In the present study we investigate the nature of the murine bone marrow cell subset responsible for the marked increase in histamine synthesis induced by interleukin-3 (IL-3). Because mast cells, and eventually their committed precursors, represent a potential source of histamine in this context, we examined our possible participation in this biologic activity with particular attention. We provide evidence that neither of these populations respond to IL-3 in terms of histamine synthesis and that other differentiated end cells or stromal components of the bone marrow are also not involved in this phenomenon. Starting from these findings, we further characterized the immature hematopoietic compartment responsible for IL-3–induced histamine synthesis using fluorescence-activated cell sorter (FACS) sorting based on rhodamine retention or wheat germ agglutinin (WGA) affinity. These procedures have allowed us to ascribe the following features to histamine-producing cells: (1) They belong to a low-density, progenitor-enriched bone marrow subset containing cells of relatively important size and internal structure. (2) The highest histamine levels are generated by the rhodamine-bright fraction of this population, while the most primitive rhodamine-dull cells do not express this biologic activity. (3) Histamine-producing cells do not copurify with colony-forming units in spleen day 7 and day 12 in WGA-bright fractions. (4) Their enrichment is associated with increased frequencies of cells forming colonies in methylcellulose (CFU-C), suggesting the involvement of several progenitors with partially limited differentiation potential in this biologic activity.

MATERIALS AND METHODS

Animals. Male or female 6- to 8-week-old C57 Bl/6 mice, bred in our own facilities under pathogen-free conditions, were currently used in limiting dilution experiments performed on BM and intraepithelial cells isolated from the gut mucosa. Mast cell-deficient WBB6F1/W'W' (W/W') mice and normal WBB6F1/+ (+/+) littermates were purchased from C.S.E.A.L. (Orléans, France). Male 12- to 20-week-old CBA × C57 Bl/F1 mice, purchased from the Medical Biological Laboratory TNO (Rijswijk, The Netherlands) and maintained under clean conventional conditions at the Laboratory Animals Center of the Erasmus University (Rotterdam, The Netherlands) were used for most of the sorting procedures and the set-up of stromal cultures.

Cell preparations. Limiting dilution assays and experiments with W/W' mice were performed with BM suspensions obtained by flushing of femurs and tibiae with ice-cold Hanks' Balanced Salt Solution (GIBCO, Grand Island, NY). For most of the sorting experiments BM cells were prepared by cleaning femurs and tibiae from muscles and tendons and grinding them in a mortar using phosphate-buffered saline (GIBCO).
saline (PBS), as previously described. The cell suspensions were sieved over a nylon filter (mesh size, 100 μm), centrifuged, and adjusted to concentrations appropriate for the different sorting procedures.

Intraepithelial cells from the gut mucosa were obtained as previously described. After washing the gut and removing the Peyer’s patches, the small bowel was opened and flattened. The epithelium was scraped with a scalpel and fragments obtained were dissociated in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco) with 20% fetal calf serum and 100 U/ml diithiothreitol using a magnetic stirrer. The washed suspension was then shaken vigorously on a vortex (Scientific Industries, Bohemia, NY) and filtered quickly through a buffered glass wool column (1.8 g packed in 20-ml syringe). Dead cells and debris were eliminated on a Ficol Isopaque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden). This procedure yields around 2 × 10⁶ nonepithelial cells per mouse.

Stromal layers were set up in flat-bottomed microtiter plates as reported before. In brief, 10⁶ BM cells were plated in 0.2 mL of culture medium consisting of α-medium supplemented with 10% fetal calf serum (GIBCO), 10% horse serum (Boehringer, Mannheim, Germany), 0.5 mg/mL human transferrin (Hoechst-Behring, Amsterdam, The Netherlands), 10⁻³ mol/L hydrocortisone sodium succinate (Sigma, St Louis, MO), and 10⁻⁴ mol/L β-mercaptoethanol (Merck, Amsterdam, The Netherlands). After 10 to 11 days of culture at 33°C, 10% CO₂, the cells were exposed to a dose of 20 Gy gamma radiation to eliminate hematopoietic activity. One day later the medium was changed and cultures were maintained at 33°C, 10% CO₂ with a complete medium change per week. Before stimulation the stromal layers were washed repeatedly to eliminate granulocytes with lower affinity or fractionation on a discontinuous Ficoll gradient. For immunofluorescence labeling, 5 × 10⁷ pelleted total BM cells were resuspended in 500 μL of hybridoma supernatant containing the MoAb and incubated for 30 minutes on ice. After three washings in DMEM in the cells were incubated for a further 30 minutes with the second antibody, a rabbit anti-rat IgG-FITC (Cappel, Malvern, PA) diluted 100-fold in DMEM supplemented with 2% normal mouse serum. After a 40-fold dilution (2.5 × 10⁶/mL) the cells were processed for FACS. Three sorting windows were selected corresponding to a highly fluorescent population of monocytes (ER-MP 20⁺), a fraction with intermediate fluorescence (ER-MP 20⁻) containing granulocytes and most of the morphologically recognizable granulocyte precursors, and the remainder of negative cells (ER-MP 20⁻) consisting almost entirely of mononuclear cells.

Lin- or low-density cells, 3 × 10⁶ per milliliter, were incubated for 45 minutes at 37°C in DMEM containing 0.1 μg/mL rhodamine dye (Eastman Kodak, Rochester, NY) as previously reported. The excess internalized Rh was removed by incubating the cells for another 30 minutes in DMEM at 37°C. After two washings in DMEM and resuspension at 3 × 10⁶/mL in RPMI with 0.4% bovine serum albumin (Sigma), cells were sorted on the basis of Rh fluorescence intensity within the blast and lower PLS granulocyte window as detailed in Results.

Five microliters of WGA-FITC (1 μg/mL; Polysciences, Warrington, PA), previously diluted 100-fold in PBS, was added per 3 × 10⁶ Lin-cells suspended in 1 mL of PBS with 0.4% bovine serum albumin, previously described. WGA-FITC labeling was optimal after 15 minutes of incubation at room temperature. After sorting (see Results), 0.2 mol/L N-acetyl-D-glucosamine (Polysciences), the competitive sugar of the lectin, was added to the cell suspension to compete WGA off the cells.

Analysis and sorting of cells was performed by a FACS II (B-D Facs Systems; Becton Dickinson & Co, Sunnyvale, CA) with an argon laser set at 488 nm. Sorting speed was about 2,500 cells per second.

Limiting dilution, colony and narrow repopulating ability (MRA) assays. Limiting dilution assays for detection of mast cell precursors (MCP) were performed in supplemented DMEM with 10% fetal calf serum in 96 flat-bottomed culture plates (Falcon 3040; Becton Dickinson, Oxnard, CA). Cell populations were tested at eight appropriate concentrations plated in 12 wells per concentration. Each 200-μL well contained, in addition, 10⁶ irradiated (40 Gy) syngeneic spleen cells and an optimal concentration of WEHI-3-conditioned medium. After 7 to 12 days, cells from growing colonies were studied on May Grünwald-Giemsa-stained cytopsins. The frequency of MCP was determined by using Poisson probability distribution.

Colony-forming units (CFU-C) were quantified in a semisolid (0.8% methylcellulose, Methocel AP4 Premium; Dow Chemical, Fluka Chemie, Buchs, Switzerland) culture medium (α-modification of DMEM) at 37°C and 5% CO₂. The culture contained 20% concanavalin A (ConA)-stimulated mouse spleen conditioned medium, 10% horse serum (Boehringer), and 1% bovine serum albumin. One thousand to 10⁶ cells were plated per dish and colonies were counted on day 8 of culture.

The day 7 and day 12 CFU-S content of cell suspensions was determined by injecting appropriate dilutions into the lateral tail vein of lethally irradiated mice. The cell numbers injected were estimated from previous experiments to give between 1 and 5 surface colonies on day 7 and 0 and 6 on day 12. MRA (CFU-C) was determined by

on the basis of forward and perpendicular light scatter (FLS/PLS) in preselected areas meeting the criteria of "lymphocyte," "blast," lower PLS, and higher PLS "granulocyte" window that were set using total BM cells as a standard (see Results).

Fluorescence-activated cell sorting (FACS) was performed either after labeling of total BM cells by the ER-MP 20 rat anti-mouse monoclonal IgG2a antibody recognizing monocytes with high and granulocytes with lower affinity or after exposure of precursor-enriched BM cells to Rh or WGA-fluorescein isothiocyanate (FITC). For immunofluorescence labeling, 5 × 10⁷ pelleted total BM cells were resuspended in 500 μL of hybridoma supernatant containing the MoAb and incubated for 30 minutes on ice. After three washings in DMEM in the cells were incubated for a further 30 minutes with the second antibody, a rabbit anti-rat IgG-FITC (Cappel, Malvern, PA) diluted 100-fold in DMEM supplemented with 2% normal mouse serum. After a 40-fold dilution (2.5 × 10⁶/mL) the cells were processed for FACS. Three sorting windows were selected corresponding to a highly fluorescent population of monocytes (ER-MP 20⁺), a fraction with intermediate fluorescence (ER-MP 20⁻) containing granulocytes and most of the morphologically recognizable granulocyte precursors, and the remainder of negative cells (ER-MP 20⁻) consisting almost entirely of mononuclear cells.

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on the basis of forward and perpendicular light scatter (FLS/PLS) in preselected areas meeting the criteria of "lymphocyte," "blast," lower PLS, and higher PLS "granulocyte" window that were set using total BM cells as a standard (see Results).
assessing the number of CFU-C generated over a 12-day period in femoral BM of lethally irradiated mice after intravenous (IV) injection of sorted cells. Data were corrected for any endogeneous CFU-C regrowth in control irradiated mice and expressed as the number of colonies per femur equivalent per 10^6 cells injected. Differential counts of unfractinated or sorted BM cells were performed after standard May Grünwald-Giema staining.

**HCSA assay.** All sorted subsets were plated, in parallel with total and pre-enriched BM cells, in flat-bottomed microtiter plates (Costar, Badhoevedorp, The Netherlands) at appropriate concentrations, according to the expected enrichment factor. After 24 or 48 hours of incubation at 37°C, 5% CO2 in serum-free DMEM alone or together with 5 ng/mL of ml-IL-3, histamine concentrations were determined in the supernatants. This assay was performed either by an automated continuous flow fluorimetric technique previously described25 or using a radioimmunoassay kit purchased from Immunotech (Marseille, France.) The lower limit of sensitivity is about 0.5 ng/mL for the fluorometric assay and around 20 pg/mL for the radioimmunoassay. IL-3-induced histamine production in all cell populations tested is completely abrogated by a dose of 5 X 10^-4 mol/L α-fluoromethylhistidine (α-FMH), the specific inhibitor of histidine decarboxylase.

Preparations for electron microscopy. Rh-bright cells, 10^5 per sample (sorted as described above), were fixed in 2% glutaraldehyde in cacodylate buffer, pH 7.5 for 1 hour, postfixed in 2% osmium tetroxide, dehydrated in alcohol series and propylene oxide and embedded in Epon (Cipec, France). Between each step, cells were centrifuged at 1,200 rpm. Ultrathin sections impregnated with uranylacetate and lead citrate were analyzed using an EM 300 Philips electron microscope (Bobigny, France). The different cell types were identified and counted on micrographs made systematically in the left quarter of the grid for randomized results. The morphologic criteria of cellular identification were based on cell shape and size, characteristics of the nucleus (shape, aspect of chromatin, nucleolus), cytoplasmic components (distribution of different cellular organelles and ribosomes), and the presence or absence of electron dense granules. Two separate experiments were performed and about 200 cells were counted and typed in each sample.

**RESULTS**

Evidence against the implication of mast cells and their committed precursors in IL-3-induced histamine synthesis. We have used the mast cell-deficient W/W’ strain to verify whether HCSA can still be demonstrated in the BM of these mice. As shown in Fig 1, there is not only a significant increase in histamine synthesis in response to IL-3, but the distribution of responder cells over the different density layers of a Ficoll gradient is very similar to that observed with control mice.

We also examined the possible involvement of committed mast cell precursors isolated from the gut mucosa23 in IL-3–induced histamine synthesis. We evaluated mast cell precursor frequencies in both intraepithelial and total BM cell suspensions, using a limiting dilution assay. The frequencies per 10^6 cells calculated from four different experiments and expressed as means ± SEM are: 94 ± 10.6 for the population isolated from the gut mucosa, versus 240 ± 34.6 for unfractinated BM cells. When tested at a concentration of 2.5 X 10^5 cells/mL, the intraepithelial precursors (235 ± 25.5/mL) do not respond to IL-3 in terms of histamine production. Conversely, the growth factor promotes a very significant increase in histamine levels (138.7 ± 9.1 ng/mL; mean ± SEM from four separate experiments) in BM cells cultured at a fourfold lower concentration (0.625 X 10^6 cells/mL) to obtain similar or lower MCP frequencies. The lack of IL-3–induced histamine production by the intraepithelial population is not explained by the degradation of the amine by histaminase because even in the presence of aminoguanidine at a dose of 10^-3 mol/L, which blocks this enzymatic activity completely, there is no histamine synthesis in response to IL-3. Addition of supernatant from intraepithelial gut mucosa cells does not modulate the histamine levels produced by total or progenitor-enriched BM cells stimulated by IL-3. Furthermore, HCSA is not increased in these populations by 10^-3 mol/L aminoguanidine, suggesting that histamine is not degraded during culture.

As shown in Fig 2, histamine synthesis by progenitor-enriched BM cells stimulated for 48 hours with IL-3 does not require proliferation of histamine-producing or histamine-containing cells because irradiation of BM cell suspensions at a dose that results in the abrogation of cell growth (20 Gy) does not significantly modify this biologic activity.

Finally, an indirect argument against the participation of mast cells in IL-3–induced histamine synthesis by BM cells is provided by the fact that murine recombinant mast cell growth factor (c-kit ligand; kindly given by S. Gillis, Immunex Corp, Seattle, WA) has no effect on this activity, neither alone nor in combination with IL-3.

Lack of IL-3–induced histamine production by other differentiated BM cells. The participation of the stromal BM environment in histamine synthesis is ruled out by the fact that stromal layers, depleted for hematopoietic activity by irradiation, express this biologic activity neither sponta-
these populations comprising, in this particular experiment, three separate experiments. Control cells incubated with subsets after labeling with the MoAb ER-MP 20 that recognizes histamine production in response to the growth factor thrombomonocytes are responsible for IL-3-driven histamine synthesis. In contrast, morphologically unidentifiable blast cells are around threefold enriched in this population (29.7% ± 4.2% vs. 8.9% ± 2.0% in total BM; means ± SEM from six separate experiments) and erythrocytes have completely disappeared. In contrast, morphologically unidentifiable blast cells are around threefold enriched in this population (29.7% ± 4.2% vs. 8.9% ± 2.0% in total BM; means ± SEM from six separate experiments) and the frequency of CFU-C, CFU-S day 12 and MRA (CFU-C) is about fivefold increased (see Fig 5).

Light scatter characteristics of histamine-producing cells. The dot display of forward versus perpendicular light scatter (Fig 4) illustrates how the four areas hereafter referred to as "lymphocyte," "blast," and low and high PLS "granulocyte" window (1, 2, 3, and 4, respectively) have been set using unsorted BM cells as a standard. It should be noted that these designations only indicate a high frequency of such cells in the selected area that does not exclude the presence of other cells. The average percentages of low-density cells selected by these criteria are indicated. As also shown in Fig 4, the highest histamine levels are generated by the cells located in the low PLS granulocyte window, while in comparison with the fraction from Ficoll layer 2 + 3, histamine synthesis by cells gated in the blast window is generally less. The low biologic activity expressed by cells located in the lymphocyte window confirms that B and T cells are not involved in HCSA.

Characterization by fluorescence-activated cell sorting. We further analyzed the cells in both blast and low PLS granulocyte windows (35% and 12% of all cells, respectively) with respect to their fluorescence intensity after rhodamine staining. To this end, we divided the two light scatter areas into four equal fractions (Rh−, Rh+, Rh++, and Rh++++) and tested the sorted populations for their HCSA as compared with their incidence of CFU-C, CFU-S day 12, and MRA (CFU-C) (Fig 5). Quiescent stem cells with long-term in vivo reconstituting activity (MRA [CFU-C]) are highly enriched in the Rh-dull fraction of the blast window and, to a lesser extent, of the low PLS granulocyte window. These cells do not respond to IL-3 by increased histamine production. There is also no correlation between IL-3-induced histamine production and CFU-S day 12 frequencies that are highest in the Rh-bright fraction of the blast window. In contrast, HCSA

Table 1. Incidence of a Series of Hematopoietic Progenitor Cell Types in BM Sorted on the Basis of ER-MP 20 Expression

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Progenitors per 10^6 Cells</th>
</tr>
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<tbody>
<tr>
<td>CFU-C</td>
<td>CFU-S Day 12</td>
</tr>
<tr>
<td>Unfractionated BMC</td>
<td>259 ± 21</td>
</tr>
<tr>
<td>ER-MP 20++</td>
<td>0</td>
</tr>
<tr>
<td>ER-MP 20+</td>
<td>10 ± 10</td>
</tr>
<tr>
<td>ER-MP 20−</td>
<td>510 ± 31</td>
</tr>
</tbody>
</table>

Abbreviations: MRA (CFU-C), number of CFU-C generated by 10^6 infused cells in a femur over a 12-day period; ER-MP 20++, 5-10% of all cells, over 90% monocytes; ER-MP 20+, 50-65% of all cells, over 90% myelomonocytic cells; ER-MP 20−, 25-45% of all cells, mainly mononuclear.
was determined after a 24-hour incubation of the cell subsets with 5 ng/mL of mrlL-3. Cell concentrations were adjusted according to the relative frequencies (%) of the three subpopulations, i.e., ER-MP 20+ (5%): 5 x 10^6 cells/mL; ER-MP 20+ (45%): 45 x 10^6 cells/mL; ER-MP 20+ (50%): 50 x 10^6 cells/mL; and control bone marrow (CBM): 10^6 cells/mL. Data, calculated per 10^6 cells after subtraction of the background values, are means of triplicate histamine determinations.

Fig 3. Fluorescence distribution of unfractionated BM cells stained with the MoAb ER-MP 20. Histamine production was determined after a 24-hour incubation of the cell subsets with 5 ng/mL of mrlL-3. Cell concentrations were adjusted according to the relative frequencies (%) of the three subpopulations, i.e., ER-MP 20+ (5%): 5 x 10^6 cells/mL; ER-MP 20+ (45%): 45 x 10^6 cells/mL; ER-MP 20+ (50%): 50 x 10^6 cells/mL; and control bone marrow (CBM): 10^6 cells/mL. Data, calculated per 10^6 cells after subtraction of the background values, are means of triplicate histamine determinations.

increases with increasing retention of the dye, reaching a maximum in the Rh+++ subset of the low PLS granulocyte window, where this biologic activity is about 40-fold enhanced over unsorted BM cells.

The distribution of histamine-producing cells and CFU-C in the different fractions, as well as the enrichment factor in the Rh-bright subset of the low PLS granulocyte window, are very similar, suggesting a major involvement of this progenitor population. To give further support to the notion that CFU-S do not take part in HCSA, we have taken advantage of a different sorting procedure, based on the affinity of hematopoietic cells for WGA that has been previously shown to be a potent device for CFU-S day 7 and day 12 purification. The labeled cells selected in the blast window were divided into three fluorescent WGA+, WGA++, and WGA+++ fractions comprising respectively 1%, 2.5%, and 6.5% of all Lin- cells. The sorted subsets were tested for their CFU-C, CFU-S day 7, and day 12 frequencies in parallel with the histamine assay. It is evident from Table 2 that the important enrichment for CFU-S day 7 in the WGA+ and CFU-S day 12 in the WGA+++ population is not associated with increased IL-3—induced histamine levels relative to unfractioanted BM cells. In contrast, the distribution of CFU-C over the three sorting windows is once more very similar to that of HCSA.

Electron microscopy analysis of Rh-bright cells. According to ultrastructural features of low-density cells sorted inside the Rh-bright window four cell types can be distinguished (average percentages from two separate experiments): (1) A majority (88.5%) of large, undifferentiated blast cells with a very large, regularly shaped cytoplasm scattered with many ribosomes and polyribosomes. These cells have immature, relatively large oval-shaped nuclei with a profound indentation containing coarse chromatin and a few clumps of dense chromatin along the nuclear membrane. An enormous nucleolus is often visible. In the cytoplasm the Golgi apparatus is often missing in the plan of the section and there are only few endoplasmic reticulum lamellae. The cytoplasm contains many mitochondriae, forming frequently a picture of clusters located in the indentation of the nucleus. (2) Myeloblasts, 4.4%, and promyelocytes with a more or less immature nucleus according to the differentiation stage considered (coarse chromatin without dense clumps and unlobated nucleus in the first case and beginning of lobation in the second case). The large cytoplasm contains two or three small, very electron dense granules which appear undifferentiated. (3) Myelocytes, 7.1%, characterized by a medium size, as compared with blast cells and promyelocytes, a relatively mature polylobated nucleus with dense clumps of chromatine. Their cytoplasm is dense, containing few ribosomes and a well-developed Golgi apparatus and endoplasmic reticulum. There are also some round, rather small, dense granules. These cells are maturing into granulocytes but it is impossible to identify them as progenitors of basophilic granulocytes. (4) The few, more or less mature, small lymphocytes present in the two samples have not been included in the differential counts because of their very low number (about 10 cells in the two experiments).

DISCUSSION

Starting from the observation of Kahlson and Rosengren showing that a histamine analogue can trigger CFU-S into cycle. More recently, Shounan and Xu have confirmed and extended these data, using exogenous histamine to induce CFU-S proliferation. In addition, some clinical reports have described cases of agranulocytosis and leukopenia subsequent to the therapeutic use of histamine antagonists and histamine-induced proliferation and differentiation of granulocyte precursors in vitro has been demonstrated. Still more recently, Brandes et al have described a new intracellular receptor for histamine, postulating that intracellular histamine may mediate proliferation as a second messenger for multiple growth-promoting receptors.

To exert its functions as a mediator of proliferation and/or differentiation, histamine has to be produced during cell development. We have shown that this is indeed the case during IL-3—induced hematopoiesis, which is preceded by a
rapid generation of large amounts of histamine in the BM.33 Furthermore, we have provided evidence that one of the specific biologic activities of the growth factor, ie, triggering of histamine synthesis and the producer cells could not be assigned to a particular progenitor subset.

Given the importance of mast cells as a potential source of histamine as well as the effect of IL-3 on mast cell differentiation, it could be assumed that HCSA was associated with the development of this lineage.11 To verify this hypothesis we first examined the contribution of mature mast cells to IL-3-induced histamine synthesis. We could not detect differentiated cells of this lineage in the populations enriched for histamine-producing cells, including the most purified rhodamine-bright subset, on the basis of morphologic criteria. Electron microscopic analysis of this latter fraction showed 88.5% undifferentiated blasts, 4.4% myeloblasts and promyelocytes, as well as 7.1% myelocytes. Myeloblasts, promyelocytes, and myelocytes contain only a few, small, perfectly round and dense granules. These features are distinct from those ascribed to immature basophilic myelocytes that contain larger, lightly dense granules.34 Because histamine synthesis in response to IL-3 is independent from cell growth it cannot be explained by the proliferation of a few mast cells or basophils that might have escaped our observations. It is also highly improbable that increased survival of histamine-producing cells in the presence of IL-3 could account for the enhanced histamine synthesis, because HDC levels are already significantly increased after an exposure to the growth factor for only 4 hours, when the viability of control cells is not impaired.9 Additional arguments against the involvement of mature mast cells in HCSA were provided by the fact that peritoneal mast cells or mast cell lines (such as MC 9) do not respond to IL-3 in terms of histamine synthesis, whereas BM cells from the mast cell-deficient strain W/Wv express this biologic activity.

Having thus discarded the participation of mature mast cells in IL-3–induced histamine production, we addressed the question of whether precursors of this lineage might be involved. Mast cell precursors contained in the intraepithelial population isolated from the gut mucosa respond to IL-3 by differentiating exclusively into mast cell colonies in the limiting dilution assay. Such lineage-committed progenitors may actually not be present in the BM. However, the fact that these cells do not express HCSA rules out the involvement of members of the mast cell lineage in late differentiation stages in histamine production. This is in agreement with the fact that W/Wv BM cells respond to IL-3 by enhanced histamine production, though mast cell-committed progenitors, as defined by their capacity to proliferate and differentiate in response to MGF (c-kit ligand), are lacking in the BM of this strain.35 Conversely, the frequency of primitive, CFU–S–derived mast cell progenitors,36 which cannot be distinguished from the subset giving rise to GM colonies in the limiting dilution assay, are normal or even higher in W/Wv than in C57BL/6 BM.37 This finding argues against the exclusive contribution of this progenitor subset to IL-3–induced histamine production which is significantly lower in the W/Wv than in C57BL/6 BM.

The conclusion that the mast cell or basophil lineage alone is responsible for increased histamine production by BM cells

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**Fig 4.** (A) Dot display of forward versus perpendicular light scatter (FLS/PLS) of low-density BM cells from Ficoll layers 2 + 3. The percentages of cells in the areas 1, 2, 3, and 4 are, respectively, 40%, 35%, 12%, and 13%. 1, lymphocyte window; 2, blast window; 3, low PLS granulocyte window; 4, high PLS granulocyte window. (B) Histamine production by sorted cells, incubated for 24 hours with 5 ng/mL of m rIL-3. Each column represents the mean of triplicate histamine determinations after subtraction of the background values. Similar results were obtained in one other experiment.
CHARACTERISTICS OF HISTAMINE-PRODUCING CELLS

Fig 5. Distribution of histamine-producing cells, CFU-C, CFU-S day 12, and MRA(CFU-C) over blast and low PLS granulocyte sorting windows divided into four equal fractions each, on the basis of the Rh fluorescence of BM cells from layers 2 + 3 (L 2 + 3) of the Ficoll gradient. BMC, bone marrow cells; BW, blast window; L-GW, low PLS granulocyte window; Rh−, rhodamine-dull; Rh+, Rh++, and Rh+++ represent cells with increasing fluorescence intensity. Histamine was assayed after a 48-hour incubation with mrlL-3 (5 ng/mL). Data represent means from triplicate determinations ± SEM after subtraction of spontaneous histamine production. Progenitor frequencies are expressed as means ± SEM from three separate experiments, excepting the data for CFU-S day 12 and MRA(CFU-C) in the low PLS granulocyte window corresponding to one experiment.

is also not in accordance with the induction of increased histamine synthesis by GM-CSF. Indeed, this factor does not support the development of BM-derived mast cells or even the expression of IgE receptors associated with the early differentiation stages of this lineage.38 We have been unable to enrich histamine-producing cells by sorting them on the basis of IgER expression. Using a labeling procedure with biotinylated IgE followed by phycoerythrin-streptavidine staining, we have found no consistent expression of this receptor on fresh BM cells, in agreement with the data reported by Rottem et al.39 Furthermore, we did not observe any enrichment for IgER+ cells during the purification procedures used herein, even in the Rh-bright subset (data not shown). The discrepancy between these data and those reported by Seder et al,40 who detected an IgER+ population even in unfractionated BM cells, might be explained by a very low expression of IgE receptors on progenitor cells, requiring an amplification procedure to obtain a significant staining. The histamine-producing cells might share some characteristics with the non-B non-T cells described by these investigators. Yet, it is hard to believe that the IgER+ subset alone accounts for our phenomenon because the histamine levels generated by IL-3-

Table 2. Histamine Production and Incidence of CFU-C, Day 7 and Day 12 CFU-S in Lin− Cell Preparations

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Increase in Histamine Synthesis (ng/10^6 cells)*</th>
<th>Progenitors per 10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CFU-C*</td>
</tr>
<tr>
<td>Unfractionated BMC</td>
<td>65 ± 4</td>
<td>287 ± 23</td>
</tr>
<tr>
<td>Lin−</td>
<td>323 ± 9</td>
<td>2,385 ± 607</td>
</tr>
<tr>
<td>Blast window</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WGA+</td>
<td>311 ± 17</td>
<td>2,414 ± 208</td>
</tr>
<tr>
<td>WGA++</td>
<td>335 ± 11</td>
<td>3,903 ± 369</td>
</tr>
<tr>
<td>WGA+++</td>
<td>89 ± 6</td>
<td>837 ± 380</td>
</tr>
</tbody>
</table>

Histamine was assayed in culture supernatants after a 24-hour incubation with mrlL-3 (5 ng/mL). 1, 2, and 6.5% of all Lin− cells were sorted in WGA+, WGA++, and WGA+++ windows, respectively.

* Means ± SD from two separate experiments.
† Means ± SEM from three separate experiments.
induced Rh-bright cells are much higher than those reported by Seder et al. after a 60-hour stimulation of cells expressing the IgE receptor.

The participation of other mature cells as well as stromal elements in IL-3-induced histamine synthesis could be excluded by positive or negative selection of these BM components. Thus, the lack of responder cells among T and B lymphocytes could be assessed by monoclonal paramagnetic removal of these populations and their physical sorting based on forward and perpendicular light scatter characteristics. In a similar way, the involvement of monocytes, granulocytes, and their late precursors could be discarded because IL-3-induced histamine production is enhanced by the depletion of these populations that do not respond to the growth factor when positively sorted after immunolabeling. This is also true for marrow-derived stromal layers that produce histamine neither spontaneously nor after exposure to IL-3.

Once we had established that histamine-producing cells belong to the immature compartment of the BM, we sorted this population on the basis of rhodamine retention. It is clear that the most immature stem cells in G0 sorted in the Rh-dull fraction of either blast or low PLS granulocyte window are not involved in our phenomenon. Conversely, histamine production increases with the increasing ability of these cells to retain rhodamine. So far, it has been assumed that the low Rh uptake in primitive stem cells results from the presence of very few or inactive mitochondria in these cells, which is in accordance with our ultrastructural observations, showing many mitochondriae in the cytoplasm of the most purified Rh-bright cells. A more recent report suggests that the initial dye accumulation could be modified by a P-glycoprotein efflux pump eliminating Rh from quiescent stem cells.

Though depleted for cells with marrow reconstituting activity, the Rh-bright fraction sorted within the low PLS granulocyte window (about 40-fold enriched for histamine-producing cells) still comprises various progenitors, including CFU-S. However, two lines of evidence argue against a major participation of this latter population in HCSA. On the one hand, CFU-S day 12 and histamine-producing cells do not copurify in the different Rh subsets of either blast or low PLS granulocyte window, the cells responsible for HCSA being optimally enriched in the Rh-bright fraction of the low PLS granulocyte window, whereas the incidence of CFU-S is highest in the corresponding fraction of the blast window. On the other hand, the purification of CFU-S day 7 and day 12 in the subsets sorted according to their WGA affinity is not correlated with the enrichment for histamine-producing cells or for CFU-C.

In conclusion, our data support the notion that the target cells of IL-3 in terms of histamine production belong to an immature subpopulation of the BM at an intermediary stage of development between pluripotent stem cells and lineage-committed precursors. Considering our previous findings showing that histamine is requisite for IL-3-induced CFU-S cell cycling and those of Nakaya and Tasaka suggesting a role of histamine in granulocyte development, we speculate that IL-3 might induce the synthesis of this mediator by immature cells to amplify its own biologic activities. Indeed, in emergency situations leading to endogenous IL-3 production, the striking amounts of histamine produced by some immature cells, while differentiating in response to the growth factor, might provide a second signal activating either their immediate progenitors, such as CFU-S, or their granulocytic progeny.

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