Establishment in Culture and Characterization of a Strain With Mast Cell and Monocytic Properties From the Bone Marrow of a Child With Diffuse Cutaneous Mastocytosis

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Bone marrow was isolated from a child with congenital mastocytosis. Upon prolonged in vitro culture, initially in the presence of interleukin-3 (IL-3), a population of relatively large fusiform, strongly adherent cells grew out plus a subpopulation of smaller nonadherent cells. The morphology of the adherent cells was not typical of fibroblasts, epithelial cells, nor of standard hematopoietic cell types, whereas the morphology of the nonadherent cells resembled mast cells. Neither cell type required the presence of IL-3 nor a feeder layer of fibroblasts for continued growth. Attempts to isolate the two populations were unsuccessful. This cell strain comprised of both cell populations has been termed human bone marrow-derived mastocytosis cells (HBM-M). These cells were found to possess some of the cytochemical, ultrastructural, and surface phenotypic features of degranulated mast cells. They reacted with the mast cell marker, monoclonal antibody YB5.B8, but not with the basophil specific monoclonal antibody BAp-1 and released the inflammatory mediators histamine, leukotriene C4, prostaglandin D2, and platelet-activating factor constitutively. This release was not potentiated by immunologic- or nonimmunologic-activating stimuli. In addition, they exhibited cytochemical and surface phenotypic features of monocytes. Our results indicate that a population of abnormal proliferative cells exist in the marrow of this patient; that these cells may be responsible for the patient's pronounced systemic proliferation of mast cells and the associated symptoms; and that the cell's mast cell, monocyte properties may be indicative of a common bone marrow-derived mast cell/monocyte precursor.

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Mastocytosis is the collective name for a spectrum of clinical abnormalities in which there is mast cell hyperplasia generally involving extensive tissue infiltration by mast cells and the associated release of chemical mediators by those cells. Mastocytosis is classified as systemic when there is involvement of internal organs and/or bone marrow. Mastocytosis may be broadly classified into juvenile- and adult-onset varieties, separated by major differences in their prognoses and age of onset. Diffuse cutaneous mastocytosis is a form of mastocytosis that is relatively rare and usually presents in early infancy. It is characterised by extensive infiltration of the skin, erythroderma, blistering, and pruritis. There may also be dissemination to internal organs such as the gastrointestinal tract, liver, spleen, lymph nodes, and bone marrow. The nature of the cellular abnormality involved in this condition is unknown.

Both in the mouse and human recent experimental evidence from in vivo and in vitro culture systems has indicated the hematopoietic origin of mast cells, the bone marrow being the apparent site of production. Following the early report by Ginsburg and Sachs of mast cells arising in suspension culture, there has been a variety of evidence for the derivation of mast cells. However, the precise lineage derivation of mast cells is still obscure with reports citing evidence for the relationship of mast cells to granulocytes, monocytes, erythrocytes, lymphocytes, megakaryocytes, basophils, and eosinophils. There is a degree of heterogeneity with different mast cell populations in both mice and humans.

Mast cells are IgE receptor-bearing, metachromatic staining cells that, when stimulated, release a range of inflammatory mediators, in particular, histamine, prostaglandin D2 (PGD2), and leukotrienes. Studies of the normal function of mast cells (including mediator release and the role of mast cells in pathologic disorders in humans) have been hampered by the difficulty in isolating pure preparations of these cells and establishing them in long-term culture. Two previous reports have described the growth of cells with mast cell characteristics in long-term culture. Horton and O'Brien grew cells from the bone marrow of a patient with mastocytosis in the presence of a fibroblast-adherent overlay that also derived from the bone marrow. However, these cells were dependent on the presence of the overlay and attempts to culture them separately were unsuccessful. In addition, Butterfield et al have described the establishment of leukemic cells resembling immature mast cells in culture using a leukopheresis sample from a patient with mast cell leukemia.

We have previously described a case of diffuse cutaneous mastocytosis with systemic involvement in which high levels of immunoreactive PGD2 and histamine as well as platelet-activating factor (PAF) and a novel endogenous inhibitor of platelet aggregation were found in the blister fluid. In the present study we sought to isolate and grow cells from the bone marrow of this patient. We have succeeded in establishing a cell strain from the patient's marrow comprised of large fusiform adherent cells and a subpopulation of

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nonadherent cells that appeared to be derived from the adherent cells. Both cell types exhibited certain mast cell as well as monocytic characteristics. This cell strain is neither transformed nor tumorigenic in nude mice. Our experiments provide evidence for an atypical though non-tumorigenic cell population in this patient with mast cell and monocyte properties. In this report we describe aspects of the cytochemistry, surface phenotype, ultrastructure, and mediator release of these cells.

**MATERIALS AND METHODS**

The patient. The patient HB (a male neonate) has been previously described in detail. In summary, the patient presented with diffuse cutaneous mastocytosis that developed within 48 hours of birth. A skin biopsy showed an intense infiltration of mast cells (which stained with toluidine blue) throughout the papillary and reticular dermis. A diagnosis of diffuse cutaneous mastocytosis with systemic involvement was made. By 20 weeks of age, through the use of appropriate treatment (sodium cromoglycate and clemastine followed by prednisone, ketotifen, and cimetidine) the systemic symptoms had improved; however, diffuse cutaneous infiltration remained. The patient had 1% to 3% mast cells in his bone marrow. At 7 months of age, a 1.5-ml bone marrow sample was taken for cell culture and at a later time (2 years and 2 months) an explant was cultured from a skin nodule. At both times Ethics Committee approval was obtained.

Cell culture. All cell culture was conducted in either RPMI 1640 or Dulbecco’s modified Eagle’s medium (DMEM; Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated (56°C, 30 minutes) fetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Australia) plus penicillin (500 IU/mL) and streptomycin (500 µg/mL). Unless otherwise noted, the medium based on RPMI was routinely used and will be referred to as growth medium (GM) throughout the supplements included phytohemagglutinin (PHA)-stimulated human T-lymphocyte-conditioned medium (PHA-TLCM); WEHI-3B-conditioned medium (WCM); conditioned medium from the human bladder cell line 5637 cell line (5637 CM); pokeweed-stimulated murine spleen cell-conditioned medium (PM-SCCM); and murine macrophage-conditioned medium (MCM). PHA-TLCM and WCM were used because they contain the growth factors interleukin-3 (IL-3) (PHA-TLCM and WCM) and IL-4 (PHA-TLCM). PHA-TLCM also contains granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, and interferon γ. 5637 CM contains IL-1, granulocyte-CSF (G-CSF), GM-CSF, and an “IL-3 like” activity. PM-SCCM contains IL-3 and IL-4, as well as G-CSF and GM-CSF. Other components tested were insulin (250 µU/L) and β-mercaptoethanol (β-ME) (1·10⁻⁴ mol/L). All cells were cultured in a humidified environment with 5% CO₂ at 37°C. When adherent cells were subcultured this was accomplished using trypsin (0.05%); EDTA (0.02%).

Culture of mastocytosis cells from the marrow of the patient. A 1.5-ml bone marrow fine needle aspirate was obtained from the infant at 7 months of age. This yielded a total of 3·10⁶ nucleated cells. All cells were placed in culture at 3·10⁶ nucleated cells per 60-mm dish in RPMI 1640 (GIBCO) growth medium (GM) with combinations of the following supplements: heat-inactivated FCS; WCM; PHA-TLCM; insulin; and β-ME. As previously noted, these conditioned media contain the growth factors IL-3 (WCM and PHA-TLCM) and IL-4 (PHA-TLCM) reported to promote mast cell growth. No Ficoll purification was conducted nor were the red blood cells (RBCs) lysed initially to attempt to maintain the total bone marrow population and to avoid cell clumping. However, the day after seeding, the RBCs were lysed by three representative plates and all plates were monitored daily for growth and viability for a further 5 days.

**Phenotypic characterization.** Cells were harvested by the use of trypsin and concentrated onto slides by cytocentrifugation or grown directly on eight-chamber glass slides (Lab-Tek; Nunc, Inc, Napierville, IL). Slides were then stained with May-Grünwald/Giemsa and metachromasia was assessed by staining with 1% toluidine blue in methanol at pH 2.5 or 6.0, 0.5% alcian blue in 0.3% acetic acid and 0.1% safranin in 1% acetic acid. Other cytochemical stains were performed using standard methods and appropriate hematopoietic cells as well as human umbilical vein endothelial cells (HUVEC) and 3T3 mouse fibroblasts were used as positive and negative controls.

Immunocytochemistry was performed using the following primary antibodies: YB5.B8 and Sal-2 (provided by Dr L. Ashman, University of Adelaide, Adelaide, Australia); Bsp-1 (provided by Dr M. Bodger, Christchurch Hospital, Christchurch, New Zealand); H4, a monoclonal antibody (MoAb) against tryptase supplied by Dr L.B. Schwartz (Departments of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA); CD2 (Dakopatts A/S, Denmark); CD11b (purified from hybridoma line M1/70.15.11.5.HL, American Type Culture Collection, Rockville, MD); CD11c (Becton Dickinson, Knoxville, Victoria, Australia); CD13 (Coulter Immunology, Hialeah, FL, and hybridoma supernatant WM-15 from Dr K. Bradstock, Westmead Hospital, Sydney, Australia); CD14 (hybridoma supernatant FMC-17 from Dr Heddy Zola, Flinders’ Medical Centre, Adelaide, Australia); CD23 (Coulter Immunology); CD33 (Coulter Immunology and hybridoma supernatant WM-53 from Dr K. Bradstock); CD34 (Coulter Immunology); HLA-DR (Dakopatts); CD19, CD25, CD41, CD45 (Dakopatts); CD11b (Becton Dickinson); von Willebrand factor (Dakopatts); neutrophil elastase, fibronectin, vimentin (Dakopatts); serotonin (Seralabs, United Kingdom); F4/80 (Dr D. Hume, University of Queensland, Queensland, Australia). The secondary antibodies used were goat antimouse, rabbit antirabbit, or rabbit antirat IgGs, conjugated to either horseradish peroxidase or alkaline phosphatase. Standard techniques were used throughout.

Binding of fluorescein isothiocyanate (FITC)-conjugated avidin (Becton Dickinson, Mountain View, CA) was demonstrated using a published method and a purified monoclonal human IgE (obtained from the serum of a patient with an IgE myeloma, a gift from Dr G. Layton) was used in the detection of binding of IgE. Slides were fixed for 10 minutes in acetone, incubated 30 minutes at 37°C with 50 µg IgE in phosphate-buffered saline (PBS) rinsed three times and then incubated 30 minutes at 37°C with FITC-rabbit anti-human IgE (Dakopatts). Slides were rinsed in PBS and examined by epifluorescence using a Zeiss photomicroscope. Nonadherent cells were pelleted (10 minutes, 50g) and smears were air-dried before staining as described above.

**Ultrastructural morphology.** A confluent 25-cm² tissue culture flask was flooded with 2.5% gluteraldehyde in 0.1 mol/L cacodylate buffer (pH 7.3) and fixed for 1 hour. Subsequent processing and embedding in Spurr’s resin was performed in the flask with cells still attached to the culture surface. After polymerization, the flask was cut open with a band saw and the cell-coated surface cut into small pieces for sectioning on a Reichert-Jung Ultracut E ultramicrotome (FDR). Following sectioning, the material was picked up on uncoated copper grids, stained with lead citrate and uranyl acetate, and viewed in a Philips 110 electron microscope (Holand). In addition, nonadherent cells shed from the monolayer during culture were pelleted in a 1.5-ml microtuge tube, resuspended in 2.5% gluteraldehyde, and processed as above in a cell pellet. Following removal from the culture surface with trypsin,
adherent cells were also fixed in 2.5% osmium tetroxide and set in 1 to 2 drops of 4% agar. The cell and agar pellet was cut into 1-mm cubes and stained with 2% uranyl acetate before dehydration and polymerization in Spurr’s resin.

**Release of inflammatory mediators.** Cells were grown to confluence in 6-well plates or 25-cm² flasks as required and washed three times with either Hank’s balanced salt solution containing calcium and magnesium (HBSS; Gibco) or HEPES-buffered saline (125 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L glucose, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂), pH 7.4. Cells were incubated in 1 mL of buffer containing 0, 2, 10, 40, or 100 μmol/L calcium ionophore A 23187 (Calbiochem, La Jolla, CA) for 40 minutes at 37°C. A stock solution of 4 mmol/L A 23187 was prepared in dimethyl sulfoxide (DMSO) and diluted in buffer immediately before addition to the cells. Cell viability at the completion of incubation was assessed using trypan blue exclusion. Supernatants from individual incubations were analyzed for histamine, eicosanoids, PAF, and a platelet aggregation inhibitor as described below.

Histamine content was measured in duplicate 200-μL aliquots, using a histamine radioimmunoassay kit (Pharmacia, Uppsala, Sweden). Supernatants from 6-well plates were extracted into 80% ethanol, and the protein precipitate was removed by centrifugation (2000g; 10 minutes at 4°C). The solvent was evaporated in a SpeedVac concentrator (Savant Instruments, Farmingdale, NY) and reconstituted in 450 μL histamine radioimmunoassay buffer. Buffer treated in an identical manner served as a negative control. The limit of detection of histamine by this method was 1 ng.

For the determination of eicosanoid levels, supernatants from the stimulation experiments described above were extracted with ethanol, evaporated, reconstituted in 220 μL radioimmunoassay buffer, and assayed in duplicate using a tritiated radioimmunoassay kit for the estimation of PGD₂ (Amersham, UK). The limit of detection was 6 pg PGD₂, and results were expressed as immunoreactive PGD₂ equivalents. Cross-reactivity of the PGD₂ antiserum at 50% displacement was stated by the manufacturer to be: PGD₂ (100%), PGJ₂ (7%), PGF₂α (0.4%), 9α,11β-PGF₁α (0.12%), tromboxane B₂ (0.5%), other F-ring compounds <0.02%. To verify that the product measured was authentic PGD₂, certain supernatants were subjected to reverse phase–high-performance liquid chromatography (RP-HPLC) to separate the various prostaglandins and leukotrienes before radioimmunoassay. Samples were applied to an ODS Ultrasphere 4.6 mm × 25 cm column (Beckman Instruments, San Ramon, CA) and eluted at 1 mL/min with a stepwise gradient of methanol in 0.1% acetic acid in water, pH 5.6, as previously described. Tritium-labeled 6-keto-PGF₁α, thromboxane B₂, PGD₂, PGE₂, PGE₁, leukotriene (LT) C₄, LTD₄, LTE₄, LTB₄, 15-, 12-, and 5-hydroxyeicosatetraenoic acid (5HETE), 12-HETE, 15-HETE, N-acetylseryl-leucyl-prolinyl-taurine (SPLT, DE) as well as unlabeled 9α, 11β-PGF₁α (Cayman Chemical Co, Ann Arbor, MI) extracted from HBSS in a manner identical to the samples used to calibrate the column each day. The column eluate was monitored with a 990+ diode array detector (Waters Associates, Millford, MA) and radioactivity was monitored with a FlowOne Beta, model CT detector (Radiomatic Instruments & Chemical Co, Tampa, FL). Recovery of leukotrienes was improved by periodic washing of the column with 0.5% EDTA in 10% methanol. When radioimmunoassay was to be performed, eluted fractions (2 mL) with retention times equivalent to prostaglandins (0 to 28 minutes) were evaporated and tested for immunoreactivity in the PGD₂ radioimmunoassay. Fractions with retention times corresponding to leukotrienes (30 to 60 minutes) were evaporated and assessed for immunoreactivity with a rabbit antiserum that binds the sulfidoepoxide leukotrienes LTC₄, D₄, and E₄ with equal affinity. The limit of detection was 1 pg LTC₄, and results are expressed as immunoreactive LTC₄ equivalents.

To determine the ability of cells to metabolize arachidonic acid they were grown to confluence in 25-cm² flasks and washed three times with 1 mL HBSS. The cells were then labeled with 2.5 μCi [³H]-arachidonic acid (Amersham, TRK-S08, specific activity 205.1 Ci/mmol) in 1 mL HBSS containing 0.01% fatty acid free bovine serum albumin for 2 hours at 37°C. After washing three times with 1 mL HBSS, the cells were incubated for 15 minutes in HBSS with or without 0.5 μmol/L A 23187. The supernatants were recovered and the cells collected into 1 mL fresh HBSS by mechanical means. For studies using immunologic stimulus, [³H]-arachidonic acid-labeled cells were washed three times with calcium- and magnesium-free HBSS (HBSS-CMF) and incubated for 60 minutes with 30 μg human IgE in 1 mL HBSS-CMF. The cell layer was washed once with 0.5 mL HBSS-CMF and then incubated with rabbit antihuman IgE (ε-chain-specific) antisera (Dakopatts). Both cells and supernatants were extracted with ethanol and subjected to RP-HPLC as described above, and the eluate monitored for radioactivity. Cultured HUVEC treated in an identical manner served as controls.

As an additional check to assess the capacity of the cells to metabolize exogenous arachidonic acid, confluent 25-cm² flasks were washed and incubated with 1 μmol/L unlabeled arachidonic acid (Cayman) for 60 minutes at 37°C in 1 mL HBSS. Cells were removed by mechanical means and combined with the supernatant. One half of each sample was extracted with 80% ethanol, evaporated, and subjected to RP-HPLC separation before radioimmunoassay for PGD₂, and the sulfidopeptide leukotrienes. Cell-free samples and cultured HUVEC treated in an identical manner served as controls.

**Identification of PAF and a platelet-aggregation inhibitor.** To assess PAF activity, samples were extracted for lipids according to the method of Bligh and Dyer. Extracts were evaporated and subjected to HPLC to separate PAF and platelet-aggregation inhibitor activity from other phospholipid classes as previously described. A Resolve 5-μm silica column (Waters) was eluted with chloroform:methanol:water (60:50:5) at 1 mL/min and 0.5 mL fractions collected. Eluted fractions were evaporated to dryness and reconstituted in 100 μL PBS containing 2.5 mg/mL bovine serum albumin and assayed for PAF activity using a platelet-aggregation bioassy. Each reconstituted fraction was added to 450 μL human platelet-rich plasma (2 × 10⁹ platelets/mL) in a stirred aggregometer cuvette and platelet aggregation monitored. The degree of platelet aggregation (PAF activity) recovered from the cell supernatant was compared with authentic PAF (Sigma Chemical Co, St Louis, MO) and expressed as nanograms PAF (C-16:0) equivalents. Platelet-aggregation inhibitor activity was assessed by inhibition of PAF-induced platelet aggregation. Platelet-rich plasma was reincubated for 60 seconds with HPLC fractions (treated as above) before the addition of authentic PAF and inhibition of platelet aggregation assessed.

**Cell cloning.** Two methods were used to attempt to clone the cells cultured from the patient’s marrow: growth in semi-solid medium and limiting dilution. In the first method, cells (5 × 10⁵ to 1 × 10⁶/dish) were plated in 0.85 mL soft (0.3%) agar (Difco) containing DMEM and 20% FCS over a 2.5 mL hard (0.5%) agar (Difco) feeder layer containing DMEM, 20% FCS, and WCM (final concentration 30%) in 35-mm petri dishes. In addition to WCM, other CM used were: the cells own CM (30% final concentration) and PM-SCCM (30% final concentration). In all cases, a transformed growth factor-independent murine monocytic cell line (EM myel) was used as a positive control for the formation of colonies. The dishes were examined microscopically the day after seeding, then once a week for 3 weeks. For limiting dilution, the number of cells seeded in microwells was 1 × 10⁴ down to 1 × 10¹ cells/well by serial factor-of-ten dilutions. These dilutions were set up with WCM alone or WCM plus self-CM.
either in microwells or in Millicell-HA culture plate inserts (Millipore Products Division, Bedford, MA) over a feeder-layer of mastocytosis cells in microwells. The latter well inserts were examined periodically using the standard fixation and hematoxylin staining procedure recommended (Millipore Products Division).

Karyotypic analysis. Cells in culture were blocked in metaphase by adding colchicine to a final concentration of 1 μg/mL for 3 to 6 hours. The cells were harvested using trypsin, washed in calcium- and magnesium-free PBS (CMF-PBS) by centrifugation and subjected to standard treatment with hypotonic solution and fixation. Slides were made from these fixed preparations and analyzed microscopically (according to ISCN, 1985) after chromosome G-banding using a modification of Seabright's trypsin technique.

Reverse transcriptase assay. To determine whether the mastocytosis cells were actively producing retrovirus, reverse transcriptase activity was assayed in the cellular supernatant. The method used was based on a published method\(^6\) and used solutions containing either Mn\(^{2+}\) or Mg\(^{2+}\), because retroviruses vary in the dependence of their reverse transcriptase on these cations.

Tumorigenicity in nude mice. To determine whether the mastocytosis cells were tumorigenic, 5 \(\times 10^5\) and 1 \(\times 10^7\) cells were injected subcutaneously into a total of eight female BALB/c nu/nu (nude) mice. Cells were harvested by trypsinization, washed in PBS, counted, and resuspended in a drop of PBS for injection. Before injection they were kept on ice to prevent cell clumping. Tumorigenicity was monitored by palpation at the site of injection. Mice were also examined internally when killed at 6 to 10 months following injection.

RESULTS

Establishment of cells from the patient's marrow in long-term culture. During the initial period of observation, a relatively normal appearance for human bone marrow culture was observed; monocytes, neutrophils, eosinophils, lymphocytes, blasts, as well as granulated mast cells and basophils were present. By day 6 all RBCs that had lysed though those cultures treated at day 2 (plates 5, 6, and 8, Table 1) had the greatest outgrowth of metachromatically staining granulated cells (Table 1). These three cultures (plates 5, 6, and 8, Table 1), containing WCM, PHA-TLCM, or both, were maintained by scraping and splitting at 1:2 on day 6, and a further 1:3 on day 7. At this time the relative importance of WCM, PHA-TLCM, insulin, and β-ME was determined. There was no significant difference in the proportion of metachromatically staining granulated cells among variations in these conditions.

By day 9, adherent fusiform cells were observed in the cultures in addition to the metachromatically staining granulated cells that floated freely or in clusters in the supernatant medium. The adherent cells appeared in significant numbers at approximately the same time that the number of granulated cells started to decrease in number and became irregular in shape (around day 14, Fig 1). Initially, these fusiform adherent cells were regarded as contaminants. To select against their presence, the cultures were passed by pipetting only the supernatant. However, this procedure did not remove the adherent cells, which appeared in increasing numbers through day 25 and following, suggesting that they may have been continuously generated from the supernatant cells.

From approximately day 14 it was also noted that the adherent cells appeared to be producing a subpopulation of nonadherent cells. Following subculture of only the adherent cell population, it was found that if the supernatant medium, containing the floating cells, was removed and replaced with fresh medium, the floating cells reappeared in the same proportion as previously observed (approximately 5% of the total cell population), indicating that the new nonadherent population was generated by the adherent cells. The derivation of the cells is outlined diagrammatically in Fig 1.

From this point on the cells were passaged by centrifugation of the supernatant medium at 1,000 rpm for 5 minutes to collect the nonadherent cells, which were then pooled with the adherent cells removed from the original culture vessel by treatment with trypsin-EDTA for 15 minutes. This procedure was the one routinely used and by 2 months in culture the cells had a stable morphology. It was at this time that the bone marrow-derived cells were designated human bone marrow-derived mastocytosis cells (HBM-M). The morphology and growth pattern of the HBM-M strain consisting of adherent fusiform cells and smaller nonadherent cells is shown in Fig 2A. The adherent cells contained some cytoplasmic granules and vacuoles and released granular material into the culture medium, which was

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*This refers to the relative growth of nonadherent metachromatically staining cells. Scoring was conducted at day 6 from seeding (see text).
DAY 0
Bone Marrow Fine Needle Aspirate

Total Blood Cells to Culture with IL-3

Relatively Normal Appearance for Human Bone Marrow Culture (Monocytes, Neutrophils, Eosinophils, Lymphocytes, Metachromatically Staining Granulated Cells)

DAY 6

DAY 9
Appearance of Adherent, Fusiform Cells

DAY 14
Decreasing Number of Granulated Cells
Increasing Number of Adherent Cells

DAY 25
Observation that Adherent Cells Producing Non-Adherent Granulated cells

Fig 1. Establishment of the HBM-M strain. The figure summarizes the observations made in establishing the HBM-M cell strain. At day 0, a bone marrow fine needle aspirate was taken from the patient HB and the cells cultured. Initially, the culture was observed to be relatively normal in appearance with monocytes, neutrophils, eosinophils, lymphocytes, and metachromatically staining granulated cells present. However, by day 9 the appearance of large, adherent, fusiform cells was noted, and by day 14 there were increasing numbers of these cells present. This increase corresponded with a decrease in the number of granulated cells. By day 25, these fusiform cells had overtaken the culture and at relatively low frequency appeared to be producing a new subpopulation (approximately 5%) of somewhat granulated nonadherent cells.

visible using light microscopy. The release of granules did not appear to be due to cell death. Both populations of cells were demonstrated to be viable by the exclusion of trypan blue stain (0.2% wt/vol in PBS).

Growth behavior and morphology of the HBM-M cells. The HBM-M strain was comprised of a heterogeneous cell population consisting of the spreading adherent fusiform cells and a nonadherent population of smaller round cells comprising approximately 5% of the total population. Attempts to grow the nonadherent cells in the absence of the adherent cell population were unsuccessful. They survived without detectable proliferation for 24 to 48 hours but then died, despite the addition of various growth factors including WCM (30%), PHA-TLCM (30%), 5637 CM (30%), MCM (30%), insulin (250 U/L), in the presence or absence of β-ME (10⁻⁴ mol/L). This result indicated that the growth and viability of the nonadherent cells was dependent on the adherent cell population.

The HBM-M cells were relatively slow growing with a doubling time of 3 to 4 days when passage nos. 12 to 22 were used. For cells of this passage number the cell density at confluence was approximately 3 × 10⁶ cells/cm² and they were routinely split at 1:3 to give a seeding density of approximately 1 × 10⁴ cells/cm². The maximum split ratio that could be used was 1:5. Below this ratio the cells grew more slowly and would not reach confluence. These properties were not altered by the addition of 5367 CM, which contains IL-1, G-CSF, GM-CSF, and an “IL-3 like” activity; PM-SCCM, which contains IL-3, IL-4, G-CSF, and GM-CSF; nor MCM. Attempts to clone the cells (described below) were unsuccessful; this appeared to be related to their inability to grow at low cell numbers. The HBM-M cells were significantly slower in their growth after approximately 28 passages, and by 30 passages the cells had clearly senesced. For this reason, all experiments were conducted with cells of passage number less than 28 (generally 12 to 22).

Attempts at cloning HBM-M were performed both in semi-solid medium and by limiting dilution. In the former case, cells were seeded in soft agar over a hard agar feeder layer with or without CM. In no case were colonies obtained. Cloning by limiting dilution was also unsuccessful even when WCM with or without HBM-M CM was added. Once there were less than 10 cells per well the cells did not
adhere to the culture vessel and subsequently died. In an attempt to overcome the lack of growth of HBM-M cells at low density, feeder layers of HBM-M cells under Millicell HA well inserts were used to provide self-CM. The rationale was that the underlay would both provide a continuous supply of factors from the HBM-M cells as well as physical proximity (though not direct contact) with growing HBM-M cells. However, this procedure too with or without WCM and HBM-M CM was unsuccessful.

The large, adherent population of HBM-M cells had several elongated cytoplasmic processes, abundant vacuolation, and a round nucleus with multiple, prominent nucleoli. The nonadherent cell population were more typical of the appearance of mast cells and tended to contain more granular material visible by May-Grünwald/Giemsa, toluidine blue, and alcian blue staining. Staining with May-Grünwald-Giemsa showed the heterogeneity of the cell population; some showed intracellular granules with the majority devoid of granules (Fig 2B and C).

When examined in situ by electron microscopy, the adherent HBM-M cells were found to have an elongated appearance with flattened ovoid nuclei, a moderate amount of granular endoplasmic reticulum, and cytoplasmic protrusions. There was some evidence for the apparent budding of smaller cells into the culture medium (Fig 3A). When adherent cells were removed from the culture surface and examined as a pellet, many microvilli were seen. The nonadherent HBM-M cells showed microvilli on the surface, and numerous cytoplasmic inclusions with a granular pattern consistent with the appearance of degranulated mast cells (Fig 3B).

**Cytochemical and immunocytochemical characterization of HBM-M cells.** HBM-M cells stained strongly positive with periodic acid Schiff and both acid and alkaline phosphatases. They stained weakly positive for specific (naphthol AS-D-chloroacetate) and nonspecific (naphthol AS-D-acetate) esterases (Table 2). Apparently due to degranulation, the majority of the cells were devoid of granules and did not stain metachromatically with toluidine blue at pH 2.5 or pH 6.0, nor with alcian blue and safranin. The small nonadherent HBM-M cell subpopulation was morphologically more typical of mast cells than the adherent cell population. A small proportion of the nonadherent cells contained granules, and stained metachromatically with toluidine blue. Some also stained with alcian blue, and the occasional safranin-positive granule was seen. Although the adherent layer of cells was unable to bind conjugated avidin, the nonadherent cells exhibited strong fluorescence when granules were present.

To characterize the immunophenotype of the HBM-M cells, a panel of immunocytochemical stains was used. The results are summarized in Table 3. It can be seen that the cells stained positively for a number of markers. These markers included CD11b, CD11c, CD13, HLA-DR-DP-DQ, F4/80, vimentin, and fibronectin. They also stained positively with the MoAb YB5.B8 (anti-gp150), reported to selectively recognize mast cells in tissue sections. Except for fibronectin, these markers tend to be of mast cell or monocyte/macrophage specificity. Neither cell type reacted with the basophil-specific MoAb, Bsp-1. Other markers, including those for basophils, B and T lymphocytes, erythroid cells, neutrophils, platelets, blast cells, and endothelial cells, were negative. Neither population was stained by the monoclonal anti-tryptase antibody H6. Excluding fibronectin, none of these antibodies exhibited positive staining with the negative controls used, namely MRC-5 human lung fibroblasts, NIH3T3 murine fibroblast cells, and HUVEC. The expression of IgE Fc receptors by these cells was assessed by pretreatment with human IgE and probing with fluorescent-labeled antihuman IgE. In both the adherent and nonadherent HBM-M cells, fluorescence was weakly positive when compared with HBM-M cells processed identically, but omitting incubation with human IgE (Table 3).

**Mediator production of HBM-M cells.** When the release of inflammatory mediators was examined, it was found that HBM-M cells produced significant amounts of histamine, LTC₄, PGD₂, and PAF (Table 4). There was no increase in histamine or immunoreactive PGD₂ release following incubation of the cells with 2, 10, 40, or 100 μmol/L A 23187, indicating that the constitutive synthesis of histamine could not be augmented by calcium ionophore. Cell viability following 40 minutes incubation with these doses of calcium ionophore was 85% to 95%. To ascertain whether this immunoreactivity was due to authentic PGD₂ or its major metabolite 9α,11β-PGF₂, supernatants from unstimulated HBM-M cell cultures in 25-cm² flasks were subjected to RP-HPLC followed by radioimmunoassay (Fig 4). This analysis quantitated the levels of extracellular PGD₂ and 9α,11β-PGF₂ as 115 and 178 pg PGD₂ immunoreactive equivalents per 10⁶ cells, respectively. In addition, there was also a more polar metabolite eluting between 4 and 6 minutes that was equivalent to 104 pg PGD₂. Addition of 1 μmol/L arachidonic acid resulted in a fourfold increase in synthesis of PGD₂, without significantly altering the level of 9α,11β-PGF₂. Similar results were obtained in three separate experiments. When RP-HPLC was used to determine levels of immunoreactive sulfidopeptide leukotrienes in the supernatant of HBM-M cells, 132 ng LTC₄ equivalents/10⁶ cells were found in the absence of a stimulus. As for histamine and PGD₂, there was no increase in leukotriene release following the addition of 2, 10, 40, or 100 μmol/L A 23187.

The HBM-M cells were also found to produce PAF and an inhibitor of platelet aggregation. Lipid extracts of cell supernatants were resolved into two classes of biologically active components by HPLC (Fig 5). The first of these, with an elution volume of 2.5 mL, was capable of inhibiting PAF-induced aggregation of human platelet-rich plasma. The second, a platelet-aggregating activity, eluted in two peaks: a major peak at 5.5 mL and a minor peak at 8.5 mL. When authentic PAF was extracted from buffer in an identical manner, biologic activity eluted in two peaks with the same retention times as the platelet-aggregating activity recovered from the cell supernatants. Unstimulated HBM-M cells (passage 13 or 21) released into the supernatant 62 to 98 ng PAF (C-16:0) equivalents/10⁶ cells and this was not
increased when stimulated with 2, 10, 40, or 100 μmol/L A23187. Thus, for each of the mediators, release was constitutive and could not be augmented by specific stimuli.

Transformation-related and tumorigenic properties of HBM-M. As described previously, the HBM-M cell strain grew as an adherent cell population with a minor population of small nonadherent cells. The adherent population did not overgrow confluence and could not be single cell cloned; nor did the HBM-M cells exhibit growth in semi-solid medium. The karyotype was found to be that of a normal male. HBM-M cells were found to be negative for reverse transcriptase activity in the cellular supernatant, indicating no production of retroviruses. These properties indicate that the cells were not transformed. To determine whether the HBM-M cell strain was tumorigenic, cells of the strain were injected into BALB/c nu/nu "nude" mice. Mice were monitored for 6 to 10 months and it was found that no tumors developed at the site of injection. In addition, no signs of tumorigenesis were found by internal examination at the time of killing. These results indicate that the HBM-M cell strain is neither transformed nor tumorigenic.
Fig. 2. (Cont'd) (C) Morphology of a nonadherent cell. Cells were collected from the supernatant of HBM-M growth, cytopun onto coverslips, and stained with May-Grünwald/Giemsa. This cell was photographed with a 60 x objective.

**DISCUSSION**

The present report describes the long-term growth, morphologic, and functional characteristics of a human cell strain from the bone marrow of an infant with diffuse cutaneous mastocytosis. This strain consisted of two cell types: the majority of the cells were large, fusiform adherent cells with a minor nonadherent population of smaller cells. The morphology of the adherent cells was not typical of fibroblasts, epithelial cells, nor of standard hematopoietic cell types, whereas the morphology of the nonadherent cells had some features of mast cells. Both bone marrow-derived cell types appeared to exhibit apparent spontaneous degranulation in culture. The nonadherent cells appeared to be derived from the adherent cells and were absolutely dependent on the presence of the adherent population for viability. The requirement for the adherent underlay could not be replaced by culturing the nonadherent cells with the growth factors IL-1, IL-3, IL-4, IL-5, G-CSF, GM-CSF, or self-CM. This cell strain composed of both cell populations has been termed HBM-M. While the marrow was initially cultured in the presence of the growth factor IL-3, the bulk population of cells of the HBM-M strain did not require any exogenous growth factors.

Cells of the HBM-M strain were found to have a limited life span, senescing at passage number 28 to 30. They have a normal karyotype, do not produce retroviral particles, and are not tumorigenic in nude mice. In addition, other criteria indicate that HBM-M cells are not transformed; they do not overgrow a monolayer, are incapable of anchorage-independent growth (in semi-solid medium), and do not grow at low cell density. Because we were unable to clone HBM-M cells from the bulk population either in semi-solid medium or by limiting dilution, the cell strain currently exists as a heterogeneous population.

Cytochrome analysis showed a variable, albeit very weak degree of granulation. This degree was reflected by weak staining of the nonadherent population with toluidine blue, alcian blue, and safranin and the binding of FITC avidin by the nonadherent cells. In addition, the cells stained positively for Periodic and Schiff and chloroacetate esterase (albeit weakly). These properties are indicative of a mast cell phenotype. There was also staining for nonspecific esterase and acid and alkaline phosphatase, indicating a hematopoietic origin for the cells. While the adherent cells were found to have relatively few metachromatic granules, electron microscopy showed a mast cell-like ultrastructure with cell surface vili and some cytoplasmic granules. These granules did not have the typical crystalline structure usually seen, and there was evidence for extensive degranulation in the form of vacuoles. The subpopulation of nonadherent cells had more typical morphologic characteristics of human mast cells, exhibiting metachromatic staining (albeit weakly) electron dense granules and the ability to bind conjugated avidin. These patterns and the clear degree of degranulation seen in tissue culture are consistent with spontaneous degranulation. Such degranulation has been reported for systemic mastocytosis mast cells,\(^{1-3,8,18}\) in other conditions such as mast cell leukemia,\(^{20,37}\) mast cell sarcoma\(^{1}\) and scleroderma,\(^{36}\) and in rat mast cells cocultured with fibroblasts in vitro.\(^{39}\)

When the immunophenotype of the HBM-M cells was examined, they were found to be positive for the myeloid/monocyte markers CD11b, CD11c, CD13, F4/80, vimentin, and the mast cell markers YB5.B8 and IgE receptor. YB5.B8 binds to mast cells but not to mature basophils.\(^{22}\) In addition, they were not positive for the basophil marker Bsp-1.\(^{40}\) When characterized biochemically, the cells were found to constitutively secrete the mast cell mediators histamine, LTC\(_3\), PGD\(_2\), PAF, and an inhibitor of PAF-induced platelet aggregation. Of these mediators, only histamine and LTC\(_3\) are synthesized by human basophils.\(^{24}\) The amounts of these mediators produced was not increased upon stimulation and it was noted that the synthesis of these mediators decreased as the cells approached senescence. The cell strain has also shown to constitutively release heparin-like secretory granule proteoglycans, another property of human mast cells (Krilis SA, Austen KF, Macpherson JL, Nicodemus CF, Gurish MF, and Stevens RL, submitted). As far as we are aware, this is the first study of lipid-derived inflammatory mediator release by cells with mast cell properties in long-term culture.

It thus appears that the HBM-M cells possess certain (weak granulation and associated spontaneous degranulation, positive cytochrome staining for periodic acid Schiff and chloroacetate esterase, the ability to bind FITC-avidin, positive immunocytochemical staining for the mast cell marker YB5.B8, the IgE receptor, and vimentin) though not all (H\(_4\) tryptase) mast cell characteristics as well as certain (nonspecific esterase, acid and alkaline phosphatase, positive immunocytochemical staining with CD11b,
CD11c, CD13, and F4/80) monocyte characteristics. Tryptase levels were not examined in one previous study using mastocytosis cells and only low levels of tryptase-positive cells (5%) were found in a study of an immature mast cell line derived from a patient with mast cell leukemia. The HBM-M cells were also positive for the intermediate filament, vimentin, which has previously been reported in normal and neoplastic tissue mast cells and in

Fig 3. Electronmicroscopic analysis of HBM-M cells. (A) Electron micrograph showing section through plastic (X) on which a monolayer of cells was growing. This shows an adherent cell (A) stretched out on the surface of the flask with an adjacent cell bud (B) exhibiting granular cytoplasmic inclusions. The arrow indicates the point of attachment. Original magnification ×5,740. (B) Electron micrograph of nonadherent cell showing mast cell-like granules (G), and lipid bodies (L). Original magnification ×4,300.
immunophenotype and colony formation is somewhat at odds with earlier evidence for a relationship of mast cells to granulocytes, erythrocytes, lymphocytes, megakaryocytes, eosinophils, and basophils.

In addition, an emerging view is that mast cell disorders may be viewed as myeloproliferative disorders. This view is based on recent data showing (1) the derivation of mast cells from immature bone marrow cells; (2) the high distribution of systemic mastocytosis cells in hematopoietic and lymphoid tissues; (3) the frequent association of systemic mastocytosis with a variety of myeloid (including both dysplastic and neoplastic) disorders; (4) increased in vitro colony growth from the bone marrow of patients with systemic mastocytosis; and (5) the bone marrow origin of mast cell leukemia. Our results, showing the existence of proliferative cells with mast cell/monocytic properties in the infant's bone marrow, support this view.

While unusual, an adherent fusiform cell population with mast cell characteristics is not totally unexpected. There has been a report describing cells of similar appearance in culture when serum was removed from a mast cell line established from a human mast cell leukemia and description of fusiform mast cells in tissues including bone marrow and skin from patients with systemic mastocytosis and in the bone marrow of certain patients with lymphoproliferative disorders.

### Table 2. Cytochemical Features of HBM-M Cells

<table>
<thead>
<tr>
<th>Cytotoxic Stain</th>
<th>Adherent</th>
<th>Nonadherent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluidine blue pH 2.5</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Alcian blue</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Safranin</td>
<td>±</td>
<td>ND</td>
</tr>
<tr>
<td>Periodic acid Schiff</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Sudan black</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chloroacetate esterase</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acid esterase</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Key to scoring of reaction: (-) all cells negative; (?) some cells weakly positive; (+) most cells weakly positive; (++) most cells strongly positive; (+++) all cells strongly positive.

Abbreviation: ND, not determined.

### Table 3. Immunocytochemical Characterization of HBM-M Cells

<table>
<thead>
<tr>
<th>Antigen Cluster Designation</th>
<th>Common Names</th>
<th>Cellular Specificities</th>
<th>HBM-M Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>Leu5, T11</td>
<td>Pan-T cell (sheep RBC receptor)</td>
<td>-</td>
</tr>
<tr>
<td>CD11b</td>
<td>Leu15, M01</td>
<td>Granulocytes, monocytes, natural killer cells</td>
<td>+</td>
</tr>
<tr>
<td>CD11c</td>
<td>LeuM5, S-HCL-3</td>
<td>Monocytes, histiocytes, hairy cells</td>
<td>+</td>
</tr>
<tr>
<td>CD13</td>
<td>MY7, DUHL60-4</td>
<td>Monocytes, granulocyte progenitors, some CFC</td>
<td>++</td>
</tr>
<tr>
<td>CD14</td>
<td>MY4, Mo2</td>
<td>Differentiated monocytes, follicular B cells</td>
<td>-</td>
</tr>
<tr>
<td>CD19</td>
<td>Leu12, B4</td>
<td>Pan-B cell (earliest pan-B ag)</td>
<td>-</td>
</tr>
<tr>
<td>CD25</td>
<td>Anti-TAC, IL-2 receptor</td>
<td>Activated T cells, B cells, monocytes</td>
<td>-</td>
</tr>
<tr>
<td>CD33</td>
<td>MY9, LA4F3</td>
<td>Monocytes, granulocyte progenitors, some CFC</td>
<td>-</td>
</tr>
<tr>
<td>CD34</td>
<td>HPCA1 (My10), 3C5, 12.8</td>
<td>Blast cells, CFC</td>
<td>-</td>
</tr>
<tr>
<td>CD41</td>
<td>J15 (gp lbs/lbs)</td>
<td>Platelets</td>
<td>-</td>
</tr>
<tr>
<td>CD45</td>
<td>HLe-1, T200 leukocyte common antigen</td>
<td>All leukocytes, CFC</td>
<td>-</td>
</tr>
<tr>
<td>CD71</td>
<td>Transferrin receptor (TPR), T9</td>
<td>Erythroid precursors, activated or proliferating cells, macrophages</td>
<td>-</td>
</tr>
<tr>
<td>HLA-DR,-DP,-DQ</td>
<td>Class II histocompatibility complex antigen</td>
<td>on diverse cell types and stages</td>
<td>+/-</td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>Neutrophils</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F4/80</td>
<td>Murine myelomonocytes</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>YB5.88</td>
<td>Mast cells, acute myeloid leukemia</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Sal-2</td>
<td>None</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IgE binding</td>
<td>Mast cells, basophils, monocytes, macrophages, platelets, eosinophils</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>Tryptase</td>
<td>Mast cells</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Bas-1</td>
<td>Basophil</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>Endothelial cells</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>Mesenchymal cells, mast cells, macrophages</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Adherent cells</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Serotonin</td>
<td>Appendices, platelets, neuronal cells</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

The table shows the antibodies tested by immunocytochemistry. Both common names and antigen cluster designations (where appropriate) are given as well as the cellular specificities. Adherent cells were tested and the criteria used for staining is as follows: (-) no staining seen; (+/-) some cells weakly positive; (+) a majority of cells weakly to moderately positive; (++) a majority of cells strongly positive.
liferative disorders.\textsuperscript{45} In addition, mast cells vary greatly in size in lymph nodes of patients with reactive mastocytosis\textsuperscript{5} and in skin cells of patients with systemic mastocytosis, in the latter case being, in general, larger than normal.\textsuperscript{43,44} These results correlate with our findings of variation in the size of mast cells present in the skin nodules of the patient under study and in a punch biopsy of another infant patient with diffuse cutaneous mastocytosis (S. Warneford, M. Townsend, A. Kemp, and G. Symonds, unpublished).

For murine bone marrow culture, IL-3 has been shown to stimulate the outgrowth of mast cells, slow growing nonadherent cells with a typical mast cell morphology.\textsuperscript{16,17,46} The growth and viability of these murine mast cells is absolutely dependent on the presence of the growth factor and, even with growth factor present, cells have a very limited proliferative capacity. In contrast to murine mast cells, it has proven difficult to grow normal human mast cells in culture. The seeding of normal human bone marrow in suspension culture with IL-3 gave rise to the outgrowth of basophils\textsuperscript{47,49} as well as other cell types, including eosinophils, neutrophils, and macrophages\textsuperscript{48} but not mast cells.\textsuperscript{47,48} One of these studies\textsuperscript{48} also used bone marrow from a patient with systemic mastocytosis, but in that case too no mast cells grew out. Reports have described limited growth of cells with mast cell characteristics from suspension culture of umbilical cord blood mononuclear cells.\textsuperscript{50,51} In the former case, the cells were described as mast cells/basophils and a source of IL-3 was used,\textsuperscript{52} while in the latter case an underlay of mouse-derived 3T3 fibroblasts was required.\textsuperscript{53} Relatively small numbers of mast cells have also been detected in normal human bone marrow following agar culture\textsuperscript{52,54} in the presence of IL-3. In addition, there has been one report of the growth of basophil/mast cell colonies from human peripheral blood of patients with systemic mastocytosis and chronic myeloid leukemia.\textsuperscript{55} Sources of IL-3 were also used in that study. By contrast to those studies requiring IL-3,\textsuperscript{47-49} selective outgrowth of basophils from human fetal liver required no growth factor.\textsuperscript{56}

It is significant that this is only the third report describing the long-term culture of human cells with some mast cell characteristics. In the two cases previously described, mast cells were grown out from a patient with mastocytosis and from a patient with mast cell leukemia.\textsuperscript{18,19} In the first report, Horton and O"Brein\textsuperscript{18} established cells in culture from the marrow of a patient with mastocytosis. While morphologically and phenotypically more closely resembling mast cells than HBM-M cells, particularly in terms of the cells' degree of granulation (though variation was also found in that study, indicating spontaneous degranulation), the cells in that case were dependent on an adherent layer of fibroblasts. The investigators state that the adherent fibroblast layer may have been required because the true mast cell progenitor had not been introduced into culture from the patient's bone marrow. It is of note that the cells described in that study (in contrast to HBM-M cells) were found to be negative for a range of monocytic markers. It may be that cells of the HBM-M strain are the putative progenitor cell referred to by those investigators. The second study\textsuperscript{19} described the establishment of an immature mast cell line HMC-1 from a patient with mast cell leukemia. There are some similarities to the HBM-M strain described in this report: (1) electron microscopy showed cells with granules, the structure of which only rarely resembled those of mature mast cells; (2) the cells pos-
essed elongated cytoplasmic processes; (3) the cells were adherent under certain culture conditions; (4) the growth of these cells was not dependent on either an adherent cell underlay nor a source of exogenous growth factor; and (5) the cells released histamine, albeit at lower levels than HBM-M cells. In addition, the majority of cells were negative for tryptase. However, unlike HBM-M cells, HMC-1 cells did not possess IgE receptors, were karyotypically abnormal, and clearly transformed and tumorigenic. Thus, apart from the synthesis of histamine, mediator release was not examined in either of the two previous studies.

The fact that we were able to culture cells with mast cell characteristics from the bone marrow of this child with a nonmalignant proliferative disorder without any exogenous growth factors indicates that HBM-M cells appear to have lost the requirement for any such additional influences. Whether this event/events occurred within the child, or by the selective pressures of the culture conditions, is difficult to determine. However, it may be that growth factor-independent cell growth, or a predisposition to it, is one of the fundamental aberrations leading to the marked cellular proliferation seen in vivo in diffuse cutaneous and other forms of mastocytosis. It will be of interest to determine whether the HBM-M cells produce an autocrine growth factor as has been shown in murine myeloid leukemic^{57} and tumorigenic mast{cell lines and whether they can be immortalized by the use of oncogenic retroviruses{or SV40 large T antigen.}

Mastocytosis, in its benign form, can progress to a malignant form (solid tumor or mast cell leukemia).{1,4,9,37,42} While this progression occurs in up to one-third of all cases in adults, it is rarer in children, where there is a high degree of spontaneous resolution of the disease, especially if the disease presents within the first 2 years of life. To our knowledge, there are no reports of mast cell or other forms of leukemia developing in patients presenting in infancy with diffuse cutaneous mastocytosis. In the case presented here the patient’s disease has not progressed to malignancy over a period of 3 years. The nonmalignant nature of the disease is supported by the findings in the cell strain HBM-M, which has been shown to be nontumorigenic in nude mice and not transformed by the criteria discussed. Despite the fact that certain of the phenotypic characteristics of cell strain HBM-M are not those of normal mast cells and that clearly the mast cells in the child have an abnormal proliferative capacity, our evidence indicates that these cells require additional genetic events to occur before they become tumorigenic.

A unique feature of the cultured cells in this study is that they spontaneously release histamine, and the inflammatory mediators PAF,\(^{61,62}\) LTC\(_4\), and PGD\(_2\) in the absence of activating stimuli. Eicosanoids and PAF are derived from membrane phospholipids following activation of phospholipase A\(_2\) with agonists that stimulate calcium mobilization. A variety of cells involved in the inflammatory process produce PAF via the phospholipase A\(_2\) pathway, but certain mammalian cells also possess the necessary enzymes to produce PAF via a de novo biosynthetic route involving a dithiothreitol-insensitive cholinephosphotransferase and a cytidine diphosphate:phosphocholinecytidytransferase.\(^{64}\) It is possible that HBM-M cells have the enzymes to synthesize PAF via the de novo pathway. An inhibitor of PAF-induced platelet aggregation was also identified in the cell supernatant; it has an elution volume on HPLC identical to that of the endogenous inhibitor of platelet aggregation previously demonstrated in blister fluid from the same subject.\(^{77}\) We have previously suggested that a balance between PAF, PGD\(_2\), and a novel inhibitor of platelet aggregation could contribute to the episodic nature of the systemic mastocytosis in this child.\(^{27}\)

It may be that PAF acts as an autocoid in these cells, causing further stimulation of cells to produce PAF and PGD\(_2\) via calcium-dependent pathways. There is evidence to suggest that the release of PAF into the surrounding medium is tightly linked to augmented and sustained PAF synthesis.\(^{62}\) The PAF produced by most cell types in response to stimuli remains largely cell-associated.\(^{28,61}\) The release of PAF into the surrounding medium that we see here is unusual, but is consistent with our earlier findings of PAF in blister fluid from the same child.\(^{27}\) The expansion of the population of mast cells seen in vivo, coupled with the spontaneous release of mediators in the absence of activating stimuli, could lead to the the continuing inflammation observed in the skin. Variable granulation was noted in skin-derived mast cells from this patient and another patient with diffuse cutaneous mastocytosis (S. Warneford, M. Townsend, A. Kemp, G. Symonds, unpublished).

Our results indicate that these cells will be of value for studying the biology and lineage derivation of human mast cells, as well as the biochemical mechanisms involved in the production and secretion of mediators. They may provide an opportunity for this not afforded by the cell types developed in previous studies. Their abnormal behaviour with constitutive release of secretory granule mediators may provide further understanding of the pathogenesis of congenital mastocytosis and other mast cell disorders. In addition, our results support the view that mastocytosis may be viewed as proliferation of bone marrow-derived cells and, hence, as a myeloproliferative disorder.

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REFERENCES


25. Holgate ST, Robinson C, Church MK: The contribution of mast cell mediators to acute allergic reaction in human skin and airways. Allergy 43:22, 1988 (suppl 5)


34. Krilis SA, Macpherson JL, de Carle DJ, Daggard GE, Talley...


Establishment in culture and characterization of a strain with mast cell and monocytic properties from the bone marrow of a child with diffuse cutaneous mastocytosis

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