number of mast cell colonies in a dose-dependent manner, whereas NGF alone (50 ng/ml) had no effect on development of mast cell colonies (Table 2). The effect of NGF was abolished by anti-NGF mAb. Cytochrome C neither enhanced nor suppressed development of mast cell colonies.

Fluorescent Intensity of Mast Cells Stained with Berberine Sulfate. Cells in mast cell colonies that developed in cultures of BMCMC or NBMC in the presence of 50 U/ml IL-3 were stained with alcian blue (94% of cells in positive) but not with berberine sulfate (100% of cells in negative), as reported by several investigators (2–4, 7). When both 50 U/ml IL-3 and 50 ng/ml NGF were added to the cultures of BMCMC or NBMC, the cells in mast cell colonies were stained not only with alcian blue (97% of cells in positive) but also with berberine sulfate (92% of cells in positive).

Fluorescent intensity of each cell in mast cell colonies was measured by the systemphotometer. The fluorescent intensity of murine PMC, typed as CTMC, was decreased after treatment with 0.5 IU/ml heparinase for 30 min, but not after treatment with 5 IU/ml chondroitinase ABC for 4 h (Table 3). Addition of 50 ng/ml NGF to 50 U/ml IL-3 in-

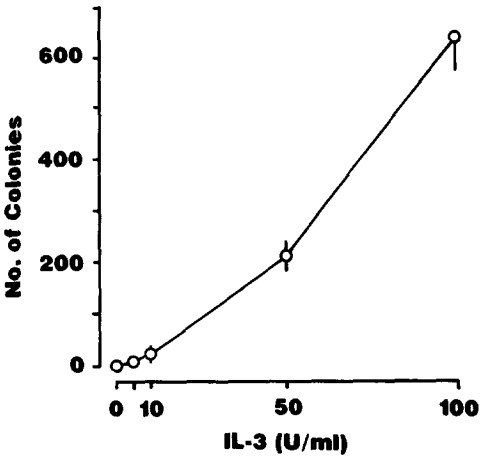

Figure 1. Colony formation of BMCMC in the presence of various concentrations of IL-3. 10^4 BMCMC were cultured in methylcellulose, and colonies were counted 20 d of the culture. Each value is expressed as means ± SE of three separate experiments performed in duplicate.

Table 1. *Colony Formation by BMCMC Promoted by 2.5S NGF*

Added factor(s)*	No. of colonies
	mean ± SE
None	0
NGF (50 ng)	0
Cytochrome C (50 ng)	0
IL-3 (50 U)	94 ± 18
IL-3 (50 U) + NGF (50 ng)	146 ± 9‡
IL-3 (50 U) + NGF (500 ng)	313 ± 15§
IL-3 (50 U) + cytochrome C (50 ng)	85 ± 11
IL-3 (50 U) + NGF (50 ng) + anti-NGF mAb (20 μg)	69 ± 6‖

BMCMC were cultured in methylcellulose at a density of 10^4 cells/ml with factor(s), and colonies were counted 20 d after the incubation. Each value was obtained from three to five separate experiments performed in duplicate.

* Amounts are per milliliter.

‡ $p < 0.05$, when compared with IL-3 alone by Student's t test.

§ $p < 0.001$, when compared with IL-3 alone.

‖ $p < 0.001$, when compared with IL-3 + NGF (50 ng).

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Table 2. *Mast Cell Colony Formation by NBMC Promoted by 2.5S NGF*

Added factor(s)*	No. of colonies
	mean ± SE
None	0
NGF (50 ng)	0
Cytochrome C (50 ng)	0
IL-3 (50 U)	3.0 ± 0.7
IL-3 (50 U) + NGF (50 ng)	6.0 ± 0.2‡
IL-3 (50 U) + NGF (500 ng)	12.6 ± 1.2§
IL-3 (50 U) + cytochrome C (50 ng)	2.8 ± 1.0
IL-3 (50 U) + NGF (50 ng) + anti-NGF mAb (20 μg)	3.2 ± 1.4‖

NBMC were cultured in methylcellulose at a density of 5 × 10^4 cells/ml with factor(s), and mast cell colonies were counted 20 d after the incubation. Each value was obtained from four to eight separate experiments performed in duplicate.

* Amounts are per milliliter.

‡ $p < 0.01$, when compared with IL-3 alone.

§ $p < 0.001$, when compared with IL-3 alone.

‖ $p < 0.05$, when compared with IL-3 + NGF (50 ng).

creased the fluorescent intensity of the mast cells developing from either BMCMC or NBMC, and the value was comparable with that of PMC. The fluorescence was abolished by heparinase treatment, but not by chondroitinase ABC treatment (Table 3). The effect of NGF was abolished by addition of anti-NGF mAb (Table 3).

Electron Microscopic Features of the Cells in Mast Cell Colonies. PMC and cultured mast cells were examined by the electron microscope. PMC contained numerous homogeneous electron-dense granules, but mast cells developed in the culture of BMCMC or NBMC stimulated by IL-3 alone possessed few dense granules and cytoplasmic vacuoles containing flocculent materials (Fig. 2 *A*). In contrast, when BMCMC or NBMC were stimulated by both IL-3 and NGF, developing mast cells became larger in size and contained abundant electron-dense granules, and the electron microscopic features resembled those of PMC (Fig. 2 *B*).

Effect of NGF on Histamine Contents of BMCMC. Because histamine contents of CTMC are higher than those of BMCMC or MMC (3, 34), we estimated histamine contents in BMCMC cultured for 2 wk in liquid medium containing IL-3 and NGF. BMCMC cultured with a fixed dose of IL-3 (50 U/ml) and increasing doses of NGF showed a dose-dependent increase of histamine contents to fivefold levels (Table 4). In contrast, addition of 50 ng/ml cytochrome C

Table 3. *Fluorescent Intensity of Mast Cells Stained with Berberine Sulfate*

Cells	Added factor(s)*	Enzyme treatment‡	Fluorescent intensity
			rlu/cell
PMC	–	–	3.722 ± 0.106§
	–	Heparinase	1.310 ± 0.198
	–	Chondroitinase ABC	3.393 ± 0.189§
Cells from	IL-3 (50 U)	–	1.360 ± 0.018
BMCMC	IL-3 (50 U) + NGF (50 ng)	–	3.124 ± 0.084‖
colony	IL-3 (50 U) + NGF (50 ng)	Heparinase	1.339 ± 0.106
	IL-3 (50 U) + NGF (50 ng)	Chondroitinase ABC	2.579 ± 0.128‖
	IL-3 (50 U) + NGF (50 ng) + anti-NGF mAb (20 μg)	–	1.189 ± 0.061
Cells from	IL-3 (50 U)	–	1.084 ± 0.018
NBMC	IL-3 (50 U) + NGF (50 ng)	–	2.606 ± 0.055‖
colony	IL-3 (50 U) + NGF (50 ng)	Heparinase	0.983 ± 0.078
	IL-3 (50 U) + NGF (50 ng)	Chondroitinase ABC	2.496 ± 0.202‖
	IL-3 (50 U) + NGF (50 ng) + anti-NGF mAb (20 μg)	–	0.805 ± 0.051

BMCMC and NBMC were cultured in methylcellulose at a density of 10^4 and 5 × 10^4 cells/ml, respectively, and mast cell colonies were picked up 20 d after the culture. The cells were stained with berberine sulfate, and fluorescent intensity of each cell was examined using the systemphotometer. Each value represents mean ± SE of 100–250 cells.

* Amounts are per milliliter.

‡ Before staining with berberine sulfate, the cells were treated with either heparinase or chondroitinase ABC.

§ $p < 0.001$, when compared with PMC which received heparinase treatment.

‖ $p < 0.001$, when compared with cells harvested from colonies that were stimulated by IL-3 alone.

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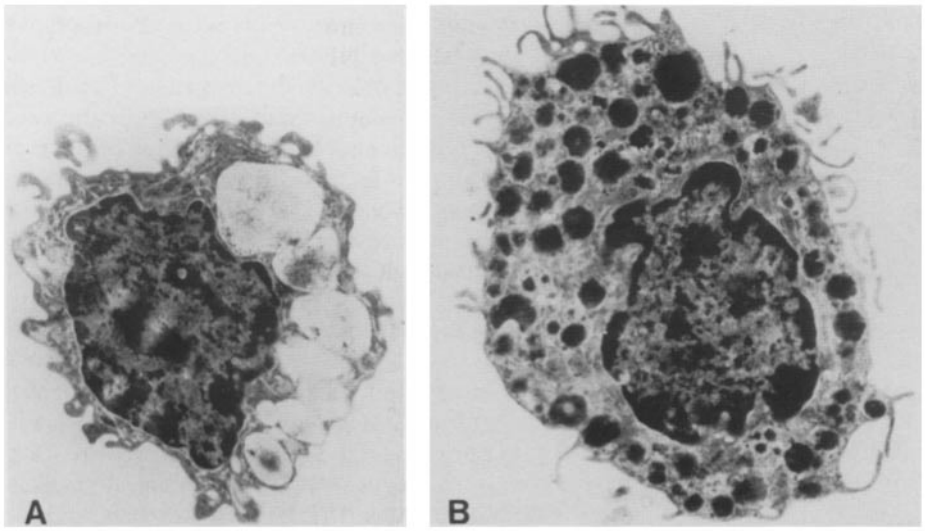

Figure 2. Electron microscopic features of cells in mast cell colonies that developed after plating 5 × 10^4 NBMC in methylcellulose: (*A*) culture medium containing IL-3 (50 U/ml) alone; (*B*) culture medium containing both IL-3 and NGF (50 ng/ml). The former mast cell contains several large vacuoles with flocculent materials. In contrast, the latter mast cell contains nearly homogeneous electron-dense granules (×10,000).

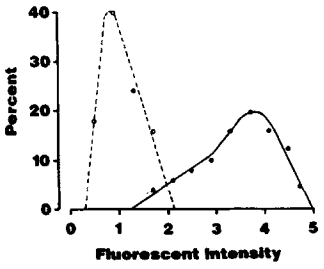

Figure 3. Effect of anti-NGF mAb on the fluorescent intensity of mast cells stained with berbertine sulfate. BMCMC were cultured with fibroblasts and IL-3, with (O) and without (●) anti-NGF mAb.

to 50 U/ml IL-3 did not increase histamine contents of BMCMC. The addition of anti-NGF mAb abolished the effect of NGF on histamine contents (Table 4).

Effect of Various Cytokines on the Phenotypic Change. To assess the specificity of NGF, we further examined the effect of other cytokines (IL-1β, IL-4, IL-5, EGF, GM-CSF, and TNF-α) in methylcellulose culture of BMCMC. A suboptimal dose of IL-3 (50 U/ml) was present in all cultures. The addition of 100 U/ml IL-1β, 50 U/ml IL-5, 500 ng/ml EGF, or 10 ng/ml TNF-α neither increased the number of colonies, nor induced development of berberine sulfate–positive mast cells (Table 5). The number of mast cell colonies was increased in the culture supplemented with 100 U/ml IL-4 and 50 U/ml IL-3 by more than three times as compared with IL-3 alone, and the number was decreased in the culture supplemented with 50 U/ml GM-CSF and 50 U/ml IL-3 by half (Table 5). Neither IL-4 nor GM-CSF affect the proportion of berberine sulfate–positive mast cells.

Table 4. *Increase in Histamine Contents of BMCMC Induced by 2.5S NGF*

Added factor(s)*	Histamine contents
	ng/10^5 cells
IL-3 (50 U)	19 ± 2
IL-3 (50 U) + NGF (5 ng)	41 ± 4‡
IL-3 (50 U) + NGF (50 ng)	82 ± 5§
IL-3 (50 U) + NGF (500 ng)	100 ± 6§
IL-3 (50 U) + cytochrome C (50 ng)	16 ± 4
IL-3 (50 U) + NGF (50 ng) + anti-NGF mAb (20 μg)	18 ± 1‖

BMCMC were incubated for 2 wk in 35-mm culture dishes at a density of 10^5 cells/ml in α-MEM containing 10% FCS, antibiotics, and factor(s). Half of the medium was replaced 1 wk later. The histamine content (mean ± SE) of the cells was determined for four separate experiments.
* Amounts are per milliliter.
‡ $p < 0.01$, when compared with IL-3 alone.
§ $p < 0.001$, when compared with IL-3 alone.
‖ $p < 0.01$, when compared with IL-3 + NGF (5 ng).

Table 5. *Colony Formation by BMCMC cultured with 50 U/ml IL-3 and Various Cytokines*

Added cytokines*	No. of colonies
	mean ± SE
IL-3	135 ± 9
IL-3 + IL-1β (100 U)	112 ± 8
IL-3 + IL-4 (100 U)	514 ± 10‡
IL-3 + IL-5 (50 U)	141 ± 4
IL-3 + EGF (500 ng)	139 ± 8
IL-3 + GM-CSF (50 U)	60 ± 6§
IL-3 + TNF-α (10 ng)	159 ± 21

BMCMC were cultured in methylcellulose at a density of 10^4 cells/ml with factor(s), and colonies were counted 20 d after the incubation. Each value was obtained from two separate experiments performed in duplicate.
* Amounts are per milliliter.
‡ $p < 0.01$, when compared with IL-3 alone.
§ $p < 0.05$, when compared with IL-3 alone.

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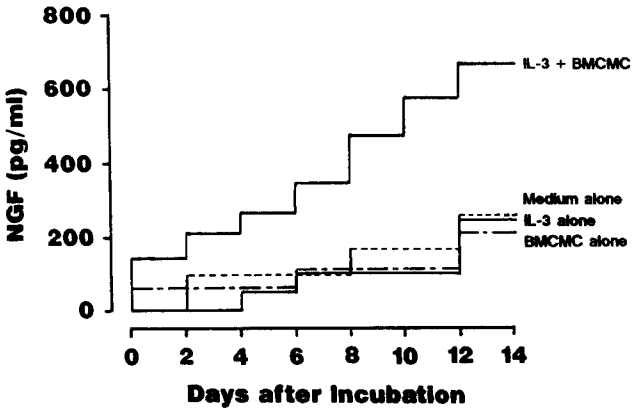

Figure 4. Accumulative NGF levels in the culture medium of fibroblast monolayers. Four different conditions were examined: (*a*) fibroblast monolayers; (*b*) IL-3 alone added to fibroblast monolayers; (*c*) BMCMC alone added to fibroblast monolayers; (*d*) both IL-3 and BMCMC added to fibroblast monolayers. Results are expressed as the mean values of three separate experiments performed in duplicate.

Production of NGF by Fibroblasts. We examined whether NGF produced by fibroblasts is actually involved in the phenotypic change of mast cells. BMCMC and 3T3 fibroblasts were cocultured with or without IL-3. 2 wk after the initiation of the coculture, mast cells harvested from the coculture containing IL-3 were strongly stained with berberine sulfate, but mast cells harvested from the coculture without IL-3 were not. When anti-NGF mAb was added to the coculture containing IL-3, no mast cells were stained with berberine sulfate (Fig. 3).

NGF levels were assessed by an ELISA in four different culture conditions: (*a*) confluent monolayers alone; (*b*) IL-3 (50 U/ml) added to the monolayers; (*c*) BMCMC added to the monolayers; (*d*) both BMCMC and IL-3 added to the monolayers. NGF levels increased significantly only when both BMCMC and IL-3 were added (Fig. 4).

Discussion

Although NGF alone neither supported colony formation of BMCMC nor induced development of mast cell colonies from NBMC, addition of NGF to the suboptimal dose of IL-3 increased the numbers of mast cell colonies produced by BMCMC or NBMC. For the proliferation of BMCMC, IL-4 has been reported to act synergistically with IL-3 (35). To our knowledge, the present report is the first one to describe the synergistic action of NGF.

The second effect of NGF on differentiation of mast cells is induction of CTMC-like mast cells. When NGF was added to IL-3, mast cells developing in methylcellulose cultures appeared to contain more heparin than mast cells developing in methylcellulose cultures without NGF. The histamine content of BMCMC maintained by IL-3 was increased by addition of NGF. Although IL-4 increased the number of mast cell colonies, all cytokines examined in the present experiment, including IL-4, did not induce the expression of CTMC-like phenotype.

Combination of staining with berberine sulfate and treatment with heparinase or chondroitinase ABC is a useful method to classify the subpopulation of mast cells. CTMC are berberine sulfate positive, and the fluorescence is abolished by the treatment of heparinase but not of chondroitinase ABC (10, 29). This suggests that the heparinase-sensitive fluorescence after staining with berberine sulfate represents the content of heparin in mast cell granules. We quantitatively measured the fluorescent intensity by using the systemphotometer. This system enables us to estimate the phenotype of individual mast cells.

Levi-Schaffer et al. (11) first reported that BMCMC acquired CTMC-like phenotype when cocultured with 3T3 fibroblasts. Although Fujita et al. (32) also cocultured BMCMC with 3T3 fibroblasts, the efficiency of the phenotypic change was much higher in the former report than in the latter report. These two experiments are principally different in one point; IL-3 was added in the former experiment but not in the latter experiment. We added IL-3 to the coculture and obtained the result that was comparable with the result of Levi-Schaffer et al. (11). This implies the involvement of NGF in the phenotypic change of BMCMC induced by fibroblasts. The amount of NGF released into the medium was increased significantly when fibroblasts were cultured in the presence of BMCMC and IL-3. Neither BMCMC alone nor IL-3 alone increased the concentration of NGF in the medium. There is a possibility that BMCMC activated by IL-3 enhances the production and/or release of NGF by fibroblasts. When anti-NGF mAb was added to the coculture containing BMCMC, fibroblasts, and IL-3, the phenotypic change of BMCMC did not occur. This also suggests that NGF produced by fibroblasts plays an important role in the phenotypic change of BMCMC. The effect of mast cells on fibroblasts has been reported by Levi-Schaffer et al. (36); they described that rat PMC stimulated migration and proliferation of 3T3 fibroblasts.

We thank Drs. J. Bienenstock, J. Marshall, and A. M. Stanisz (McMaster University, Ontario, Canada) for helpful discussions and supplying pure NGF, and Dr. Ihle (National Cancer Institute, Frederick, MD) for providing pure IL-3. We also thank Dr. E. M. Shooter (Stanford University) for providing anti-NGF mAb.

This work was supported by a grant from the Ministry of Education, Science and Culture, Japan, and by the Basic Research Core System, the Special Coordination Fund for Promoting Science and Technology, Japan.

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Received for publication 13 February 1991.

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