Immunology 1992 **77** 136–143

Characterization of human mast cells developed *in vitro* from fetal liver cells cocultured with murine 3T3 fibroblasts

A. A. IRANI,* S. S. CRAIG,† G. NILSSON,‡ T. ISHIZAKA§ & L. B. SCHWARTZ‡ **Department of Pediatrics, ‡Medicine, and †Anatomy, Medical College of Virginia, Richmond, Virginia and the §Division of Allergy, La Jolla Institute for Allergy and Immunology, La Jolla, California, U.S.A.*

Accepted for publication 28 April 1992

SUMMARY

Cocultures of dispersed human fetal liver cells with murine Swiss 3T3 fibroblasts resulted in the development of human mast cells after 1 to 4 weeks of culture. Mast cells were detected by immunohistochemistry using a murine monoclonal anti-tryptase antibody, before metachromasia appeared with toluidine blue. When subjected to double immunohistochemistry using murine monoclonal anti-chymase and anti-tryptase antibodies, 94% ± 10% (SD) of the mast cells seen at day 30 of culture were of the MC_T type. These results contrast with those obtained with human mast cells derived from cord blood mononuclear cells cocultured with murine 3T3 fibroblasts which are comprised of substantially greater numbers of MC_{TC} cells, averaging 48% ± 31% (SD) at day 30 of culture. Mast cells developed *in vitro* from fetal liver cells or cord blood mononuclear cells contained similar amounts (± SD) of histamine (0·9 ± 0·5 pg/cell and 1·1 ± 1 pg/cell, respectively) and tryptase (1·7 ± 0·4 pg/cell and 1·9 ± 1·2 pg/cell, respectively) on day 30 of culture. Fetal-liver-derived mast cells from a 30-day-old culture were identified by immunoelectron microscopy using gold-labelled anti-tryptase antibody. Typically, these mast cells appeared immature as they had large nuclear to cytoplasmic ratio and a small number of ill-formed cytoplasmic granules. For both fetal-liver- and cord-blood-derived mast cells, there was no evidence of conversion of the MC_T type into the MC_{TC} type provided by this study. These results suggest that commitment to develop as an MC_T or MC_{TC} type of mast cell may have occurred in mast cell precursors present in fetal liver and cord blood mononuclear cells, prior to granulation.

INTRODUCTION

Two types of human mast cells have been identified based on their composition of neutral proteases.[1-5] MC_T cells contain tryptase in their secretory granules and are the predominant type of mast cell seen in alveolar walls of the lung and in the small intestinal mucosa. MC_{TC} cells contain tryptase in addition to chymase, a cathepsin G-like proteinase[6] and human mast cell carboxypeptidase.[7] MC_{TC} cells are the predominant type of mast cell seen in the skin and in the submucosa of the small intestine. Ultrastructural differences between MC_T cells and MC_{TC} cells have also been described[8] and characteristic ultrastructural features of the secretory granules have been correlated to the protease composition.[9] A selective depletion of MC_T cells was demonstrated in the small intestinal mucosa of subjects with congenital combined immunodeficiency disorders and with the acquired immunodeficiency disease syndrome, suggesting a role for T lymphocytes in the normal development of MC_T cells but not MC_{TC} cells.[10] Immature mast cells, identified by ultrastructural morphologic criteria of a large nuclear/cytoplasmic ratio and small granules, are also distinguished by their neutral protease compositions and ultrastructural characteristics, like their mature counterparts.[11] This latter finding indicates that, at the time granule formation begins, MC_T and MC_{TC} cells appear to mature along distinct pathways of differentiation.

The development of an *in vitro* system for differentiation of human mast cells from precursors found in cord blood mononuclear cells has provided a powerful tool to examine mast cell lineage.[12] In this system, cord blood mononuclear cells are cocultured with mouse-skin-derived 3T3 fibroblasts. After 7–8 weeks of coculture, mast cells develop, 85% of which are of the MC_{TC} type and display the characteristic gratings and lattice pattern associated with this mast cell type. Addition of recombinant interleukins -1, -2, -3, -4, -5, -6, and granulocyte/macrophage colony-stimulating factor, individually or in combination, did not facilitate the differentiation of human mast cells in suspension cultures of cord blood or bone marrow cells.[12] On the other hand, cultures of cord blood or bone marrow cells in the presence of culture supernatant of phytohaemagglutinin-stimu-

Abbreviations: MC_T cells, tryptase-positive, chymase-negative mast cells; MC_{TC} cells, tryptase-positive, chymase-positive mast cells.

Correspondence: A.-M. Irani, MD, Medical College of Virginia, MCV Station, Box 225, Richmond, VA 23298, U.S.A.

lated T cells resulted in the development of basophils.[13] These results suggest that the growth factors and microenvironment necessary for the development of human mast cells from progenitor cells are different than those required for the development of human basophils.

In the current study, we report on the development of human mast cells *in vitro* from dispersed fetal liver cells, using a similar coculture technique. The phenotype and mediator content of these *in vitro*-derived human mast cells is analysed and a model for differentiation of MC_T and MC_{TC} types of human mast cells is proposed.

MATERIALS AND METHODS

Cell Cultures

Human fetal livers of 18–21 weeks gestational age were obtained at the time of therapeutic abortions. The experimental protocol was reviewed and approved by the Human Studies Committee at Virginia Commonwealth University. Fetal liver cells were dispersed by mechanical mincing of the tissue, resuspended in 40 cc of RPMI-1640 supplemented with 10% fetal calf serum, 10 mM Hepes, 0·1 mM non-essential amino acids, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (50 μg/ml) and amphotericin B (250 ng/ml) (complete RPMI; Sigma Chemical Co., St Louis, MO), and filtered through a no. 80 mesh stainless steel sieve to separate cells from tissue aggregates; the cell suspension was layered over an equal volume of Histopaque density gradient medium and centrifuged at $500 \times g$ for 20 min. Cells recovered at the Histopaque–buffer interface were washed and resuspended at 1×10^6 cells/ml in complete RPMI. Cells were plated into 25 cm^2 flasks (5–10 ml per flask), or on 6-well plates (6 ml per well), with or without a 1-week-old confluent layer of the contact-inhibited murine Swiss 3T3 fibroblast (ATCC, Rockville, MD) and cultured in a Napco 5410 incubator at 37° and in an atmosphere containing 5% CO_2. Half the medium was changed twice weekly.

Umbilical vein cord blood was collected from full-term newborn infants at delivery in heparinized tubes and diluted in 1 vol of phosphate-buffered saline. Mononuclear cells were recovered by differential centrifugation over Histopaque. Cells were plated as above, with or without a confluent layer of mouse 3T3 fibroblast.

In some cocultures, the dispersed fetal liver cells or the cord blood mononuclear cells were placed in a millicell chamber (Millipore, Bedford, MA) with a pore size of 0·4 μm, over 6-well plates seeded with a confluent layer of 3T3 fibroblasts. The pore size allows nutrients and secreted factors to equilibrate between the two cell populations while preventing cell traffic and contact between the two chambers.

Preparation of lymphocyte-conditioned medium

Peripheral blood mononuclear cells were isolated by Histopaque density gradient sedimentation, and resuspended at $0{\cdot}5 \times 10^6$ cell/ml in complete RPMI with 20% FCS. The cells were then cultured for 48 hr in the presence of phytohaemagglutin (10 μg/ml) (Sigma Chemical Co.) at 37° and in 5% CO_2. Culture supernatants were harvested, centrifuged at $400 \times g$ to remove cellular material, and added to complete RPMI at a concentration of 10% (v/v).

Analysis of the cocultures

Cell culture supernatants were recovered at weekly intervals and frozen at −70° for future determinations of mediator content. Adherent and non-adherent cocultured cells were also recovered at weekly intervals by treatment with 0·25% trypsin in 1 mM EDTA for 5 min at 37°. The cells were then washed with PBS; viability was determined by trypan blue exclusion. Cytocentrifuge preparations were obtained with a Shandon cytocentrifuge, using 10^5 cells per slide, and were stored at room termperature for 1–2 weeks until used for immunohistochemistry as described below. An aliquot of 1×10^6 cells was pelleted and frozen at −70° for future determination of mediator contents. Cytocentrifuge preparations and frozen cell pellets were obtained at days 0, 7, 14, and 30.

Immunohistochemistry

Cytocentrifuge preparations were fixed in Carnoy's fluid (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 15 min at room temperature. Immunohistochemistry for detection of MC_T and MC_{TC} cells was performed as previously described.[4] In brief, slides were incubated in methanol with 0·6% hydrogen peroxide for 30 min at room temperature in order to inhibit endogenous peroxidase. The slides were then washed in distilled water and incubated—first, with a biotinylated murine monoclonal anti-chymase antibody (B7-B, 2·6 μg/ml) followed by peroxidase-conjugated streptavidin (1:50 dilution; Sigma Chemical Co.) and 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% hydrogen peroxide. At this stage, MC_{TC} cells were stained a reddish-brown colour by virtue of their content of chymase. The slides were then incubated with alkaline phosphatase-conjugated murine monoclonal anti-tryptase antibody (G3-AP, 6 μg/ml), followed by fast-blue RR in naphthol AS-MX phosphate (0·2 mg/ml in 0·1 M Tris buffer, pH 8·2), which stains MC_T cells blue. Alternatively, cytocentrifuge preparations were incubated with toluidine blue (0·5% in 0·5 N HCl) for 2 hr at room temperature for detection of metachromatic cells. Cytocentrifuge preparations of dispersed human lung mast cells were stained by immunohistochemistry and with toluidine blue in parrallel with the cultured fetal liver cells and served as positive controls.

Mediator assays

Frozen cell pellets were thawed and resuspended at 3×10^6 cells/ml in 0·01 M MES buffer, pH 6·5, 1 M NaCl. Cell extracts were prepared by sonication of the cell pellets at 4° with a Branson Sonifier and microtip at power setting of 3–4, on a 50% pulse cycle, with three periods of sonication, each consisting of 20 pulses. Solubilized material was obtained from the supernatant after centrifugation of the sonicate at $40{,}000 \times g$ for 45 min at 4°.

Histamine determinations were performed with a commercially available radioimmunoassay (AMAC Inc, Westbrook, ME). Tryptase assays were performed by a specific sandwich enzyme-linked immunosorbent assay, utilizing a polyclonal goat anti-tryptase antibody and a murine monoclonal antibody termed G5.[14]

Electron microscopy

In one coculture of fetal liver cells and murine 3T3 fibroblasts, an aliquot of the cells recovered on day 29 was fixed in a modified Karnovsky's solution (1% glutaraldehyde, 3% para-

formaldehyde) in 0·1 M cacodylate buffer, pH 7·2 for 45 min at 4°, washed in 0·1 M cacodylate buffer with 5% sucrose and post-fixed in 1% osmium tetroxide in cacodylate buffer at 4° for 1 hr. The specimen was then dehydrated in graded ethanol, transferred through propylene oxide and embedded in araldite. Thin sections of 60–100 nm were cut on a Sorvall Mt 2 ultramicrotome. Immunogold staining with the G5 murine monoclonal anti-tryptase antibody was performed as previously described,[8] followed by sequential staining with uranyl acetate and lead citrate.

RESULTS

Cultures of fetal liver cells

Fetal livers obtained at 18–21 weeks gestational age weighed around 3 g each, and yielded between 1×10^7 to 3×10^7 dispersed mononuclear cells per gram of tissue. The viability of the dispersed cells, as assessed by trypan blue exclusion was over 95%. Cytocentrifuge preparations of the dispersed fetal liver cells were uniformly negative when stained with toluidine blue, and with anti-tryptase (G3) and anti-chymase (B7) antibodies.

Cultures supplemented with lymphocyte-conditioned media

An earlier study by Seldin *et al.* reported the development of human basophilic cells from dispersed fetal liver cells cultured in the presence of mouse conditioned medium rich in interleukin-3.[15] In order to analyse whether these cells were tryptase-positive or not, cultures were prepared by plating six different dispersed fetal liver cell preparations in 75 cm^2 flasks at 1×10^6 cells/ml in complete RPMI supplemented with 10% lymphocyte-conditioned media. Aliquots of the cell suspensions were obtained at weekly intervals and cytospins were prepared for analysis of staining with toluidine blue and with anti-tryptase antibody. Analysis of mediator content in frozen cell pellets were not performed for these cultures. Half the medium was replaced at weekly intervals with complete RPMI supplemented with 10% lymphocyte-conditioned media. In all six cultures, metachromatic cells developed by the first week and comprised an average of $34\% \pm 25\%$ of total cells by the fourth week of culture. However, tryptase-positive cells were totally absent in three of the cultures and comprised less than 0·5% of total cells in the remaining three cultures. Since all human mast cells have been shown to contain large amounts of tryptase,[16] while human basophils contain very little tryptase and are undetectable by immunohistochemistry using anti-tryptase antibodies,[17] these results indicate that the toluidine-blue-positive cells developing in the presence of lymphocyte-conditioned media were basophils.

Cocultures with murine 3T3 fibroblasts

Cocultures with fibroblasts versus buffer alone

The purpose of our initial experiments was to compare development of mast cells *in vitro* when fetal liver cells were cultured alone *versus* when cocultured with murine 3T3 fibroblasts. Dispersed fetal liver cells were obtained from two specimens. In each case, the resulting cell suspension was divided into two equal parts: half of the cells (approximately 2×10^7 cells in 20 ml of complete RPMI) was plated into a 75 cm^2 flask, and the other half was plated into a 75 cm^2 flask with a 1-week-old confluent layer of murine 3T3 fibroblasts. Aliquots of the non-adherent cells were recovered every 3–4 days and examined for staining with toluidine blue and with anti-tryptase antibody.

After 3 weeks of cultures, substantial percentages of metachromatic cells developed in the two flasks containing fetal liver cells cultured alone (30% and 15%, respectively), while tryptase-positive cells were absent. In contrast, in the two fetal liver cell preparations cocultured with murine 3T3 fibroblasts, less than 1% of the total cells showed metachromasia with toluidine blue while tryptase-positive cells accounted for 3% and 4% of the total cells, respectively. The latter cocultures were maintained for 2 months at which time metachromatic cells still comprised less than 1% of the total cells, but higher numbers of tryptase-positive cells (28% and 16%, respectively) were demonstrated. Because mast cells developed only when fetal liver cells were cocultured with murine 3T3 fibroblasts, all subsequent experiments were performed under the coculture setting.

Direct contact with fibroblasts

Dispersed fetal liver cells were resuspended at $0{\cdot}2\text{–}1 \times 10^6$ cells/ml in complete RPMI and plated onto 4–6 25 cm^2 flasks over confluent layers of murine 3T3 fibroblasts. Mast cell development was monitored by staining cytocentrifuge preparations of the combined adherent and non-adherent cell populations obtained at weekly intervals with anti-tryptase antibody. Table 1 shows the results of six different experiments. Tryptase-positive cells were first visualized at day 7 in three cultures, at day 14 in another two cultures, and at day 30 in one culture. Not shown in the table, toluidine-blue positive cells were absent; thus, under these experimental conditions, tryptase expression is an earlier event in mast cell ontogeny than the development of metachromasia.

Both the percentages as well as the total number of tryptase-positive cells increased over time (Table 1). For individual cultures, the ratio of the mast cell number at each time-point over the total number of cells initially plated varied between 0·02 to 0·33 with a mean of 0·04 at day 7, 0·02 at day 14, and 0·12 at day 30. The total number of viable cells in the cultures decreased over time, suggesting a selective effect on development, maintenance and/or proliferation of mast cells.

Table 1. Time-course of development of tryptase-positive cells (MC) *in vitro* from dispersed fetal liver cells cocultured with mouse 3T3 fibroblasts

Culture number	Mast cells $\times 10^{-3}$ (% mast cells) (day)			
	7	14	30	60
1	560 (4)	284 (4)	704 (9)	
2	0	226·5 (8)	630 (21)	
3	304 (4)	ND	580·5 (14)	
4	0	6·4 (2)	221 (13)	
5	38 (1)	38 (2)	132 (3)	324 (4)
6	0	0	136·5 (4)	150·8 (5)

ND, not done.

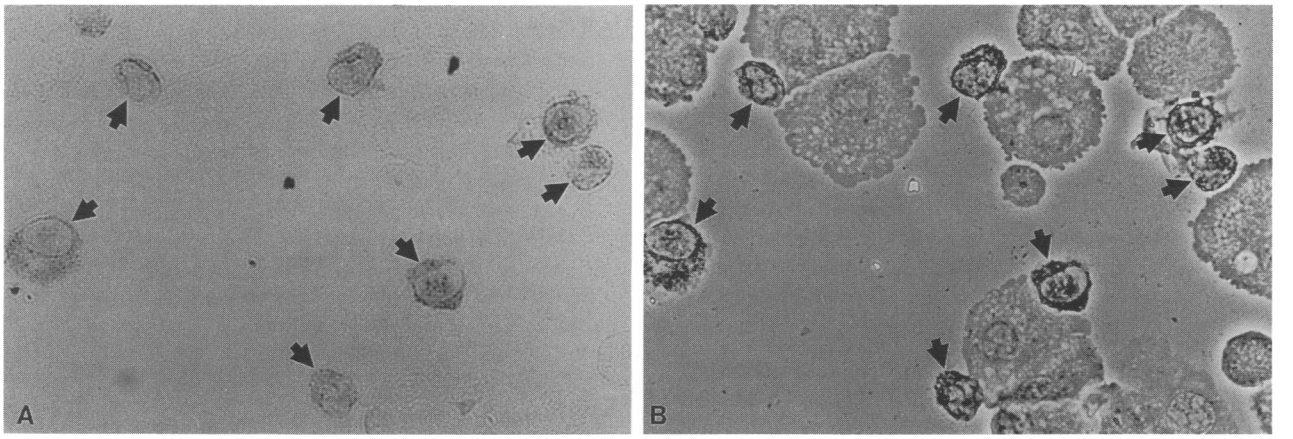

Figure 1. (A) Cytocentrifuge preparations of a 30-day-old coculture of dispersed fetal liver cells and murine 3T3 fibroblasts stained sequentially with anti-chymase and anti-tryptase antibodies. Numerous MC_T cells (arrows) are stained in blue. (B) Phase-contrast microscopy of the same field shown in (A); ×2100.

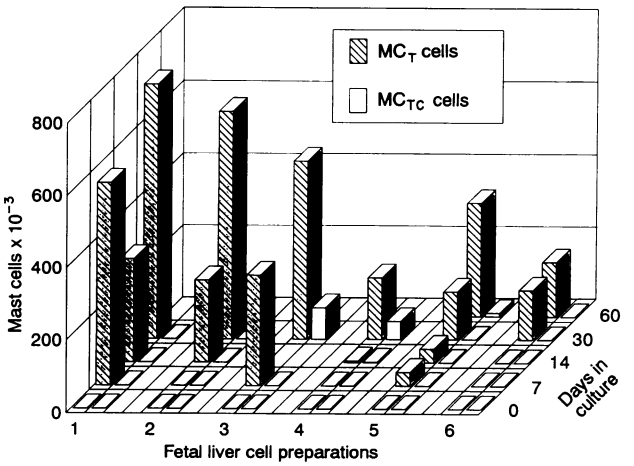

Figure 2. Distribution of MC_T and MC_{TC} cells in six experiments at various periods of coculture of fetal liver cells with murine 3T3 fibroblasts.

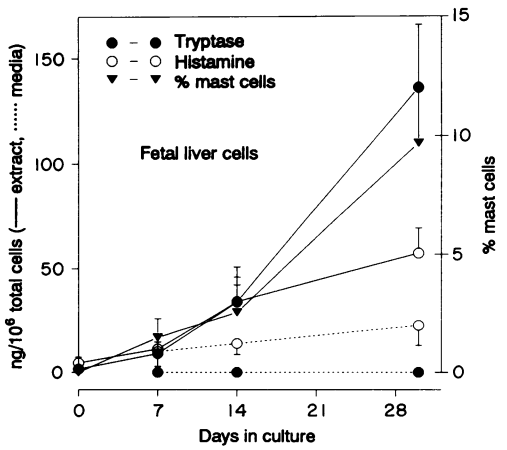

Figure 3. Levels of tryptase and histamine in cell extracts and culture medium of fetal liver cells cocultured with murine 3T3 fibroblasts for various time periods. Data are expressed as mean ±SD. For cell extracts, $n=2$ for day 0, $n=4$ for days 14 and 30. For culture supernatants, $n=5$ at all time-points.

Distribution of MC_T and MC_{TC} cells

Analysis of the mast cell phenotype with respect to neutral protease content was performed in all cultures. MC_T cells were the predominant type of mast cell seen and accounted for 94–100% of all the mast cells in a given culture (Fig. 1), with a mean value of 94% MC_T cells at day 30 of culture. Although MC_{TC} cells appeared later than MC_T cells, the distribution of these two types of human mast cells did not change significantly at the various time-points examined. In two experiments where MC_T cells accounted for 100% of the mast cells developing in the culture by day 30, the distribution of MC_T and MC_{TC} cells was also determined on day 60 of culture and found to be almost unchanged with 100% MC_T cells in one, and 97% MC_T cells in the other. As shown in Fig. 2, the total number of MC_T cells also increased over time, whether or not MC_{TC} cells appeared in the cultures. Occasional toluidine-blue-positive cells were seen on day 60, comprising less than 1% of the total cells.

Mediator content

The amounts of tryptase and histamine measured in the cell extracts and culture supernatants at various time points of the coculture experiments are shown in Fig. 3. As expected, these values increased with increasing numbers of mast cells developing in the cultures. At day 30 of coculture, and assuming that these mediators are found exclusively in the mast cells present in the culture, mast cells contained a mean ±SD of 1·7±0·4 pg of tryptase per cell and of 0·9±0·5 pg of histamine per cell. The supernatant fluid from the cocultures contained low levels of histamine at day 7 (10·4±14·3 ng per 10^6 total cell equivalents) and undetectable levels of tryptase (<1·8 ng/10^6 total cell equivalents). Control culture medium did not contain any detectable histamine. These levels did not change significantly throughout the period of culture.

Electron microscopy

In experiment no. 1, fetal liver cells cocultured with murine 3T3 fibroblasts for 29 days were analysed by electron microscopy (Fig. 4). Approximately 50 mast cells were studied. Mast cells appeared immature, with a large nuclear to cytoplasmic ratio, small granules that were more concentrated at the periphery of

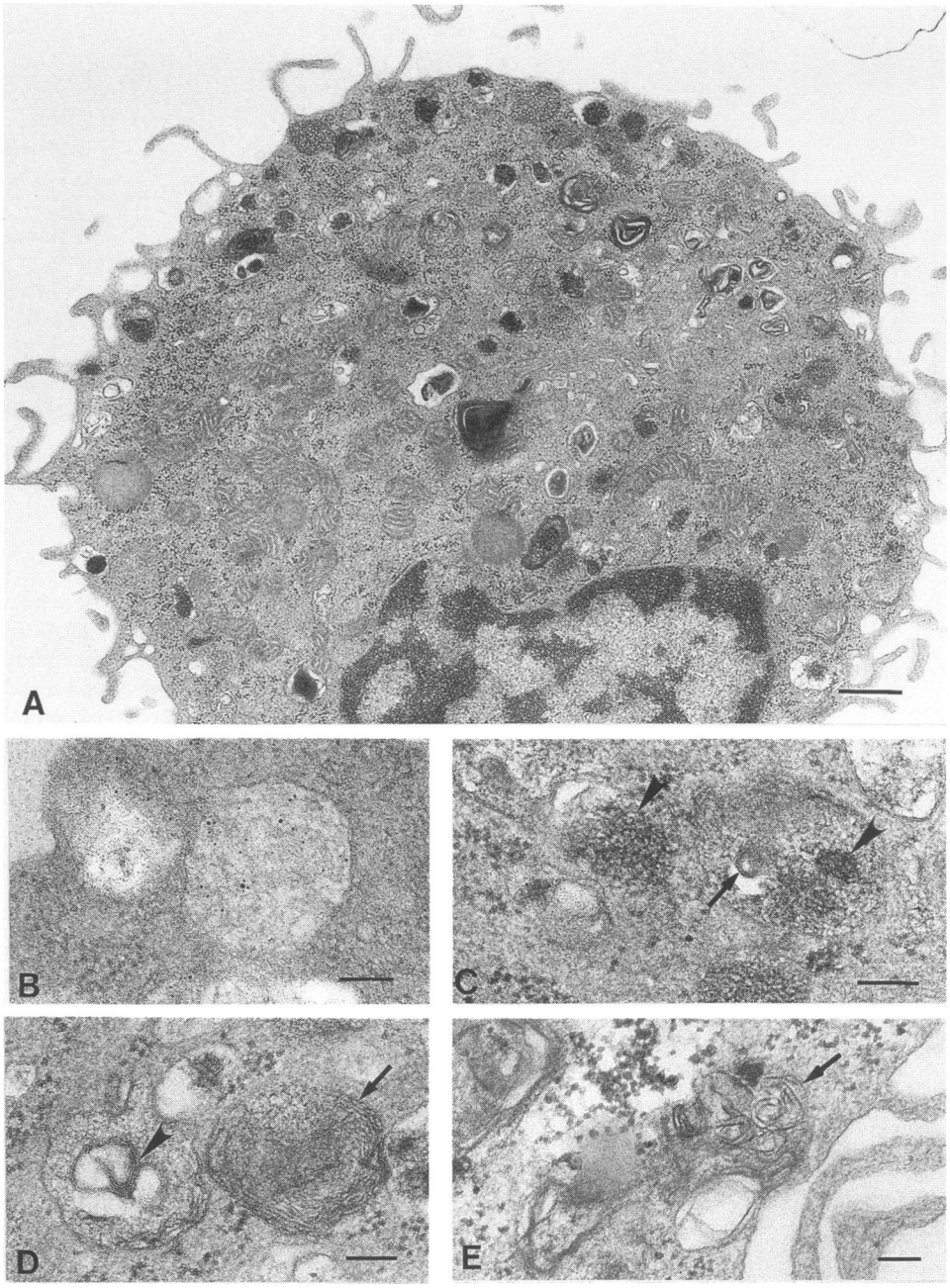

Figure 4. Ultrastructural appearance of mast cells derived *in vitro* from a 30-day-old coculture of fetal liver cells and murine 3T3 fibroblasts. Numerous cytoplasmic surface folds and small granules are seen in (A). (B) Immunogold staining of a mast cell granule with anti-tryptase antibody is shown. Mast cell granules containing a single scroll formation (arrow) and electron-dense particulate material (arrowheads) are seen in (C). (D) Parallel lamellae are seen at the periphery (arrow), and in the matrix (arrowhead) of granules. (E) A granule with an irregular scroll pattern is demonstrated. Bar = 0·5 μm in (A), and 0·1 μm in (B) to (E). (A) × 24,900; (B) × 102,000; (C) × 120,000; (D) × 100,000; (E) × 82,000.

the cell, and numerous narrow surface folds of the cytoplasmic membrane (Fig. 4A). Mast cells were identified by the presence of tryptase in the cytoplasmic granules as assessed by immunogold staining with anti-tryptase antibody (Fig. 4B). In contrast, negligible labelling was seen when an irrelevant murine antibody, MPC 11, was used (data not shown). The granules were polymorphic in appearance containing particulate material (Fig. 4C), lamellar formations (Fig. 4D), and irregular scroll formations (Fig. 4E).

Cultures im millicell chambers

In three experiments, the dispersed fetal liver cells were cultured in millicell chambers over a confluent layer of 3T3 fibroblasts. Tryptase-positive mast cells were detected in all cultures on day

7 and comprised up to 21% of the total cells in one culture by day 30. Toluidine-blue-positive cells were not seen. Of note is that MC_{TC} cells were completely absent from all three cultures.

Cultures of cord blood mononuclear cells with 3T3 fibroblasts

A total of five cultures were analysed. In four of them, direct contact between the cord blood mononuclear cells and the 3T3 fibroblasts occurred. A millicell chamber was used to separate the two different cell populations in the fifth culture. Tryptase-positive mast cells were first detected on day 14 in two cultures and on day 30 in the remaining three. By day 30, mast cells comprised 8% ± 3% of the total cells with a range of 4 to 12% (Table 2), while toluidine-blue-positive cells comprised 2% ± 3% of the total cells (data not shown).

In contrast to mast cells developing from dispersed fetal liver cells, numerous MC_{TC} cells were usually seen in direct cocultures of cord blood mononuclear cells and 3T3 fibroblasts (Table 2). In two such cocultures, MC_{TC} cells were the predominant type seen on day 30, comprising 84% and 57% of total mast cells, respectively. In the other two direct cocultures, MC_{TC} cells accounted for 41% and 9%, respectively, of the total mast cells seen at day 30. Culture no. 3 containing mostly MC_T cells (91%) was maintained and analysed through to day 90. The percentage of MC_T cells did not change significantly (95% at day 60 and 89% at day 90). In culture no. 5 where cord blood mononuclear cells were placed in millicell chambers over confluent layers of murine 3T3 fibroblasts, tryptase-positive mast cells were first seen on day 30 of the coculture and comprised 12% of the total cells. All mast cells were of the MC_T type and remained chymase-negative when maintained in culture for 60 days.

Table 2. Types of mast cell developing *in vitro* from cord blood mononuclear cells cocultured with murine 3T3 fibroblasts for 30 days

Culture number	Mast cell × 10^{-3} (% mast cells)	$\%MC_T/\%MC_{TC}$
1	28 (7)	43/57
2	10·4 (4)	16/84
3	66·24 (5)	91/9
4	327 (10)	59/41
5*	134·56 (12)	100/0

*Cord blood mononuclear cells were placed in millicell chambers over murine 3T3 fibroblasts.

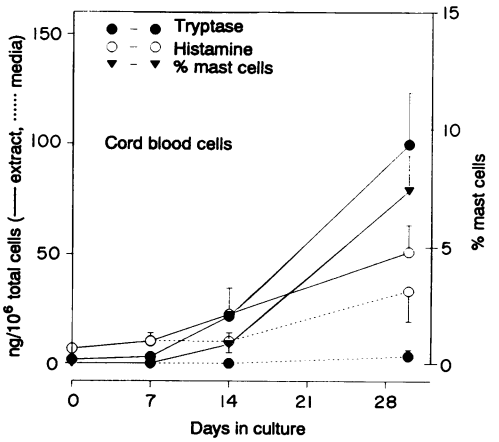

Figure 5. Levels of tryptase and histamine in cell extracts and culture medium of cord blood mononuclear cells cocultured with murine 3T3 fibroblasts for various time-periods. Data represent mean ± SD, $n = 5$ for days 7 and 14, $n = 4$ for day 30.

The amount of tryptase and histamine was measured in cell extracts and culture supernatants of cocultured cells and was comparable with that found in mast cells developing from fetal liver cells (Fig. 5). In 30-day-old cocultures, mast cells contained a mean (± SD) of 1·9 ± 1·2 pg of tryptase per cell and 1·1 ± 1 pg of histamine per cell. Low amounts of histamine (10·4–32·9 ng/10^6 cell equivalents) and no detectable tryptase were found in the coculture supernatants.

DISCUSSION

This study demonstrates that human mast cells can develop *in vitro* from precursor cells found in fetal livers of 18–21 weeks gestational age when cocultured with confluent murine 3T3 fibroblasts. Mast cells do not develop when fetal liver cells are cultured without fibroblasts or in the presence of lymphocyte-conditioned media. In the latter conditions, numerous metachromatic cells develop which are tryptase-negative, suggesting that these cells are basophil leucocytes. This result is consistent with an earlier study that reported the development of human basophilic cells from fetal liver cells cultured in the presence of mouse conditioned medium rich in interleukin-3.[15] In addition, dispersed fetal liver cells cocultured with murine 3T3 fibroblasts have a 5- to 10-fold increase in survival rate (data not shown). These results are similar to those reported by Levi-Schaffer *et al.*, who found improved viability of human lung mast cells cocultured with murine 3T3 fibroblasts.[18] In the current study, mast cells were visualized by immunohistochemistry with an anti-tryptase antibody and were detected as early as the 7th day of culture, prior to the development of metachromasia. Histamine also was detected in cultured cells. Although mast cells have been traditionally identified by their metachromatic staining properties, it is now clear that metachromasia is affected by fixation methods, and is not specific for mast cells since it is also a property of basophil leucocytes. On the other hand, detection of tryptase in cytoplasmic granules has been shown to be specific for mast cells.[16,17] Having detected tryptase in the cultured fetal liver cells by immunohistochemistry, by immunogold staining at the electron microscopy level, and by immunoassay of cell extracts, and having demonstrated the characteristic ultrastructural features of narrow surface folds of the cytoplasmic membrane as well as the presence of cytoplasmic granules with lamellar and scroll formation, we are confident in referring to these cells as mast cells, in spite of the lack of metachromasia. These results suggest that tryptase expression and histamine synthesis occur with or soon after granule formation begins. The presence of tryptase at this stage of mast cell development is consistent with a previous ultrastructural study of immature mast cells *in vivo*.[11] The lack of metachromatic staining in these immature mast cells developing *in vitro* suggests that the proteoglycans presumed responsible for this staining property either have not yet been incorporated into the granules, are present at levels below those needed for the

cells to exhibit metachromasia, or are otherwise masked. Although mast granule proteases are believed to be stored intracellularly tightly bound to proteoglycans, there are known exceptions: Rat skin tryptase, for example, is not bound to heparin,[19] and rat intestinal mast cell protease II is soluble at physiologic ionic strength, suggesting it is not bound to chondroitin sulphate E.[20] Therefore, immunohistochemistry using an anti-tryptase antibody is a more sensitive test for detection of developing mast cells than the traditionally employed metachromatic dyes. In a single experiment, the addition of recombinant human IL-3 fetal liver cells cocultured with murine 3T3 fibroblasts for 4 weeks did not appear to affect either the number of tryptase-positive cells developing in the culture or their lack of metachromasia (data not shown). Also of interest is the observation by A. A. Irani and L. B. Schwartz (unpublished results) that mast cells which develop from fetal liver cells in the presence of recombinant human stem cell factor (Kit ligand) do show metachromasia as well as intense staining for tryptase.

The ratio of mast cell numbers developing in 30-day-old cultures over the total number of fetal liver cells initially plated averaged 0·125 or one mast cell per 8 fetal liver cells placed in culture. Since it is unlikely that such a high proportion of dispersed fetal liver cells normally consists of committed mast cell precursors, these results suggest that proliferation of mast cells or of mast cell precursors may have occurred in the coculture setting. Another possibility is that otherwise uncommitted progenitor cells become committed to the mast cell lineage when cocultured with murine 3T3 fibroblasts. The majority of the mast cells developing *in vitro* from dispersed fetal liver cells cocultured with murine 3T3 fibroblasts were of the MC_T type. In two experiments where the cocultures were maintained for 60 days, the predominance of MC_T cells was confirmed on day 7, when mast cells were first detected, and again at the termination of the cocultures. The apparent lack of conversion of MC_T cells into MC_{TC} cells in the coculture system, although not definitive, is consistent with the hypothesis that MC_T and MC_{TC} cells follow separate differentiation pathways, at least from the time granule formation begins.[21] The development of small numbers of MC_{TC} cells along with MC_T cells in the same coculture is consistent with the presence in fetal liver of two different types of progenitor cells, each already committed to develop as either MC_T or MC_{TC} cells. Alternatively, accessory cells present among the dispersed fetal liver cells may selectively facilitate the development of MC_T cells or suppress development of MC_{TC} cells, from a common progenitor.

An earlier report by Furitsu *et al.* indicated that mast cells developed *in vitro* from cord blood mononuclear cells cocultured with murine 3T3 fibroblasts were mostly of the MC_{TC} type.[12] Accordingly, we initiated cocultures of cord blood mononuclear cells and murine 3T3 fibroblasts to allow comparison of mast cell types developed in the same laboratory from fetal liver to those from cord blood cells. Again, substantial numbers of MC_{TC} cells developed from the cord blood mononuclear cells, ranging from 9% to 84% of all mast cells by day 30. Interestingly, MC_{TC} cells developing *in vitro* were seen at the earliest time that tryptase-positive mast cells were detected and, therefore, did not appear to pass through an MC_T cell stage. Furthermore, in one culture where MC_T cells were the predominant type, the distribution of MC_T and MC_{TC} cells did not change significantly from day 14 to day 90 of culture, indicating that, as MC_T cells mature *in vitro*, they are not converted to the MC_{TC} phenotype. This conclusion, in turn, is consistent with similar observations derived from cultures of fetal liver cells.

In four experiments, contact between fetal liver cells ($n=3$) or cord blood mononuclear cells ($n=1$) and murine 3T3 fibroblasts was prevented by the use of millicell chambers, which allow free flow of nutrients and soluble factors between the two cell populations in coculture. Interestingly, only MC_T cells developed in such cultures. Although the number of experiments is small, these results would suggest that a soluble factor secreted by murine 3T3 fibroblasts participates in the development of mast cells. Whether expression of chymase or development of MC_{TC} cells is influenced by cognate interactions between mast cell progenitors and murine 3T3 fibroblasts must be examined in future experiments.

The increase in histamine levels in cell extracts as the number of tryptase-positive cells increased (Figs 3 and 4) suggests that the histamine is derived from the tryptase-positive cells, although we cannot rule out that other tryptase-negative cells in the cultures also contain histamine. Assuming that all the histamine is found in the tryptase-positive cells, mast cells developed *in vitro* from fetal liver cells and cord blood mononuclear cells contained similar amounts of histamine ($0·9 \pm 0·5$ pg/cell and $1·1 \pm 1$ pg/cell, respectively) and tryptase ($1·7 \pm 0·4$ pg/cell and $1·9 \pm 1·2$ pg/cell, respectively), on day 30 of culture. The tryptase levels in 30-day-old *in vitro*-derived mast cells are significantly lower than those found in mature human lung mast cells (11 ± 7 pg/cell), mature human skin mast cells (35 ± 12 pg/cell) and immature mast cells from newborn foreskin (12 ± 7 pg/cell),[2] probably reflecting an immature stage of mast cell development. In contrast, the histamine levels in the same *in vitro*-derived mast cells are not significantly different from histamine levels found in mature human lung mast cells ($1·5 \pm 0·7$ pg/cell), mature human skin mast cells ($1·9 \pm 0·8$ pg/cell) or immature newborn foreskin mast cells ($1·6 \pm 0·8$ pg/cell),[2] suggesting that, under the experimental conditions used in the current study, synthesis of histamine reaches maximum capacity at very early stages in mast cell development. Interestingly, low levels of histamine were consistently detected in the supernatants from early as well as 30-day-old cocultures, while tryptase levels were undetectable. These results may reflect histamine 'leakage' from immature mast cells as was demonstrated in immature rat mast cells which do not acquire the ability to sequester newly formed histamine until the number of intracellular granules and the supply of sulphated mucopolysaccharide within them increases.[22] Alternatively, the presence of histamine but not tryptase in the culture supernatants may reflect synthesis and release of histamine from cell types other than mast cells, such as basophils, lymphocytes[23] or macrophages.[24]

In one experiment, cells recovered from a 30-day-old coculture of fetal liver cells and murine 3T3 fibroblasts were identified as mast cells by immunoelectron microscopy using gold-labelled anti-tryptase antibody. These mast cells appeared to be immature, with a large nuclear to cytoplasmic ratio and low numbers of small cytoplasmic granules with visible lamellar formation. In addition, irregular scroll formations were observed which were reminiscent of similar structures observed in immature lung mast cells (Craig S., unpublished observations). No granules containing multiple discrete scrolls as seen *in situ* in mature MC_T cells were observed. Further ultrastructural

characterization of *in vitro*-derived mast cells at various periods of culture is in progress.

Recently, the product of the SI gene, which is expressed on the surface of murine 3T3 fibroblasts from the mast cell-deficient SI/SI^d mouse, was shown to be identical to stem cell factor (SCF), a mast cell growth factor and the ligand for a transmembrane tyrosine kinase receptor encoded for by the c-*kit* gene.[25,26] Since the W locus contains the c-*kit* gene,[27] the defect in mast-cell-deficient W/W^v mice seems to be at the growth factor receptor level (Kit), while the defect in the SI/SI^d mouse is at the level of production of the stem cell growth factor (Kit ligand). These findings raise the possibility that the human mast cell growth factor secreted by the Swiss murine 3T3 fibroblasts may be identical to SCF. A recent report by Ishizaka *et al.*,[28] showing *in vitro* development of human mast cells from cord blood mononuclear cells cultured in the presence of recombinant human SCF, supports this hypothesis. Whether SCF is the only factor responsible for the development of human mast cells *in vitro* remains to be clarified.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants A1-20487 and A1-27517, the Small Grant Award Program of Virginia Commonwealth University, and by a grant from the United Scleroderma Foundation, Inc. The authors would like to thank Christopher L. Kepley and Scott Howard for expert technical assistance and Ms Gloria Smith for excellent secretarial assistance.

REFERENCES

1. Irani A.A., Schechter N.M., Craig S.S., Deblois G. & Schwartz L.B. (1986) Two types of human mast cells that have distinct neutral protease compositions. *Proc. Natl. Acad. Sci. USA*, **83,** 4464.
2. Schwartz L.B., Irani A.M.A., Roller K., Castells C. & Schechter N.M. (1987) Quantitation of histamine, tryptase and chymase in dispersed human T and TC mast cells. *J. Immunol.* **138,** 2611.
3. Osman I.A.R., Garrett J.R. & Smith R.E. (1989) Enzyme histochemical discrimination between tryptase and chymase in mast cells of human gut. *J. Histochem. Cytochem.* **37,** 415.
4. Irani A.-M.A., Bradford T.R., Kepley C.L., Schechter N.M. & Schwartz L.B. (1989) Detection of MC$_T$ and MC$_{TC}$ types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies. *J. Histochem. Cytochem.* **37,** 1509.
5. Irani A.A. & Schwartz L.B. (1989) Mast cell heterogeneity. *Clin. Exp. Allergy*, **19,** 143.
6. Schechter N.M., Irani A.-M.A., Sprows J.L., Abernethy J., Wintroub B. & Schwartz L.B. (1990) Identification of a cathepsin G-like proteinase in the MC$_{TC}$ type of human mast cell. *J. Immunol.* **145,** 2652.
7. Irani A.-M.A., Goldstein S.M., Wintroub B.U., Bradford T. & Schwartz L.B. (1991) Human mast cell carboxypeptidase: Selective localization to MC$_{TC}$ cells. *J. Immunol.* **147,** 247.
8. Craig S.S., Schechter N.M. & Schwartz L.B. (1988) Ultrastructural analysis of human T and TC mast cells identified by immunoelectron microscopy. *Lab. Invest.* **58,** 682.
9. Craig S.S. & Schwartz L.B. (1990) Human MC$_{TC}$ type of mast cell granule: the uncommon occurrence of discrete scrolls associated with focal absence of chymase. *Lab. Invest.* **63,** 581.
10. Irani A.M., Craig S.S., Deblois G., Elson C.O., Schechter N.M. & Schwartz L.B. (1987) Deficiency of the tryptase-positive, chymase-negative mast cell type in gastrointestinal mucosa of patients with defective T lymphocyte function. *J. Immunol.* **138,** 4381.
11. Craig S.S., Schechter N.M. & Schwartz L.B. (1989) Ultrastructural analysis of maturing human T and TC mast cells in situ. *Lab. Invest.* **60,** 147.
12. Furitsu T., Saito H., Dvorak A., Schwartz L.B., Irani A.A., Burdick J.F., Ishizaka K. & Ishizaka T. (1989) Development of human mast cells in vitro. *Proc. Natl. Acad. Sci. USA*, **86,** 10039.
13. Ishizaka T., Conrad D.H., Huff T.F., Metcalfe D.D., Stevens R.L. & Lewis R.A. (1985) Unique features of human basophilic granulocytes developed in in vitro culture. *Int. Archs. Allergy Appl. Immunol.* **77,** 137.
14. Wenzel S., Irani A.M., Sanders J.M., Bradford T.R. & Schwartz L.B. (1986) Immunoassay of tryptase from human mast cells. *J. Immunol. Methods*, **86,** 139.
15. Seldin D.C., Caulfield J.P., Hein A., Osathanondh R., Nabel G., Schlossman S.F., Stevens R.L. & Austen K.F. (1986) Biochemical and phenotypic characterization of human basophilic cells derived from dispersed fetal liver with murine T cell factors. *J. Immunol.* **136,** 2222.
16. Schwartz L.B. (1985) Monoclonal antibodies against human mast cell tryptase demonstrate shared antigenic sites on subunits of tryptase and selective localization of the enzyme to mast cells. *J. Immunol.* **134,** 526.
17. Castells M.C., Irani A.M. & Schwartz L.B. (1987) Evaluation of human peripheral blood leukocytes for mast cell tryptase. *J. Immunol.* **138,** 2184.
18. Levi-Schaffer F., Austen K.F., Caulfield J.P., Hein A., Gravellese P.M. & Stevens R.L. (1987) Co-culture of human lung-derived mast cells with mouse 3T3 fibroblasts: morphology and IgE-mediated release of histamine, prostaglandin D2, and leukotrienes. *J. Immunol.* **139,** 494.
19. Braganza V.J. & Simmons W.H. (1991) Tryptase from rat skin: Purification and properties. *Biochemistry*, **30,** 4997.
20. Woodbury R.G. & Neurath H. (1978) Purification of an atypical mast cell protease and its levels in developing rats. *Biochem.* **17,** 4298.
21. Irani, A.A. & Schwartz, L.B. (1990) Neutral proteases as indicators of human mast cell heterogeneity In: *Neutral Proteases of Mast Cells* (ed. L. B. Schwartz) p. 146. Karger, Basel.
22. Mussie E.W., Aiken D. & Beaven M.A. (1986) Evidence for rapid histamine turnover and loss of histamine from immature rat mast cells. *Biochem. Pharmacol.* **35,** 903.
23. Aoi R., Nakashima I., Kitamura Y., Asai H. & Nakano K. (1989) Histamine synthesis by mouse T lymphocytes through induced histidine decarboxylase. *Immunology*, **66,** 219.
24. Okamoto H. & Nakano K. (1990) Regulation of interleukin-1 synthesis by histamine produced by mouse peritoneal macrophages per se. *Immunology*, **69,** 162.
25. Martin F.H., Suggs S.V., Langley K.E., Lu H.S., Ting J., Okino K.H., *et al.* (1990) Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell*, **63,** 203.
26. Huang E., Nocka E., Beir D.R., Chu T.-Y., Buck J., Lahm H.-W., Wellner D., Leder P. & Besmer P. (1990) The hematopoietic growth factor k1 is encoded by the sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell*, **63,** 225.
27. Chabot B., Stephenson D.A., Chapman V.M., Besmer P. & Bernstein A. (1988) The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. *Nature*, **335,** 88.
28. Ishizaka, T., Furitsu, T., Inagaki, N., Tagaya, Y., Mitsui, H., Takei, M. & Zsebo, K.M. (1992) Development of human mast cells *in vitro* In: *Proceedings of the XIV International Congress of Allergology and Clinical Immunology* (eds T. Miyamoto & M. Okuda) Hogrefe and Huber, Toronto. (in press)