INTERLEUKIN 4 AS AN ESSENTIAL FACTOR FOR IN VITRO CLONAL GROWTH OF MURINE CONNECTIVE TISSUE–TYPE MAST CELLS

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Mast cell precursors are derived from multipotential hematopoietic stem cells, migrate in the blood stream, and enter the tissue where they differentiate into morphologically identifiable mast cells (reviewed in reference 1). The differentiated mast cells may be classified into at least two phenotypically distinct populations; connective tissue–type mast cells (CTMC) and mucosal mast cells (MMC) (2–4). CTMC occur in the skin and peritoneal cavity, among other sites. Classically, CTMC contain heparin proteoglycan and store relatively a large amount of histamine. By contrast, MMC are prominent in the mucosal layer of the gastrointestinal tract, and contain chondroitin sulfate proteoglycan and relatively small quantities of histamine (2–4).

Investigations about differentiation of mast cells have recently been promoted by development of culture techniques. Cells with many features of MMC developed in suspension cultures of normal mouse hematopoietic cells containing mast cell growth factors (reviewed in references 2–4). Both MMC and cultured mast cells are exquisitely sensitive to T cell regulation. Striking proliferation of MMC occurs during T cell–dependent responses to certain intestinal parasites in normal mice and rats (5, 6). By contrast, athymic nude mice lack such a response (6). The nude mouse intestinal mucosa contains MMC precursors (5), however, and MMC proliferation does occur if nude mice are reconstituted with T cells before intestinal infection (6). Cultured mast cells proliferate in response to mast cell growth factors, which can be elaborated by T cells (reviewed in references 2–4).

Two distinct T cell factors are known to support proliferation of cultured mast cells. Ihle et al. (7) purified IL-3 from WEHI-3 cell supernatants. A mast cell growth factor produced by a concanavalin A–stimulated helper T cell line was demonstrated to be identical with IL-3 (8, 9). Smith and Renick (10) recently identified another mast cell growth factor in supernatants of the same T cell line; Lee et al. (11) isolated and characterized cDNA clone that encoded this mast cell growth factor. Unexpectedly, the nucleotide sequence of the cDNA was identical with that of cDNA encoding the IgG1-inducing factor isolated by

Noma et al. (12). Because a peptide encoded by this cDNA showed multiple biological activities, the peptide was designated as interleukin 4 (IL-4) (10–12).

In contrast with the exquisite sensitivity of MMC and cultured mast cells to T cell regulation, CTMC have been considered to show little or no dependence on T cell–derived factors (2, 3). Numbers of CTMC in the skins and peritoneal cavities of nude mice are comparable to those observed in control normal animals (6). However, Kobayashi et al. (13) and Nakahata et al. (14) recently demonstrated that pure mast cell colonies developed when peritoneal mast cells of mice were plated in methylcellulose containing pokeweed mitogen–stimulated spleen cell–conditioned medium (PWM-SCM).

In the present study, we investigated growth factors that supported clonal proliferation of CTMC, and demonstrated that PWM-stimulated spleen cells contain mRNA for both IL-3 and IL-4, and that the presence of both interleukins was necessary for development of mast cell clusters from CTMC.

Materials and Methods

Cell Suspension.   Mice of (WB × C57BL/6)F_1 were raised in our laboratory, and used at 8–10 mo of age. The method to prepare cell suspensions has been described (4).

Purification of Peritoneal Mast Cells.   The major compenents of mouse peritoneal cells are macrophages and small lymphocytes. They were removed according to the method described by Yurt et al. (15). Peritoneal cells from 10 mice were collected by lavage of peritoneal cavity of each mouse with 5 ml of Tyrode's buffer containing 0.1% gelatin (Sigma Chemical Co., St. Louis, MO). The cells were sedimented at 400 g for 15 min at room temperature, and washed twice with Tyrode's buffer containing 0.1% gelatin. ~6 × 10^7–10^8 cells in 1 ml of the Tyrode's buffer were layered on 2.0 ml of 22.5% wt/vol metrizamide (density, 1.125 g/ml) and centrifuged at room temperature for 15 min at 400 g at the buffer-metrizamide interface. The cells remaining at this interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1.0 ml of Tyrode's buffer. Of the nucleated cells in this preparation, ~80% were mast cells. To obtain mast cell suspension of 99% purity, the above-mentioned procedure was repeated by using the 80%-pure mast cell suspension.

Conditioned Medium.   PWM-SCM was prepared according to the method described by Nakahata and Ogawa (16).

Lymphokines.   IL-3 purified from WEHI-3 supernatant was a gift of J. N. Ihle (National Cancer Institute, Frederick, MD) (7). We synthesized IL-4 according to the method described previously (12). Briefly, pSPKmIL4-374 plasmid containing the full-length IL-4 cDNA was cleaved with SalI to be linealized. Capped IL-4 mRNA was synthesized using SP6 RNA polymerase. The synthesized mRNA was injected into *Xenopus* oocytes, and translation products secreted into the incubation media were collected. We used this incubation media as recombinant IL-4 (rIL-4).

Northern Blot Hybridazation.   Total cellular RNA was extracted from cells using the guanidium thiocyanate method (12), and was separated on a agarose/formaldehyde gel and then transferred to a nitrocellulose filter.

Clonal Cell Culture.   Methylcellulose culture were carried out by using a modification of the technique described previously (13). 1 ml of culture mixture containing 10^3 peritoneal mast cells, α-medium (Flow Laboratories), 1.0% methylcellulose (Sigma Chemical Co.), 30% FCS (Flow Laboratories), 1% deionized BSA (Sigma Chemical Co.), 10^{-4} M 2-ME (Sigma Chemical Co.), and 10% of PWM-SCM or 10% of α-medium containing various amounts of lymphokines was plated and incubated at 37°C in humidified atmosphere flushed with 5% CO_2 in air. Large clusters containing ≥32 cells and small clusters containing 4–31 cells were counted on day 14.

Staining of Cells.   Cells from individual colonies were collected as described by Kobayashi et al. (13). After washing with PBS, samples were immediately spun in a cytocentrifuge
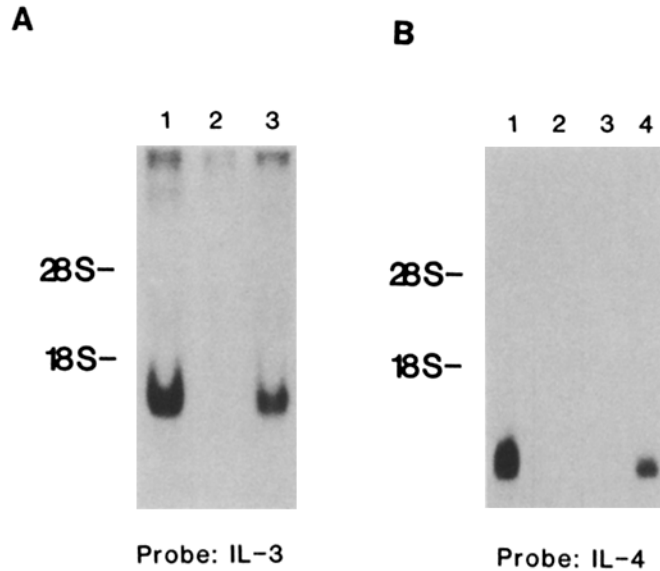
A

B

Probe: IL-3   Probe: IL-4

FIGURE 1.   (A) RNA blot analysis of 25 μg of total cellular RNA isolated from WEHI-3 cell line (lane 1) or unstimulated (lane 2) or PWM-stimulated (lane 3) spleen cells. 1.1 kb Eco RI fragment of a mouse IL-3 clone (9) was used as a probe. (B) RNA blot analysis of 25 μg of total cellular RNA isolated from 2.19 T cells (12) (lane 1) or WEHI-3 cell line (lane 2), or unstimulated (lane 3) or PWM-stimulated (lane 4) spleen cells. 360 bp Rsa I fragment of a mouse IL-4 clone (12) was used as a probe.

(Cytospin; Shadon Southern, Elliott, IL) at 600 rpm for 5 min and were stained with May-Grünwald-Giemsa, alcian blue, or berberine sulfate. Berberine sulfate is a fluorescent dye that binds to heparin proteoglycan in granules of mouse CTMC (4).

Results

PWM-SCM is probably a mixture of various growth factors. We examined whether PWM-stimulated spleen cells produced IL-3 and IL-4 by Northern blot hybridization. PWM-stimulated spleen cells contained both IL-3 and IL-4 mRNA, but the control nonstimulated spleen cells contained neither. Since supernatants of WEHI-3 cells are frequently used as a source of mast cell growth factor, RNA from WEHI-3 cells was also examined. As shown in Fig. 1, WEHI-3 cells contained mRNA for IL-3 but not for IL-4.

In the next experiment, we investigated the effect of purified IL-3 and rIL-4 on proliferation of mast cells collected from the peritoneal cavities, a classical source of CTMC. Since major components of murine peritoneal cells are lymphocytes and macrophages, mast cells were highly purified (99%) and used as the target in order to minimize the effect of factors secreted by lymphocytes and macrophages. 10^3 peritoneal mast cells were plated with PWM-SCM, IL-3, or rIL-4. PWM-SCM supported proliferation of mast cells; about one-fifth of mast cells produced small (4–31) or large (≥32) clusters in methylcellulose (Table I). IL-3 alone induced development of a small number of clusters, and most of them were small in size. No clusters appeared in dishes supplemented with rIL-4 alone. However, when rIL-4 was added to IL-3 (50 U), the number and size of clusters were comparable to the values observed in dishes supplemented with PWM-SCM

TABLE I

Mast Cell Clusters 14 d after Plating 10^3 Peritoneal Mast Cells with Various Growth Factors

Expt.*	Factors† added to growth medium			Number of clusters		
	PWM-SCM	IL-3	rIL-4	Small (4–31)	Large (≥32)	Total
	%	U	%			
1	0	0	0	0	0	0
	10	0	0	137	65	202
	0	50	0	16	0	16
	0	50	5.0	190	11	201
	0	0	5.0	0	0	0
2	0	0	0	0	0	0
	10	0	0	158	81	239
	0	50	0	27	1	28
	0	50	0.5	218	83	301
	0	50	2.0	217	109	326
	0	50	5.0	262	118	380
	0	50	10.0	175	84	259
	0	0	5.0	0	0	0
	0	0	10.0	0	0	0

* Results are the mean of four dishes in exp. 1, the mean of two dishes in exp. 2.
† Units of IL-3 as defined by Ihle et al. (7); percentage of PWM-SCM or rIL-4 to total culture medium.

(Table I). Although a small quantity (0.5%) of rIL-4 was enough to synergize with IL-3, control supernatant of Xenopus eggs that had not been injected with IL-4 mRNA did not show any activities even in a large quantity (10%) (data not shown).

Cells from mast cell clusters were stained with various dyes; granules of the majority of the cells stained with either May-Grüwal-Giemsa or alcian blue. About 70% of cells contained granules that stained with berberine sulfate, suggesting the presence of heparin in granules of developing mast cells.

Discussion

Factors that support the growth of mast cells in culture have extensively been investigated. Purified IL-3 supports development of mast cells from bone marrow precursors in either liquid (2, 3) or semisolid (17) culture medium. When mast cells lines are used as the target, either IL-3 or IL-4 supports the growth (10, 11, 18). Since phenotypes of bone marrow–derived cultured mast cells, including mast cell lines, are similar to MMC (2, 3), these results have been considered as evidence for T cell dependency of MMC. In contrast to MMC, CTMC have been supposed to be independent on T cells (2, 6). However, the present results clearly demonstrated that the in vitro growth of CTMC may also be induced by combination of IL-3 and IL-4.

IL-3 alone supported development of a small number of clusters from peritoneal mast cells, typical CTMC, but the addition of rIL-4 to IL-3 increased the number of clusters by a factor of 10. Since no clusters developed in dishes supplemented with rIL-4 alone, IL-4 seems to act as a cofactor of IL-3. This probably reflects a physiological function of IL-4 to CTMC.

Differentiation of mast cells have a unique feature. The progeny of multipotential hematopoietic stem cells, such as erythrocytes, neutrophils, and platelets, leave the bone marrow after maturation, and they do not divide thereafter.

Although mast cells are progeny of stem cells as well, either CTMC or cultured mast cells have extensive proliferation activity even after morphological differentiation (1, 13, 14, 19). Since CTMC rarely divide in physiological conditions (2, 19), the present result suggests that IL-4 may be an immunological signal that initiates the division of CTMC. IL-4 was reported to activate resting B cells (18). There is a possibility that the same mechanism may be used for activation of both CTMC and resting B cells.

IL-4 also enhances IgE production by B cells (10, 11, 18). There is a possibility that IL-4 secreted by T cells may locally induce proliferation of mast cells and IgE production at the same time. This may facilitate a local defense mechanism operated by IgE and mast cells, since high-affinity IgE receptors are present on the surface of mast cells.

Summary

We investigated the biological activity of IL-4 to murine connective tissue–type mast cells (CTMC). When purified peritoneal mast cells, typical CTMC, were incubated with pokeweed mitogen–stimulated spleen cell–conditioned medium (PWM-SCM) in methylcellulose, about one-fifth of mast cells showed clonal growth. Recombinant IL-4 alone did not stimulate the clonal growth, and purified IL-3 alone induced development of a small number of tiny clusters. In contrast, addition of IL-4 to IL-3 increased the number of clusters by a factor of 10. The number and size of clusters induced by the combination of IL-3 and IL-4 were comparable to those of mast cell clusters induced by PWM-SCM. The present results indicate that IL-4 is an essential factor for in vitro clonal growth of CTMC.

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