Identification of FcϵRI^{neg} Mast Cells in Mouse Bone Marrow Cell Cultures. Use of a Monoclonal Anti-p161 Antibody

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Summary

A monoclonal hamster antibody (K-1) specific for a 161-kD mast cell surface glycoprotein was derived. p161 is expressed on normal and cultured mast cells and on some macrophages, but not on basophils or other hematopoietic cells. A population of FcϵR^{neg} cells expressing p161 was found in short term cultures of bone marrow cells grown in interleukin (IL)-3. These cells were purified and propagated for extended periods in IL-3. They express c-*kit* and FcγRII/III, contain alcian blue–positive granules and histamine, and secrete IL-3 in response to ionomycin treatment. Their morphology is consistent with that of mast cells. We propose that they represent FcϵRI^{neg} mast cells that can be detected and purified because of their p161 expression.

Mast cells express the high affinity receptor for the Fc portion of IgE (FcϵRI) (1), possess characteristic granules containing histamine and other preformed mediators (2), contain a set of specific proteases (3), and express large amounts of c-*kit* (4). Here we describe the derivation of a mAb (K-1) specific for a 161-kD membrane protein (p161) expressed on mouse mast cells and on some macrophages but not on other hematopoietic cells. Since p161 is not expressed on basophils, K-1 can be used to distinguish mast cells and basophils. In short term cultures of mouse bone marrow grown in IL-3, ~10% of the p161^{pos} cells failed to bind IgE. During long-term culture in IL-3, these cells maintain the phenotype of being p161^{pos}/FcϵR^{neg}. They express c-*kit* and FcγRII/III and contain histamine and alcian blue–positive granules. When stimulated with ionomycin, they secrete IL-3. These p161^{pos} cells represent a population of FcϵRI^{neg} mast cells that appear regularly in short-term cultures of mouse bone marrow cells in IL-3.

Materials and Methods

Animals. Virus-free female Armenian hamsters (*Cricetulus migratorius*), 6–12 wk old, were obtained from Cytogen Research and Development, Inc. (West Roxbury, MA). Female BALB/c and CBA/JCR mice, 8–12 wk old, were obtained from the Frederick Cancer Research and Development Center (Frederick, MD) and the Jackson Laboratories (Bar Harbor, ME), respectively.

Culture Conditions. IL-3–dependent, bone marrow–derived cells and long-term IL-3–dependent cell lines were cultured in RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% FBS, 2-ME, L-glutamine, penicillin-streptomycin, sodium pyruvate (complete RPMI), plus 10–25% WEHI 3 supernatant as an IL-3 source.

Immunization and Preparation of Hybridomas. An Armenian hamster was injected intraperitoneally with cells of the CFTL.12 mast cell line (2.5 × 10^7) in emulsified CFA (Difco Laboratories, Detroit, MI). It was boosted twice, at 10-d intervals, with CFTL.12 cells (2.5 × 10^7) first emulsified in IFA (Difco Laboratories) and then suspended in saline. 3 d later, the hamster was killed, the spleen was removed, and a single cell suspension in HBSS (Biofluids, Inc.) was prepared. The cells were fused with SP2/0 cells and placed in 96-well plates as described (5), and a mAb (K-1) was obtained based on its ability to bind to CFTL.12 cells but not cells of the T cell line CT.4S (6).

Staining. Purified K-1 was either labeled with FITC or biotinylated. The latter was used with streptavidin-PE (SA-PE) (Southern Biotechnology Associates, Inc., Birmingham, AL) or streptavidin TRI-COLOR® (Caltag Laboratories, San Francisco, CA). IgE receptors were identified with purified mouse IgE (10 μg/ml) (7) followed by FITC– or PE–anti-IgE (Southern Biotechnology Associates). FITC-labeled anti–c-*kit*, FITC-2.4G2 (anti-FcγRII/III), and FITC anti–mouse CD11b (anti–Mac-1) were obtained from PharMingen (San Diego, CA). 2.4G2 ascitic fluid was used in staining reactions to block binding of immunoglobulins to FcγRII/III.

For alcian blue staining, 3–5 × 10^4 cells were deposited on glass slides by a cytocentrifuge and heat fixed. Staining was carried out with 0.5% alcian blue 8GX (Sigma Chemical Co., St. Louis, MO) in deionized H_2O, pH 1, for 45 min followed by two rinses with distilled H_2O. Slides were blotted dry and then counter-stained for 15 min in 0.1% safranin O (Sigma Chemical Co.) in 1% acetic acid.

Cell Lines and Cell Populations. 32D cells and 32D cells transfected with cDNA for c-*kit* were obtained from Dr. J. Pierce (National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD) and Dr. R. Seder (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). IC2 cells and transfectants of them expressing epidermal growth factor receptors (IC2E) were obtained from Dr. J. Schreurs (DNAX Research Institute, Palo Alto, CA). CFTL.12 and CFTL.15 cells were obtained from Dr. J. Pierce. PT18 cells were a gift of Dr. D. Pluznik (Food and Drug Administration, Bethesda, MD). The pre-B cell lines 1881 and 300-18 were kindly provided by Dr. F. Alt (Harvard Medical School, Boston, MA).

Mast Cell and Macrophage Purification. Mast cells were purified from peritoneum of CBA/JCR mice as described (8). To induce an enriched macrophage population in the peritoneal cavities of BALB/c mice, 3 ml of sterile fluid thioglycollate (29.8 gm/liter; NIH media unit) was injected intraperitoneally. After 72 h, the peritoneal exudate cells were harvested, treated with ACK lysing buffer (B & B Research Laboratories, Fiskeville, RI), washed three times, and FACS® analyzed.

Results

Distribution of K-1^{pos} Cells. Myeloid (e.g., 32D, a c-*kit*^{pos} 32D transfectant and FDC-1), macrophage (P388D1 and WEHI-3), pre-B cell (1881 and 300-18), and T cell (OE-4, EL-4 and CT.4S) lines failed to be stained by K-1. One exception were DA-1 cells, a population of primitive retrovirally transformed myeloid cells (9) whose growth is IL-3 dependent. By contrast, all mast cell lines tested (MC/9, CFTL.12, CFTL.15, P815, PT18, IC2 [FcεRI^{neg}] and IC2E [FcεRI^{pos}]) were stained brightly by K-1.

The analysis of these cell lines allow us to distinguish the antigen recognized by K-1 from FcεRI and from c-*kit*. Both FcεR^{neg} and FcεR^{pos} variants of the IC2 mast cell line stain with K-1, indicating that FcεRI and the K-1 antigen are not the same. The antigen recognized by K-1 can be distinguished from c-*kit*. Both wild-type (c-*kit*^{neg}) 32D cells and 32D cells that express c-*kit* as a result of stable transfection are K-1^{neg}. Furthermore, cells of the c-*kit*^{neg} mast cell line CFTL.15 are K-1^{pos}.

In a preparation of peritoneal CBA/JCR mast cells enriched to a purity of 95% by density gradient centrifugation, all FcεR^{pos} cells were brightly K-1^{pos}, implying that all peritoneal mast cells are K-1^{pos} (Fig. 1).

K-1 failed to stain freshly prepared bone marrow or lymph node cells. Weak staining of a small subpopulation of spleen and blood cells was observed. Two-color immunofluorescence analysis showed that they were a minor fraction of the cells that expressed Mac-1. However, thioglycollate-induced peritoneal exudate cells were brightly stained by K-1. Similarly, macrophage-enriched cell populations prepared by culture of bone marrow cells in CSF-1 or GM-CSF for 10 d contained Mac-1^{pos} cells that were K-1^{pos} (data not shown).

p161 is the Antigen Recognized by K-1. Surface iodinated CFTL.12 cells were extracted with NP-40 (10). The lysate

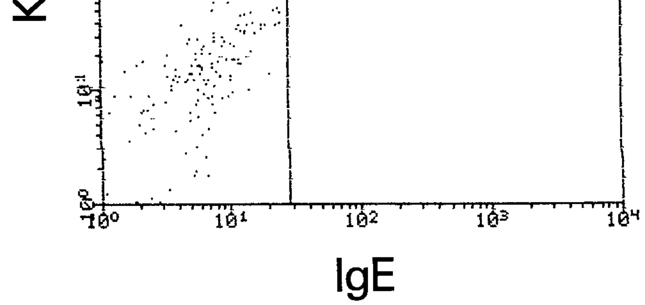

Figure 1. Peritoneal mast cells bind K-1. Mast cells were purified from the peritoneal cavities of CBA/J mice. Cells were stained with IgE/FITC–anti-IgE and with biotinylated K-1/SA-PE and analyzed on a FACScan®.

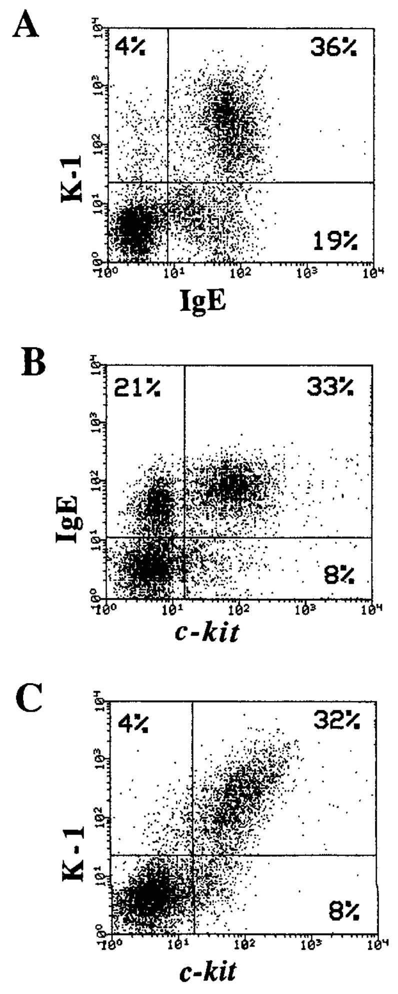

Figure 2. K-1 and anti-c-*kit* stain a similar population of FcεR^{pos} cells from short-term cultures of bone marrow cells in IL-3. Bone marrow cells were cultured in IL-3 for 10 d. The resultant cells were stained with (*A*) biotinylated K-1/SA-PE and IgE/FITC–anti-IgE; (*B*) IgE/PE–anti-IgE and FITC–anti-c-*kit*; (*C*) biotinylated K-1/SA-PE and FITC–anti-c-*kit*.

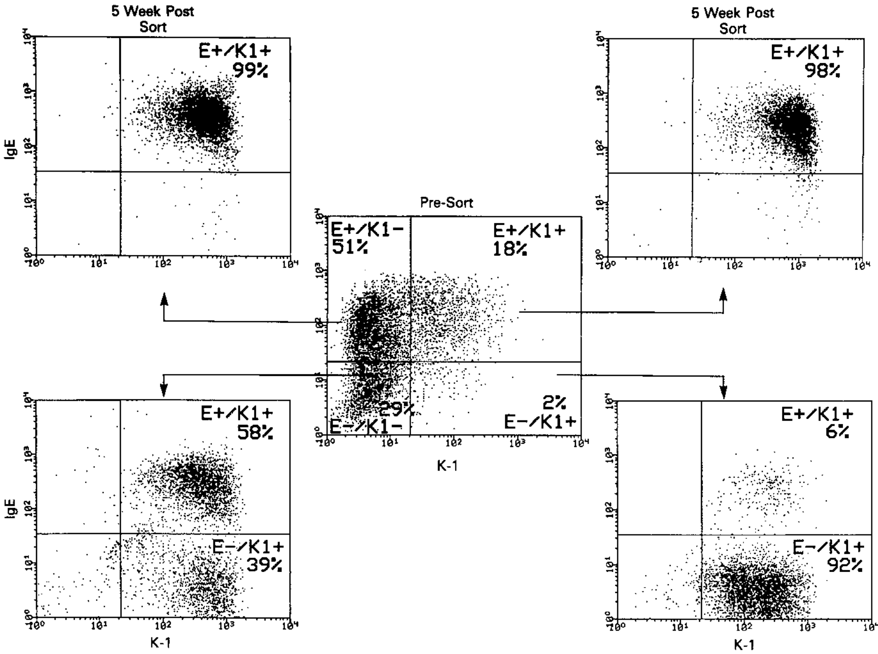

Figure 3. The p161^{pos}/FcεR^{neg} phenotype is stable. Bone marrow cells cultured for 10 d in IL-3 were stained with K-1/FITC–anti–hamster IgG and IgE/PE–anti-IgE. Four cell populations, as indicated in the figure, were purified by sorting and were cultured in IL-3 for an additional 5 wk. Each cell population was reanalyzed by staining, as described above. The central two-color dot plot represents the cell population before sorting. The four other dot plots represent the individual cell populations after the additional 5-wk culture in IL-3.

was precleared twice by incubation with protein A–Sepharose beads and once with protein A–Sepharose beads to which a control hamster mAb had been bound. The residual supernatants were incubated with K-1 bound to protein A–Sepharose beads. A major 161-kD band was observed (data not shown). Consequently, we have designated this molecule p161.

Short-term Cultures of Bone Marrow Contain Both p161^{neg}/FcεR^{pos} Cells and p161^{pos}/FcεR^{pos} Cells. BALB/c bone marrow cells were placed into culture with IL-3 for 21 d. The great majority of these cells expressed FcεR. All FcεR^{pos} cells were p161^{pos} and had a morphology consistent with that of mast cells (data not shown). Bone marrow cells that had been cultured for 10 d with IL-3 had a more heterogenous pattern; 55% were FcεR^{pos}. Of the FcεR^{pos} cells, ∼65% also stained positively with K-1 (Fig. 2 *A*). Interestingly, ∼10% of the cells that stained with K-1 were FcεR^{neg}.

Two-color analysis of FcεR and c-*kit* (Fig. 2 *B*) revealed a coexpression pattern similar to that of FcεR and p161. Furthermore, a comparison of staining with K-1 and anti–c-*kit* revealed that cells were generally doubly positive or doubly negative (Fig. 2 *C*). Thus, FcεR^{pos} cells can be subdivided into p161^{pos} and p161^{neg} cells; FcεR^{pos} cells that are p161^{pos} are also c-*kit*^{pos}.

Bone marrow cells cultured with IL-3 for 10 d were sorted into populations that were p161^{neg}/FcεR^{pos} or p161^{pos}/FcεR^{pos} and stained with alcian blue/safranin red. The p161^{pos}/FcεR^{pos} cells displayed a uniform morphology. They were relatively large with monomorphic nuclei; most expressed alcian blue–positive granules, consistent with their being mast cells (data not shown). The p161^{neg}/FcεR^{pos} cells were more heterogenous, with many relatively small cells with lobulated nuclei. Many of these cells contained very small alcian blue–positive granules. These results are consistent with the presence of substantial numbers of basophils in the p161^{neg}/FcεR^{pos} cell population. Thus, p161 expression can be used to distinguish mature mast cells from basophils.

p161^{pos}/FcεR^{neg} Mast Cells Can Be Grown from Normal Bone Marrow Cells. BALB/c bone marrow cells cultured with IL-3 for 10 d were stained with IgE/FITC anti-IgE and with biotinylated K-1/SA-PE and sorted into four populations (Fig.

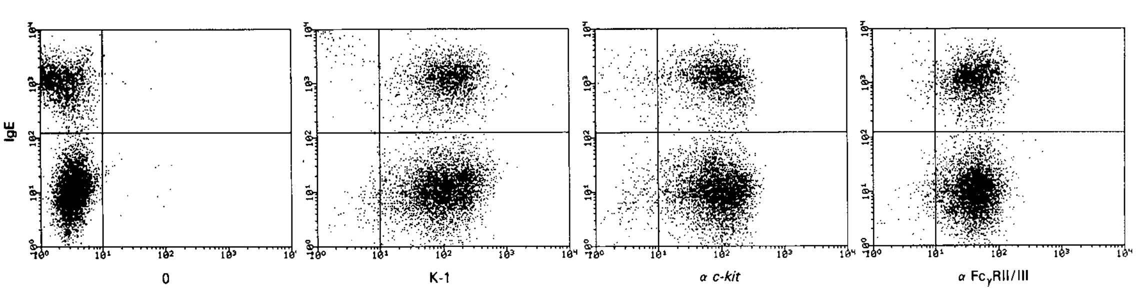

Figure 4. p161^{pos}/FcεR^{neg} cells express c-*kit* and FcRγII/III. The cell population illustrated in Fig. 3 that contained both p161^{pos}/FcεR^{neg} and p161^{pos}/FcεR^{pos} cells after 5 wk of culture were stained with IgE/PE-anti-IgE plus nothing, K-1/FITC–anti–hamster IgG, FITC–anti–c-*kit*, or FITC-2.4G2.

p161pos/FcεRneg Cells

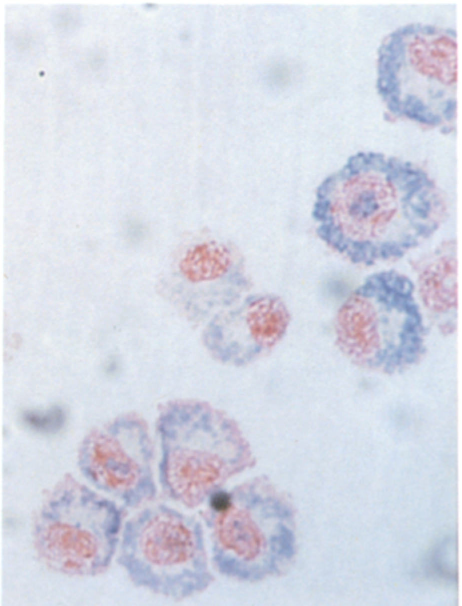

Figure 5. Alcian blue/safranin red staining of long-term cultured p161pos/FcεRneg cells. Cytospins of p161pos/FcεRneg cells that had been cultured for 10 mo in IL-3 were stained with alcian blue/safranin red. ×630.

3). As noted above, there was a small population of p161pos/FcεRneg cells (2% of total; 10% of p161pos cells). Each cell population was cultured for an additional 5 wk in IL-3 and then reanalyzed by two-color staining for FcεR and p161 expression. The p161pos/FcεRIpos cells retained their phenotype during the 5-wk culture in IL-3, as did the p161pos/FcεRneg cells. The p161neg/FcεRpos cells gave rise to p161pos/FcεRpos cells, indicating that the p161neg population contained precursors for p161pos cells. Purified double-negative cells cultured for 5 wk gave rise to a mixture of p161pos/FcεRpos and of p161pos/FcεRneg cells. These results indicate that mast cell precursors may be found among the p161neg population in short-term bone marrow cultures and that stable populations of p161pos/FcεRneg cells can be propagated, either as independent cell populations or growing together with p161pos/FcεRpos cells.

Characterization of Cultured p161pos/FcεRneg Mast Cells. 5 wk after sorting, the p161pos/FcεRneg cells were further analyzed. Expression of c-*kit* and of FcγRII/III on both p161pos/FcεRneg and p161pos/FcεRpos was examined using a cell population that had been derived by culture of double-negative cells in IL-3 (Fig. 4). Both the FcεRpos and the FcεRneg cells were p161pos, c-*kit*pos, and FcγRII/IIIpos. Purified p161pos/FcεRneg cells obtained by sorting of cells with this phenotype (see Fig. 3) also expressed c-*kit* and FcγRII/III. The expression of c-*kit* and FcγRII/III on p161pos/FcεRneg cells has been verified by studying resorted populations that have been grown in culture for more than 2 yr. When stained with alcian blue, pure p161pos/FcεRneg cells had a morphology consistent with that of mast cells (Fig. 5). These cells expressed small amounts of histamine when compared to bone marrow cells cultured in IL-3 for 11 d; however, their histamine content was slightly greater than that of comparably cultured p161pos/FcεRpos cells. When stimulated with ionomycin, p161pos/FcεRneg cells secreted IL-3 (Table 1).

Table 1. *IL-3 Production and Histamine Content of FcεRIneg/p161pos and FcεRIpos/p161pos Cells*

IL-3 production in response to 1 μM Ionomycin (three separate experiments)

Cells	IL-3
	U/10,000 cells
IL-3–cultured bone marrow cells*	6.7; <1; 8.0
FcεRIneg/p161pos cells§	4.0; 4.0; >15
FcεRIpos/p161pos cells‡	8.0; 2.7; >15

Histamine content

Cells	Histamine content
	ng/10⁶ cells
IL-3–cultured bone marrow cells*	236
FcεRIneg/p161pos cells‡	21
FcεRIpos/p161pos cells§	6.5

* Bone marrow cells were cultured in WEHI 3–conditioned medium for 11 d.

‡§ Bone marrow cells were cultured in WEHI 3–conditioned medium for 7 d. FcεRIneg/p161pos and FcεRIpos/p161pos cells were purified by cell sorting and cultured 7 d and repurified by a second cell sorting. They were cultured for an additional 14 d and analyzed.

Discussion

Among hematopoietic cell lines, p161 is found on both freshly isolated and cultured mast cells. It is found on some macrophages but is absent from cells of other hematopoietic lineages. In particular, FcεRIpos cells that lack c-*kit* and are enriched in cells with basophil-like morphology are p161neg. Expression of p161 on macrophages appears to be associated with the differentiated state of the cells. The great majority of resident Mac-1pos cells in spleen and blood are p161neg, and the small percent of such cells that are p161pos stain weakly with K-1. By contrast, macrophages found in peritoneal exudates of mice injected with thioglycollate are very strongly positive for p161, and macrophages derived by culture of bone marrow cells in CSF-1 are uniformly p161pos.

The detection of a cell population, derived by culturing bone marrow cells in an IL-3–enriched supernatant, that expresses p161 but fails to stain with IgE/FITC–anti-IgE is consistent with the existence of p161^{pos}/FcϵRI^{neg} mast cells. These cells stain with 2.4G2, and thus express FcγRII and/or FcγRIII, and with anti–c-*kit*, consistent with their being mast cells. p161^{pos}/FcϵRI^{neg} cells express messenger RNA for FcϵRI α and β chains but are strikingly deficient in message for FcϵRI γ chain, further consistent with their being FcϵRI^{neg} mast cells (11). Cultured macrophages, which may be p161^{neg} or p161^{pos}, fail to express mRNA for either α or β chains of FcϵRI, strongly arguing that the cultured p161^{pos}/FcϵRI^{neg} cells are not macrophages (11). Efforts to find naturally occurring populations of these cells are underway. Our ability to routinely isolate these cells by flow cytometric sorting from 7–10-d cultures of bone marrow cells grown in IL-3 strongly suggests that they are a physiologic population rather than an aberrant set of cells that have extinguished the expression of FcϵRI γ chain in the course of maintenance in tissue culture.

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