

Disialoganglioside GD3 is selectively expressed by developing and mature human mast cells

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Background: Disialoganglioside GD3 is expressed on the surface of selected cell types. Anti-GD3 mAb administered to human subjects with malignant melanoma produces signs and symptoms of immediate hypersensitivity reactions.

Objective: The expression of GD3 by human mast cells was assessed during mast cell development in vitro and in samples of lung and skin.

Methods: GD3 on tissue- and in vitro-derived mast cells was analyzed after double labeling of cells for tryptase (G3 mAb) or Kit (YB5.B8 mAb) and GD3 (R24 mAb). Glycolipids in extracts of fetal liver-derived mast cells were examined by using high-performance thin-layer chromatography.

Results: Flow cytometry showed that the percentage of GD3⁺ cells increased in parallel to Kit⁺ cells during the recombinant human stem cell factor-dependent development of fetal liver-derived mast cells. Double-labeling experiments showed that GD3⁺ cells were also surface Kit⁺ and granule tryptase positive, identifying them as mast cells in preparations of lung-, skin-, fetal liver-, and cord blood-derived cells. The major acidic glycolipid detected was NeuAc α 2-8NeuAc α 2-3Gal β 1-4Glc β 1-1'Cer (GD3). Among peripheral blood leukocytes, only basophils and about 10% of the T cells were labeled with anti-GD3 mAb. Anti-GD3 mAb-conjugated magnetic beads were used to purify mast cells to greater than 90% purity from dispersed skin cells enriched to approximately 12% purity by means of density-dependent sedimentation but were less proficient for dispersed human lung mast cells, most likely because of other cell types that express GD3.

Conclusion: GD3 is expressed on the surface of developing human mast cells in parallel to tryptase in secretory granules and, like Kit, can serve as a target for their enrichment by immunoaffinity techniques. (*J Allergy Clin Immunol* 2001;107:322-30.)

Key words: GD3, mast cells, tryptase, Kit

Mast cells and basophils are the principal effector cells of immediate hypersensitivity in human subjects. Mast cells in peripheral tissues are derived from hematopoietic

Abbreviations used

HFH buffer: HBSS containing 1% FCS and 10 mmol/L HEPES
PE: Phycoerythrin
PHABS: PBS containing 10% human AB serum
rhSCF: Recombinant human stem cell factor
TRITC: Tetramethylrhodamine isothiocyanate

progenitors that circulate as multipotential progenitor cells until they are recruited into tissues, where they complete their differentiation and maturation. Mature mast cells can be distinguished from other cell types by expression of high levels of surface Fc ϵ RI and Kit and of granule tryptase. Two types of human mast cells, MC_T and MC_{TC}, have been identified on the basis of protease compositional differences. MC_T cells contain tryptase in their secretory granules and are the predominant type of mast cell in the lung. MC_{TC} cells contain chymase, carboxypeptidase, and a cathepsin G-like protease in addition to tryptase and are the predominant mast cell type in the skin. Basophils complete their differentiation and maturation in the bone marrow and circulate as mature cells until recruited into tissues at sites of inflammation. Basophils are the only cell type in peripheral blood from healthy subjects that expresses substantial amounts of surface Fc ϵ RI, but they express little, if any, Kit and only small amounts of tryptase.

Glycosphingolipids are amphipathic membrane-associated molecules that consist of 3 regions: a carbohydrate, a fatty acid, and a sphingoid base. Sialic acid-containing glycosphingolipids (gangliosides) are known to be involved in diverse cellular processes, including differentiation,^{1,2} proliferation,³⁻⁵ survival,⁶ and activation.^{5,7} Of potential interest are clinical studies with R24, a murine IgG3 mAb specific for GD3. GD3 is a disialylganglioside derived from GM3 by using GD3 synthase (sialyl transferase II). Intravenous administration of R24 consistently was associated with immediate hypersensitivity reactions (urticaria and bronchospasm).⁸⁻¹⁰ Because of the potential involvement of gangliosides with different aspects of human mast cell biology, the gangliosides present in in vitro- and tissue-derived human mast cells were examined in the current study. GD3 was found to be selectively expressed by nearly all mast cells, and anti-GD3 mAb was an effective agent for the immunoaffinity purification of mast cells dispersed from human tissues.

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METHODS

Materials

Histopaque-1077, Dulbecco's modified essential medium, minimal essential medium, RPMI-1640, HBSS, L-glutamine, nonessential amino acids, heat-inactivated controlled process serum replacement 3, penicillin-streptomycin, human AB serum, FCS, type 1S hyaluronidase, type 1A collagenase, horseradish peroxidase-conjugated streptavidin, 30% hydrogen peroxide, DNase I, soybean trypsin inhibitor, 3-amino-9-ethylcarbazole, naphthol AS-MX phosphate, fast blue RR, mAb MOPC-31C (mouse IgG1, κ), DEAE-Sephadex (A-25), Percoll, and LH-20 (Sigma Chemical Co, St Louis, Mo); mouse IgG3 (A112-3), FITC-conjugated goat anti-mouse Ig, and FITC-conjugated rat anti-mouse IgG3 (BD Pharmingen, San Diego, Calif); type 2 collagenase and hyaluronidase (Worthington Biochemical Corporation, Lakewood, NJ); and high-performance thin-layer chromatography (HPTLC) silica gel plates (E Merck, Darmstadt, Germany) were obtained as indicated. RhSCF was a gift from Amgen (Thousand Oaks, Calif). The murine antichymase mAb B7 (biotin-conjugated) and anti-tryptase mAb G3 (alkaline phosphatase or tetramethylrhodamine isothiocyanate [TRITC] conjugated) were obtained and prepared as described previously.¹¹ The murine mAb YB5.B8 against human Kit¹² was obtained from Dr Leonie K. Ashman (Institute of Medical and Veterinary Science, Adelaide, Australia) and used as previously described,¹³ whereas phycoerythrin (PE)-labeled YB5.B8 and mouse mAb against human CD3 were purchased from BD Pharmingen. Anti-Fc ϵ RI α mAb 22E7 was used as previously described.¹⁴ MAb R24 (IgG3) was a gift from Dr P. O. Livingston (Memorial Sloan-Kettering Cancer Center, New York, NY) and has been shown to selectively recognize the disialoganglioside GD3.¹⁵ All of the glycolipid standards were made as described by Zeng et al.²

Cultures of dispersed fetal liver and cord blood cells

Human fetal livers, 16 to 21 weeks of gestational age, were obtained at therapeutic abortions, and cord blood was obtained at normal deliveries. The experimental protocol was approved by the Human Studies Institutional Review Board of Virginia Commonwealth University. Fetal liver-derived mast cells¹⁶ and cord blood-derived mast cells¹⁷ were obtained as previously described. Cell viability was determined by using trypan blue exclusion. Cells harvested at various times were collected by means of centrifugation for immediate analysis of surface Kit, Fc ϵ RI, and GD3 by means of flow cytometry. Cytocentrifuge preparations also were made with a Cytospin 2 (Shandon Southern Products, Cheshire, United Kingdom) at the initiation of the culture and at various time points thereafter, air-dried, and stained with toluidine blue or stored until ready for immunocytochemical staining.

Dispersal and purification of human skin- and lung-derived mast cells with the anti-GD3 mAb

Skin- and lung-derived mast cells were dispersed with type 2 collagenase and hyaluronidase and enriched by using Percoll density-dependent sedimentation essentially as described previously.^{18,19} Fresh samples of skin were obtained after mastectomies performed on patients with breast cancer or from abdominoplasties or through the Cooperative Human Tissue Network (Columbus, Ohio), as approved by the Human Studies Institutional Review Board at Virginia Commonwealth University. Surgical lung tissue samples (lung cancer or emphysema) were obtained from the Department of Pathology at Virginia Commonwealth University (Richmond, Va) or through the Cooperative Human Tissue Network, as approved by

the Human Studies Institutional Review Board at Virginia Commonwealth University. Lung- and skin-derived mast cells enriched by density-dependent sedimentation were then purified by using an immunoaffinity technique with anti-GD3 mAb. To reduce nonspecific binding of anti-GD3 mAb, cells were incubated in PBS containing 10% human AB serum (PHABS) for 30 minutes. Anti-GD3 mAb (3 μ g) was added to 1×10^7 washed cells, which were incubated at 4°C for 30 minutes. Excess mAb was removed by washing the cells with HBSS containing 1% FCS and 10 mmol/L HEPES (HFH buffer). Cells were then incubated with microbead-conjugated anti-mouse IgG antibody (Miltenyi Biotec, Auburn, Calif) at 4°C for 15 minutes. After washing with HFH buffer, the resuspended cells were applied to the separation column and placed in a magnetic field, as recommended by the manufacturer. Unbound cells were removed; bound cells were then eluted by removing the column from the magnetic field. Mast cell purities were initially determined in cytospin preparations stained with toluidine blue. Total cell viabilities and numbers were determined by using trypan blue exclusion. Mast cell purities also were determined by using double labeling for tryptase and chymase¹¹ after first blocking with normal mouse IgG to eliminate binding of primary antibodies to bead-derived anti-mouse IgG. Control results with alkaline phosphatase- and biotin-conjugated nonimmune mouse IgG were negative.

Separation of human white blood cells

Human white blood cells were separated by means of Percoll density-dependent sedimentation, as previously described.²⁰ Briefly, human peripheral blood was diluted 1:1 with HBSS and layered onto 45%, 58.5%, and 65% Percoll cushions. After centrifugation at 700g for 20 minutes at room temperature, cells from the upper 45% Percoll interface contained predominantly monocytes and lymphocytes, cells from the 45%/58.5% interface contained basophils and mononuclear cells, and cells from the 58.5%/65% interface contained predominantly eosinophils and neutrophils. Percoll-enriched cells were washed with PBS twice and then subjected to immunocytochemical or flow cytometric studies.

Immunocytochemistry

Mast cells were subjected to double-immunocytochemical labeling with anti-tryptase (TRITC-conjugated G3) and anti-GD3 (R24) mAbs. For skin and lung tissue, 4- μ m sections from paraffin-embedded 4% buffered formalin-fixed tissue samples were mounted on glass slides, blocked in PHABS, incubated with anti-GD3 mAb (diluted 1:100 with PHABS) or control mouse IgG3 for 1 hour at room temperature, washed in PBS, and then incubated with FITC-conjugated goat anti-mouse Ig for 1 hour. After blocking with normal mouse IgG, cells were further incubated with TRITC anti-tryptase mAb (1:100) or TRITC-conjugated control mouse antibody for 1 hour at room temperature and examined by means of fluorescent microscopy. For cytocentrifuge preparations of fetal liver-derived and dispersed human skin and lung mast cells, cells were fixed with 4% buffered formalin for 30 minutes and then washed in PBS. After blocking with PHABS for 30 minutes, slides were labeled as described above. For dispersed cytocentrifuged cells, at least 200 cells per field, 4 to 8 fields per slide, and 1 to 3 slides per sample were examined, such that a minimum of 100 mast cells per sample were assessed. For tissue sections, at least 100 tryptase-positive cells were examined for GD3 positivity per sample.

Flow cytometry

The presence of Kit, Fc ϵ RI, and GD3 on the surface of cells was assessed by using flow cytometry. Cells to be labeled (0.2×10^6) were incubated for 30 minutes at 4°C in PHABS and washed in PBS containing 1% FCS and 0.1% sodium azide. Cells then were incubated with anti-Kit mAb (diluted 1:2000 with PHABS), anti-Fc ϵ RI α mAb

(1:100), anti-GD3 mAb (1:100), or nonimmune mouse IgG for 30 minutes at 4°C, washed as above, incubated with FITC-conjugated goat anti-mouse Ig at 4°C for 15 minutes, washed as above, resuspended in PBS containing 1% paraformaldehyde, and analyzed with a FACScan flow cytometer and Cell Quest software (BD Immunocytometry Systems, San Diego, Calif). Before analysis, propidium iodide was added to cell suspensions to label dead cells. For each sample, 5000 gated cells, excluding dead cells, were analyzed. The percentage of positive cells was calculated by subtracting the percentage of cells labeled with an isotype-matched negative control mAb (MOPC-31C or IgG3, <5% positive cells) from the percentage of cells labeled with experimental mAb. The human mast cell leukemia cell line HMC-1 was a gift from Dr G. Gleich and Dr J. Butterfield (Mayo Clinic, Rochester, Minn).²¹ The human basophil leukemia cell line KU812 was obtained from Dr G. Nilsson (University of Uppsala, Uppsala, Sweden).²² HMC-1 cells and KU812 cells were used as positive controls for surface Kit and FcεRI surface expression, respectively.

GD3 expression on different types of peripheral blood leukocytes separated by Percoll gradient sedimentation also was assessed by flow cytometry, as described above. Because substantial FcεRI is only expressed on basophils in peripheral blood, this cell type was analyzed after gating on the window of the forward and side light scatter of the FcεRI⁺ cells. Some of the Percoll-separated cells were further incubated with mouse nonimmune IgG, washed, and then labeled with PE-conjugated anti-CD3 mAb.

Extraction and purification of glycosphingolipids

Total lipids were extracted, as previously described,²³ from each sample of fetal liver–derived mast cells (10⁷ cells collected on days 1, 14, 21, and 28) by mixing the cells with 5 mL of chloroform/methanol (1:1, vol/vol) and stirring this mixture at room temperature for 24 hours. After extraction, mixtures were centrifuged at 2000 rpm for 10 minutes, supernatants were collected, and the residue was extracted twice with 5 mL of chloroform/methanol/H₂O (30:60:8, vol/vol/vol; solvent A). The extracts from each sample were combined and adjusted to equal the chloroform/methanol/H₂O composition of solvent A, applied to a DEAE-Sephadex column (acetate form; bed volume, 2 mL), and washed with 10 volumes of solvent A. Wash fractions contained neutral lipids. The acidic lipids were then eluted with 10 volumes of chloroform/methanol/0.8 mol/L aqueous sodium acetate (30:60:8, vol/vol/vol).

The neutral lipid fraction and a small amount of the acidic lipid fraction were treated with 0.2 N methanolic NaOH at 37°C for 2 hours to hydrolyze contaminating phospholipids. After each sample was neutralized with 0.2 N HCl, the salts in the neutral lipid fraction were removed by using Folch partitioning,²⁴ and those in the acidic lipid fraction were removed by means of passage through a C18 Sep-Pak Cartridge (Waters Associates, Inc, Milford, Mass). Bound gangliosides were eluted first with 2 mL of methanol and then with 20 mL of chloroform/methanol (1:1, vol/vol). Eluted fractions were combined. After desalting, the glycosphingolipid samples were dried under a stream of nitrogen and redissolved in a small amount of chloroform/methanol (1:1, vol/vol).

HPTLC

The ganglioside and neutral glycolipid fractions were examined by means of thin-layer chromatography with HPTLC plates of Silica Gel 60. The plate for gangliosides was developed with chloroform/methanol/0.5% aqueous CaCl₂·2H₂O (55:45:10, vol/vol/vol). For neutral glycolipids, the plate was developed with chloroform/methanol/water (60:35:8, vol/vol/vol). The glycolipid patterns were detected by spraying the plate with orcinol-H₂SO₄ reagent and then heating the plate at 95°C for 30 minutes. A bovine brain ganglioside mixture was used as a standard for gangliosides and for

neutral glycolipids. The amount of each ganglioside and neutral glycolipid was estimated by using densitometry with a Hewlett Packard 6200C scanner and the Un-Scan-It software program (Silk Scientific, Inc, Orem, Utah).

RESULTS

During recombinant human stem cell factor (rhSCF)–dependent development of fetal liver–derived mast cells, the percentages of cells expressing surface GD3 increased in proportion to those stained metachromatically with toluidine blue and those expressing surface Kit and intracellular tryptase, approximating 90%, 75%, 94%, and 70%, respectively, by day 28 (Fig 1, A). At no time point were FcεRI⁺ cells detected (data not shown), which is consistent with previous reports.^{14,18} On day 1, 6% of the cells were weakly Kit⁺, but essentially none (<2%) were metachromatic, tryptase positive, or GD3⁺. The parallel increase in cells coexpressing Kit and tryptase and staining metachromatically has been reported,¹³ whereas that of GD3 is new. Flow cytometry patterns for GD3 (R24) and Kit (YB5.B8) expression on fetal liver cells cultured in rhSCF are shown for a representative experiment in Fig 1, B. On day 1, no GD3⁺ cells were detected, even though small numbers of weakly Kit⁺ cells were observed. Thereafter, both the percentages of GD3⁺ cells and the mean fluorescence intensities of those cells progressively increased. By day 28, there was a uniphasic shift of the cells after labeling either with anti-Kit mAb or anti-GD3 mAb, suggesting all of these Kit⁺ mast cells expressed surface GD3, even though a portion of the anti-GD3–labeled cells overlap with the shaded negative control. Thus increasing amounts of surface GD3 appear on the surface of mast cells as they develop in vitro from fetal liver progenitors.

To directly assess GD3 expression by mast cells, fetal liver cells cultured for 28 days with rhSCF were double labeled for surface GD3 (FITC-conjugated goat anti-mouse Ig or a negative control) and Kit (PE-YB5.B8 or a negative control). As shown in Fig 2, A (*upper right quadrant*), essentially all GD3⁺ cells were Kit⁺, and approximately 34% of the Kit⁺ cells appeared to be GD3⁺. However, the anti-GD3 mAb-labeled Kit⁺ cells exhibited a monophasic shift, suggesting that the entire population of Kit⁺ cells expressed GD3, albeit weakly in some cases.

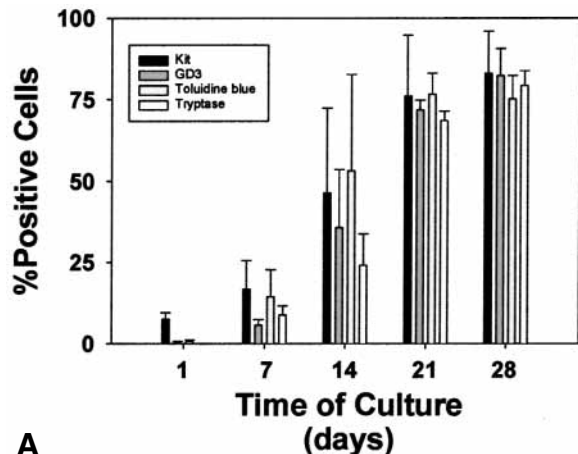
Preparations of fetal liver–derived mast cells were double labeled with anti-GD3 mAb/FITC-rat IgG anti-mouse IgG3 and then with TRITC-anti-tryptase mAb, as shown in Fig 2, B (*top and bottom*, respectively). Substituting nonimmune mouse IgG3 for anti-GD3 mAb did not result in FITC labeling, and substitution of TRITC-MOPC-31C for TRITC-anti-tryptase mAb did not result in TRITC labeling (not shown). All GD3-labeled cells (Fig 2, B, *top panel*) were colabeled for tryptase (Fig 2, B, *bottom panel*). However, a small portion of the tryptase-labeled cells (Fig 2, B, *arrows*) showed negligible labeling for GD3. The data indicate that among tryptase-positive mast cells (92% of total cells), at least 90% also expressed GD3. When cord blood–derived mast cells obtained and purified to greater than 98%, as

previously described,¹⁷ were examined for GD3 expression by means of cytofluorescence, they too were mostly positive (not shown). Thus GD3 appears to be expressed in nearly all of the in vitro-derived mast cells.

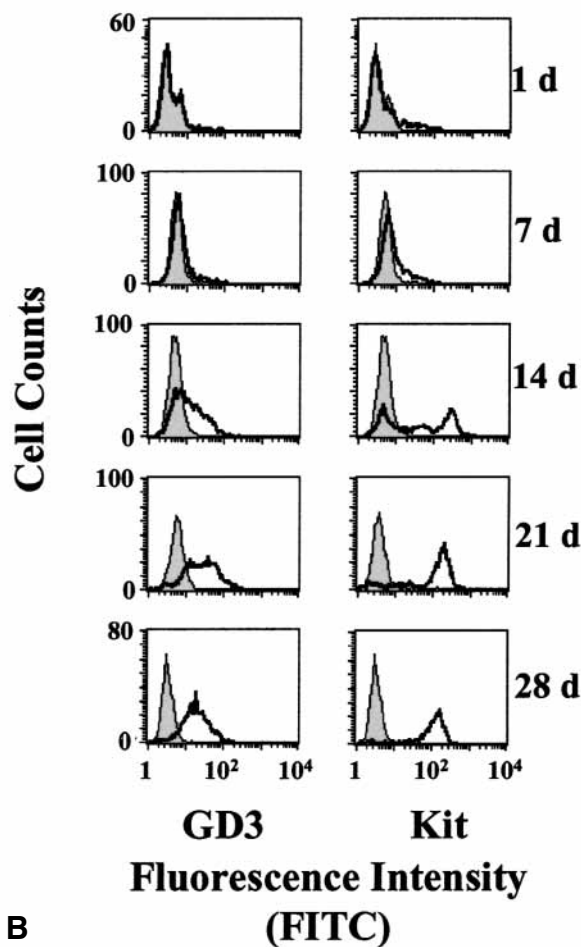
Mast cells derived from lung and skin also were assessed for GD3 expression. As shown in Fig 3, essentially all tryptase-positive mast cells (Fig 3, *B, D, F, and H*) derived from skin (tissue, Fig 3, *A and B*; dispersed cells, Fig 3, *C and D*) and lung (tissue, Fig 3, *E and F*; dispersed cells, Fig 3, *G and H*) were GD3⁺ (Fig 3, *A, C, E, and G*). Although there were regions of noncellular staining of lung and skin (eg, keratin layer of epidermis in Fig 3, *A*, *filled arrowhead*) tissues with anti-GD3 mAb, most of the cells labeled with this mAb were also tryptase-positive mast cells. In skin sections (Fig 3, *A and B*) labeled cells were observed almost exclusively in the dermis, whereas in bronchial (Fig 3, *E and F*) and alveolar (not shown) regions of the lung, there were GD3⁺ and tryptase-positive cells, which is consistent with the widespread distribution of mast cells. Among dispersed skin (Fig 3, *C and D*) and lung (Fig 3, *G and H*) cells, all tryptase-positive mast cells were labeled by anti-GD3 mAb, even though this cell type accounted for only about 10% to 20% of the cell population. Highly purified mast cells from these sources also were determined to be GD3⁺ by using R24 labeling (not shown). Among dispersed cells that were GD3⁺, essentially all such cells derived from skin were tryptase positive (Fig 3, *C and D*), whereas only a little over 50% of those derived from lung tissue were tryptase positive (Fig 3, *G and H*, *closed arrowheads*). Other lung-derived GD3⁺ cells (Fig 3, *open arrowheads*) were tryptase negative. Also, because nearly all mast cells from skin were chymase positive, whereas most of those from lung were chymase negative (not shown), we concluded that both MC_T and MC_{TC} types of mast cells express GD3.

To chemically confirm GD3 expression on human mast cells, neutral and acidic glycosphingolipids, separated by DEAE-Sephadex, were examined by using HPTLC. Fig 4, *A*, shows HPTLC profiles of neutral glycosphingolipids extracted from fetal liver cultures on days 1 (<1% mast cells), 14, 21, and 28. Little, if any, neutral glycosphingolipids were detected on day 1. Lactosyl ceramide was the principal neutral glycolipid expressed in samples obtained on days 14 through 28 and therefore may be expressed by mast cells, as well as other cells in the preparation. Expression by mast cells is expected because lactosyl ceramide is a precursor of the acidic glycosphingolipid GM3, which, in turn, can give rise either to GM2 or GD3.

Gangliosides are shown in Fig 4, *B*. Little, if any, acidic glycosphingolipid was detected at day 1. Although GM3 was the principal ganglioside detected at day 14, by day 28, when most of the cells were mast cells, GD3 was the major ganglioside. Lesser amounts of GM2 also were detected. The ganglioside compositions of day-28 fetal liver cultures composed of approximately 80% tryptase-positive mast cells were determined in 2 separate samples by using scanning densitometry and revealed an



A



B

FIG 1. Expressions of ganglioside GD3 on fetal liver-derived cells. **A**, Surface GD3⁺ cells increase along with those positive for surface Kit, granule tryptase, and metachromasia. Cultures of fetal liver cells with rhSCF were analyzed after 1, 7, 14, 21, and 28 days. Three different preparations of fetal liver were used for each time point. **B**, Histograms of fetal liver cells labeled either with anti-GD3 mAb (*left*) or anti-Kit mAb (*right*) during culture with rhSCF (*unshaded area*) compared with nonimmune control mAbs (*shaded area*).

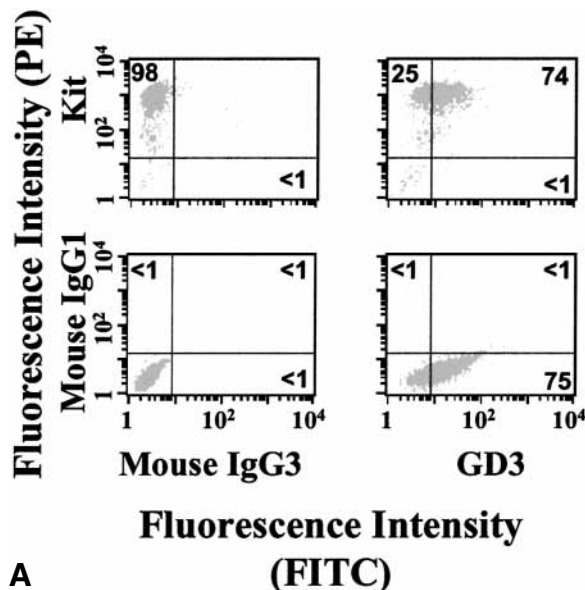
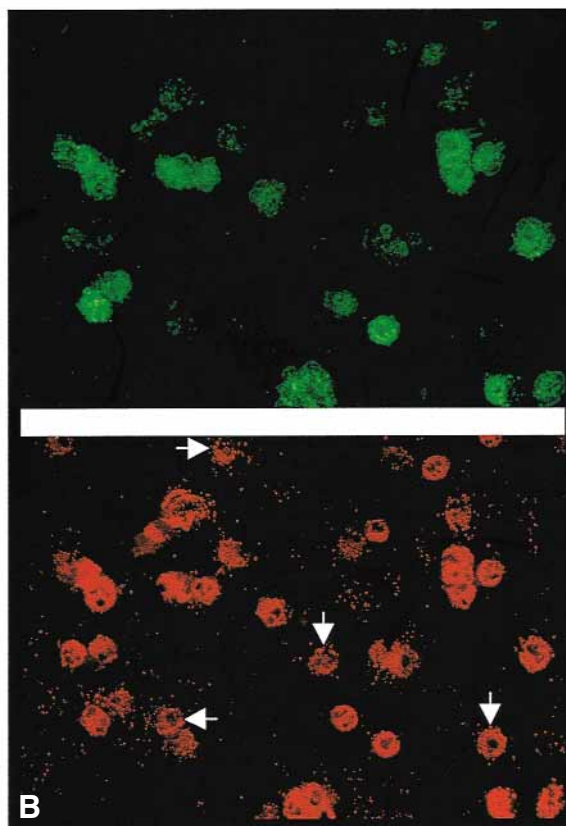
**A**

FIG 2. Characterization of GD3 expression on fetal liver-derived mast cells by double labeling. **A**, GD3 resides on Kit⁺ mast cells. FACS analysis was performed on fetal liver-derived cells after 28 days of culture with rhSCF. Cells were double labeled with anti-GD3 mAb/FITC-conjugated goat IgG against mouse Ig and PE-conjugated anti-Kit mAb. Neither nonimmune PE-conjugated IgG1 nor IgG3, when substituted for the corresponding immune mAb, labeled these cells. **B**, GD3 resides in tryptase-positive mast cells. Fetal liver-derived mast cells at culture day 28 were cyto-centrifuged, fixed in 4% buffered formalin, washed in PBS, and labeled with anti-GD3 mAb/FITC-conjugated rat anti-mouse IgG3 (*top*) and TRITC-anti-tryptase mAb (*bottom*). The same field of view is shown in the *top* and *bottom* panels. Immunofluorescence was analyzed with a fluorescence microscope (model BX50, Olympus Optical Co Ltd) and U-MNG and U-MNB filters to show only FITC and TRITC fluorescence, respectively, and photographed by using the PM-30 exposure control unit. Substitution of TRITC-conjugated mouse IgG1 for G3 anti-tryptase or mouse IgG3 for R24 anti-GD3 each failed to label cells with the double-labeling procedure.

average of 71% of the acidic glycosphingolipids to be GD3, 17% to be GM3, and 12% to be GM2. These data support the immunologic evidence that R24 correctly recognizes GD3 on human mast cells.

Because urticaria has been reported when R24 anti-GD3 mAb was administered to patients with melanoma, the degranulating activity of the mAb was tested in vitro with human skin-derived mast cells and fetal liver-derived mast cells. For a skin-derived mast cell preparation of greater than 90% purity and 95% viability, 0.03 to 1 $\mu\text{g/mL}$ R24, 1 $\mu\text{g/mL}$ anti-Fc ϵ RI α mAb, or isotype-matched control mAb was incubated with cells for 15 minutes at 37°C in Tyrode's buffer containing 10 mmol/L HEPES (pH 7.4), 1mg/mL gelatin, 10 mg/mL DNase, 1 mmol/L MgCl₂, and 2.5 mmol/L CaCl₂. Activation reactions were stopped with 7 volumes of ice-cold incubation buffer lacking divalent cations, cells were separated from buffer by centrifugation, and the amounts of tryptase in supernatants and cell lysates were measured with an ELISA. With spontaneous release being 5% on



average (range, 4%-7%), the net percentage release values were 12% with anti-Fc ϵ RI α mAb and 2% or less at each dose of anti-GD3 mAb. A similar activation experiment was performed with fetal liver-derived mast cells (>90% purity) that had been developed with rhIL-4 and rhSCF and activated as previously described.¹⁶ With spontaneous release again being 5% on average, net percentage release values for anti-GD3 mAb (1 $\mu\text{g/mL}$) of -1% and for anti-Fc ϵ RI α mAb of 22% were obtained. These data suggest that R24 is not a direct degranulating agent for human mast cells.

Selective expression of GD3 by mast cells may be of practical utility for purifying these cells. This possibility was first tested with dispersed skin cells containing 12% $\pm$ 2% (mean $\pm$ SD) mast cells ($n = 5$). Magnetic bead affinity purification of skin-derived mast cells with anti-GD3 mAb resulted in mast cell purities of 90% or greater in each case (92% $\pm$ 2%). The mast cell yields ranged from 6% to 55% (34% $\pm$ 17%), and viabilities assessed by exclusion of trypan blue from cells were greater than 90%. Immunocytochemical staining with mAb G3 against tryptase and mAb B7 against chymase showed that the purified mast cells were all of the MC_{TC} type. The purification procedure was then applied to 2 different preparations of dispersed human lung cells containing 8% and 10% mast cells. Mast cell purities of 58% and 49% were obtained. The protease phenotype of the mast cells before and after their purification revealed no substantial difference, with the MC_T type accounting for 70% to 90% of the mast cells in both cases.

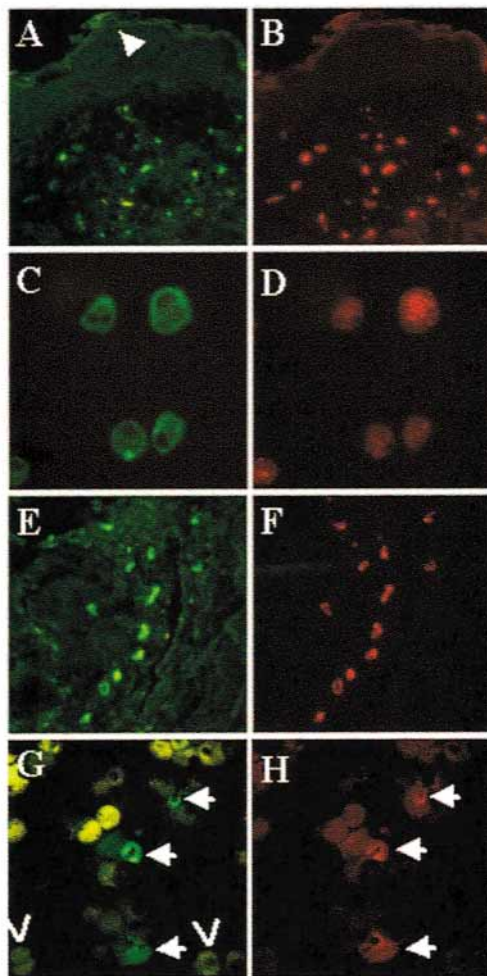


FIG 3. GD3 resides in mast cells from skin (A-D) and lung (E-H) tissue. Sections from tissues fixed in formalin (A, B, E, and F) or cytospin preparations fixed in formalin (C, D, G, and H) were double labeled with TRITC-conjugated anti-tryptase mAb and anti-GD3 mAb/FITC-conjugated goat anti-mouse Ig and analyzed as in Fig 2, B. Note that A and B show the same field, as do pairs C and D, E and F, and G and H. In A the arrowhead points to keratin layer of epidermis. In G and H filled arrowheads point to double-labeled cells. In G open arrowheads point to cells labeled with anti-GD3 mAb but not anti-tryptase mAb. Yellow-colored cells in G are autofluorescent and easily distinguished from green FITC-labeled cells.

GD3 expression on peripheral blood leukocytes was assessed by means of flow cytometry. Analysis of the monocyte-lymphocyte population from the 43% Percoll interface in Fig 5, A, showed that 11% of the CD3⁺ lymphocytes ($[9/72] \times 100$) and about 6% of the CD3⁻ monocytes ($[1/18] \times 100$) expressed surface GD3. A similar analysis of eosinophils and neutrophils from the 58%/65% Percoll interface in Fig 5, B, revealed no (<1%) positive cells. The 43%/58% Percoll interface contained the majority of basophils (35% purity) along with monocytes and lymphocytes. As shown in Fig 6, FcεRI⁺ cells were labeled with the anti-FcεRIα mAb to establish a gate based on forward scatter (Fig 6, A) and side scatter (Fig 6, B) that would encompass primarily

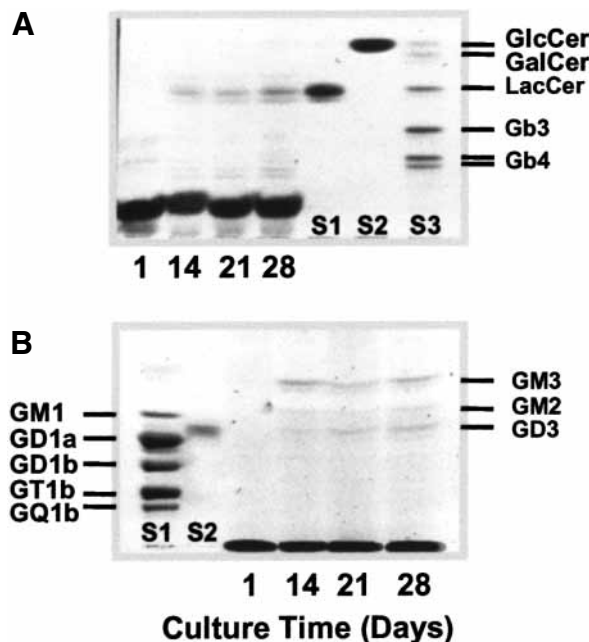


FIG 4. HPTLC of glycolipids from fetal liver-derived cells. **A**, HPTLC of neutral glycosphingolipids. Extracts were prepared from fetal liver-derived cells cultured with rhSCF at 1, 14, 21, and 28 days and analyzed. Experimental samples are in lanes labeled by the day of harvest. Lane S1, Lactosylceramide (LacCer); lane S2, glucosylceramide (GlcCer); lane S3, neutral glycolipid mixture: glucosylceramide, galactosylceramide (GalCer), lactosylceramide, Gb3, and globoside (Gb4). Each experimental lane contains glycolipids from 10^6 cells. The bands were visualized by spraying with the orcinol- H_2SO_4 reagent. **B**, HPTLC of gangliosides from fetal liver-derived mast cells. Lane S1, Human brain-derived ganglioside mixture used as standard; lane S2, ganglioside GD3 purified from bovine buttermilk. Lanes 1, 14, 21, and 28 reflect the day of harvest for the experimental samples. Each lane contains gangliosides from 10^7 cells. The bands were visualized by spraying with the orcinol- H_2SO_4 reagent.

basophils. Among the cells within this gate, 94% were FcεRI⁺ (Fig 6, C). Among the gated population (Fig 6, D), 78% were labeled with anti-GD3 mAb, suggesting that basophils, like mast cells, express GD3.

DISCUSSION

Ganglioside GD3 joins FcεRI and Kit as a surface marker that is preferentially, although not exclusively, expressed on the surface of human mast cells. This was shown by immunocytochemical and flow cytometric techniques for mast cells derived in vitro from progenitors in fetal liver and cord blood and for those from lung (predominantly MC_T type) and skin (MC_{TC} type) tissue. The portion of Kit⁺ mast cells detected as GD3⁺ is greater when analyzed with immunocytofluorescence than with flow cytometry. This result may occur because immunocytofluorescence detects total cellular GD3, whereas flow cytometry detects only cell-surface GD3. Because little, if any, GD3 expression was detected on the surface of freshly dispersed fetal liver cells or by chemical techniques in

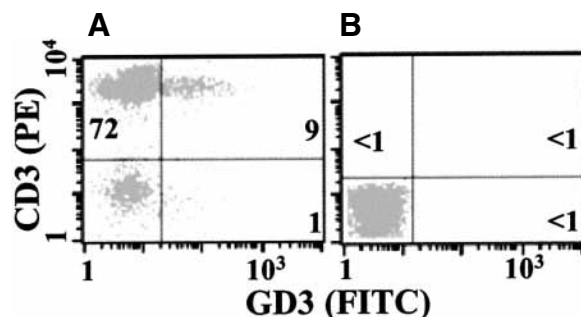


FIG 5. Surface GD3 expression on human peripheral leukocytes determined by using flow cytometry. **A**, GD3 is expressed on small portions of the CD3⁺ lymphocytes and the CD3⁻ monocytes. In the preparation shown, 24% of the cells were predominantly monocytes, 76% were lymphocytes, and 0.3% were other myeloid or erythroid cells, as determined by staining with May-Grünwald and Giemsa dyes. Cells from this mononuclear cell preparation were double labeled with anti-CD3 and anti-GD3 mAbs, as previously described. **B**, GD3 is not expressed on neutrophils and eosinophils. Cells from the neutrophil-eosinophil layer contained 90% neutrophils, 9% eosinophils, 0.4% basophils, and 0.2% lymphocytes, as determined by staining with May-Grünwald and Giemsa dyes and were double labeled with anti-CD3 and GD3 mAbs. Numbers in the upper left and right panels and in the lower right panel depict the percentage of cells in the corresponding quadrant of **A** and **B**.

extracts of such cells, it appears this ganglioside is expressed during mast cell development in parallel to tryptase and is most abundant on more mature mast cells. The distribution of GD3 expression in normal skin and lung tissues tracked most closely with that of tryptase-positive mast cells. Conversion of GM3 to GD3 by developing and malignant cells in the central nervous system^{25,26} is somewhat analogous to the situation for mast cells but differs from the mast cell scenario in that expression of GD3 in the central nervous system generally declines with cell maturation. The diffuse staining of the epidermis is consistent with previous reports finding weak expression of GD3 in skin associated with keratinocytes and melanocytes,^{27,28} with the latter cell type also expressing Kit. However, the ability to purify mast cells from dispersed skin cell preparations suggests that little of the GD3 expressed by keratinocytes or melanocytes is available on the surface of these cells to bind to anti-GD3 mAb and that GD3-expressing cells other than mast cells might have been removed by the initial isolation procedures. In contrast to normal melanocytes, malignant melanoma cells express substantial amounts of surface GD3. Consequently, patients with malignant melanoma have been treated experimentally with R24 anti-GD3 mAb.^{8,9,29,30} In normal human lung tissue, details on the distribution of GD3 expression are scant, but small cell lung cancers have been reported to express GD3.³¹ The purities of mast cells from lung tissue after anti-GD3 mAb immunoaffinity purification are lower than those purified from skin. This may reflect the presence of greater numbers of GD3⁺ nonmast cells in lung preparations (Fig 3, *G* and *H*), including lym-

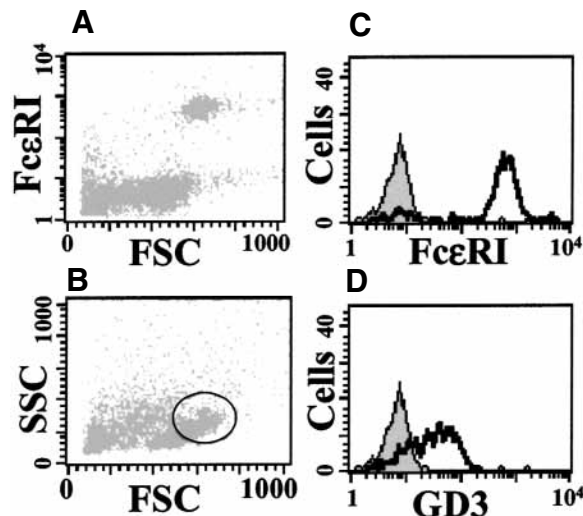


FIG 6. GD3 is expressed on human peripheral basophils. In **A** basophils were identified by FcεRI surface expression with anti-FcεRIα mAb. In **B** this information was used to establish a basophil-selective gate (encircled area). **C** shows that the vast majority of cells within this gate are basophils. **D** shows the presence of anti-GD3 mAb-labeled cells within this gate; GD3 expression was observed on most of the cells. A modest portion of the cells outside of this gate also expressed GD3 (approximately 10%).

phocytes, monocytes, or malignant pulmonary cells. Overall, abundant cell-surface GD3 in normal skin and lung tissue is preferentially found on mast cells.

Immunoaffinity purifications of human mast cells also have been achieved with anti-Kit and anti-FcεRI antibodies. Anti-Kit mAbs in some cases may compete with SCF for binding to Kit, potentially impairing survival of mast cells placed into culture. Anti-FcεRI mAbs may activate or desensitize mast cells during their purification, a potentially undesirable functional effect. Anti-GD3 mAb does not appear to degranulate human skin-derived or fetal liver-derived mast cells nor does it affect the survival of fetal liver-derived mast cells (data not shown). In some experimental situations, the lack of these effects by anti-GD3 mAb may be advantageous.

Among peripheral blood leukocytes, GD3 expression on a subset of normal T cells (both CD4⁺ and CD8⁺) has been reported previously,^{4,5,32-34} which is consistent with the finding in the current study of surface GD3 detected on about 10% of the CD3⁺ cells. GD3 also is expressed on T-cell, but not B-cell, leukemias,^{35,36} which is consistent with the theme that malignant transformation upregulates GD3 expression in certain cell types. The current study also demonstrates GD3 expression on human basophils, although not on eosinophils, which are closely related by lineage to basophils, and neutrophils. The expression of GD3 on both mast cells and basophils, the 2 principal effector cells of immediate hypersensitivity, is of potential biologic interest.

The functional consequence of GD3 expression on mast cells has yet to be determined. GD3 has been impli-

cated in a variety of biologic pathways, including cell activation, proliferation, signaling, and apoptosis. For example, R24 anti-GD3 provides a costimulation signal to T cells activated through their T-cell receptor to prevent anergy.³³ Apoptosis of hematopoietic cells through CD95 and ceramide pathways requires newly synthesized GD3, which in turn appears to disrupt the mitochondrial transmembrane potential.⁶ This GD3 is generated from ceramides released by acidic sphingomyelinase.³⁷ Functional involvement of glycolipids in mast cells has been suggested in studies of mouse bone marrow-derived mast cells, in which ganglioside GM3 inhibits proliferation.³⁸ Antibody against GD1b (AA4) labels the surface of the rat mucosal mast cell line RBL-2H3, upregulates mediator release induced by calcium ionophore, downregulates mediator release induced through FcεRI, and coimmunoprecipitates GD1b, FcεRI, the tyrosine kinase Lyn, and a serine kinase.³⁹⁻⁴² Interestingly, an mAb against GD3 did not reveal an association of tyrosine and serine kinases with this ganglioside in RBL-2H3 cells. In studies of the activation of human HMC-1 cells, basophils, and skin-derived mast cells, addition of GM2, GM3, and GD1a each augment IgE-dependent mediator release.^{43,44} These latter gangliosides, along with GM1 (but not GD3), were detected in HMC-1 cells.⁴⁵ When R24 was administered to human subjects during therapy of malignancies, immediate hypersensitivity events were consistently reported.^{8-10,29,30} However, our preliminary results indicate that R24 anti-GD3 does not induce either fetal liver-derived mast cells or skin-derived mast cells to degranulate in vitro (unpublished data). Thus the functional role of GD3 on mast cells and the in vivo observations during R24 administration remain to be clarified.

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