Development of human mast cells *in vitro*

(coculture/3T3 fibroblasts/cord blood cells/tryptase/chymase)

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ABSTRACT Nucleated cells of human umbilical cord blood were cocultured with mouse skin-derived 3T3 fibroblasts. After 7–8 weeks in culture, when the number of the other hematopoietic cells declined, metachromatic granule-containing mononuclear cells appeared in the cultures, and the number of the cells increased up to 12 weeks. After 11–14 weeks in culture, the metachromatic mononuclear cells comprised a substantial portion of the cultured cells. These cells contained 1.8–2 μg of histamine per 10^6 cells and bore receptors for IgE. All of the cells contained tryptase in their granules. Electron microscopic analysis showed that these cells were mature human mast cells, clearly different from the basophilic granulocytes or eosinophils that arise in a variety of circumstances in cord blood cell cultures. Most of the cultured mast cells expressed some granules with regular crystalline arrays and contained both tryptase and chymase, and thus resembled human skin mast cells.

In the murine system, recombinant interleukin 3 (IL-3) promotes the proliferation of mouse mast cell-line cells (1) and facilitates the formation of mast cell colonies in semisolid cultures of mouse bone marrow (BM) cells (2). Suspension culture of murine BM cells with recombinant IL-3 results in the development of a pure mast cell population (3). These IL-3-dependent, BM-derived mast cells appear to be analogous to mast cells in the gastrointestinal mucosa and different from mast cells in connective tissue (4). However, Levi-Schaffer *et al.* (5) as well as Dayton *et al.* (6) demonstrated that coculture of the BM-derived, IL-3-dependent mast cells with 3T3 fibroblasts in the presence of IL-3-containing conditioned medium resulted in a phenotype change to those resembling connective-tissue mast cells.

In contrast to the murine mast cell system, requirements for the differentiation and proliferation of human mast cells are unknown. In the past 10 years, numerous attempts were made to develop human basophils and mast cells *in vitro*. Several groups, including ours, demonstrated *in vitro* development of human basophils from progenitors in BM, umbilical cord blood, and fetal liver (7–10). However, reproducible success in *in vitro* development of mature human mast cells has not been achieved. Recombinant human IL-3 promoted the differentiation of human basophils from precursors in BM and cord blood cells, but neither IL-3 nor IL-4 induced the differentiation of human mast cells (11–13). We anticipated that a feeder layer or cytokines from non-T cells might facilitate the differentiation of human mast cells *in vitro*. In the present experiments, nucleated cells from cord blood were cocultured with various human and murine fibroblasts. Prolonged coculture of cord blood cells with mouse 3T3

fibroblasts resulted in the development of mature human mast cells *in vitro*.

MATERIALS AND METHODS

Cell Cultures. Total nucleated cells were recovered from heparinized umbilical cord blood by differential centrifugation in 73–75% Percoll (Pharmacia) at $300 \times g$ for 20 min at room temperature. In some experiments, a portion of nucleated cells was applied to a Sephadex G-10 column to deplete adherent cells. Total nucleated cells or nonadherent cells (10^6 per ml) were resuspended in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), and gentamicin (25 $\mu\text{g}/\text{ml}$) and were cocultured with Swiss albino mouse skin-derived 3T3 fibroblasts or one of three human fibroblast lines, Detroit 551, MRC-9, or IMR-90 (American Type Culture Collection). The 3T3 cells were seeded in a flat-bottomed tissue culture well (24-well plate, Flow Laboratories) at a density of 10^3 cells per ml and cultured until they became confluent (≈ 2 weeks), and cord blood nucleated cells (10^6 per ml) were seeded on the confluent 3T3 fibroblasts. In the case of human fibroblasts, 10^4 cells were placed in a 2-ml well and incubated for 4 hr at 37°C prior to seeding of cord blood cells. In some cultures, culture medium was enriched with a fraction of conditioned medium (CM) that was obtained from culture supernatant of human T lymphocytes stimulated with phytohemagglutinin (Electro-Nucleonics) as described (8). The fraction was concentrated to 1/10th the volume of the original culture supernatant and was added to the culture at a final concentration of 3% (vol/vol). The cultures were incubated at 37°C in a humidified atmosphere of 6% CO_2 and 94% air. Half the culture medium was replaced twice a week with freshly prepared medium.

Human lung mast cells were obtained from normal lung tissues resected during surgery of lung carcinoma. The finely fragmented tissues were digested with Pronase, chymopain, and collagenase (14), and a suspension of lung cells was centrifuged on 23.5% (wt/vol) metrizamide. Aliquots of a lung cell suspension ($1\text{--}1.5 \times 10^6$ cells per ml), which contained 6.2–15.5% mast cells, were cocultured with various fibroblasts in tissue culture wells (15).

Microscopic Analysis of Cells Developed in the Cultures. Nucleated cells cocultured with fibroblasts were recovered by treatment for 10 min at 37°C with 0.25% trypsin/1 mM EDTA. In some experiments, cells were resuspended in RPMI-1640 medium with 10% fetal bovine serum and placed in a Petri dish. After incubation for 2 hr at 37°C , nonadherent cells were recovered. Viability of the cells was determined by trypan blue exclusion. Differential count of the total cells or

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Abbreviations: BM, bone marrow; M-cells, mast cell-like cells; CM, T-cell conditioned medium; IL-*n*, interleukin *n*.

nonadherent cells was performed in a preparation centrifuged onto microscope slides and stained with May-Grünwald and Giemsa reagents. The metachromatic granule-containing cells were identified by staining with toluidine blue. For electron microscopy, nonadherent cells recovered from the coculture of cord blood cells with 3T3 fibroblasts were fixed in a dilute mixture of aldehydes, washed in 0.1 M sodium cacodylate buffer, and spun through molten agar to form agar blocks containing the cells. The blocks were processed for electron microscopic studies as described (16).

Determination of the Binding of IgE to Cultured Cells. The nonadherent cells harvested from the tissue culture wells were incubated for 1 hr at 4°C with purified human E myeloma protein (PS, 50 µg/ml) or overnight with IgE (10 µg/ml). After washing, the cells were incubated for 1 hr at 4°C with rabbit antibodies (10 µg/ml) specific for IgE. The cells were washed again and incubated for 30 min with fluoresceinated anti-rabbit IgG (Melyo Laboratories). Surface staining of the cells was evaluated by fluorescence microscopy.

Characterization of Proteases in the Cultured Mast Cells. Cytospin preparations of cultured cells were air-dried, fixed in Carnoy's fluid (60% chloroform/30% ethanol/10% glacial acetic acid) for 15 min at room temperature, and subjected to the sequential double-labeling procedure for the detection of trypsin and chymase associated with granules (17). Smears were incubated overnight at 4°C with biotinylated mouse monoclonal anti-chymase, B7 (8 µg/ml). Binding of the antibody was detected by treatment with peroxidase-conjugated streptavidin followed by a freshly prepared solution of 3-amino-9-ethylcarbazole (0.2 mg/ml) in 0.1 M acetate buffer (pH 5.2) with 0.01% H₂O₂. Alkaline phosphatase-conjugated murine monoclonal anti-trypsin, G3 (0.7 µg/ml), was then applied overnight at 4°C and developed at room temperature with fast blue RR (1 mg/ml) in 0.1 M Tris buffer (pH 8.2) containing naphthol AS-MX phosphate (0.2 mg/ml). Chymase-containing granules were stained reddish-brown, while those containing trypsin but no chymase (MC_T) were stained blue. Cells containing at least 10 clearly stained reddish-brown granules were counted as MC_{TC} cells, even when higher numbers of blue granules were present.

Histamine Release and Histamine Content. Lung mast cells were sensitized with IgE (10 µg/ml) overnight at 37°C. After washing, the cells were challenged with an optimal concentration of anti-IgE for histamine release. Histamine in the supernatant and cell pellet was assayed by the automated technique of Siraganian (18). Total histamine content in

cultured cord blood nucleated cells was measured by the same technique.

RESULTS

Selection of Fibroblasts for Coculture. To select a human fibroblast cell line that may facilitate the development of human mast cells, three human fibroblast lines, Detroit 551, MRC-9, and IMR-90, were tested for the ability to maintain viability and functions of human lung mast cells. Since Levi-Schaffer *et al.* (15, 19) had shown that coculture of human lung mast cells with mouse 3T3 fibroblasts maintained viability of the cells and responsiveness for anti-IgE for mediator release, mouse 3T3 fibroblasts were also employed as a feeder layer. Cells were recovered after 4 weeks of culture and examined for the percentage of mast cells, viability, and histamine content. Contrary to what one might expect, coculture with 3T3 fibroblasts was most effective for maintenance of mast cells and viability of lung cells. In two separate experiments of the same design, mast cells cocultured with 3T3 fibroblasts had the highest histamine content and gave maximum responses to anti-IgE for histamine release.

Long-Term Coculture of Cord Blood Cells with Fibroblasts. Aliquots of the total nucleated cell preparations of cord blood were cocultured with 3T3, MRC-9, Detroit 551, or IMR-90 fibroblasts in the presence or absence of T-cell CM. Cells recovered from the wells after 2, 4, 8, and 11 weeks in culture were examined for viability, the number of viable cells, and differential count (Table 1). In the cocultures with human fibroblasts, metachromatic granule-containing cells developed and survived over 8 weeks. All of these cells were human basophils, 80% of which were mature basophils having a segmented nucleus. After 8 weeks, eosinophils became predominant in these cultures, and no mast cell-like mononuclear cells were detected. Although only the results obtained with MRC-9 human fibroblasts are shown in Table 1, coculture of cord blood cells with Detroit 551 and IMR-90 gave similar results. Culture of the nucleated cells without fibroblasts in the presence or absence of CM resulted in a marked decrease in viable cells; the majority of the remaining cells were macrophages.

In the coculture of the same nucleated cell preparation with mouse 3T3 fibroblasts, only small numbers of basophils and eosinophils developed within 4 weeks. After 8 weeks, mononuclear cells containing metachromatic granules appeared and the number of such cells increased with culture period (Table 1). Each of the cells contained a large oval or round nucleus and cytoplasm filled with metachromatic granules (Fig. 1).

Table 1. Long-term cocultures of cord blood cells with fibroblasts

Fibroblasts	CM	Weeks	Viable cells per well	Viability, %	Differential count, %							
					Ba	Eo	Neu	Mac	Ly	Bla	M-cells	Fib
None	—	0	10 ⁶	99	0.5	1	62.5	7.5	26	0.5	0	0
MRC-9†	3%	4	1.8 × 10 ⁶	90	6	44	22.5	23	0.5	0	0	4‡
		8	3.5 × 10 ⁵	76	26.5	58.5	1.5	12	1	0	0	0.5
		11	2.5 × 10 ⁵	66	44.5	42.5	2	4	0.5	0	0	6.5
MRC-9†	—	4	1.1 × 10 ⁶	90	6	11.5	52.5	24	1	1	0	4‡
		8	2.1 × 10 ⁵	62	3	16	0	67.5	1	0	0	12.5
		11	4.5 × 10 ⁴	27	14.5	28	0.5	16.5	3	0	0	37.5
3T3	3%	4	8.9 × 10 ⁵	79	8	25	4.5	6.5	3	0.5	0	52.5
		8	4.8 × 10 ⁵	69	1.4	3	2	11.8	3.2	0	3	75.6
		11	3.0 × 10 ⁵	68	0.5	0	0	6.5	0.5	0	17.5	75
3T3	—	4	7.4 × 10 ⁵	92	2.8	4.8	1.6	20.4	6	0.4	0	64
		8	2.5 × 10 ⁵	89	1	0.2	3.8	28.2	4.6	0.4	1.6	60.2
		11	2.9 × 10 ⁵	90	0	0	0	7.6	2.8	0	8	81.6

*Cells were recovered by trypsin treatment of the wells and stained with May-Grünwald and Giemsa reagents. Morphology of 200 non-fibroblasts was examined. Ba, basophils; Eo, eosinophils; Neu, neutrophils; Mac, macrophages; Ly, lymphocytes; Bla, blasts; M-cells, mast cell-like cells; Fib, fibroblasts and fibroblast-like cells.

†Coculture with Detroit 551 and IMR-90 gave similar results.

‡Fibroblasts that had overgrown and detached from the well were not dispersed by trypsin digestion.

FIG. 1. Mast cells developed in a long-term (80-day) coculture of cord blood nucleated cells with 3T3 cells. (May-Grünwald/Giemsa staining, $\times 3750$.)

They were morphologically distinguishable from mature basophils remaining in the culture and appeared to be mast cells. After 11 weeks of coculture with 3T3 in the presence of CM, 40–70% of the total cells other than fibroblasts were mast cell-like (M-cells) (Table 1). Measurements of histamine content in nonadherent cells recovered at 11 weeks indicated that M-cells contained 1.8–2.0 μg of histamine per 10^6 cells. Furthermore, Fc_ϵ receptors were demonstrated by immunofluorescence on M-cells recovered after 84 days and 74 days of coculture with 3T3 fibroblasts. Nevertheless, the intensity of the fluorescence on M-cells was less than that of human lung mast cells sensitized with IgE.

Since previous studies showed that precursors of human basophils in cord blood are nonadherent cells (20), nucleated cell preparations were fractionated on a Sephadex G-10 column to obtain nonadherent populations. The yield of nonadherent cells was 11–31% of total nucleated cells in five cord blood cell preparations. Coculture of the nonadherent cell population with 3T3 fibroblasts over 8 weeks resulted in the development of M-cells, whereas coculture of the same nonadherent cells with Detroit 551, IMR-90, or MRC-9 failed

to develop M-cells even after 12 weeks. Coculture of total nucleated cells and nonadherent cells from the same cord blood specimens with 3T3 fibroblasts suggested that precursors of M-cells were enriched in nonadherent cells. Thus, subsequent cocultures were carried out using nonadherent cells of cord blood. Although the number of M-cells developed in the cultures varied greatly from one cord blood specimen to another (3–20%), development of M-cells was observed in coculture with 3T3 cells for each of three total nucleated cell preparations and four nonadherent cell populations of cord blood.

Characterization of Proteases in the M-Cells. Secretory granules of human mast cells can be stained by monoclonal antibodies against tryptase and/or those against chymase, whereas human basophils, which contain very small amounts of tryptase, are not stained with either antibody under standard conditions (21). Thus, we determined whether M-cells recovered at 12 weeks from the cocultures with 3T3 cells contained tryptase/chymase. Among the cells recovered from the coculture of cord blood cells in the presence or absence of CM, 20% of the cells ($n = 1289$) and 21% of the cells ($n = 1365$), respectively, were identified as human mast cells on the basis of tryptase or chymase positivity. Representative MC_{TC} and MC_{T} M-cells are shown in Fig. 2. Whereas 84% (146/174) of the tryptase-positive M-cells in the CM-containing culture were of the MC_{TC} type, 94% (131/139) of the M-cells in the culture without CM were of the MC_{TC} type.

Electron Microscopic Analysis of M-Cells. Total nucleated cells of cord blood were cocultured with 3T3 fibroblasts for 12 weeks, and the cells were recovered by trypsin treatment. Electron microscopic analysis of the nonadherent cells in the preparation revealed the presence of mature human mast cells (Fig. 3). These cells conformed to published descriptions of human mast cells studied *in vivo* and those in isolated, purified preparations from multiple organ sources (22). Mast cells in the cultures were generally smaller than those noted in previous *in vivo* studies, varying in size between 7 and 12 μm . They were mononuclear and displayed partially condensed chromatin. Many cells had large nucleoli. Cytoplasmic contents included many tubular mitochondria, free ribosomes, and variable numbers of membrane-bound granules. Narrow surface folds were regularly distributed over the cell surface. In the cultured cells, previously reported substructural granule patterns were evident (22, 23). These patterns included crystalline arrays of several periodicities, spirals

FIG. 2. Cord blood cells cocultured with 3T3 cells in the presence of CM were centrifuged onto a glass slide and subjected to sequential double labeling with biotin-anti-chymase and alkaline phosphatase-anti-tryptase antibodies. (Left) Two MC_{TC} cells and one MC_{T} cell are seen by standard light microscopy. (Right) Phase-contrast microscopy of the field shown in A, showing numerous unstained cells in addition to the stained mast cells. ($\times 1490$.)

FIG. 3. (A) Human mast cell developed from cord blood cells shows monolobed nucleus with partially condensed peripheral chromatin, narrow surface folds, intracytoplasmic canaliculi (arrow), a lipid body, and numerous membrane-bound granules. Substructure of granules delineated by boxes in A are seen at higher magnification in B and C. (B) A crystal granule is seen in cross-section adjacent to a mixed granule with homogeneous dense contents and peripheral scrolls or lamellae. (C) A mixed granule shows parallel crystalline array and homogeneous dense contents adjacent to a dense lipid body with focal peripheral lucency. (A, $\times 16,000$; B, $\times 86,500$; C, $\times 89,000$.)

and scrolls, particles, and homogeneously dense areas (Fig. 3). Many granules contained mixtures of these patterns. Most mast cells in the culture expressed some granules with regular crystalline arrays resembling those frequently present in human skin biopsy samples and in mast cells isolated from skin specimens (ref. 24; A.M.D., W. Massey, J. Warner, S. Kissell, A. Kagey-Sobotka, and L. Lichtenstein, unpublished data). Lipid bodies were rarely evident, a feature also noted for human skin mast cell populations (22). The mast cells that developed in these cultures were clearly different, by ultrastructural criteria, from the mature basophils (13, 25), basophilic myelocytes (13), mature eosinophils (13), and eosinophilic myelocytes (11, 13, 25) arising in a variety of circumstances of cultured human cord blood cells.

DISCUSSION

The failure of human mast cells to grow *in vitro* under conditions in which successful growth of basophils from cord blood and BM cells was achieved (11, 12) suggested that the growth factors and microenvironment essential for the differentiation of human mast cells from progenitors are distinct from those required for the development of basophils. Since

our experiments indicated that recombinant IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, and granulocyte/macrophage colony-stimulating factor, individually or in combinations, did not facilitate differentiation of human mast cells in suspension culture of cord blood or BM cells (unpublished results), we anticipated that feeder layers might support the differentiation of mast cell progenitors. After a prolonged coculture of total nucleated cells of cord blood with 3T3 fibroblasts, metachromatic granule-containing mononuclear cells (M-cells) appeared in the culture and the number of the cells in the culture increased with time (up to 11–14 weeks). Coculture of total nucleated cells from normal BM with 3T3 fibroblasts also resulted in the development of M-cells. However, the number of M-cells developed at 11 weeks of coculture of BM cells with 3T3 cells was 0.5–3% of the total cells (results not shown).

A major disadvantage in the use of the human fibroblast lines as feeder layer in long-term culture is that they do not exhibit contact inhibition and thus overgrow during prolonged culture. Since one of the cocultures of BM cells with human MRC-9 fibroblasts resulted in the development of a small number of M-cells after 7 weeks, human fibroblasts may facilitate the differentiation of human mast cells from their precursors.

M-cells recovered from coculture of cord blood cells with 3T3 fibroblasts for 11 weeks contained 1.8–2.0 μg of histamine per 10^6 cells and bore type I Fc_ϵ receptors. After sensitization with IgE, they released a small but significant amount of histamine upon challenge with anti-IgE. All of the M-cells could be stained by monoclonal anti-tryptase antibody as determined by immunofluorescence, whereas basophils developed in the culture could not. Further, M-cells recovered from 12-week cultures were identified as mature human mast cells by an electron microscopy. Most of the M-cells expressed some granules with regular crystalline arrays resembling those frequently present in mast cells from human skin. More than 84% of the M-cells contained both chymase and tryptase. It has been shown that mast cells containing tryptase and chymase (MC_{TC} type) are predominant among mast cells in normal dermis and intestinal submucosa. In contrast, mast cells of the MC_{T} type, which contain tryptase but little if any chymase, are predominant in the lung and small intestinal mucosa (26). It is possible that skin-derived 3T3 fibroblasts not only facilitated the differentiation of mast cell precursors to mature mast cells but also contributed to the determination of their phenotype.

Other investigators have attempted to develop human mast cells *in vitro* (27–29). Yuen *et al.* (28) observed persistent mast cell colonies from human BM and cord blood cells grown in semisolid agar cultures containing peripheral blood cells as feeder layer. Kirshenbaum *et al.* (29) reported that culture of BM cells from mastocytosis patients for 2–3 weeks in semisolid agar containing recombinant IL-3 resulted in the development of a small number of mast cell-like cells together with a large number of basophils. Reproducible development of mature human mast cells *in vitro* by a long-term coculture of cord blood cells with 3T3 fibroblasts is a major breakthrough for growth of human mast cells *in vitro*. However, many questions remain to be answered—e.g., whether contact with 3T3 cells is essential for the differentiation of mast cell progenitors, and why differentiation of mast cell progenitors begins after 6 or 7 weeks of coculture. Nevertheless, the present findings indicate that culture conditions required for the development of human mast cells are different from those for the differentiation of basophils, and suggest that mast cells have a lineage distinct from that of basophils.

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