

Kinetics of the Appearance of Fc_εRI-Bearing Cells in Interleukin-3-Dependent Mouse Bone Marrow Cultures: Correlation With Histamine Content and Mast Cell Maturation

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While it is known that mast cells arise from pluripotent hematopoietic cells and express their mature phenotypes in tissues, the sequence of events in maturation is incompletely understood. To study early mast cells, we sorted cells from interleukin-3 (IL-3)-dependent mouse bone marrow cultures on the basis of Fc_εRI and examined their morphology, histamine content, and growth characteristics. Flow cytometric analysis and sort showed that the Fc_εRI-bearing (Fc_εRI⁺) cells increased from 0% on day 0 to 90% by day 21 and that the total number of Fc_εRI⁺ cells increased from 0 at the start of culture to 3.75×10^5 cells by day 21 from an initial population of 1×10^5 cells. The dissociation rate of ¹²⁵I-labeled IgE from early cultured cells resembled the dissociation rate of mouse IgE from mature murine mast cells. Mean fluorescence intensity increased over time, reflecting an increase in IgE receptor density. Fc_εRI⁺ cells were also positive for Fc_εRII/III. Morphologic studies showed gradual acquisition of metachromatic

granules in the Fc_εRI⁺ cells, which was paralleled by an increase in histamine content. Sorted Fc_εRI⁺ cells, when placed in liquid suspension culture, gave rise to pure mast cell populations. Fc_εRI⁺ cells sorted at day 3 and cultured in agarose with IL-3 gave rise to 4,800 small and 150 medium-size mast cell colony-forming units per 10^6 cells, while Fc_εRI⁻ cells gave rise to 23 medium-size and 49 large mast cell colony-forming units per 10^6 cells. Fc_εRI⁺ cells grown in granulocyte-macrophage colony-stimulating factor (CSF) or macrophage-CSF did not give rise to colony-forming units. These results show that Fc_εRI⁺ cells have proliferative potential, but that there also is a population of mast cell progenitor cells that have not yet expressed Fc_εRI, and such individual progenitor cells have greater potential for proliferation than cells that express Fc_εRI.

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MAST CELLS ARISE from pluripotent hematopoietic stem cells¹ and reach functional maturity within tissues.² They are characterized by both the presence of high-affinity receptors for IgE (Fc_εRI) on their surface, and granules within their cytoplasm that contain histamine and exhibit metachromasia due to specific granule-associated proteoglycans.³ These mature tissue mast cells, while associated with allergic reactions, also appear to have a normal physiologic role in host defense against parasites,⁴ in connective tissue repair,⁵ and in the regulation of immune responses.⁶ Recently, it has been hypothesized that immature mast cells may also have a biologic function. It has thus, for example, been suggested that an interleukin-4 (IL-4)-producing non-T, non-B cell may be an immature mast cell⁷ that lacks a full complement of metachromatic granules that would allow its identification as a mast cell; however, there is currently only limited information on the characteristics of early mast cells and the evolution of such cells into mature mast cells.

Recently, we have reported that cells bearing Fc_εRI appear by 7 days in IL-3-dependent mouse bone marrow

(BM) cultures known to give rise to 90% to 99% mast cells by 3 weeks, with only approximately 25% of these Fc_εRI-positive cells at 7 days containing granules typical of mast cells.⁸ This observation suggested to us that a detailed study of Fc_εRI-positive cells evolving in IL-3-dependent mouse BM cultures would show that such cells, early in culture, are destined to mature into mast cells and, further, would contribute to our understanding of the evolution of mast cell progenitors into mature mast cells. To better understand the characteristics of early mast cells and follow their maturation, we thus determined the kinetics of the appearance of Fc_εRI-bearing cells, isolated the cells by use of fluorescence-activated cell sorter (FACS), and examined the morphologic characteristics, histamine content, and growth potential of both Fc_εRI-positive (Fc_εRI⁺) and Fc_εRI-negative (Fc_εRI⁻) cells from 0 to 21 days. As will be shown, mast cells progress through a developmental stage in which they exhibit high-affinity IgE receptors as well as Fc_εRII and/or Fc_εRIII (Fc_εRII/III),⁹ but are not recognized as mast cells by techniques that rely on the demonstration of cytoplasmic granules.

MATERIALS AND METHODS

Materials. The following were purchased: RPMI 1640, HEPES, Hanks' balanced salt solution (HBSS; Bio-fluids, Inc, Rockville, MD), penicillin/streptomycin, nonessential amino acids, L-glutamine, sodium pyruvate (Flow Laboratories, Inc, McLean, VA), fetal calf serum (FCS; HyClone Laboratories, Logan, UT), WEHI-3-conditioned medium as a source for IL-3 (Collaborative Research, Inc, Bedford, MA), recombinant mouse granulocyte-macrophage colony-stimulating factor (rGM-CSF), recombinant macrophage colony-stimulating factor (rM-CSF; Genzyme Corporation, Boston, MA), tissue culture flasks (Corning Inc, Corning, NY), bovine serum albumin (BSA), 2-mercaptoethanol, toluidine Blue-O (Sigma Chemical Co, St Louis, MO), fluorescein-5-isothiocyanate (FITC; Molecular Probes, Inc, Eugene, OR), Sephadex G-25M PD-10 columns (Pharmacia, Uppsala, Sweden), Wright-Giemsa stain (Port City Diagnostics, Inc, Wilmington, NC), biotin-conjugated rat antimouse monoclonal antibody (MoAb) (2.4G2)

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directed against Fc_γRII and Fc_γRIII (Fc_γRII/III),⁹ R-phycoerythrin-conjugated rat IgG_{2b},¹⁰ biotin conjugated rat IgG_{1a}, streptavidin-phycoerythrin conjugate (Pharmingen, San Diego, CA), ³H-thymidine, and ³H-uridine (DuPont, Boston, MA). The PT18 mast cell line was maintained in WEHI-3-supplemented conditioned media. Purified mouse IL-3 (30,000 U/mL stock, where a unit is defined as the amount of IL-3 in 1 mL that gives 50% maximal tritiated thymidine incorporation into factor-dependent cells)¹⁰ was kindly provided by Dr James Ihle (St Jude's Children Research Hospital, Memphis, TN). All other materials were reagent grade and were obtained commercially.

Mouse BM cultures. Six- to 10-week-old BALB/c mice were killed by CO₂ asphyxiation and BM was aseptically flushed from femurs and tibiae into RPMI 1640 medium containing 4 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.1 mmol/L nonessential amino acids (complete RPMI). The cell suspension was washed twice in complete RPMI by centrifugation at 400g for 10 minutes and finally resuspended in complete RPMI containing 10% vol/vol FCS and 10% vol/vol WEHI-3-conditioned medium and placed in 75-cm² or 175-cm² tissue culture flasks at a density of 2×10^5 cells/mL. Flasks were then incubated at 37°C in a 5% CO₂ humidified atmosphere. In stated experiments, purified mouse IL-3 or rGM-CSF was added to the cell suspension in place of the WEHI supernatant at a final concentration of 30 U/mL or 100 U/mL, respectively. Cells were centrifuged and resuspended in fresh media every 7 days to achieve a final concentration of 2×10^5 cells/mL.

FITC conjugation of IgE. Mouse monoclonal IgE was isolated from ascitic fluid of CAF₁ mice.¹¹ FITC conjugation of IgE was performed as described.¹² Briefly, 250 µL of IgE (5.9 mg/mL), 55 µL of 500 mmol/L Na₂CO₃, pH 8.9, and 160 µL of FITC were slowly mixed overnight at 4°C. The mixture was chromatographed over a PD-10 column with phosphate-buffered saline (PBS) as the running buffer and, after discarding the void volume, a single fraction of 1.3 mL was collected. The protein concentration and the fluorochrome-to-protein ratio of the conjugates were calculated on the basis of their optical density at 280 nm and 495 nm. After conjugation, the protein concentration of IgE was 0.9 mg/mL (total volume, 1.3 mL), and the ratio of moles of FITC per mole of IgE was 3.

Fluorescent ligand binding. Fluorescent staining of IgE receptors was performed using a modification of a previously described procedure.¹³ Cultured cells were washed twice by centrifugation at 800g for 10 minutes and resuspended in HBSS without calcium, magnesium, or phenol red, containing 0.2% BSA at a concentration of 10^7 cells/mL. Cells were then incubated with FITC-conjugated IgE at a concentration of 10 µg/mL for 45 minutes at 4°C. To eliminate IgE binding to low-affinity receptors,^{14,15} cells were washed three to four times in 40 vol of the above medium by centrifugation at 800g for 10 minutes and finally resuspended in HBSS with 0.2% BSA at a concentration of 5×10^6 cells/mL. Cells were analyzed and sorted by flow cytometry. Nonspecific fluorescence was assessed by preincubating an aliquot of the cells with 100 µg/mL of purified monoclonal IgE before adding the FITC-conjugated IgE. In stated experiments, instead of FITC-conjugated IgE, cells were incubated in the same conditions with 1 µg/mL of biotin-conjugated 2.4G2 MoAb (anti-Fc_γRII/III)⁹ and counterstained with streptavidin phycoerythrin conjugate. Isotype-specific antibody or preincubation with a 100-fold concentration of the nonconjugated antibody was used as a control.

Flow cytometry and cell sorting. Cell analysis and sorting was performed on an Epics 753 (Coulter Electronics Inc, Hialeah, FL) with an Argon laser (Coherent Inc, Palo Alto, CA) tuned to 488

nm, wavelength 150 mW. A 525/25 bandpass optical filter was used to screen the FITC emission. Multiparameter data acquisition display systems (MDADS) software (Coulter) with a three-decade logarithmic amplifier was used to collect the data. APR software (CSL, NIH) was used for statistical analysis and data displays. Cells were kept at 4°C during sorting with the instrument's cooling system. Sorted cells were suspended in complete RPMI.

Binding assays. Mouse IgE was iodinated by the chloramine-T method.¹⁶ Dissociation of receptor-bound IgE from cultured murine BM cells or the PT18 mast cell line was measured as described.¹⁷ In brief, cells were incubated with ¹²⁵I-labeled mouse IgE (5 µg/mL) for 20 minutes at 22°C, and nonlabeled IgE was then added at a 100-fold excess. At various times, cells were pelleted through phthalate oil¹⁸ in a Microfuge (Beckman Instruments, Inc, Fullerton, CA) and the cell-bound IgE was assayed by counting the radioactivity with a gamma counter (Auto Gamma 5000 series; Packard Instrument Co, Meriden, CT). Nonspecific binding of IgE, estimated by the addition of 100-fold excess of unlabeled mouse IgE to the cells before addition of the labeled IgE, was subtracted from the total binding. Fractional occupancy was determined from the log of the cell-bound IgE molecules. To translate the counts bound per cell into molecules of ¹²⁵I-IgE bound per cell, the measured specific activity of IgE (cpm/mg) and an assumed molecular weight of 2×10^5 were used. To determine specific activity for the preparation, protein concentrations were calculated from the absorbancy at 280 nm.

Histochemical stains. At designated times, aliquots of cultured cells were spun onto glass slides in a cytocentrifuge (Shandon Southern Instruments Inc, Sewickley, PA), allowed to air dry, and stained with Wright-Giemsa using an Ames Hematek automated slide stainer (Miles Laboratories Inc, Elkhart, IN). Acid toluidine blue (pH 1.0), which stains all mast cells, was performed as described on slides fixed in Mota's lead acetate.¹⁹ Slides were examined at high magnification ($\times 1,250$) under oil.

Histamine assays. To determine cell-associated histamine, aliquots of cells were washed and resuspended in PBS at a concentration of 5×10^4 cells/mL and placed in a water bath at 100°C for 10 minutes.²⁰ Samples were then analyzed for histamine content using an immunoassay that uses MoAbs raised against acylated histamine.²¹ Histamine in the sample was acylated using succinyl glycineamide *N*-hydroxysuccinamide ester, which was supplied with the antibody in a kit (Amac Inc, Westbrook, ME).

Colony-forming assays. Mouse BM colony-forming units were determined in a semi-solid phase agarose system in which a feeder layer of agarose is overlaid with a cell layer in six-well plates.⁸ The feeder layer in each well was composed of 1.25 mL of complete RPMI 1640 medium, 0.375 mL 2X concentrated RPMI 1640, 0.5 mL FCS, and 0.375 mL of a 3.3% (wt/vol) stock agarose solution in distilled water. IL-3, GM-CSF, or M-CSF were added to complete RPMI 1640 to give a final concentration of 30 U/mL, 100 U/mL, and 40 U/mL, respectively. The agarose was allowed to equilibrate at 37°C in 5% CO₂ for 1 hour before use. The cell layer in each well was composed of 0.36 mL of complete RPMI 1640, 0.18 mL of FCS, and 0.18 mL of a 0.9% (wt/vol) stock agarose solution in distilled water and 0.1 mL of complete RPMI 1640 containing 2.5×10^3 BM cells. Colonies were counted after 14 days. Individual colonies were transferred to glass slides, stained with acid toluidine blue-O, and examined.

Thymidine and uridine incorporation assays. The mouse BM thymidine and uridine incorporation assays have been described.²² Briefly, 5×10^5 sorted cells per well in 0.2 mL in a 96-well plate were pulsed with ³H-thymidine or ³H-uridine. The cells were pulsed for 8 hours (1 µCi per well), harvested on glass fiber filters using an automated cell harvester (PHD cell harvester; Cambridge

Technology, Inc, Watertown, MA), and assayed for radioactivity by liquid scintillation counting (Packard 2200 CA; Packard Instrument Co, Downers Grove, IL).

RESULTS

Expression of $Fc\epsilon RI$ on cells in BM cultures. We have previously examined IL-3-dependent cultures of mouse BM cells and reported that $Fc\epsilon RI$ -bearing cells can be identified at 7 days.⁸ To extend this observation, we examined IL-3-dependent BM cultures for the appearance of $Fc\epsilon RI$ -bearing cells from day 0 through day 21. The number of cells staining with FITC-conjugated IgE and relative fluorescence as an indication of $Fc\epsilon RI$ number per cell were assessed at each time point by FACS analysis. As can be seen in Fig 1, $Fc\epsilon RI$ -bearing cells could be identified in IL-3-dependent BM cultures as early as 3 days. The relative percentage of $Fc\epsilon RI$ -bearing cells progressively increased in culture through day 21, when greater than 90% of cells were positive. The fluorescence could be completely blocked by preincubation of the cells with unlabeled IgE. Washing the cells after incubation with FITC-conjugated IgE four times or incubating them with mouse IgE for 1 hour to eliminate

IgE binding to low-affinity receptors¹⁵ showed no change in fluorescence intensity.

To determine if $Fc\epsilon RI$ -bearing cells also expressed $Fc\epsilon RII/III$, we used two-color analysis, looking simultaneously at $Fc\epsilon RI$ and at $Fc\epsilon RII/III$ using FITC-conjugated IgE and biotin-conjugated 2.4G2 MoAbs⁹ counterstained with streptavidin-phycoerythrin. Rat IgG_{2b}-phycoerythrin served as control for nonspecific binding of 2.4G2 MoAbs. The results showed that all $Fc\epsilon RI^+$ cells were also positive for $Fc\epsilon RII/III$ (25% of cells at day 7). Of the remaining cells, 69% were $Fc\epsilon RI^+$, $Fc\epsilon RII/III^+$; 5% were $Fc\epsilon RI^+$, $Fc\epsilon RII/III^-$; and 1% were $Fc\epsilon RI^+$, $Fc\epsilon RII/III^-$. In all, virtually all BM cells cultured for 7 days in IL-3 were $Fc\epsilon RII/III^+$, while only 40% were positive at the beginning of the culture. Incubation of cells with a 100-fold concentration of 2.4G2 antibodies before incubation with FITC-conjugated IgE did not effect the binding of FITC-conjugated IgE to the cells, eliminating the possibility that FITC-conjugated IgE bound to cells via $Fc\epsilon RII/III$ receptors. Preincubation of the cells with unrelated Ig did not effect the percent of $Fc\epsilon RI$ -bearing cells detected by FACS analysis.

It has previously been reported that mouse BM cells cultured in GM-CSF, with or without IL-3, do not give rise to mast cells.²³ To determine if GM-CSF would, however, permit the growth of $Fc\epsilon RI$ -bearing cells without mast cell granules, we next analyzed GM-CSF-driven cultures for this receptor. As can be seen in Fig 2, no $Fc\epsilon RI$ -bearing cells were seen in cultures containing GM-CSF. Cell differentials performed on mouse BM cells placed for 10 days in media supplemented with GM-CSF confirmed that these cultures consisted of 20% blasts, 19% promyelocytes, 30% myelomonocytes, 4% bands, 15% segmented neutrophils, 9% eosinophils, and 3% monocytes/macrophages, in agreement with previous reports.²⁴ When placed in IL-3-supplemented media for the same period of time, the BM cells gave rise to 2% blasts, 2% metamyelocytes, 5% bands, 40% segmented neutrophils, 5% basophils, 2% eosinophils, 37% mast cells, 2% lymphocytes, and 5% large mononuclear cells. By day 21, differential counts of cells cultured in GM-CSF have shown 4% blasts, 8% promyelocytes, 68% myelomonocytes, 6% bands, 11% eosinophils, and 3% monocytes/macrophages. Cells cultured for 21 days in the presence of IL-3, in contrast, were made up of 99% mast cells and 1% of small mononuclear cells.

The total average number of cells in culture and of $Fc\epsilon RI$ -bearing cells as determined by repeated FACS analyses is shown in Fig 3. The total number of $Fc\epsilon RI$ -bearing cells increased from an average of 1×10^3 cells on day 3 to an average of 3.8×10^5 cells on day 21 from an initial total BM population of 1×10^5 cells. Cells cultured in the presence of purified IL-3 at 30 U/mL instead of WEHI supernatant showed identical results over 3 weeks.

Measurement of fluorescence intensity provides semi-quantitative data on the number of molecules expressed by cell populations that are reactive with the fluorescent reagent. Mean fluorescence intensity (MFI) thus reflects the relative number of IgE receptor molecules bound per cell and can be used to estimate the relative number of receptors per cell. To allow appropriate comparison of

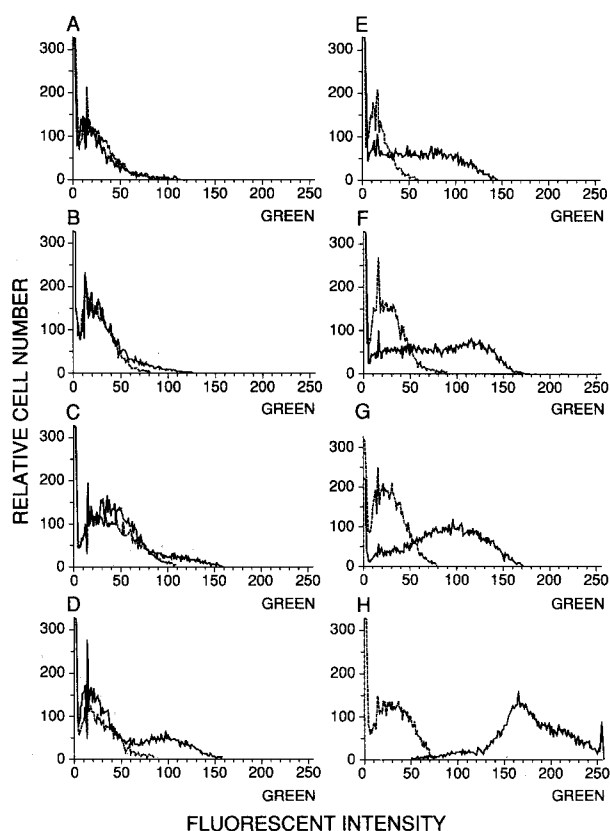


Fig 1. FACS analysis of IL-3-dependent mouse BM cultures. Mouse BM was cultured for 0 (A), 3 (B), 5 (C), 7 (D), 10 (E), 12 (F), 16 (G), and 21 (H) days in the presence of WEHI supernatant as a source for IL-3. The number of cells staining with FITC-conjugated IgE at each time point was examined using flow cytometry (—). As a control, cells were also pretreated with monoclonal IgE for 1 hour before the addition of FITC-conjugated IgE (----). GREEN stands for FITC fluorescence. Data shown are representative of three to five experiments.

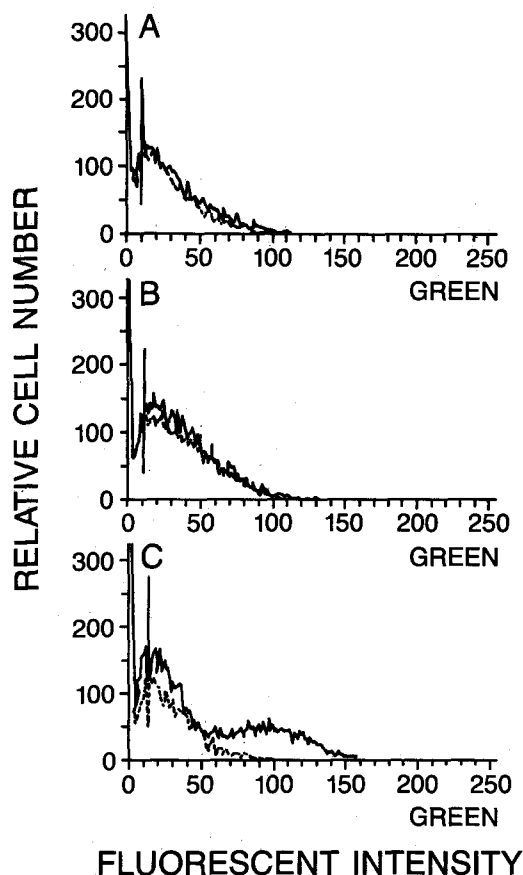


Fig 2. FACS analysis of mouse BM cells cultured for 7 days in the presence of GM-CSF alone (A), GM-CSF with IL-3 (B), or in the presence of IL-3 alone (C). The number of cells staining with FITC-conjugated IgE was examined using flow cytometry (—). As a control, cells were also pretreated with monoclonal IgE for 1 hour before the addition of FITC-conjugated IgE (----). GREEN stands for FITC fluorescence. Data shown are representative of four experiments.

fluorescent intensity between different experiments and time points, we have used an MFI ratio. The MFI ratio is determined by calculating the ratio of the MFI of FITC-conjugated IgE-stained cells to their corresponding total MFI of nonstained cells in each experiment. As can be seen in Table 1, the ratio of stained cells relative to the corresponding unstained cell population increased over 21 days from 1.20 to 3.59. The data suggest that not only does the number of Fc ϵ RI-bearing cells increase over time, but that the number of Fc ϵ RI per cell also increases.

Characterization of IgE receptors. While it has been generally assumed that FACS analysis using FITC-conjugated IgE demonstrates the presence of IgE receptors on mast cells arising in IL-3-dependent cultures, to date there has been no demonstration of high-affinity receptors in IL-3-dependent BM cultures at 7 days by binding assays. To verify that high-affinity IgE receptors exist in primary IL-3-dependent BM cultures, binding assays were performed on the total cell population at both day 7 and day 28, and the results compared with those obtained using the mouse PT18 cell line. As can be seen in Fig 4, binding assays showed that log IgE dissociation from cells cultured for 7

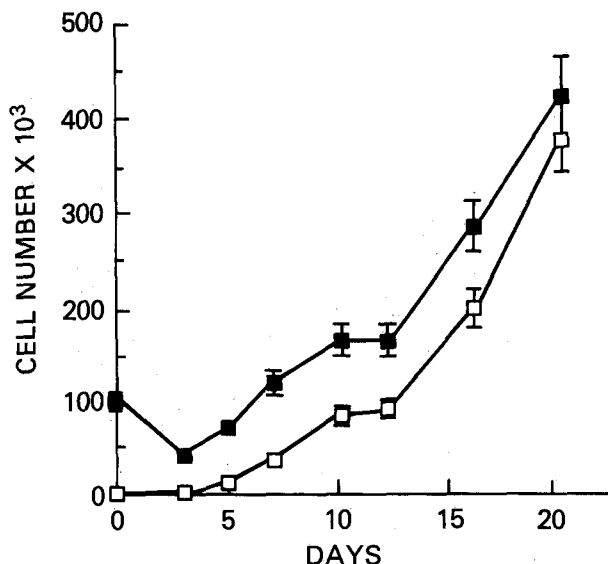


Fig 3. (■) Total cell number and (□) number of Fc ϵ RI $^{+}$ cells in IL-3-dependent BM cultures from day 0 to day 21. Each data point is the mean ($\pm$ SEM) of three to five separate experiments.

days was linear, with 76% fractional occupancy after 4 hours, similar to the rate for mature murine BM cells or PT18 mast cells. The dissociation rate of the cultured BM cells was similar and considerably slower than the dissociation rate for the low-affinity IgE receptor.^{14,15} Calculation of receptor number showed an increase in Fc ϵ RI from 3.3×10^3 per Fc ϵ RI-bearing cell on day 7 to 5.0×10^3 on day 28, consistent with the increase in MFI. Because the conditions of equilibrium in binding assays are different than the conditions in FACS analysis, the results of the two studies, although in direct relationship, are not identical. The number of receptors for PT18 cells was 2.8×10^4 per cell.

Enrichment of Fc ϵ RI-bearing cells. Cells were next sorted on the basis of FITC-conjugated IgE to yield a high purity of Fc ϵ RI-bearing cells (over 95% as estimated by FACS analysis post-sort). The sort was gated to include only live cells as assessed by propidium iodide staining and by light scatter fluorescence. In addition, forward light scatter (FLS), reflecting cell size, and perpendicular light scatter (PLS), reflecting internal complexity of the cells, were also

Table 1. MFI

Day	Control	FITC-IgE Bound Cells	MFI Ratio
3	32.59 $\pm$ 2.00	39.08 $\pm$ 2.23	1.20
5	36.78 $\pm$ 2.45	47.61 $\pm$ 2.45	1.29
7	34.71 $\pm$ 2.45	61.11 $\pm$ 2.64	1.76
10	22.33 $\pm$ 2.43	64.03 $\pm$ 2.55	2.86
12	31.76 $\pm$ 2.44	81.92 $\pm$ 2.61	2.58
16	31.22 $\pm$ 2.46	88.60 $\pm$ 2.53	2.83
21	27.41 $\pm$ 2.30	98.52 $\pm$ 2.29	3.59

MFI in IL-3-dependent mouse BM cells labeled with FITC-conjugated IgE as analyzed by flow cytometry is a reflection of the number of IgE molecules bound per cell. The MFI ratio is determined by dividing the MFI of the labeled cells by the corresponding MFI of the unlabeled cells and corrects for variation in individual experiments. Each data point represents the mean of three to five experiments ($\pm$ SEM).

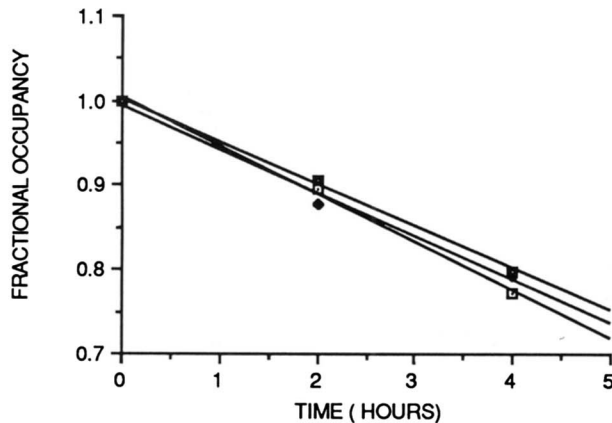


Fig 4. Dissociation kinetics for the interaction of IgE with mouse BM cells cultured for (□) 7 days or (◆) 28 days, and with (■) PT18 mast cell line. The lines drawn through the data points represent a first-order regression analysis for each of the cell populations.

assessed at each time point. The number of Fc ϵ RI-bearing cells sorted over time and the cell type in both sorted positive and sorted negative cells is shown in Fig 5. As can be seen, the number of Fc ϵ RI $^{+}$ cells increased from an average of 1.6×10^3 (4% of total) on day 3 to an average of 3.8×10^5 (90% of total) on day 21, consistent with FACS analysis (Fig 1). Mast cells, as identified through the presence of acid toluidine blue-positive granules, made up only 14% of the Fc ϵ RI $^{+}$ cells at day 3, but by day 7 made up 68% of the sorted cells, and, by day 16, 97% of the sorted cells. Figure 5 represents differential counts on the various days. Included in Fig 5 are only cell type groups that comprise greater than 5% of the population on each day. In a further breakdown of the groups, mast cells included less than 6% basophils, neutrophils included less than 5% immature basophils and eosinophils, large mononuclear cells comprising monocyte/macrophage-like cells with less than 5% myelomonocytic cells. Small mononuclear cells consisted of lymphocyte-like cells. At days 3 and 5, the majority of the cells without granules had the appearance of small to large mononuclear cells (Fig 6). Interestingly, the increase in acid toluidine blue-positive mast cells within the Fc ϵ RI $^{+}$ cell population initially increased as the number of small mononuclear cells decreased within the Fc ϵ RI $^{-}$ cells.

Light scatter of the Fc ϵ RI $^{+}$ cells increased over time, indicating an increase in cell size. Changes in PLS showed a similar but slower trend, indicating that the internal complexity of the Fc ϵ RI-bearing cells is closer in early stages to that of the Fc ϵ RI $^{-}$ cells rather than to the internal complexity of fully matured cultured mast cells (Fig 7).

It has been reported that IL-3 induces histamine synthesis in BM cultures.^{25,26} To determine if this histamine synthesis is associated with Fc ϵ RI-bearing cells, we next determined the amount of histamine in Fc ϵ RI $^{+}$ and Fc ϵ RI $^{-}$ populations. As might be expected, the majority of histamine was found in the Fc ϵ RI $^{+}$ populations (Fig 8). We next calculated the amount of histamine per cell in the Fc ϵ RI $^{+}$ population. As can be seen in the insert of Fig 8, the amount of histamine per Fc ϵ RI $^{+}$ cell increased eightfold from day 3 to day 16.

Fate of Fc ϵ RI-bearing cells. To determine if Fc ϵ RI-bearing cells at days 3, 5, and 7 would develop into pure populations of mast cells, Fc ϵ RI $^{+}$ cells were placed in IL-3 and examined after an additional 7 days in culture. Cell number in these cultures increased fourfold to fivefold during this 7-day period, after which the total cell population consisted of greater than 95% mast cells. When Fc ϵ RI $^{-}$ cells were cultured in IL-3, the percent of cells with acid toluidine blue granules also increased, while the total cell number decreased.

Using an agarose semisolid system, we have determined the number of small and large murine mast cell colony-forming units in Fc ϵ RI $^{+}$ and Fc ϵ RI $^{-}$ cell populations. Day 3 Fc ϵ RI $^{+}$ cells, when placed in agarose supplemented with IL-3 and cultured for an additional 14 days, gave rise to $4,800 \pm 30$ (N = 4) small colonies (< 5 cells per colony) and single cells and to 150 ± 20 (N = 4) medium-size colonies

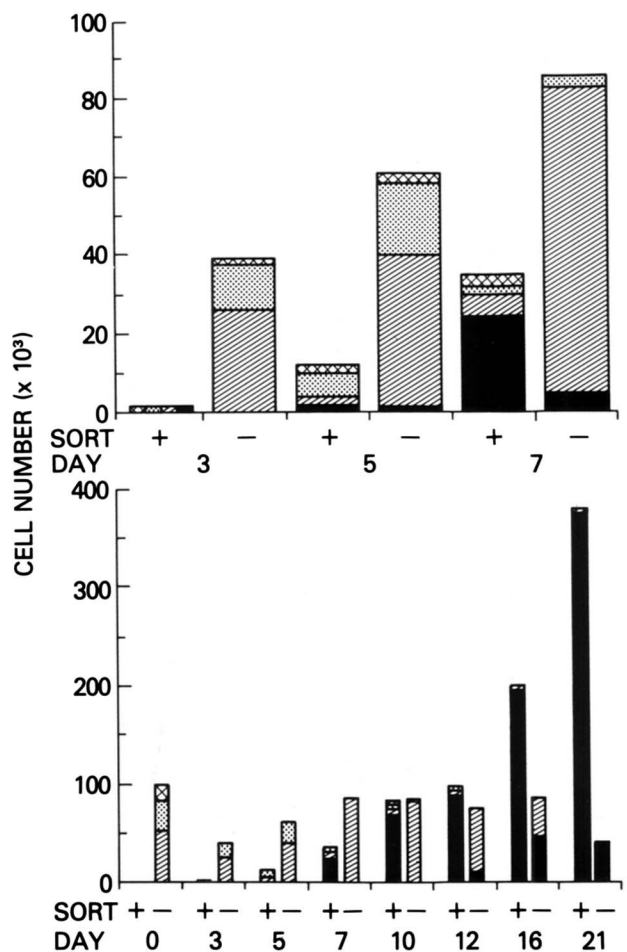


Fig 5. Cell type in Fc ϵ RI $^{+}$ and Fc ϵ RI $^{-}$ mouse BM cells in the presence of WEHI supernatant. Upper panel is an enlargement of the data for days 3, 5, and 7 from the lower panel and shows the number of (▨) large mononuclear cells (□) small mononuclear cells, (▤) polymorphonuclear cells, (■) and mast cells seen in Fc ϵ RI $^{+}$ (+) and Fc ϵ RI $^{-}$ (−) sorted cells. The lower panel shows all data from day 3 to day 21. Data presented are the average of three to five experiments. No experiment differed from another experiment by more than 10% of the mean of all experiments.

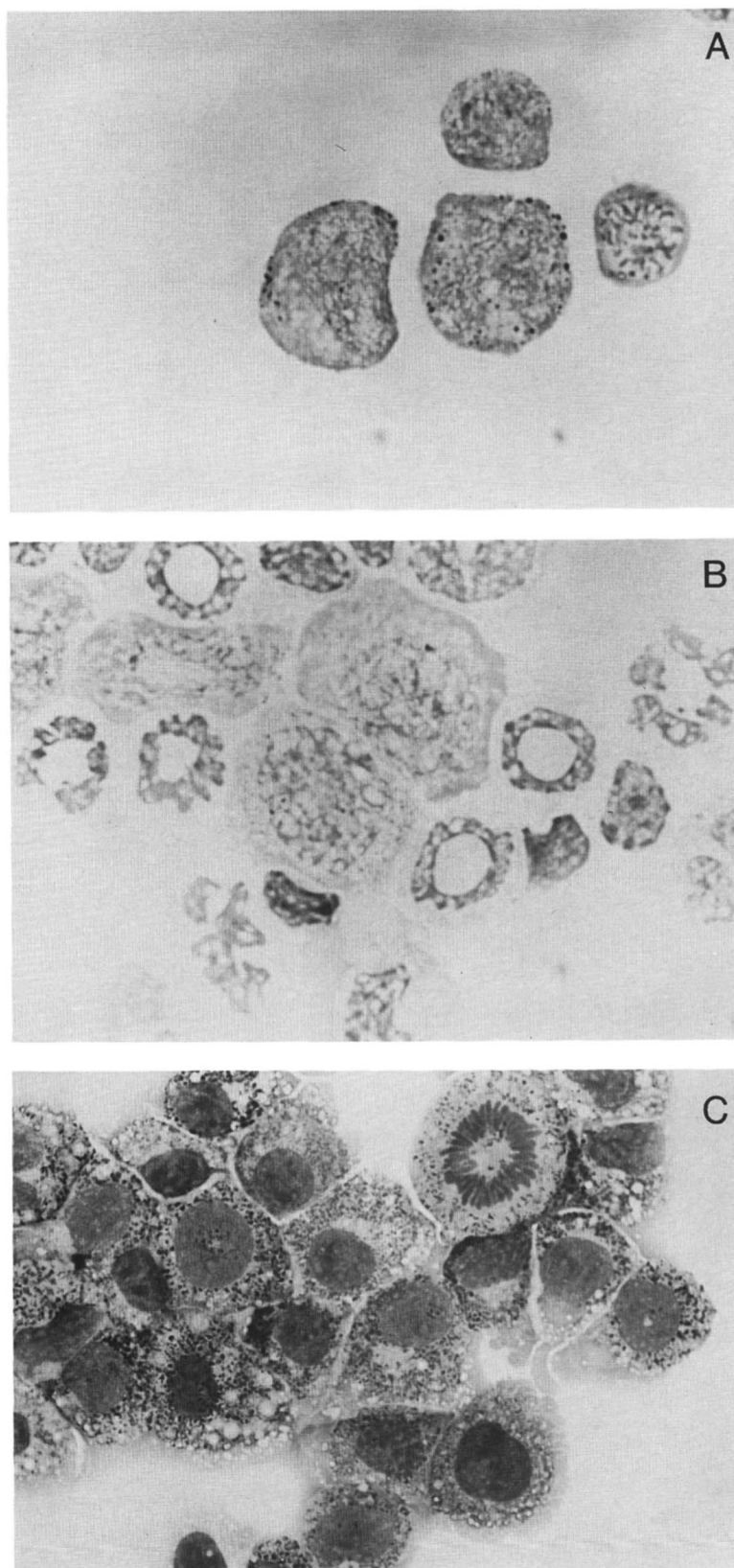


Fig 6. Comparison of $Fc\epsilon RI^+$ cells with $Fc\epsilon RI^-$ cells in early mast cell cultures. (A) BM cells cultured in the presence of IL-3 for 5 days and sorted positive on basis of $Fc\epsilon RI$. Only a few sparsely granulated mononuclear cells are seen. (B) Day 5 $Fc\epsilon RI^-$ cells, which consist primarily of polymorphonuclear cells and mononuclear cells. (C) $Fc\epsilon RI$ -bearing cells sorted at day 5 and cultured in liquid suspension for an additional 7 days in the presence of IL-3. Virtually all cells have numerous granules and may be identified as mast cells. In every case, the cell preparation was fixed in Mota's fixative and stained with acid toluidine blue.

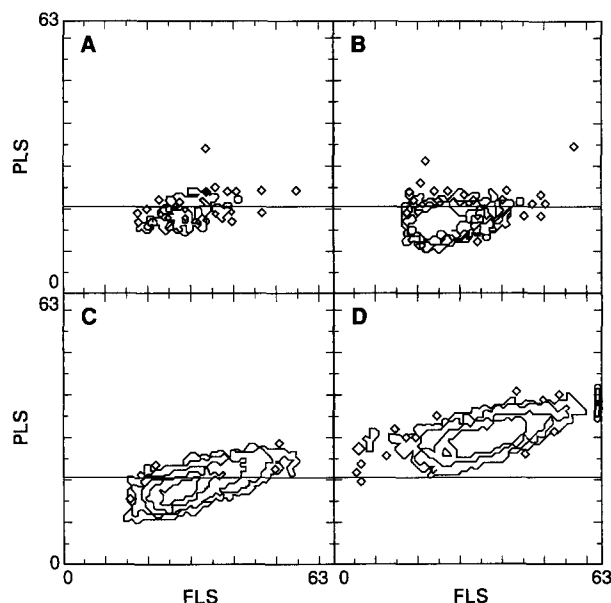


Fig 7. FACS analysis comparing PLS as a reflection of internal complexity and FLS as a reflection of size of $Fc\epsilon RI$ -bearing cells at 5 days compared with $Fc\epsilon RI$ -bearing cells at day 21 in IL-3-dependent mouse BM culture. $Fc\epsilon RI^+$ cells at day 5 (A) are similar in terms of the internal complexity and size to $Fc\epsilon RI^+$ cells at day 21 (B). The total cell population at day 5 (C) shows cells of less internal complexity and somewhat smaller size when compared with cultures of mast cells at day 21 (D). The increase in internal complexity and cell size with time is illustrated by comparing day 5 $Fc\epsilon RI^+$ cells (A) with the day 21 total cell population (D), in which virtually all cells are $Fc\epsilon RI^+$. A line drawn through the mean of the perpendicular light scatter of the $Fc\epsilon RI^+$ cells sorted at day 5 is shown for comparison in each of the other panels.

(5 to 50 cells per colony) per 10^6 cells plated. $Fc\epsilon RI^-$ cells gave rise to 23 ± 2 ($N = 4$) medium-size and 49 ± 3 ($N = 4$) large colonies (>50 cells per colony) per 10^6 cells plated. All mast cell colonies consistently contained greater than 99% mast cells and less than 1% histiocytes. These results indicate that $Fc\epsilon RI^+$ cells have the capacity to proliferate, but that a mast cell pool of mast cell progenitors exists

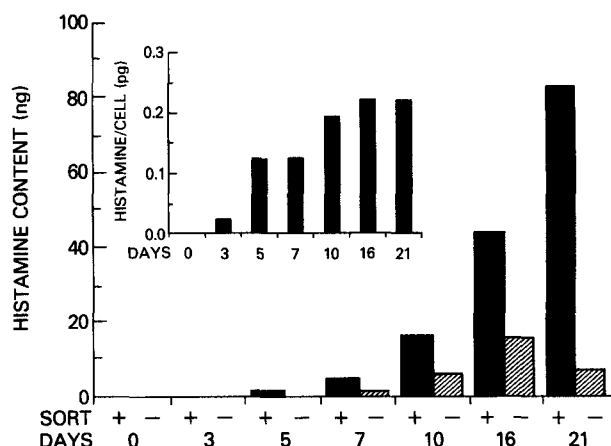


Fig 8. Total histamine content of (■) $Fc\epsilon RI^+$ (+) and (□) $Fc\epsilon RI^-$ (-) cell cultures grown in IL-3 for 0 to 21 days. Insert shows histamine content per cell of $Fc\epsilon RI$ -bearing cells. Data presented are the average of two separate cultures.

within the $Fc\epsilon RI^-$ cell population. Day 3 $Fc\epsilon RI^+$ cells, when cultured in agarose supplemented with either GM-CSF or M-CSF for 14 days, did not give rise to colonies of any type. Multiple single cells resembling macrophage or histiocyte-like cells were seen in the agarose. The total number of these cells was less than 1% of the original number of cells placed in agarose.

We next examined thymidine and uridine incorporation as a measure of DNA and RNA synthesis, respectively. Thymidine incorporation decreased from 4.7×10^4 cpm $\pm$ 0.3 for $Fc\epsilon RI$ -bearing cells sorted on day 3 to 1.7×10^4 cpm $\pm$ 0.08 by day 7. Uridine incorporation increased from 4.9×10^2 cpm $\pm$ 0.1 for $Fc\epsilon RI$ -bearing cells sorted on day 3 to 1.7×10^4 cpm $\pm$ 0.06 for $Fc\epsilon RI$ -bearing cells sorted on day 7.

DISCUSSION

Mature mast cells reside in a variety of tissues and are known for their ability to release potent chemical mediators after activation by IgE-mediated stimuli.²⁷ Inappropriate activation by these means leads to allergic diseases and, for this reason, the overwhelming majority of studies in mast cell biology has focused on the morphologic and biochemical characterization of these cells. Recently, however, several investigators have suggested that committed mast cell progenitors and mast cells early in their development, before they become resident in tissues, may also exhibit biologic function.^{7,8} There is limited information concerning the early developmental stages of mast cells. Flow cytometric analysis of murine BM cells in liquid culture in the presence of IL-3 suggested that Thy 1.2-positive precursors give rise to granulocytes and mast cells.²⁸ Recently, we reported that message for the three subunits of the $Fc\epsilon RI$ receptor could be identified in cultured BM cells grown in IL-3. Further, $Fc\epsilon RI$ -bearing cells could be identified as early as 7 days in mouse BM cultures, but less than 25% of these cells had the morphologic characteristics of mast cells.⁸ We thus hypothesized that $Fc\epsilon RI$ -bearing cells were, in fact, mast cell progenitors, and that the sequential study of the kinetics of appearance of such cells in culture and their morphologic features and growth characteristics would contribute to our understanding of early events in mast cell maturation.

Extending the previous reports,^{8,28} we first performed FACS analysis of IL-3-driven mouse BM cultures. These studies showed a distinct population of $Fc\epsilon RI$ -bearing cells as early as 3 days of culture (Fig 1), and that these cells were also positive for $Fc\epsilon RII/III$. Preincubation of the cells with unlabeled unrelated antibody did not effect the ability to detect $Fc\epsilon RI$ cells. Preincubation of the cells with 2.4G2 MoAb directed against $Fc\epsilon RII/III$ at a 100-fold concentration before incubation with FITC-conjugated IgE also did not effect the number of $Fc\epsilon RI^+$ cells identified; however, $Fc\epsilon RI^+$ cells were not detected in cell populations preincubated with a 100-fold unlabeled IgE before incubation with FITC-conjugated IgE. These experiments clearly established the specificity of the assay. $Fc\epsilon RI$ -bearing cells by day 21 made up greater than 90% of all cells (Fig 3). FACS sorting of $Fc\epsilon RI$ -bearing cells showed that fewer than 14%

were mast cells on the basis of acid toluidine blue staining at day 3 but, by day 21, 100% of Fc ϵ RI-bearing cells had numerous acid toluidine blue-positive stained granules identifying them as mast cells (Fig 5).

While many cells sorted during the first week of culture on the basis of Fc ϵ RI receptors did not contain acid toluidine blue-positive granules (Fig 6), there is substantial evidence that these cells are mast cell progenitors. First, the Fc ϵ RI receptor density increased on these cells over time (Table 1). Second, the majority of histamine is found within the Fc ϵ RI⁺ population, and the histamine content per cell also increased over time (Fig 8). Third, the internal complexity of these cells increased in concert with the appearance of acid toluidine blue-positive granules (Fig 7). Fourth, sorted Fc ϵ RI-bearing cells obtained at 3, 5, and 7 days, when placed in liquid culture in the presence of IL-3, gave rise to pure populations of mast cells within 7 days. Finally, granulation of these isolated Fc ϵ RI⁺ cells was associated with an increase in cell number and with thymidine incorporation, suggesting that Fc ϵ RI⁺ populations were capable of continued expansion.

The growth potential of Fc ϵ RI⁺ and Fc ϵ RI⁻ cells was further assessed by determining colony-forming units in these populations in the presence or absence of IL-3 or GM-CSF. Fc ϵ RI⁺ cells gave rise to single cells, an average of 4,800 small mast cell colonies and 150 medium-size mast cell colonies per 10⁶ cells. Fc ϵ RI⁻ cells gave rise to 23 medium-size mast cell colonies and 49 large mast cell colonies per 10⁶ cells. Thus, cells acquiring Fc ϵ RI have proliferative potential. The data, however, also show that a population of cells exists in the Fc ϵ RI⁻ population that is capable of giving rise to mast cells. Indeed, the data are consistent with the existence of a pool of mast cell progenitors that have not yet acquired the Fc ϵ RI receptor.

It is clear that not only the appearance of acid toluidine blue-positive mast cells is dependent on IL-3, but also two characteristics of mast cells, ie, the acquisition by these cells of Fc ϵ RI and synthesis of histamine. Binding studies at 7 and 28 days confirmed the presence of high-affinity IgE receptors in IL-3-dependent mouse BM cultures (Fig 4). Fc ϵ RI-bearing cells were sorted under conditions known to select for cells bearing Fc ϵ RI.¹⁷ Fc ϵ RI-bearing cells could not be identified in mouse BM cultures grown in the presence of GM-CSF rather than IL-3 (Fig 2). These GM-CSF-driven cultures also did not give rise to acid

toluidine blue-positive cells, in agreement with a previous report.²³ Histamine synthesis has also been reported to be IL-3 dependent,^{25,26} and the data presented in this report strongly support the supposition that histamine synthesis and storage in IL-3-dependent BM cultures is a feature of Fc ϵ RI-bearing cells.

During the initial week of culture of mouse BM in IL-3, the appearance of Fc ϵ RI-bearing cells is accompanied by a decrease in the number of large and small mononuclear cells in the Fc ϵ RI⁻ population. These data and the data on mast cell colony-forming units reviewed above suggest that Fc ϵ RI⁺ cells develop from mononuclear cells. This finding is in agreement with the concept that mast cells arise from pluripotent hematopoietic stem cells¹ and suggests that there may be a step in mast cell development in which mast cell progenitors have not yet acquired sufficient Fc ϵ RI receptors to be selected by sorting procedures; however, the identification of Fc ϵ RI-bearing mononuclear cells lacking acid toluidine blue-positive granules and destined to become mast cells is in agreement with *in vivo* studies reporting the appearance of mast cell progenitors in blood, which were indirectly shown to be within a mononuclear cell population.²⁹ Fc ϵ RI⁺ cells cultured in GM-CSF or M-CSF did not give rise to either colonies of neutrophils or monocytes. This observation suggests that once a cell expresses Fc ϵ RI it no longer has the capacity to give rise to neutrophil or monocyte colonies in response to GM-CSF or M-CSF. Fc ϵ RI⁺ cells, however, remain responsive to IL-3 but give rise only to mast cell colonies.

In summary, the data presented clearly show that mast cells express Fc ϵ RI before they can be identified as mast cells based on morphology, and show that these cells develop into mature histamine-containing cultured mast cells. Fc ϵ RI-bearing cells also express Fc ϵ RII/III and have proliferative potential. The Fc ϵ RI⁻ cell population, however, contains more large mast cell colony-forming units than cells that have expressed Fc ϵ RI, demonstrating the existence of a pool of Fc ϵ RI⁻ progenitor cells capable of giving rise to Fc ϵ RI⁺ cells that themselves mature into mast cells. The characteristics of these Fc ϵ RI⁻ mast cell progenitors remain to be elucidated.

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