

Suspension culture of human mast cells/basophils from umbilical cord blood mononuclear cells

(cell differentiation/IgE receptors)

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ABSTRACT Selective growth of human mast cells/basophils was obtained in suspension cultures of mononuclear cells from umbilical cord blood. A fraction of culture supernatant of phytohemagglutinin-stimulated T cells, which lacked interleukin 2, was required for the selective growth of mast cells. When the mononuclear cells were cultured for 2–4 wk in the presence of the fraction, 50–90% of the total cells in the cultures contained metachromatic granules. Under the optimal culture conditions, the number of mast cells/basophils recovered from the cultures was 30–60% of the number of mononuclear cells plated. Cultured mast cells/basophils bear $1.2\text{--}3.83 \times 10^5$ IgE receptors per cell and contained $0.48\text{--}1.6 \mu\text{g}$ of histamine per 10^6 cells. The average forward rate constant, k_1 , and dissociation constant, k_{-1} , for the binding of human IgE to IgE receptors on the cells were $1.9 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ and $6.9 \times 10^{-5} \text{ sec}^{-1}$, respectively (average equilibrium constant = $2.75 \times 10^9 \text{ M}^{-1}$). Specific binding of human IgE with high affinity indicates that the cells recovered in the suspension culture are human mast cells/basophils. Cultured cells sensitized with human IgE released a substantial amount of histamine upon exposure to anti-IgE. The results indicate that human mast cells/basophils obtained in the culture are functionally mature.

Mast cells and basophils play a primary role in the clinical manifestation of atopic diseases. In reaginic hypersensitivity reactions, IgE antibodies bind to mast cells and basophils through their IgE receptors, and the reaction of allergen with cell-bound IgE antibodies induces the release of a variety of chemical mediators such as histamine and leukotrienes (1). Studies of the biochemical mechanisms of mediator release from human mast cells/basophils have been hampered by the difficulty in procuring these cells in sufficient numbers and in relatively pure cell populations. Although suspension culture and clonal culture methods provided enriched populations of mouse mast cells (2–6), such techniques have not been available for human mast cells. Razin *et al.* (7) reported selective growth of basophils in culture of human fetal liver. However, scarcity in the sources of the material for the culture limits application of the method for immunological studies.

Recently, one of us (T.N.) observed selective growth of mast cells/basophils in culture of human umbilical cord blood mononuclear cells in the presence of medium conditioned by phytohemagglutinin (PHA)-stimulated human leukocytes. In the present study, we modified culture conditions by using a fraction of PHA-stimulated human T-cell conditioned media. This paper provides a description of the cell culture conditions and

characterization of human mast cells/basophils developed in the culture.

MATERIALS AND METHODS

IgE and Anti-IgE. Human E-myeloma protein P.S. was purified as described (8). Purity of the myeloma protein was >99%. A rabbit antiserum specific for the Fc portion of human IgE has been described (9). The concentration of anti-IgE antibodies in the antiserum was 1.8 mg/ml.

Purified IgE was labeled with ^{125}I (New England Nuclear) by the method of McConahey and Dixon (10). The proportion of ^{125}I -labeled IgE (^{125}I -IgE) that binds to cultured mast cells was determined by the method of Kulczycki and Metzger (11). Seventy-three percent of the ^{125}I was bound to cultured mast cells. The concentration of ^{125}I -IgE described herein was corrected relative to the concentration that is bindable to mast cells.

Conditioned Media. Culture supernatants of human T lymphocytes stimulated with PHA [crude human T-cell growth factors (TCGF); lot no. 051-205] was purchased from Associated Biomedic Systems (Buffalo, NY). A fraction containing mast cell growth factor was obtained by the method described by Yung *et al.* (12) for the purification of growth factor for mouse mast cells. Briefly, precipitates at 80% saturation of ammonium sulfate (Aldrich), at pH 7.0, were dissolved in 10 mM phosphate buffer (pH 8.3) containing 0.002% polyethylene glycol (M_r 8,000, Sigma). After extensive dialysis, the fraction was applied to a DEAE-cellulose (DE-52 Whatman) column (2.5×20 cm) that had been equilibrated with the buffer. The column was eluted with the 10 mM phosphate buffer, followed by elution with 100 mM phosphate buffer (pH 8.3). Fractions eluted with each buffer were pooled separately, dialyzed against Hanks' balanced salt solution followed by RPMI 1640 medium, and concentrated by ultrafiltration with Diaflo YM-5 membranes (Amicon); their volumes were adjusted to 1/10th of the original conditioned medium. The fractions were kept at -70°C until use.

Cell Cultures. Heparinized cord blood was layered over Ficoll-Hypaque (Pharmacia), the tubes were centrifuged at $350 \times g$ for 30 min at room temperature, and mononuclear cells at the interface were collected. Cells were suspended in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 10% (vol/vol) fetal calf serum (M. A. Bioproducts and Flow Laboratories), $50 \mu\text{M}$ 2-mercaptoethanol, 2 mM L-glutamine, 100 units of penicillin per ml, and $100 \mu\text{g}$ of streptomycin per ml. The medium was enriched with 30–50% (vol/vol) crude TCGF or 3% (vol/vol) of the concentrated 10 mM

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Abbreviations: TCGF, T-cell growth factor(s); IL 2, interleukin 2; PHA, phytohemagglutinin.

fraction of the preparation described above. Cells were incubated at 1×10^6 cells per ml in 25-cm² tissue culture flasks at 37°C in a humidified, 5% CO₂ in air atmosphere. Half of the culture medium was replaced weekly with freshly prepared medium.

Titration of Interleukin 2 (IL 2). IL 2 in the conditioned medium was measured by the standard microassay of Gillis *et al.* (13) by using IL 2-dependent murine cytotoxic T cells, CTLL15H (14), which were kindly supplied by Kendall Smith (Dartmouth-Hitchcock Medical Center, Hanover, NH). A reference standard of IL 2 was prepared by culture of rat spleen cells (10^6 nucleated cells per ml) in RPMI medium containing 10% fetal calf serum and 5 μ g of concanavalin A per ml for 48 hr and the IL 2 activity in the culture supernatant was arbitrarily taken as 1 unit/ml (15). The IL 2 activity in samples was expressed in units/ml by comparing experimental probit data with that of a reference standard.

Binding of IgE Molecules to Mast Cells. The binding of IgE molecules to mast cells was determined by the method of Kulczycki *et al.* (16). Nonadherent cells recovered from cultures ($\approx 10^6$ cells per ml) were incubated with various concentrations of ¹²⁵I-IgE at 37°C for 90 min with constant shaking. To determine nonspecific binding, aliquots of the same cell suspension were incubated for 15 min with 1.4 mg of unlabeled IgE per ml prior to the addition of ¹²⁵I-IgE. After incubation with ¹²⁵I-IgE, 0.2 ml of each cell suspension was layered over 0.2 ml of fetal calf serum in duplicate and was centrifuged in a Beckman 152 Microfuge for 1 min. After subtraction of nonspecific binding, ¹²⁵I-IgE molecules bound per mast cell were calculated from the net count with an assumption that the M_r of human IgE is 190,000.

Association and Dissociation Kinetics. The kinetics of association and dissociation between IgE and mast cell receptors were determined by the methods described by Kulczycki and Metzger (11). To determine the forward rate constant (k_1), 5×10^5 to 10^6 mast cells per ml were incubated with 6 μ g of ¹²⁵I-IgE per ml at 37°C, and cell-bound ¹²⁵I-IgE was measured during the first 300 sec of incubation. The forward rate constant (k_1) was calculated from the following equation: $k_1 = (V_0)/(IgE_0)(R_0)$. V_0 represents the initial rate of the binding, and IgE_0 and R_0 represent the initial concentration of IgE and of receptors, respectively.

To determine the dissociation rate constant (k_{-1}), cell suspensions ($2-3 \times 10^6$ cells per ml) were first incubated with 6 μ g of ¹²⁵I-IgE per ml for 1 hr at 37°C. Cells were centrifuged and resuspended in fresh RPMI medium at a final concentra-

tion of $0.5-1 \times 10^6$ cells per ml and the cell suspension was divided into two tubes. At the beginning of the incubation, unlabeled IgE (1.4 mg/ml) was added to one tube and the same volume of medium was added to the other tube. Both tubes were incubated at 37°C with constant rotation. After various time intervals, aliquots of the cells from both experimental and control tubes were assayed for cell-bound ¹²⁵I-IgE. The dissociation rate constant (k_{-1}) was calculated by assuming that the dissociation of IgE from receptors was a simple first order of decay process: IgE receptor complex $\rightarrow$ IgE + receptor (11). Equilibrium constant (K_A) was calculated from the equation: $K_A = k_1/k_{-1}$.

Passive Sensitization and Histamine Release. To saturate IgE receptors on cultured mast cells/basophils, IgE was added to cultures at the concentration of 10 μ g/ml. After overnight incubation, nonadherent cells were harvested from the cultures and washed with RPMI 1640 to remove free IgE. The cells were then suspended in Tyrode solution (pH 7.0) containing 1 mM CaCl₂, 5 mM 2-(N-morpholino)ethanesulfonic acid, 5 mM Hepes, 10 μ g of phosphatidylserine (Supelco, Bellefonte, PA) per ml, and 0.5 g of gelatin/liter. Phosphatidylserine was dispersed in the solution by sonication. The cell suspensions containing $3-5 \times 10^4$ mast cells were then incubated with various concentrations of anti-IgE at 37°C. Histamine in the supernatants was measured by the automated technique of Siraganian (17).

RESULTS

Selective Growth of Mast Cells/Basophils. Mononuclear cells from cord blood were cultured in the presence of crude TCGF and cells recovered at 3 wk were examined by direct staining of cell suspensions with toluidine blue (18). In four separate experiments, 17-53% of nonadherent cells in the cultures contained metachromatic granules. To separate mast cell growth factor from IL 2, the conditioned medium was fractionated by ion-exchange column chromatography (see *Materials and Methods*) and aliquots of a mononuclear cell fraction of cord blood were cultured for 3 wk in a medium enriched with either the fraction eluted with 10 mM phosphate or that eluted with 100 mM phosphate. Determination of IL 2 activity showed that the 10 mM fraction contained no detectable amount of IL 2 (<0.002 units/ml), whereas the 100 mM fraction contained 0.36 unit/ml of IL 2. In the culture containing the 10 mM fraction, nucleated cells declined approximately to one-half of the original input of mononuclear cells. However, 89% of nonadherent cells

Table 1. Time course of cell proliferation and differentiation in culture

Culture number	Days in culture	Nonadherent cells per flask, no. $\times 10^6$	Differential counts, %*							
			Mast/baso [†]	Ly	Ery	Neu	Eo	Mac	Meg	Bl
I	0	9.0		(52)	13	33	1)‡			1
	7	5.0	49.5 (48)	11	7	0.5	0.5	5.5	0	26
	14	3.5	76 (71)	1	4.5		3.5	7	3.5	4.5
	21	3.5	67 (68)	2	2	1	19	5	0.5	3.5
	28	3.6	52 (51)				35	8	2	4
II	0	10.0		(53.5)	9.4	37)‡				
	7	5.0	48 (56)	13.5	1			4	1	32.5
	14	5.3	65 (59)	4	0.5			15.5	1.5	13.5
	21	5.0	64 (57)	3	1.5		11.5	16	2	2
	28	5.3	60 (60)	3			15	17	1	4

Mast/baso, mast cells/basophils; Ly, lymphocytes; Ery, erythroblasts; Neu, neutrophils; Eo, eosinophils; Mac, macrophages; Meg, megakaryocytes; and Bl, blasts.

*Differential counting was performed on a total of 200 cells stained with May/Grünwald/Giemsa.

[†]Toluidine blue-positive cells (%) analyzed simultaneously are shown in parentheses.

[‡]Differential counts of input are those of cord blood smears.

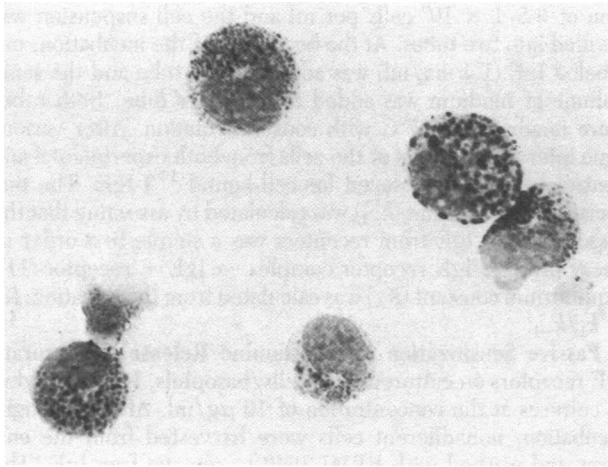


FIG. 1. A portion of a smear from a 2-wk-old culture of cord blood cells revealing cells with large basophilic granules.

in the culture contained metachromatic granules. In contrast, cultures with the 100 mM fraction contained a large number of lymphocytes, and only 21% of the total cells gave metachromatic staining. In subsequent experiments, mononuclear cells from cord blood were cultured with the 10 mM fraction. Time course observations of the cell suspension by toluidine blue staining yielded concordant data (Table 1). We were unable to classify these cells as either mast cells or basophils. Some cells had an oval nucleus with a dispersed chromatin pattern that is attributed to mast cells (7), whereas others had a segmented nucleus and dense heterochromatin. Preliminary studies by electron microscopy indicated that the granules had the particulate appearance that has been described as being characteristic of basophils (19).

Smears of the cells obtained in a 2-wk-old culture were stained with May/Grunwald/Giemsa and are presented in Fig. 1. All cells that were identified as mast cells/basophils contained basophilic granules varying in size and maturity. Simultaneous examination of the cell suspension by toluidine blue staining yielded concordant data (Table 1). We were unable to classify these cells as either mast cells or basophils. Some cells had an oval nucleus with a dispersed chromatin pattern that is attributed to mast cells (7), whereas others had a segmented nucleus and dense heterochromatin. Preliminary studies by electron microscopy indicated that the granules had the particulate appearance that has been described as being characteristic of basophils (19).

Immunological Properties of Cultured Mast Cells/Basophils. Experiments were carried out to determine that the cultured mast cells are functionally mature. Cord blood mononuclear cells were cultured for 3–4 wk in a medium containing the 10 mM fraction of conditioned medium. In seven separate experiments, in which $\approx 6 \times 10^6$ mononuclear cells were plated per culture flask, $1.2\text{--}3.8 \times 10^6$ mast cells/basophils were recovered. As shown in Table 2, the purity of mast cells/baso-

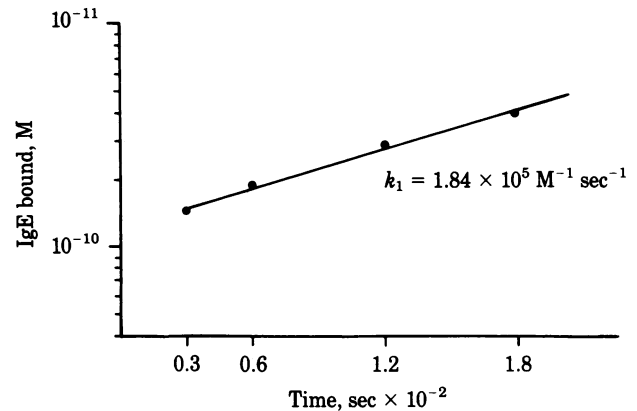


FIG. 2. Determination of the forward rate constant, k_1 , for IgE binding to cultured human mast cells/basophils. Aliquots of cultured cells were mixed with $6 \mu\text{g}$ of ^{125}I -IgE per ml and the cell suspension was kept at 37°C with constant shaking. The reaction was stopped by adding a 100-fold excess of unlabeled IgE to the tubes at the time indicated, and cell-bound IgE was determined. Each point represents the mean of duplicate samples. The average number of IgE receptors on the cells employed was 2.75×10^5 per cell.

phils in the cultures was 70–90% and their histamine content was $0.4\text{--}1.6 \mu\text{g}$ per 10^6 cells.

Because mast cells and basophils bear receptors for IgE, we determined the number of IgE receptors on the cultured mast cells. When aliquots of the cells were incubated for 90 min with $1\text{--}20 \mu\text{g}$ of ^{125}I -IgE per ml, the number of IgE molecules specifically bound to the cells increased as the concentration of ^{125}I -IgE increased and reached a plateau at $5 \mu\text{g}$ of ^{125}I -IgE per ml. To determine the total number of IgE receptors, cells were incubated at 37°C for 90 min with $10 \mu\text{g}$ of ^{125}I -IgE per ml and the radioactivity associated with the cells was determined. Preincubation of the cells with 1.4 mg of unlabeled IgE per ml decreased the binding of ^{125}I -IgE to $<8\%$. As shown in Table 2, the average number of IgE receptors per mast cell/basophil in seven different cultures was in the range of $1.2\text{--}3.83 \times 10^5$ per cell.

Binding characteristics of ^{125}I -IgE to cultured mast cells/basophils were then determined. The results of a representative experiment for the measurement of forward rate constant (k_1) are shown in Fig. 2. Determination of k_1 derived from Fig. 2 and two additional studies are shown in Table 2. An average value (mean $\pm$ SEM) of k_1 of the three experiments was $1.88 \pm 0.43 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$. The kinetics of dissociation of cell-bound ^{125}I -IgE was measured by using the same cell preparations and the results of one such experiment are illustrated in Fig. 3. An average k_{-1} (mean $\pm$ SEM) of the three exper-

Table 2. Histamine content and IgE receptors of cultured mast cells

Culture number*	Days in culture	Mast cells/basophils		Histamine per 10^6 cells, μg	IgE receptor per cell, $\times 10^5$	Binding characteristics		
		Proportion, %†	Yield/flask, $\times 10^6$			$k_1 \times 10^{-5}, \text{M}^{-1} \text{sec}^{-1}$	$k_{-1} \times 10^5, \text{sec}^{-1}$	$K_A \times 10^{-9}, \text{M}^{-1}$
III	23	89	2.3	0.52				
IV	26	89	2.8	1.60	3.83	1.47	7.54	1.95
V	29	70	1.2	0.96				
VI	26	92	3.2	1.04	2.75	1.84	6.75	2.73
VII	29	88	3.8	0.48	2.98	2.32	6.52	3.56
VIII	27	89	2.8	0.82	2.77			
IX	29	74	2.4	0.68	1.20			

Six million mononuclear cells were plated in each flask.

* Mononuclear cells from seven different cord blood specimens were cultured.

† Cells containing metachromatic granules stained by toluidine blue (18).

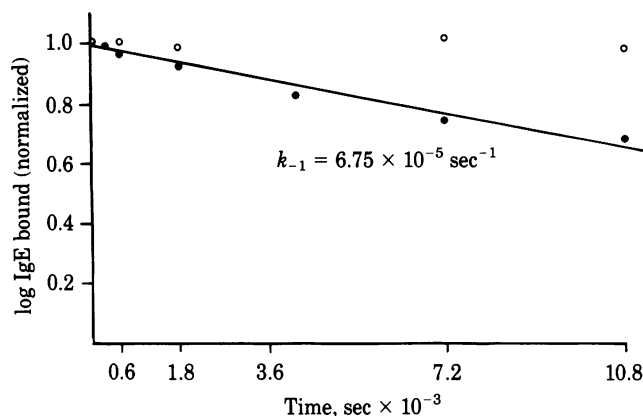


FIG. 3. Determination of the dissociation rate constant, k_{-1} , of IgE from cultured mast cells/basophils. Aliquots of a cell suspension were incubated with $6 \mu\text{g}$ of ^{125}I -IgE per ml at 37°C for 1 hr. At the initiation of the experiment (t_0), either unlabeled IgE ($\bullet$) or an equal volume of medium ($\circ$) was added to aliquots of the cell suspension. The amount of cell-bound ^{125}I -IgE was determined at the times indicated and the data were normalized to compensate for the difference in cell-bound ^{125}I -IgE concentration at t_0 . Each point represents mean of duplicate measurements.

iments was $6.93 \pm 0.54 \times 10^{-5} \text{ sec}^{-1}$ (Table 2). The equilibrium constant (K_A) (mean $\pm$ SEM) for the binding reaction calculated from the average value of k_1 and k_{-1} was $2.75 \pm 0.81 \times 10^9 \text{ M}^{-1}$.

Finally, we determined whether the cultured mast cells could be passively sensitized with human IgE for anti-IgE-induced histamine release. Cultured cells were incubated with $10 \mu\text{g}$ of IgE per ml at 37°C overnight to saturate IgE receptors. Non-adherent cells recovered from the culture were washed and then challenged with various concentrations of anti-IgE. As shown in Fig. 4, the sensitized cells released histamine upon challenge with anti-IgE, whereas the same cells cultured without IgE released $<2\%$ of histamine. The maximal histamine release from five different cell preparations was in the range of 22–72%. In all of the experiments with cultured cells, an optimal concentration of anti-IgE for the maximal histamine release was $0.45 \mu\text{g}/\text{ml}$, which was comparable to that required for maximal histamine release from human lung mast cells (9) and human basophils (20).

DISCUSSION

In this report, we have presented a simple method for obtaining large numbers of human mast cells/basophils from culture of umbilical cord blood. Because the purity of mast cells in 2- to 4-week-old cultures was 50–90% and the yield of the cells was $\approx 40\%$ of the total mononuclear cells plated (Table 2), both the proliferation and differentiation of mast cell precursors should have taken place during the culture. Successful development of mast cells in the culture depended on mast cell growth factor(s) present in the medium conditioned by PHA-stimulated human T cells and on the elimination of TCGF, which stimulates the growth of T cells. However, the mast cell growth factor-enriched fraction employed in the present experiment may contain granulocyte- and other colony-stimulating factors (21), which may account for simultaneous growth of eosinophils and other hemopoietic cells in some cultures (see Table 1). In mouse bone marrow cell cultures, Schrader and Clark-Lewis (22) as well as Yung and Moore (23) reported that mast cell growth factor is different from granulocyte colony-stimulating factor. Further purification of human mast cell growth factor may provide more consistent results for the culture of pure mast cells.

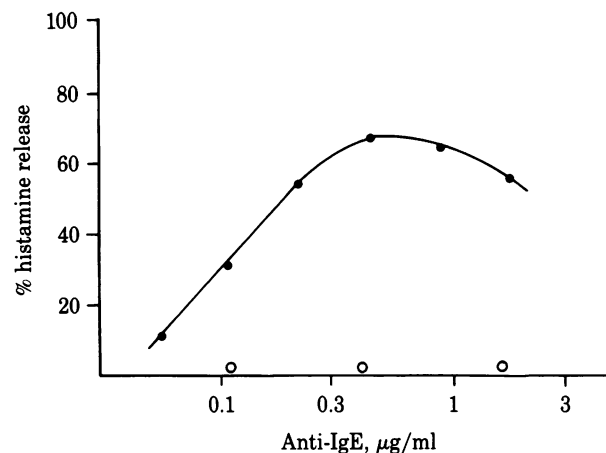


FIG. 4. Anti-IgE-induced histamine release from cultured mast cells/basophils. Cells were sensitized with IgE and aliquots of the sensitized cells ($\bullet$) and unsensitized cells ($\circ$) were incubated for 30 min at 37°C with various concentrations of anti-IgE. Each point represents mean of duplicate samples. Histamine content in the mast cells was $0.96 \mu\text{g}$ per 10^6 cells.

The present experiments also showed that the percentage of mast cells/basophils in the cultures varied from one sample of cord blood to another and appeared to be influenced by the batch of fetal calf serum. An apparent difference in the proportion of mast cells/basophils between culture I and II in Ogawa's laboratory (Table 1) and culture III–IX obtained in Ishizaka's laboratory (Table 2) is probably due to a difference in fetal calf serum, because the same batch of mast cell growth factor-enriched fraction was employed in the two laboratories. The fetal calf serum employed for cultures I and II had been selected for hemopoietic cell culture, whereas the fetal calf serum for cultures III–IX was selected for the culture of rat basophilic leukemic cells. A recent experiment comparing three different fetal calf serum lots for the culture of the same cord blood cells demonstrated a significant difference (52, 78, and 88%) in the purity of mast cells/basophils developed after 4 wk.

Cultured human mast cells/basophils are functionally mature. They bear $1.2\text{--}3.83 \times 10^5$ IgE receptors per cell (Table 2). These values are comparable to the number of IgE receptors on purified rat and mouse mast cells (24). Previous reports indicated that the number of IgE receptors on human basophils was 30,000–680,000 (25), whereas purified lung mast cells bear 4,300–395,000 IgE receptors (26) per cell. It should be noted that in both experiments, the number of IgE receptors was estimated from the numbers of IgE molecules in acid extracts of the cells.

Cultured human mast cells/basophils had receptors with high affinity for human IgE. An average equilibrium constant (K_A) (mean $\pm$ SEM) of the binding between human IgE and IgE receptors on the cultured mast cells/basophils was $2.75 \pm 0.81 \times 10^9 \text{ M}^{-1}$, which was comparable to the equilibrium constant between mouse/rat IgE and IgE receptors on rodent mast cells (11, 24). Although receptors for IgE were detected on lymphocytes (27) and macrophages (28), they have an affinity for IgE that is 1/10th to 1/100th of that for IgE receptors on mast cells. Thus, the presence of high affinity IgE receptors on cultured cells obtained in the present experiments evinces further that they are mast cells/basophils.

The histamine content of the cultured mast cells/basophils was lower than that previously reported for human basophils (29) and lung mast cells (9). However, sensitization of cultured mast cells with human IgE followed by challenge with anti-IgE resulted in the release of a substantial portion of histamine. An

optimal concentration of anti-IgE for maximal histamine release from cultured mast cells/basophils was 0.45 $\mu\text{g}/\text{ml}$, which was comparable to that of the same anti-IgE preparation to induce maximal histamine release from human basophils (20) and lung mast cells (9).

Preliminary studies revealed that sensitized cultured mast cells preincubated with [^{14}C]arachidonic acid released >50% [^{14}C]arachidonic acid upon challenge with anti-IgE. In mouse mast cells, Razin *et al.* (30, 31) established the existence of two distinct subclasses of mast cells that differ in the nature of their proteoglycan and in the character of their oxidative products of arachidonic acid. Further analysis of oxidative products of arachidonic acid and of the nature of the proteoglycan in the cultured human mast cells would provide a biochemical basis for classification of the cells.

In the past few years, attempts were made to purify human basophils or mast cells to analyze the mechanisms of IgE-dependent mediator release. MacGlashan and Lichtenstein (32) obtained up to 80% pure human basophils from normal peripheral blood by sensitization of mononuclear cells with human IgE antibodies to the benzyl penicilloyl group, followed by affinity chromatography of the sensitized cells on benzyl penicilloyl-coupled Sepharose. However, the yield of purified basophils obtained by this procedure was limited. Recently, Schulman *et al.* (33) established a procedure to obtain a large number of mast cells from human lung tissues by combinations of enzyme digestion of lung fragments, countercurrent centrifugation, and elutriation, followed by affinity chromatography on benzyl penicilloyl-Sepharose. A common disadvantage of both techniques is limitation of available anti-benzyl penicilloyl IgE antibody from patients with penicillin hypersensitivity. Although this difficulty was overcome by recent modification of the latter technique by using Percoll density gradient centrifugation rather than affinity chromatography (9), extensive digestion of lung fragments may alter plasma membranes of mast cells. We believe that high enriched human mast cells/basophils in suspension cultures will provide useful tools for characterization of IgE receptors and for biochemical analysis of mediator release.

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