

## STIMULATION OF CONNECTIVE TISSUE-TYPE MAST CELL PROLIFERATION BY CROSSLINKING OF CELL-BOUND IgE

BY MINEO TAKAGI,\* TATSUTOSHI NAKAHATA,\* KENICHI KOIKE,\*  
TOSHIMI KOBAYASHI,\* KOHICHIRO TSUJI,\* SOMEI KOJIMA,‡  
TAKAO HIRANO,§ ATSUSHI MIYAJIMA,|| KEN-ICHI ARAI,||  
AND TARO AKABANE\*

*From the \*Department of Pediatrics, Shinshu University School of Medicine, Matsumoto 390;  
the ‡Department of Parasitology, Chiba University School of Medicine, Chiba 280;  
and the §Department of Internal Medicine and Rheumatology,  
Juntendo University School of Medicine, Tokyo 113, Japan; and the  
||DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California 94304*

There are two phenotypically distinct subpopulations of mast cells in rodents, i.e., connective tissue-type mast cells (CTMC)<sup>1</sup> and mucosal mast cells (MMC). These populations differ in their location, cell size, staining characteristics, ultrastructure, mediator content, and T cell dependency (1-3). Although proliferation of MMC was known to be T cell dependent *in vivo* and thought to be IL-3 dependent *in vitro*, the factors on which CTMC proliferation depends remained elusive. We recently found that mature CTMC separated from mouse peritoneal cells could proliferate extensively in methylcellulose culture and maintain the appearance and function of CTMC (4). Clonal growth of CTMC could not be induced by IL-3 alone and required the presence of both IL-3 and IL-4/B cell stimulatory factor 1 (BSF-1) (5).

Mast cells bear high affinity IgE receptors, and crosslinking of cell-bound IgE by multivalent antigen or anti-IgE antibody triggers the release of a variety of chemical mediators (6).

In B cell proliferation, crosslinking of surface IgM on B cells by anti-IgM antibodies causes entry of resting B cells into the G<sub>1</sub> phase of the cell cycle (7). BSF-1 also plays a role in B cell proliferation as an activating factor (8).

These findings led us to examine whether crosslinking of IgE molecules bound to IgE receptors on CTMC plays some role in the proliferation of these cells. We report here that IgE-sensitized CTMC can proliferate *in vitro* after the stimulation with the specific antigen or anti-IgE antibody in the presence of IL-3. Our data indicate the possibility that antigen stimulation not only triggers the release of chemical mediators from CTMC but participates in the mechanisms of their proliferation.

---

This work was supported by grants from the Ministry of Education, Science and Culture, Japan. Address correspondence to Tatsutoshi Nakahata, Department of Pediatrics, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, 390 Japan.

<sup>1</sup> *Abbreviations used in this paper:* BSF-1, B cell stimulatory factor 1; CTMC, connective tissue-type mast cells; MMC, mucosal mast cells; Sj, *Schistosoma japonicum* antigen; 48/80, compound 48/80

## Materials and Methods

**Cell Preparation.** 20–25-wk-old male BDF<sub>1</sub> mice were obtained from the Shizuoka Experimental Animal Center (Shizuoka, Japan). Peritoneal cells were collected as described previously (9). Mature CTMC ( $\geq 98\%$  purity) were separated from peritoneal cells by the removal of phagocytes followed by Percoll (Sigma Chemical Co., St. Louis, MO) density-gradient centrifugation as described in detail (10).

**IgE Antibodies, Antigens, and Other Secretagogues.** Both mouse monoclonal anti-DNP-BSA IgE antibody (B53) (11) and mouse monoclonal anti-*Schistosoma japonicum* (Sj) IgE antibody (12) were used as diluted ascitic fluid. Anti-DNP IgE at a dilution of 1/1,000 and anti-Sj IgE at a dilution of 1/1,000 corresponded to 80 ng/ml and 100 ng/ml IgE, respectively. Purified rat anti-mouse IgE mAb (6HD5) was prepared as described previously (13). DNP-BSA contained 6.8 DNP groups per BSA molecule. Sj antigen was extracted from adult worms of Sj as described previously (12). Compound 48/80 (48/80) was purchased from Sigma.

**IL-3, IL-4, and Anti-IL-4 Antibody.** Both mouse rIL-3 (14) and rIL-4 (15) were produced in yeast cells. Their activities were determined by using P cell clone IC2 (16), and a colorimetric assay (17). Rat anti-IL-4 mAb was purified from 11B11-containing ascitic fluid (18).

**Clonal Cell Cultures.** Methylcellulose cultures were carried out by using a modification of technique described previously (9, 19). Culture mixture (1 ml) containing  $10^3$  purified CTMC,  $\alpha$ -medium (Flow Laboratories, Inc., Rockville, MD), 30% FCS (HyClone Laboratories, Inc., Logan, UT), 1% deionized BSA (Sigma Chemical, Co.),  $10^{-4}$  M 2-ME (Eastman Organic Chemicals, Rochester, NY), and 100 U IL-3 were preincubated in the presence or absence of anti-DNP IgE (or anti-Sj IgE) at 37°C in 5% CO<sub>2</sub>/95% air for passive sensitization. After 12 h, varying concentrations of specific antigen (or anti-IgE antibody) and methylcellulose (final concentration, 0.8%) (Shinetsu Chemical Co., Tokyo, Japan) were added to the preincubation mixture. This mixture was then incubated in a culture dish at 37°C in a humidified atmosphere flushed with 5% CO<sub>2</sub> in air. Mast cell colonies containing  $\geq 20$  cells were counted on day 16.

Serum free culture was carried out by using a modification of the technique described previously (20, 21). Culture mixture contained 1% crystallized globulin-free deionized BSA (Calbiochem-Behring Corp., La Jolla, CA), 300  $\mu$ g/ml fully saturated human transferrin (Sigma Chemical Co.), 160  $\mu$ g/ml soybean lecithin (Sigma Chemical Co.), 96  $\mu$ g/ml cholesterol (Nakarai Chemicals Ltd., Kyoto, Japan) and  $10^{-7}$  M sodium selenite (Sigma Chemical Co.), instead of FCS.

**Staining of Cultured Cells.** Individual colonies were lifted from the methylcellulose culture using a 3  $\mu$ l Eppendorf pipette under direct microscopic visualization and were collected in Eppendorf microcentrifuge tubes containing 0.5 ml PBS. After washing, the samples were immediately spun in a cytocentrifuge (Cytospin; Shandon Southern Instruments, Sewickley, PA) at 800 rpm for 5 min and stained with alcian blue-safranin (22) or berberine sulfate (23). Specimens stained with berberine sulfate were examined with an Olympus epifluorescence microscope. Alcian blue(–)-safranin(+) mast cells are thought to be mature CTMC (22). Berberine sulfate staining was reported to be highly specific for heparin-containing granules of CTMC (23). We confirmed this by demonstrating that the staining was blocked by treating the cells with heparinase from *Flavobacterium heparinum* (0.5 IU/ml, pH 7.0; Seikagaku Kogyo Co., Tokyo, Japan) for 30 min but not chondroitinase ABC from *Proteus vulgaris* (5.0 IU/ml, pH 8.0; Seikagaku Kogyo Co.) for 4 h (9).

**Histamine Release.** Histamine release experiments were carried out as described previously (24). Aliquots of cells sensitized with anti-DNP IgE (1/1,000) containing  $\sim 5 \times 10^4$  mast cells were incubated for 20 min with varying concentrations of DNP or anti-IgE antibody at 37°C. Histamine release from mast cells was measured by HPLC with fluorometry (25).

## Results

**Clonal Growth of CTMC Sensitized with Anti-DNP IgE by Stimulation with DNP in the Presence of IL-3.** When normal mouse CTMC sensitized with monoclonal anti-DNP IgE (B53) were stimulated by DNP and then cultured in methylcellulose medium

containing IL-3, pure mast cell colonies developed, depending on the concentration of DNP (Fig. 1 *a*). Proliferation of mast cells also depended on the concentration of anti-DNP IgE (Fig. 1 *b*). The maximal colony formation was seen with 1/1,000 dilution of anti-DNP IgE and 1  $\mu\text{g}/\text{ml}$  DNP. About one-third of mature CTMC separated from mouse peritoneal cells produced colonies in culture containing optimal concentrations of anti-DNP IgE, DNP, and IL-3. Neither DNP nor IL-3 alone stimulated clonal growth of sensitized CTMC. IL-3 and/or DNP had no effects on proliferation of nonsensitized CTMC. When sensitized cells were washed with  $\alpha$ -medium after stimulation with DNP and then cultured in methylcellulose medium containing DNP with IL-3, anti-DNP IgE with IL-3, or IL-3 alone, no mast cell colonies developed. In contrast, many mast cell colonies developed when the same washed cells were incubated with DNP, anti-DNP IgE, and IL-3 (data not shown). These results suggest that antigen stimulation only in early stage of culture is not sufficient to induce subsequent mast cell colony formation in the presence of IL-3, and that continuous presence of anti-DNP IgE, DNP, and IL-3 in culture is required for extensive proliferation of CTMC.

Cyocentrifuged preparations of mast cell colonies were individually stained with alcian blue-safranin and berberine sulfate to determine the characteristics of these

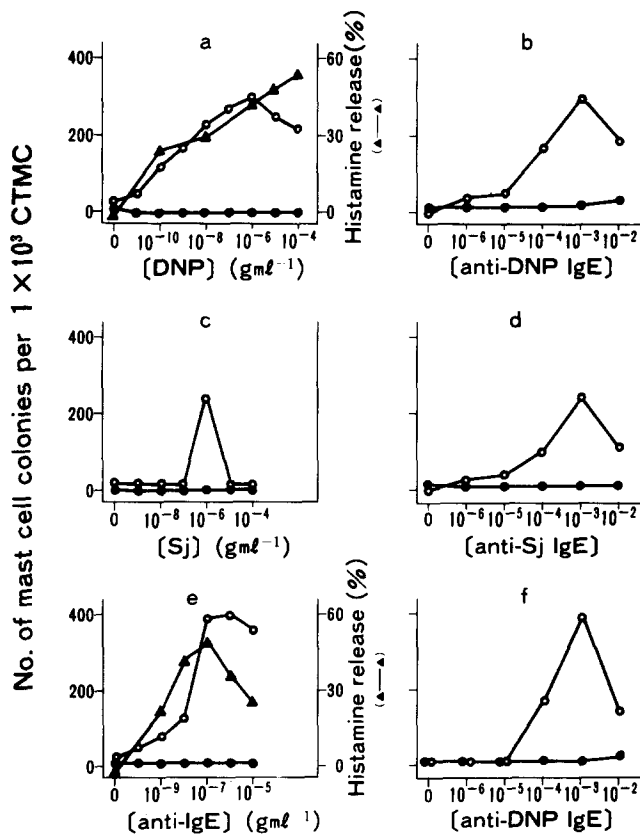


FIGURE 1. Dependence of colony growth on various stimulation. (Left) Mast cell colony growth from mature CTMC at varying concentrations of (a) DNP-BSA, (c) *Schistosoma japonicum* (Sj) antigen, and (e) anti-mouse IgE antibody. (Open circles) Cells sensitized with (a, e) anti-DNP IgE (1/1,000) or (c) anti-Sj IgE (1/1,000); (filled circles) cells without sensitization. (Right) Mast cell colony growth from cells sensitized with varying concentrations of (b, f) anti-DNP IgE and (d) anti-Sj IgE. Open circles, cells stimulated by (b) 1  $\mu\text{g}/\text{ml}$  DNP, (d) 1  $\mu\text{g}/\text{ml}$  Sj, or (f) 0.1  $\mu\text{g}/\text{ml}$  anti-IgE antibody; (filled circles) cells without stimulation. In different sets of sensitized cells, the rate of histamine release was determined. Spontaneous release was 8%. These values were subtracted from the experimental values. Data in the figure are the average of triplicated cultures. Errors are smaller than the symbols.

colonies. Most cells composing individual colonies were stained with both safranin ( $99.4 \pm 1.7\%$ ) and berberine sulphate ( $69.2 \pm 8.7\%$ ). Treatment with heparinase completely abolished the fluorescence of granules of proliferating mast cells in our culture, whereas chondroitinase ABC did not. These results suggest that most developing mast cells in culture actually contain heparin proteoglycan in their granules, and that the cells retain the characteristics in common with CTMC (4, 26). Safranin(+)-berberine sulfate(+) granules in the cells, however, were somewhat scarcer as compared with CTMC separated from peritoneal cavity. In addition, although all mast cell colonies developed in culture were considered to be derived from safranin(+)-berberine sulfate(+) mature CTMC (4), about two-thirds of the colonies contained a few alcian blue(+)-safranin(-)-berberine sulfate(-) mast cells.

To completely exclude the effects of accessory cells on colony formation in our culture system, single-cell culture was performed according to the method described previously (27). Purified CTMC from peritoneal cells were plated in methylcellulose medium. A single CTMC was identified morphologically, lifted from the medium using a fine Pasteur pipette attached to a Leitz micromanipulator, transferred to fresh culture medium, and incubated for 16 d. 14 of 48 transferred single CTMC produced colonies in the culture containing optimal concentrations of anti-DNP IgE, DNP, and IL-3. This result demonstrates that clonal growth of CTMC in our culture condition does not require the presence of accessory cells.

Serum-free culture was also carried out to exclude the effects of some factors in FCS on CTMC proliferation using a technique described in Materials and Methods. 60 ( $\pm 5$ ) mast cell colonies developed from 500 purified CTMC in the serum-free culture containing optimal concentrations of anti-DNP IgE, DNP, and IL-3.

*Effects of Sj and Anti-IgE Antibody on Colony Formation.* To examine whether mast cell colony formation induced by antigen stimulation depends on the kinds of antigens, mature CTMC sensitized with monoclonal anti-*S. japonicum* (Sj) IgE were stimulated by Sj antigen and then cultured in the presence of IL-3. Mast cell colonies also developed within a relatively small range of concentrations of Sj antigen (Fig. 1 c). Anti-Sj IgE stimulated proliferation of CTMC dose dependently in the presence of 1  $\mu\text{g/ml}$  Sj antigen (Fig. 1 d).

The effects of anti-mouse IgE mAb (6HD5) on mast cell colony formation from mature CTMC sensitized with anti-DNP IgE in the presence of IL-3 were also tested. Mast cell colonies developed, depending on the concentration of both anti-IgE antibody and anti-DNP IgE (Fig. 1, e, f).

*Histamine Release Experiments and Serial Observations of Dividing Cells.* Together with colony formation, we examined histamine release from mast cells as an index to the degree of degranulation. When the cells sensitized with anti-DNP IgE were stimulated by DNP, both colony formation and histamine release increased in a dose-dependent manner (Fig. 1 a). Anti-IgE antibody also stimulated both colony formation and histamine release at an optimal concentration of 0.1  $\mu\text{g/ml}$  anti-IgE antibody (Fig. 1 e). However, whether dividing mast cells are similarly degranulated remains unanswered. To examine this, serial observations of mast cells proliferating in culture were performed. It was found that most dividing cells in the culture containing anti-DNP IgE, DNP, and IL-3 had pericellular halos which indicated degranulation of CTMC as reported previously (28) (Fig. 2, B-D), whereas resting cells in culture containing IL-3 alone had no halos around them (Fig. 2 A).

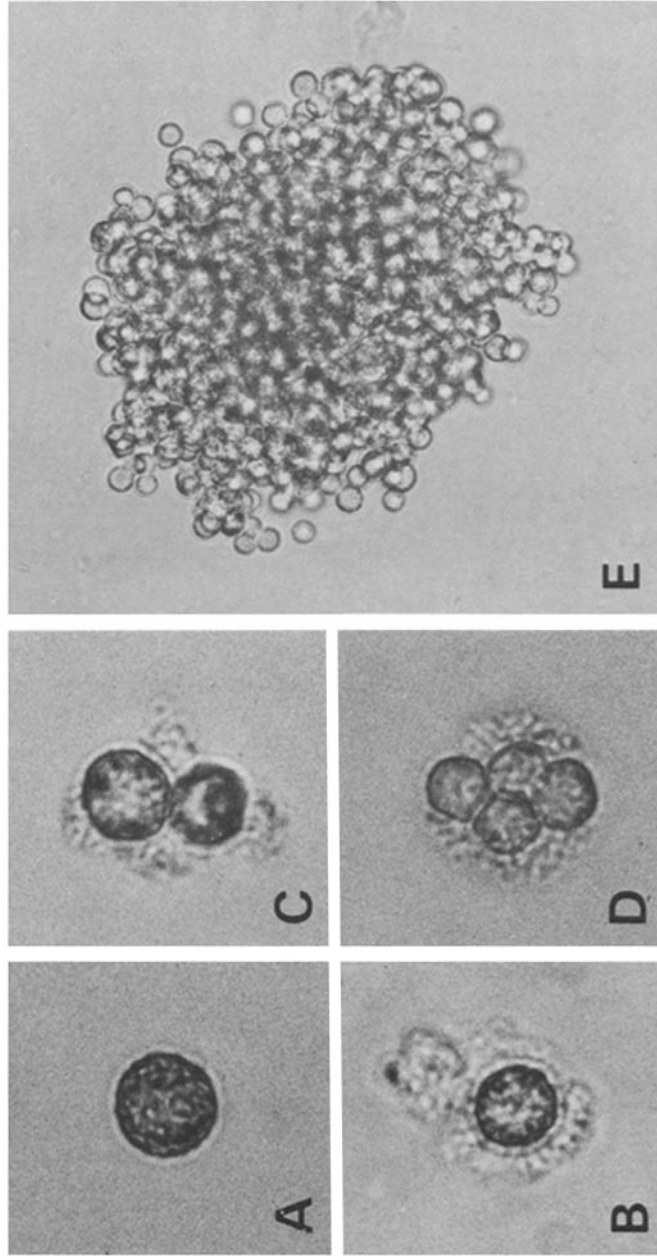


FIGURE 2. Serial observations of a mast cell proliferating in culture. A mast cell in resting stage in culture containing IL-3 alone (A). Serial observations of a mast cell stimulated by anti-DNP IgE, DNP, and IL-3; (B) 24 h, (C) 48 h, (D) 96 h and (E) 16 d of incubation. A-D, 1,300; E,  $\times 500$ .

TABLE I  
Effects of 48/80 on Mast Cell Colony Formation

Stimuli	No. of mast cell colonies per $5 \times 10^2$ CTMC
IL-3	0
48/80	0
IL-3 + 48/80	0
IgE + DNP + IL-3	$148 \pm 4$
IgE + DNP + IL-3 + 48/80	$134 \pm 1$

Data represent the number of mast cell colonies on day 16 (48/80 10  $\mu\text{g/ml}$ , IL-3 100 U/ml, anti-DNP IgE 1/1,000, DNP 1  $\mu\text{g/ml}$ ). Data shown in the table are the mean  $\pm$  SD of triplicate cultures.

*Effects of Non-IgE-mediated Stimulation on Colony Formation.* 48/80 is a secretagogue known to stimulate CTMC directly (29). Whether 48/80 could induce mast cell colony formation in the presence of IL-3 as well as stimulation via IgE receptors was tested. When CTMC were stimulated by 10  $\mu\text{g/ml}$  48/80 for 20 min, 43% total cellular histamine was released from the cells. However, in three separate experiments, no mast cell colonies developed when CTMC were cultured with 10  $\mu\text{g/ml}$  48/80 and 100 U/ml IL-3 (Table I). Addition of 10  $\mu\text{g/ml}$  48/80 neither enhanced nor inhibited proliferation of CTMC supported by anti-DNP IgE, DNP, and IL-3. These results indicate that some mediators released from mast cells can not induce their proliferation.

*Effects of a Combination of IL-4 and Antigen Stimulation on Colony Formation.* When varying concentrations of IL-4 were added to CTMC sensitized with anti-DNP IgE in the presence of IL-3, mast cell colony formation increased in a dose-dependent manner and reached a plateau at 100 U/ml IL-4. However, the combination of IL-4 and DNP increased the number of colonies exceeding by far the maximal level supported by IL-4 (Fig. 3). When cells were incubated with IL-4 and IL-3 without sensitization, the dose-response curve was about equal to that of the sensitized cells (data

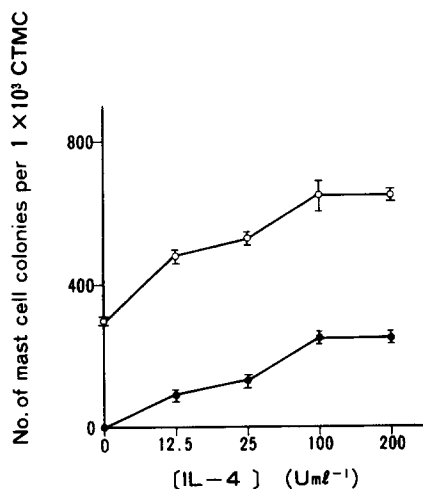


FIGURE 3. The effects of a combination of IL-4 and DNP on colony formation.  $10^3$  CTMC sensitized with anti-DNP IgE (1/1,000) were cultured at varying concentrations of IL-4 with (open circle) or without (closed circle) stimulation of DNP (1  $\mu\text{g/ml}$ ) in the presence of IL-3 (100 U/ml). Data shown in the figure are the mean  $\pm$  SD of triplicated cultures.

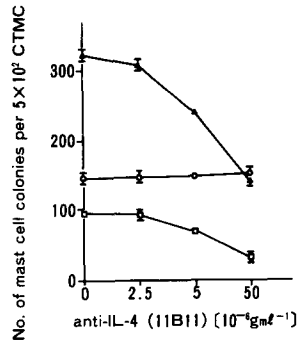


FIGURE 4. The effects of anti-IL-4 antibody on colony formation.  $5 \times 10^2$  CTMC were incubated with; DNP + anti-DNP IgE + IL-3 (circles), IL-4 + IL-3 (squares) or anti-DNP IgE + DNP + IL-4 + IL-3 (triangles) (anti-DNP IgE 1/1,000, DNP 1  $\mu\text{g/ml}$ , IL-3 100 U/ml, IL-4 100 U/ml). Varying concentrations of anti-IL-4 antibody were added to the cultures on day 0. Data shown in the figure are the mean  $\pm$  SD of triplicated cultures.

not shown). When sensitized cells were incubated with both IL-4 and DNP in the absence of IL-3, no mast cell colonies developed. It is of interest that the combination of IL-4 and DNP in the presence of IL-3 not only increased the number of colonies but greatly enlarged both the colony size and the cell volume (data not shown). Addition of anti-IL-4 antibody, 11B11, to the culture containing IL-4 (100 U/ml) and IL-3 (100 U/ml) resulted in a dose-dependent decrease of clonal growth of CTMC (Fig. 4). About 70% of the colonies were suppressed by the addition of 50  $\mu\text{g/ml}$  anti-IL-4 antibody. On the other hand, in culture containing optimal concentrations of anti-DNP IgE, DNP, and IL-3, their growth was not affected by the addition of anti-IL-4 antibody at the same concentration. Anti-IL-4 antibody also neutralized effects of a combination of IL-4 and DNP on proliferation of sensitized CTMC and depressed numbers of mast cell colonies to the level supported by anti-IgE DNP, DNP, and IL-3.

**Replating Experiments.** Whether both IL-4 and antigen stimulation could exert colony-inducing effects on a single CTMC was also examined. Since it is impossible

TABLE II  
Replating Studies of Mast Cell Colonies

Colony no.	Colony size	Primary stimuli	Secondary stimuli		
			IgE + DNP + IL-3	IL-4 + IL-3	IL-3
1	170	IgE + DNP + IL-3	13	12	0
2	210	IgE + DNP + IL-3	17	24	0
3	260	IgE + DNP + IL-3	37	61	1
4	110	IL-4 + IL-3	6	8	0
5	470	IL-4 + IL-3	66	61	2
6	1,000	IL-4 + IL-3	99	237	2

Mast cell colonies supported by IL-4 with IL-3, or anti-DNP IgE and DNP with IL-3 were individually lifted from the culture on day 16, suspended in  $\alpha$ -medium and gently pipetted. Aliquots of the cell suspension were added to the secondary culture medium containing: anti-DNP IgE + DNP + IL-3, IL-4 + IL-3, or IL-3 (anti-DNP IgE 1/1,000, DNP 1  $\mu\text{g/ml}$ , IL-3 100 U/ml, IL-4 100 U/ml). One quadrant of the sample was stained with alcian blue-safranin and berberine sulphate to determine the characteristics of the colony. Data represent the number of mast cell colonies on day 16 of the secondary culture.

to culture a CTMC with two different stimuli, individual colonies, which derived from an ancestor cell, supported by IL-4 with IL-3 or anti-DNP IgE and DNP with IL-3 were divided and replated in secondary cultures containing different stimuli. All of the mast cell colonies supported by IL-4 with IL-3 produced many secondary mast cell colonies in culture containing anti-DNP IgE, DNP, and IL-3. The reverse relation was also observed as shown in Table II. It should also be noted that CTMC can not proliferate extensively without successive stimulation of anti-DNP IgE and DNP or IL-4 in the culture containing IL-3.

### Discussion

The present experiments show that crosslinking of cell-bound IgE on mature CTMC separated from mouse peritoneal cells by multivalent antigen or anti-IgE antibody, which leads to degranulation, also induces clonal growth of CTMC in the presence of IL-3. When CTMC sensitized with anti-DNP IgE were stimulated by DNP in the presence of IL-3, mast cell colonies developed in methylcellulose culture, depending on the concentration of both DNP and anti-DNP IgE. Our various preincubation studies revealed that antigen stimulation only in early stage of culture is not sufficient to induce extensive proliferation of CTMC in the presence of IL-3, and that continuous presence of IgE, antigen, and IL-3 was required for the proliferation of CTMC. These results suggest that IgE-antigen complexes remaining in culture play some role in proliferation of CTMC. Evidences for a direct effect of anti-DNP IgE and DNP on proliferation of CTMC were provided by the experiments of both single cell culture and serum free culture. Neither accessory cells nor some factors in FCS were not required for clonal growth of CTMC in our culture condition.

Cytological analyses revealed that all mast cell colonies developed in our culture consisted of cells with CTMC-like features. However, safranin(+)-berberine sulfate(+) granules in a cell were somewhat scarcer than those in the donor cells. In addition, about two-thirds of the colonies contained a few alcian blue(+)-safranin(-)-berberine sulfate(-) mast cells. It is of interest that proliferating CTMC in culture containing PWM-stimulated spleen cell-conditioned medium (30) or IL-3 and IL-4 (5) similarly lose some of their staining with safranin and berberine sulfate. These findings may suggest the possibility of transdifferentiation from mature CTMC to alcian blue(+)-safranin(-)-berberine(-) mast cells in methylcellulose culture in agreement with the *in vivo* observation by Sonoda et al. (31).

Crosslinking of cell bound IgE by S<sub>j</sub> antigen or anti-IgE antibody in the presence of IL-3 also induced clonal growth of CTMC. However, no mast cell colonies developed when CTMC were directly stimulated by 48/80. These results suggest that stimulation of CTMC via their IgE receptors causes proliferation of CTMC. It seems unlikely that some mediators released from CTMC by crosslinking of cell-bound IgE induce clonal growth of CTMC.

Histamine release experiments showed that the concentrations of antigen and anti-IgE antibody for optimal proliferation of CTMC approximately correspond to those for maximal degranulation. In addition, serial observations of cultures on an inverted microscope revealed that most dividing cells stimulated by antigen had pericellular degranulation halos, suggesting that crosslinking of IgE molecules bound to



high affinity IgE receptors on CTMC can stimulate both degranulation and proliferation of these cells in methylcellulose culture. Recently, however, a second class of receptors with a low affinity for the Fc portion of IgE (Fc $\epsilon$ R2) has been identified on macrophages, platelets, eosinophils, B lymphocytes, and T lymphocytes (32, 33, 34). Antibody to CD23 antigen on B cells, which is known to be equivalent to Fc $\epsilon$ R2 (35), promote DNA synthesis in B cells activated with 12-*O*-tetradecanoylphorbol 13-acetate (36). Although presence of Fc $\epsilon$ R2 on mast cells has not yet been reported, our data do not preclude a possibility that crosslinking of Fc $\epsilon$ R2 on CTMC participates in the mechanisms of their proliferation. Further studies will be needed to investigate the possible role of Fc $\epsilon$ R2 in the proliferation of CTMC.

Replating experiments suggested that both IL-4 and antigen stimulation can exert colony-inducing effects on a single CTMC. It seems unlikely that there are two subpopulations in CTMC, i.e., one can proliferate by stimulation with IL-4 and another with IgE-antigen complexes. Replating experiments also indicated that IL-3 can not support successive cell division without IL-4 or IgE-antigen complexes in culture.

A recent study by Brown et al. (37) has shown that IL-4 mRNA is expressed by IL-3-dependent nontransformed mast cell lines. A question remains whether IL-4 is involved in the mechanisms of proliferation of CTMC stimulated via their IgE receptors. The present experiments show that the combination of IL-4 and antigen stimulation increased the numbers of mast cell colonies, exceeding by far the maximal level supported by IL-4. In addition, clonal growth of CTMC supported by antigen stimulation was not neutralized by the addition of anti-IL-4 antibody. These results may suggest that IL-4 and antigen stimulation support proliferation of CTMC by different mechanisms. However, another possibility: that crosslinking of cell-bound IgE generates IL-4 by CTMC in an autocrine manner and IL-4 in turn supports their proliferation cannot be ruled out. Although our preliminary Northern blot hybridization analysis could not demonstrate IL-4 mRNA in antigen-stimulated mouse peritoneal CTMC, further investigations, using more sensitive techniques and larger numbers of cells, are required to answer the question.

In any case, the present experiments show a dual effect of antigen stimulation on IgE-sensitized mouse mature CTMC, i.e., stimulating both degranulation and proliferation. Intestinal nematode infection is associated with both potentiation of IgE production (38) and hyperplasia of immature mucosal mast cells with active secretory functions (39). A recent study by Czarnetzki et al. (40) has shown that the number of CTMC in biopsied skin of sensitized rats increased after multiple injection of *Ascaris* antigen into the skin. Although they speculated that mast cell precursors immigrate into tissue sites in response to chemotactic factors to differentiate there into mature cells under the influence of specific growth factors, our results provide another possibility: that increased CTMC in the skin results from proliferation of CTMC by antigen stimulation. IL-4 secreted by T cells also enhances IgE production by B cells (38, 41), and locally produced IgE binds to the IgE receptors on CTMC. Crosslinking of cell-bound IgE by multivalent antigen may induce proliferation of CTMC in combination with IL-3, while triggering the release of various chemical mediators and cytolytic factors (42). Our findings should allow the elucidation of many pathophysiological mechanisms involving the CTMC.

### Summary

Crosslinking of cell-bound IgE on mouse connective tissue-type mast cells (CTMC) by multivalent antigen or anti-IgE antibody induced clonal growth of CTMC in methylcellulose culture containing IL-3. Continuous presence of antigen, IgE antibody, and IL-3 in culture was required for extensive proliferation of CTMC. Optimal concentrations of antigen and anti-IgE antibody for proliferation of sensitized CTMC approximately corresponded to those for maximal histamine release from the cells, and it was observed that most dividing cells stimulated by antigen had pericellular degranulation halos in culture. Experiments of both single cell culture and serum free culture provided evidence for a direct effect of antigen stimulation on proliferation of CTMC. Neither accessory cells nor some factors in FCS were required for the clonal growth of CTMC in our culture condition. Compound 48/80, a direct stimulator of CTMC, also triggered histamine release from CTMC but failed to support their proliferation. These results suggest that stimulation of CTMC via IgE receptors not only triggers the release of chemical mediators from the cells but induces clonal growth of CTMC in the presence of IL-3. Our data indicate the possibility that antigen stimulation may play another role in the proliferation of CTMC.

The authors thank Dr. Allan Waitz (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA) for a constructive review of the manuscript and Drs. Shigeru Ikeda and Hidetada Komatsu (Central Research Laboratories of Kissei Pharmaceutical Co., Matsumoto, Japan) for their assistance in measurements of histamine.

Received for publication 24 January 1989 and in revised form 4 April 1989.

### References

1. Enerback, L. 1981. The gut mucosal mast cell. *Monogr. Allergy*. 17:222.
2. Bienenstock, J., A. D. Befus, F. Pearce, J. Denburg, and R. Goodacre. 1982. Mast cell heterogeneity: derivation and function, with emphasis on the intestine. *J. Allergy Clin. Immunol.* 70:407.
3. Galli, S. J., A. M. Dvorak, and H. F. Dvorak. 1984. Basophils and mast cells: morphologic insights into their biology, secretory patterns, and function. *Prog. Allergy*. 34:1.
4. Nakahata, T., T. Kobayashi, A. Ishiguro, K. Tsuji, K. Naganuma, O. Ando, Y. Yagi, K. Tadokoro, and T. Akabane. 1986. Extensive proliferation of mature connective-tissue type mast cells in vitro. *Nature (Lond.)*. 324:65.
5. Hamaguchi, Y., Y. Kanakura, J. Fujita, S. Takeda, T. Nakano, S. Tarui, T. Honjo, and Y. Kitamura. 1987. Interleukin 4 as an essential factor for in vitro clonal growth of murine connective tissue-type mast cells. *J. Exp. Med.* 165:268.
6. White, J. R., D. H. Pluznik, K. Ishizaka, and T. Ishizaka. 1985. Antigen-induced increase in protein kinase C activity in plasma membrane of mast cells. *Proc. Natl. Acad. Sci. USA*. 82:8193.
7. Defranco, A. L., E. S. Raveche, R. Asofsky, and W. E. Paul. 1982. Frequency of B lymphocytes responsive to anti-immunoglobulin. *J. Exp. Med.* 155:1523.
8. Rabin, E. M., J. Ohara, and W. E. Paul. 1985. B-cell stimulatory factor 1 activates resting B cells. *Proc. Natl. Acad. Sci. USA*. 82:2935.
9. Kobayashi, T., T. Nakano, T. Nakahata, H. Asai, Y. Yagi, K. Tsuji, A. Komiyama, T. Akabane, S. Kojima, and Y. Kitamura. 1986. Formation of mast cell colonies in methylcellulose by mouse peritoneal cells and differentiation of these cloned cells in both the skin and the gastric mucosa of W/W<sup>v</sup> mice: evidence that a common precursor can give

- rise to both connective tissue-type and mucosal mast cells. *J. Immunol.* 136:1378.
10. Sonoda, T., Y. Kanayama, H. Hara, C. Hayashi, M. Tadokoro, T. Yonezawa, and Y. Kitamura. 1984. Proliferation of peritoneal mast cells in the skin of W/W<sup>v</sup> mice that genetically lack mast cells. *J. Exp. Med.* 160:138.
  11. Böttcher, I., M. Ulrich, N. Hirayama, and Z. Ovary. 1980. Production of monoclonal mouse IgE antibodies with DNP specificity by hybrid cell lines. *Int. Arch. Allergy Appl. Immunol.* 61:248.
  12. Kojima, S., M. Niimura, and T. Kanazawa. 1987. Production and properties of a mouse monoclonal IgE antibody to *Schistosoma japonicum*. *J. Immunol.* 139:2044.
  13. Hirano, T., H. Miyajima, H. Kitagawa, N. Watanabe, M. Azuma, O. Taniguchi, H. Hashimoto, S. Hirose, H. Yagita, S. Furusawa, Z. Ovary, and K. Okumura. 1988. Studies on murine IgE with monoclonal antibodies. *Int. Arch. Allergy Appl. Immunol.* 85:47.
  14. Yokota, T., F. Lee, D. Rennick, C. Hall, N. Arai, T. Mosmann, G. Nabel, H. Cantor, and K. Arai. 1984. Isolation and characterization of a mouse cDNA clone that expresses mast-cell growth-factor activity in monkey cells. *Proc. Natl. Acad. Sci. USA.* 81:1070.
  15. Lee, F., T. Yokota, T. Otsuka, P. Meyerson, D. Villaret, R. Coffman, T. Mosmann, D. Rennick, N. Roehm, C. Smith, A. Zlotnik, and K. Arai. 1986. Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor 1 activities and T-cell- and mast-cell-stimulating activities. *Proc. Natl. Acad. Sci. USA.* 83:2061.
  16. Koyasu, S., H. Nakauchi, K. Kitamura, S. Yonehara, K. Okumura, T. Tada, and I. Yahara. 1985. Production of interleukin 3 and  $\gamma$ -interferon by an antigen specific mouse suppressor T cell clone. *J. Immunol.* 134:3130.
  17. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J. Immunol. Methods.* 65:55.
  18. Ohara, J., and W. E. Paul. 1987. Receptor for B-cell stimulatory factor-1 expressed on cells of haematopoietic lineage. *Nature (Lond.)* 325:537.
  19. Nakahata, T., S. S. Spicer, J. R. Cantey, and M. Ogawa. 1982. Clonal assay of mouse mast cell colonies in methylcellulose culture. *Blood.* 60:352.
  20. Iscove, N. N., L. J. Guilbert, and C. Weyman. 1980. Complete replacement of serum in primary cultures of erythropoietin-dependent red cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acid, lecithin and cholesterol. *Exp. Cell Res.* 126:121.
  21. Koike, K., T. Nakahata, M. Takagi, T. Kobayashi, A. Ishiguro, K. Tsuji, K. Naganuma, A. Okano, Y. Akiyama, and T. Akabane. 1988. Synergism of BSF-2/interleukin 6 and interleukin 3 on multi-potential hemopoietic progenitors in serum-free culture. *J. Exp. Med.* 168:879.
  22. Pretlow, T. G., and I. M. Cassady. 1970. Separation of mast cells in successive stages of differentiation using programmed gradient sedimentation. *Am. J. Pathol.* 61:323.
  23. Enerback, L. 1974. Berberine sulphate binding to mast cell polyanions: a cytofluorometric method for quantitation of heparin. *Histochemistry.* 42:301.
  24. Ishizaka, T., F. Hirata, K. Ishizaka, and J. Axelrod. 1980. Stimulation of phospholipid methylation, Ca<sup>2+</sup> influx, and histamine release by bridging of IgE receptors on rat mast cells. *Proc. Natl. Acad. Sci. USA.* 77:1903.
  25. Yamatodani, A., K. Maeyama, T. Watanabe, H. Wada, and Y. Kitamura. 1982. Tissue distribution of histamine in a mutant mouse deficient in mast cells. *Biochem. Pharmacol.* 31:305.
  26. Nakano, T., T. Sonoda, C. Hayashi, A. Yamatodani, Y. Kanayama, T. Yamamura, H. Asai, T. Yonezawa, Y. Kitamura, and S. J. Galli. 1985. Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal and intravenous transfer into genetically mast cell deficient W/W<sup>v</sup> mice. Evidence that cultured mast cells can give rise to both connective tissue-type and mucosal mast cells. *J. Exp. Med.* 162:1025.
  27. Nakahata, T., K. Tsuji, A. Ishiguro, O. Ando, N. Norose, K. Koike, and T. Akabane.

1985. Single-cell origin of human mixed hemopoietic colonies expressing various combinations of cell lineages. *Blood*. 65:1010.
28. Fernandez, J. M., E. Neher, and B. D. Gomperts. 1984. Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature (Lond.)*. 312:453.
29. Johnson, A. R., and N. C. Moran. 1969. Selective release of histamine from rat mast cells by compound 48/80 and antigen. *Am. J. Physiol.* 216:453.
30. Kobayashi, T., and T. Nakahata. 1989. Stochastic model for mast cell proliferation in culture of murine peritoneal cells. *J. Cell. Physiol.* 138:24.
31. Sonoda, S., T. Sonoda, T. Nakano, Y. Kanayama, Y. Kanakura, H. Asai, T. Yonezawa, and Y. Kitamura. 1986. Development of mucosal mast cells after injection of a single connective tissue-type mast cells in the stomach mucosa of genetically mast cell deficient  $W/W^v$  mice. *J. Immunol.* 137:1319.
32. Capron, M., T. Jouault, L. Prin, M. Joseph, J.-C. Ameisen, A. E. Butterworth, J.-P. Papin, J.-P. Kusnierz, and A. Capron. 1986. Functional study of a monoclonal antibody to IgE Fc receptor (FcεR2) of eosinophils, platelets, and macrophages. *J. Exp. Med.* 164:72.
33. Spiegelberg, H. L. 1984. Structure and function of Fc receptor for IgE on lymphocytes, monocytes, and macrophage. *Adv. Immunol.* 35:61.
34. Yodoi, J., and K. Ishizaka. 1979. Lymphocytes bearing Fc receptor for IgE. 1. Presence of human and rat T lymphocyte with Fcε-receptor. *J. Immunol.* 122:2577.
35. Yukawa, K., H. Kikutani, H. Owaki, K. Yamasaki, A. Yokota, H. Nakamura, E. L. Barsumian, R. R. Hardy, M. Suemura, and T. Kishimoto. 1987. A B cell-specific differentiation antigen, CD23, is a receptor for IgE (FcεR) on lymphocytes. *J. Immunol.* 138:2576.
36. Gordon, J., A. J. Webb, L. Walker, G. R. Guy, and M. Rowe. 1986. Evidence for an association between CD23 and the receptor for a low molecular weight B cell growth factor. *Eur. J. Immunol.* 16:1627.
37. Brown, M. A., J. H. Pierce, C. J. Watson, J. Falco, J. N. Ihle, and W. E. Paul. 1987. B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells. *Cell*. 50:809.
38. Azuma, M., T. Hirano, H. Miyajima, N. Watanabe, H. Yagita, S. Enomoto, S. Furusawa, Z. Ovary, T. Kinashi, T. Honjo, and K. Okumura. 1987. Regulation of murine IgE production in SJA/9 and nude mice: potentiation of IgE production by recombinant IL-4. *J. Immunol.* 139:2538.
39. Woodbury, R. G., H. R. P. Miller, J. F. Huntley, G. F. J. Newlands, A. C. Palliser, and D. Wakelin. 1984. Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode infections in rat. *Nature (Lond.)*. 312:450.
40. Czarnetzki, B. M., and G. Mecklenburg. 1987. In vivo increase of cutaneous mast cells in response to specific mediators and ascaris antigen. *Int. Arch. Allergy Appl. Immunol.* 82:259.
41. Coffman, R. L., J. Ohara, M. W. Bond, J. Carty, A. Zlotnik, and W. E. Paul. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* 136:4538.
42. Young, J. D. E., C. C. Liu, G. Butler, Z. A. Cohn, and S. J. Galli. 1987. Identification, purification, and characterization of a mast cell-associated cytolytic factor related to tumor necrosis factor. *Proc. Natl. Acad. Sci. USA*. 84:9175.