Original article

Identification of basophils by a mAb directed against pro–major basic protein 1

Douglas A. Plager, PhD,[a] Ellen A. Weiss,[a] Gail M. Kephart, BS,[a] Robert M. Mocharla,[a] Ryoji Matsumoto, MD, PhD,[a] James L. Checkel,[a] Lawrence B. Schwartz, MD, PhD,[b] Gerald J. Gleich, MD,[a,c] and Kristin M. Leiferman, MD[a,c] *Rochester, Minn, Richmond, Va, and Salt Lake City, Utah*

Background: Basophils possess characteristics of both mast cells and eosinophils, and all 3 cell types often are found together, particularly during allergic reactions. A mAb (J175-7D4) generated against the recombinant pro-form of human eosinophil granule major basic protein 1 (rproMBP1) appeared to stain only basophils in tissue specimens.
Objective: We investigated J175-7D4 to characterize its specificity for basophils.
Methods: Fluid-phase immunoprecipitation, Western blotting, and immunocytochemistry and immunohistochemistry were used to establish the specificity of J175-7D4.
Results: First, J175-7D4 binds to various glycosylated and proteolytically processed forms of rproMBP1, but not to major basic protein. Second, cells transfected with the rproMBP1 gene and human placental tissue (known to express the pro-form of major basic protein 1 [proMBP1]) stain specifically with J175-7D4. In contrast, although mature eosinophils contain substantial major basic protein, they lack proMBP1 and do not stain. Neutrophils, lymphocytes, monocytes, and skin mast cells also are not stained. However, blood basophils are stained by J175-7D4, anti-IgE, Wright-Giemsa (metachromatically), and a previously characterized basophil-specific mAb, 2D7. Finally, formaldehyde-fixed, paraffin-embedded basophils are identically detected by J175-7D4 and 2D7, and J175-7D4 also recognizes putative basophils in formaldehyde-fixed, paraffin-embedded tissue specimens from inflammatory dermatoses, such as atopic dermatitis and delayed pressure urticaria.
Conclusion: The J175-7D4 mAb recognizes proMBP1 as a novel marker for human basophils. J175-7D4 should prove useful for characterizing basophil involvement in human health and disease. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Basophils, immunofluorescence, immunohistochemistry, monoclonal antibody, pro–major basic protein*

Allergic diseases continue to increase in prevalence and have a major socioeconomic effect.[1-3] Because of their expression of cell surface high-affinity IgE receptor (FcεRI), basophils, like mast cells, have been implicated in the pathogenesis of allergic inflammation.[4] Basophils release biologically active substances that are important mediators of allergic inflammation, including lipid mediators (eg, platelet activating factor and leukotriene C_4), proteases (eg, tryptase), histamine, and cytokines (eg, IL-4 and IL-13).[4,5] Furthermore, basophils can induce human IgE synthesis in B cells.[6,7] In cutaneous allergic disease, basophils infiltrate the skin of patients with allergic contact dermatitis,[8] chronic idiopathic urticaria,[9] and atopic dermatitis (after allergen-induced late phase reactions).[10-13] They also infiltrate allergen-challenged allergic airways.[14] Thus, the evidence for the basophil's role in allergic inflammation is compelling. The basophil may also play a protective role in host defense[5,15] (eg, against ticks).[16,17] Unfortunately, more direct proof of the basophil's involvement has been lacking, in part because of the difficulty detecting basophils in tissues. Therefore, a method to detect basophils rapidly and specifically in routinely processed tissue samples would aid in the evaluation of the basophil's role in health and disease.

Basophil-specific antibodies that have been reported include BSP-1,[18] 2D7,[19] 97A6,[20,21] and BB1+.[22,23] BSP-1 and 97A6 recognize basophil surface molecules and are relatively insensitive[19] or concomitantly recognize mast cells[20] in immunohistochemistry, respectively. In contrast, the 2D7 and BB1+ mAbs specifically detect basophils in formalin-fixed tissue specimens;[12,22,24] however, the molecular ligands for these antibodies have not been conclusively identified.[19,23]

Although mast cells and basophils express some of the same molecules and some findings suggest that they originate from the same lineage,[5,20,25] most cell differentiation

From [a]the Departments of Dermatology, Immunology, and Medicine, Mayo Clinic Rochester; [b]the Department of Internal Medicine, Virginia Commonwealth University; and [c]the Department of Dermatology, University of Utah.

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Reprint requests: Kristin M. Leiferman, MD, Department of Dermatology, University of Utah Health Sciences Center, 4B454 School of Medicine, 30 North 1900 East, Salt Lake City, UT 84132-2409. E-mail: kristin.leiferman@hsc.utah.edu.

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Abbreviations used
AML: Acute myelogenous leukemia
CHO: Chinese hamster ovary
MBP: Major basic protein
proMBP1: Pro-form of major basic protein 1
rproMBP1: Recombinant pro-form of major basic protein 1
Sf9: *Spodoptera frugiperda* 9

studies indicate that the basophil is more closely related to the eosinophil.[4,26-29] Consistent with this are eosinophil-like recruitment of basophils—for example, via interaction of eotaxin (CC-chemokine ligand 11[CCL11]) and CC-chemokine receptor 3(CCR3),[5,30] and basophil expression of a molecule immunochemically indistinguishable from eosinophil major basic protein (MBP).[31,32] Serendipitously, we found that a mAb (designated J175-7D4) directed against the recombinant pro-form of MBP1 (rproMBP1) readily stains basophils by indirect immunofluorescence. Because the pro-form of MBP1 (proMBP1) has a highly restricted expression profile[33,34] and is not detectable in mature eosinophils,[35] it represents a potentially useful basophil marker. Here we characterize the basophil-specific mAb, J175-7D4, assess the expression of proMBP1 in peripheral blood leukocytes and selected tissues, and demonstrate the usefulness of J175-7D4 in the study of basophils in human tissues.

METHODS

Human MBP and rproMBP1

Human eosinophil MBP was purified from peripheral blood eosinophils from patients with the hypereosinophilic syndrome, as previously described.[36] Human rproMBP1 was expressed in Chinese hamster ovary (CHO) cells and purified as previously described.[37] This purification yielded heterogeneous, highly glycosylated rproMBP1 (approximately 40-70 kd)[35,38] and minimally glycosylated rproMBP1 (approximately 36 kd). Subsequent spontaneous degradation of isolated rproMBP1 led to formation of intermediate rproMBP1 (approximately 22 kd; confirmed by N-terminal amino acid sequencing).[35] Human rproMBP1 was also expressed in *Spodoptera frugiperda* 9 (Sf9) insect cells (Invitrogen, San Diego, Calif) using a previously described approach.[39] Briefly, proMBP1 cDNA was inserted into a baculovirus transfer vector, pVL1393 (PharMingen, San Diego, Calif), downstream of the polyhedrin gene promoter. This transfer vector was mixed with linearized BaculoGold DNA (PharMingen) and calcium phosphate, and the resultant DNA solution was transfected into Sf9 insect cells by a calcium phosphate method. A baculovirus construct expressing proMBP1 cDNA was identified by a plaque assay, amplified, and used to infect High Five insect cells (Invitrogen) to express rproMBP1. After 4 days of culture, infected and noninfected High Five cells were collected by centrifugation for cytospin preparations. Sf9 cells and High Five cells were cultured in TNM-FH medium (Mediatech, Herndon, Va) supplemented with 10% heat-inactivated FBS and in Ultimate Express protein-free medium (Invitrogen), respectively, at 27°C.

Anti-rproMBP1 mAb production

Anti-rproMBP1 mAb, J175-7D4, was produced essentially as described previously.[34,37] Briefly, 50 μg purified rproMBP1 (minimally glycosylated, approximately 36 kd) was injected intraperitoneally into BALB/c mice once a month for 3 months followed by a final intravenous injection (50 μg rproMBP1 in saline) 3 days before harvesting spleens. Spleen cells were fused with FO myeloma cells, and media conditioned by these hybridoma cells were tested for reactivity with ^{125}I-rproMBP1 by using a Falcon Assay Screening Test system (Becton-Dickinson, Oxnard, Calif). The rproMBP1-reactive hybridoma cell lines were subcloned by limiting dilution, and additional testing identified the J175-7D4 mAb (IgG_1 isotype, κ light chain).

Radioimmunoprecipitation assay

Radiolabeled (^{125}I) MBP or minimally glycosylated rproMBP1 (from CHO cells) was mixed overnight at 4°C with diluted normal mouse serum (diluted 20-fold) or with diluted normal mouse serum containing 500 ng of a mAb generated against MBP or rproMBP1. Burro antimouse IgG antibody was added, and the mixture was left at room temperature for 3 hours and centrifuged at 1000*g* for 15 minutes. Supernatant was carefully drained away for 1 minute, and the pelleted immunoprecipitate was analyzed for radioactivity.

Electrophoresis and Western blotting

NuPAGE precast polyacrylamide gels were used in the associated XCell electrophoresis system (Invitrogen/Novex, Carlsbad, Calif). Cells tested in Western blotting were lysed in denaturing and reducing sample buffer at a concentration of approximately 1×10^7 cells/mL. Cell lysates, molecular weight markers, or differentially glycosylated or degraded forms of purified rproMBP1 were heated in denaturing and reducing sample buffer at 100°C for 5 minutes before loading and running SDS-polyacrylamide gel electrophoresis. Polyacrylamide gels were either stained with GelCode Blue (Pierce, Rockford, Ill) or used to transfer protein to polyvinylidene fluoride membranes for Western blotting per the manufacturer's instructions (Invitrogen/Novex). Membranes with transferred proteins were initially blocked by soaking in a dried milk solution with gentle mixing. Membranes were soaked in dried milk solution containing anti-MBP antibody (J6-8A4, 0.17 μg/mL) or anti-proMBP1 antibody (J163-15E10, 0.17 μg/mL; J175-7D4, 2 μg/mL) or both. This was followed by soaking with horseradish peroxidase–labeled antimouse IgG antibody and Western blot detection using ECL Plus reagent (Amersham Biosciences, Piscataway, NJ) and BioMax autoradiography film (Eastman Kodak Co, New Haven, Conn).

Peripheral blood basophil, neutrophil, and eosinophil isolations

For basophil enrichment, 12.5 mL EDTA-anticoagulated blood was diluted 1:1 with 12.5 mL 1X PBS containing 1% controlled process serum replacement-type 3 heat inactivated Hybri-Max (CPSR-3; Sigma) and 0.01 mol/L EDTA and carefully layered on top of a 2-layer Percoll (Sigma) density gradient (10 mL 1.070 g/mL upper layer and 15 mL 1.080 g/mL lower layer with osmolarities of approximately 315 mOsm). The layered sample was centrifuged at 1000*g* for 30 minutes at 4°C. Enriched basophils were collected from the plasma/1.070 density interface or, more optimally, from the 1.070/1.080 interface and washed with ice-cold PBS, 1% CPSR-3, 0.01 mol/L EDTA, and if necessary, contaminating erythrocytes were lysed by hypotonic shock. Neutrophils were isolated by underlayering 10 mL of 1.085 g/mL Percoll below 30 mL total volume of peripheral blood mixed 1:1 with 1X isotonic Pipes solution and by centrifuging at 2000*g* for 30 minutes at 4°C. All layers, except the bottom layer containing primarily erythrocytes, eosinophils, and neutrophils, were carefully aspirated away. Erythrocytes were lysed by 2 rounds of hypotonic shock, and the remaining cell mixture (approximately 90% neutrophils) was used for preparation of neutrophil-

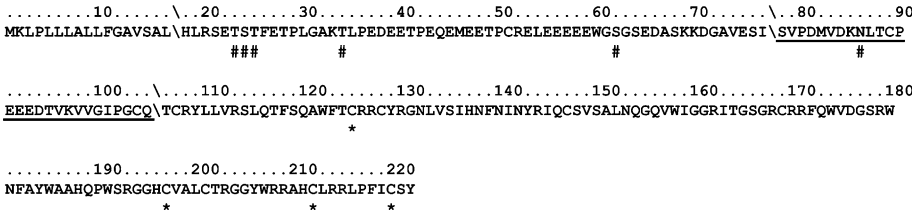

FIG 1. Amino acid sequence of the signal sequence (pre) and human proMBP1. Sequential proteolytic cleavage sites of preproMBP1 (National Center for Biotechnology Information accession number = NP_002719) are indicated by backslashes and give rise to proMBP1 (18-222), intermediate proMBP1 (78-222), and MBP1 (106-222). Six glycosylation sites[38] and the disulfide bond half-cystines determined for MBP1[51] are designated by number symbols (#) and asterisks (*), respectively, under the modified amino acids. The 28 N-terminus amino acids of intermediate proMBP1 contributing to the J175-7D4 epitope are underlined.

enriched cytospins. To isolate eosinophils (>95% purity), an enriched neutrophil preparation (as just described) was subjected to immunomagnetic, negative selection of eosinophils using CD16 MicroBeads (Miltenyi Biotec, Auburn, Calif).[40]

Immunocytochemistry and immunohistochemistry

Cytospins of the following cells were prepared by centrifugation of 1×10^5 to 2×10^5 cells per positively charged microscope slide: acute myelogenous leukemia (AML)14.3D10 (kindly provided by Dr Cassandra C. Paul), rproMBP1-transfected CHO, eosinophil-derived neurotoxin-transfected CHO, rproMBP1-transfected High Five insect, nontransfected High Five insect, enriched basophils, enriched neutrophils, and purified eosinophils. Cytospin slides were fixed in methanol at −20°C for 3 minutes and dried before indirect immunofluorescent staining. For fibrin clot specimens, cell pellets enriched in basophils by Percoll density gradient (as described) were resuspended in EDTA-anticoagulated plasma in a glass tube, and excess calcium was added to induce clotting. Fibrin clot specimens and tissue specimens of nasal polyp, placenta, and skin were fixed in 10% buffered formalin and embedded in paraffin before sectioning for immunohistochemistry.

Tissue sections (5 μm) were processed for immunohistochemistry by mounting serial sections on positively charged microscope slides, deparaffinizing in xylene, and rehydrating stepwise in descending concentrations of ethanol in water.[41,42] For indirect immunofluorescence, slides were incubated in 1 mg/mL trypsin for 1 hour at 37°C and washed. Trypsinized tissue sections (after blocking with normal goat serum) or dried methanol-fixed cytospins were overlain with mAb J175-7D4, J163-15E10, or isotype-matched control (IgG_1, κ, clone MOPC-31C [Sigma]) at 100 μg/mL or with anti-MBP polyclonal antibody or normal rabbit IgG control antibody at 40 μg/mL for 30 minutes at 37°C. Slides were washed 3 times with PBS and treated with 1% chromotrope 2R (J. T. Baker, Phillipsburg, NJ) to eliminate nonspecific eosinophil staining.[43] After 3 PBS washes, slides were overlain with fluorescein isothiocyanate–labeled goat antimouse IgG antibody for 30 minutes at 37°C. Microscope slides were mounted in a glycerol solution containing *p*-phenylenediamine to prevent fading.[44] Representative photomicrographs were taken with a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with excitor barrier filter set: blue BP450/490-LP520/560 (N 487910).

For chromogen-based immunohistochemistry, antigen retrieval was performed as follows: slides were incubated in 1 mg/mL bovine pancreas protease, Type I (Sigma) in 10 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween 20, pH 7.4, for 20 minutes at room temperature and washed 5 times with distilled water; or, for antichymase staining, slides were heated in a steamer in 10 mmol/L sodium citrate, pH 6.0, for 20 minutes, cooled to room temperature, and washed twice for 5 minutes in PBS. The UltraVision LP Detection System kit was then used essentially as instructed by the manufacturer (Lab Vision, Fremont, Calif); all of the following steps were performed at room temperature unless noted otherwise. Processed tissue sections were overlaid with Hydrogen Peroxide Block reagent (Lab vision) for 15 minutes and washed with PBS. Ultra V Block reagent (Lab vision) was applied for 5 minutes and then blotted away. Primary mAbs (10 μg/mL 2D7 (prepared as described[19]), 10 μg/mL J175-7D4, 0.1 μg/mL antichymase CC1 (Novus Biologicals, Littleton, Colo), or 10 μg/mL MOPC-31C) were applied overnight at 4°C on tissue processed by proteolytic antigen retrieval, or, for antichymase staining and heat-based antigen retrieval, antibodies were applied for 30 minutes at room temperature. Slides were washed for a total of 15 minutes in four fresh PBS solutions. Primary Antibody Enhancer reagent (Lab vision) was applied for 20 minutes, followed by 4 PBS washes. In the dark, HRP Polymer reagent (Lab vision) was applied for 30 minutes, slides were washed 4 times in PBS, AEC Single Solution reagent (Lab vision) was applied for 10 minutes, and slides were washed 4 times in deionized water. Slides were subsequently counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, Calif) and coverslipped by using Faramount aqueous mounting media (DakoCytomation, Carpinteria, Calif).

For sequential double staining, indirect immunofluorescence was performed first by using J175-7D4 mAb as described. After images were taken, coverslips were removed by soaking in PBS, the slides were washed in PBS for 30 minutes 3 times to remove J175-7D4 mAb, and then chromogen-based staining using 2D7 mAb (or leaving out 2D7 as a negative control experiment) was performed as described.

RESULTS

mAb J175-7D4 binds rproMBP1 and intermediate rproMBP1, but not MBP

Mature MBP1 is derived from a prepro-form (Fig 1) that is proteolytically processed in a stepwise manner within the eosinophil secondary granule core.[35] To distinguish proMBP1 from MBP1, mAbs were prepared against MBP and rproMBP1 (see Methods). By radioimmunoprecipitation (Fig 2, *A*), anti-MBP1, J13-6B6, preferentially bound radiolabeled MBP, whereas 2 antibodies generated against rproMBP1, J163-15E10 and J175-7D4, only recognized rproMBP1. By Western blot (Fig 2, *B*), J175-7D4 also recognized various forms of rproMBP1, including intermediate rproMBP1 (approximately 22 kd; Fig 1 legend), highly glycosylated

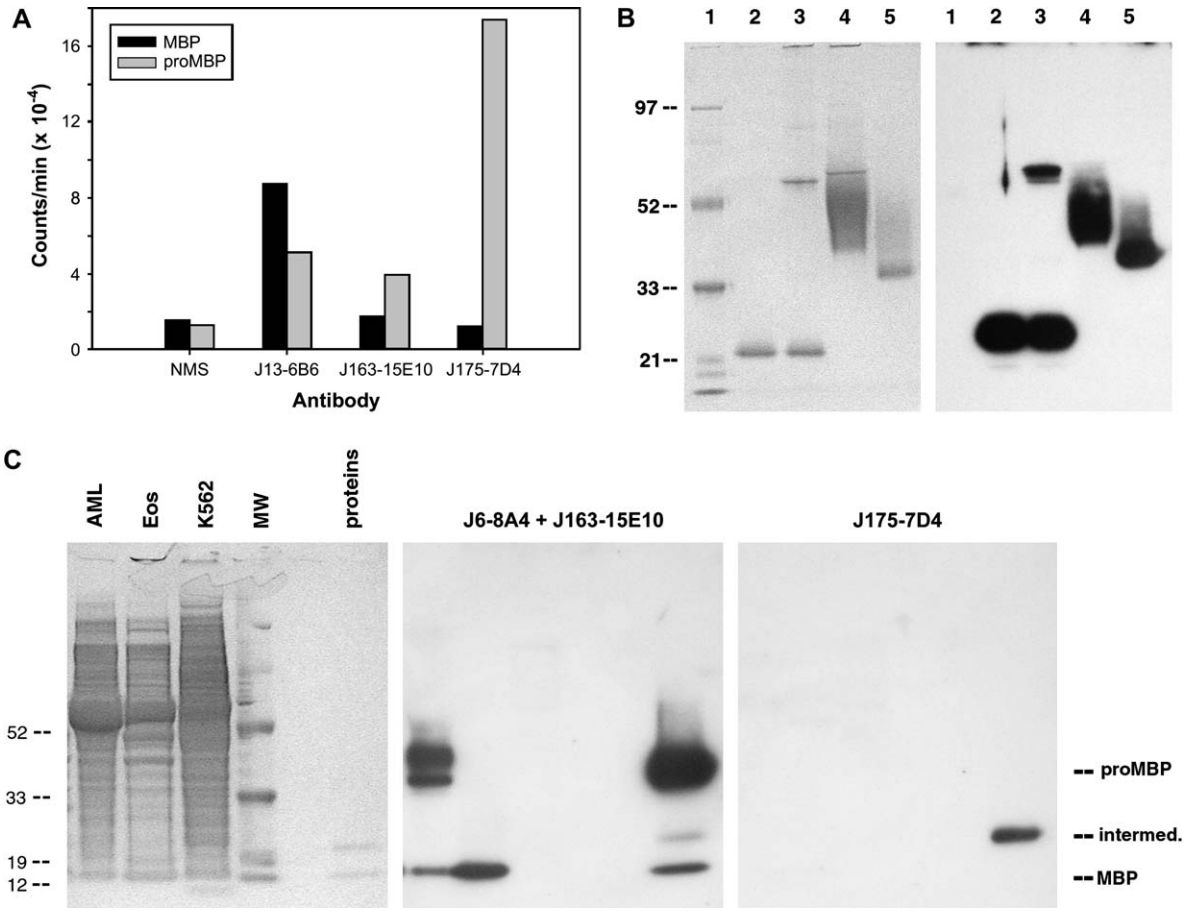

FIG 2. J175-7D4 binds rproMBP1 and intermediate rproMBP1, but not MBP. **A,** Purified MBP or rproMBP1 labeled with iodine-125 was mixed with anti-MBP (J13-6B6), anti-proMBP1 (J163-15E10 and J175-7D4), or normal mouse serum only (negative control), and γ-radiation of the burro antimouse IgG immunoprecipitate was measured. **B,** Different preparations of rproMBP1 (see Methods) were identically loaded and fractioned using 2 SDS-polyacrylamide gels. *Left panel,* GelCode Blue-staining of molecular weight markers *(lane 1)* and various preparations of intermediate rproMBP1 *(lanes 2 and 3)* and rproMBP1 (*lane 4,* highly glycosylated form; *lane 5,* minimally glycosylated form). Marker masses in kilodaltons are indicated on the *left.* The upper band in *lane 3* has not been characterized. *Right panel,* Western blot autoradiograph with J175-7D4 (2 μg/mL) detection. **C,** *Left panel,* GelCode Blue-stained SDS polyacrylamide gel with lysates of cells containing both MBP and proMBP (*AML;* AML14.3D10 cells), MBP only (*Eos;* eosinophils), or neither protein (K562 cells). A mixture of purified MBP (13.8 kd; 250 ng) and minimally glycosylated rproMBP1 (~36 kd) that had substantially degraded to intermediate rproMBP1 (~22 kd; 375 ng total of minimally glycosylated and intermediate rproMBP1 [intermed.]) is also shown *(proteins).* The degraded minimally glycosylated proMBP1 used in the *proteins* lane is the same as that used in *B, lane 2.* Marker *(MW)* masses in kilodaltons are indicated on the *left. Middle panel,* Western blot of an identically loaded gel with detection using a mixture of previously characterized anti-MBP mAb, J6-8A4 (0.17 μg/mL), and anti-proMBP1 mAb, J163-15E10 (0.17 μg/mL). *Right panel,* Western blot of an identically loaded gel with detection using J175-7D4 (2 μg/mL).

rproMBP1 (approximately 40-70 kd), and minimally glycosylated rproMBP1 (approximately 36 kd). However, in Western blots, a comparison between J163-15E10 and J175-7D4 showed the latter to be substantially less sensitive for detecting AML14.3D10-expressed proMBP1 or minimally glycosylated rproMBP1 (Fig 2, *C*). Indeed, J175-7D4 showed no reactivity for proMBP1 between 33 kd and 52 kd (Fig 2, *C, right panel*), whereas a 10-fold lower concentration of J163-15E10 showed extensive reactivity in this region in the 2 lanes expected to contain small quantities of proMBP1 or rproMBP1 (Fig 2, *C, middle panel*). Another mAb, J6-8A4, generated against MBP and commonly used in Western blot detection, recognized purified MBP, MBP in lysates of cells anticipated to contain MBP, and, to a lesser extent, intermediate rproMBP1[33] (Fig 2, *C, middle panel,* and data not shown). In contrast, J175-7D4 mAb, again, detected intermediate rproMBP1, but it did not detect mature MBP in cell lysates or purified MBP (Fig 2, *C, right panel*).

mAb J175-7D4 stains cells and tissue expressing proMBP1

Although the J175-7D4 mAb did not detect proMBP1 in AML14.3D10 cell lysates by Western blotting, it

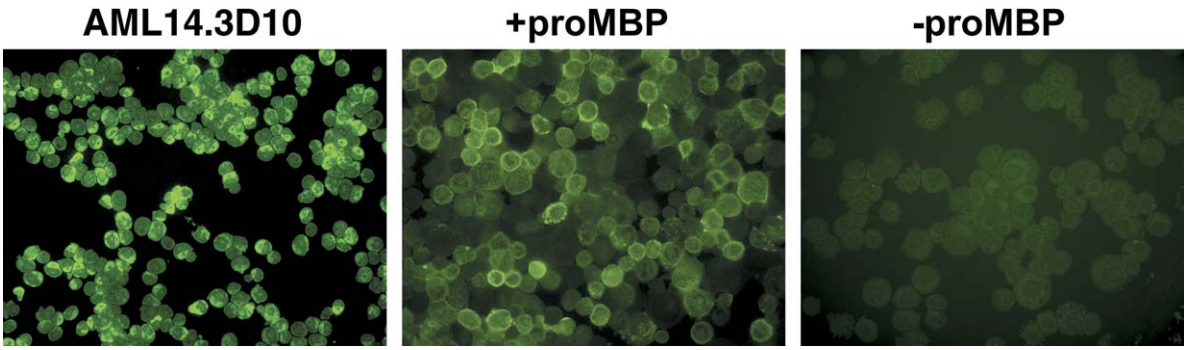

FIG 3. J175-7D4 stains cells expressing human proMBP1. Photomicrographs show indirect immunofluorescence staining of AML14.3D10 cells *(left)* and insect cells with *(middle)* and without *(right)* transfected rproMBP1. J175-7D4 mAb (100 μg/mL) was the primary antibody.

TABLE I. Comparison of histologic and J175-7D4 immunofluorescence cell identification

Sample	Cell differential by histologic staining (%)*					J175-7D4 positive (%)†
	Eosinophils	Neutrophils	Lymphocytes	Monocytes	Basophils	
Enriched basophils(donor 1—low-density cut)‡‖	0	2	47	15	36	43
Enriched basophils(donor 1—mid-density cut)§‖	0	4	18	4	74	72
Enriched basophils(donor 2—mid-density cut)	1	8	51	8	32	28
Purified eosinophils	~95	ND	ND	ND	ND	~1
Enriched neutrophils	ND	~90	ND	ND	ND	~1

ND, Not determined.
*Cell differential by Wright-Giemsa or hematoxylin-eosin staining (200 cells counted).
†Percent of cells positively stained by indirect immunofluorescence (200 cells counted).
‡Low-density cut = cells collected from the plasma-1.070 g/mL Percoll interface.
§Mid-density cut = cells collected from the 1.070 g/mL-1.080 g/mL Percoll interface.
‖See Fig 4.

readily stained the AML14.3D10 cells by using an indirect immunofluorescence technique (Fig 3). Similarly, the J175-7D4 specifically stained insect cells (Fig 3) and CHO cells (data not shown) engineered to express human rproMBP1. J175-7D4 also stained human placental tissue, previously shown to express proMBP1[33,45,46] (data not shown). Notably, J175-7D4 typically produced more intense indirect immunofluorescence staining than J163-15E10.

mAb J175-7D4 specifically stains peripheral blood and tissue basophils

In initial experiments using J175-7D4, nasal polyp tissue and atopic dermatitis skin showed only staining of granular cells that had the appearance of basophils. To test whether basophils were the cells staining in these tissues, peripheral blood basophils were enriched by using a 2-layer density gradient. Cytospins of basophil-rich, neutrophil-rich, and eosinophil-rich preparations were prepared and stained (Table I). Comparable proportions of peripheral blood cells from 2 donors were stained with J175-7D4 and metachromatically with Wright-Giemsa (Table I and Fig 4) and with anti-IgE (data not shown). Mature eosinophils do not contain proMBP1, and they did not stain with J175-7D4 (Table I and Fig 5, *A and B*). Furthermore, although multiple chymase-positive mast cells were present, no cells were stained by J175-7D4 in normal skin (Fig 5, *C and D*). In contrast, several cells stained positively with J175-7D4 in serial sections of a cutaneous late-phase response 24 hours after allergen injection (data not shown). In these 24-hour late-phase response serial sections, a similar number of cells also stained positively with the basophil-specific 2D7 mAb; however, alignment of the few cells stained by 2D7 and J175-7D4 was equivocal. To compare J175-7D4 and 2D7 staining more definitively, we used formaldehyde-fixed, paraffin-embedded fibrin clots of basophil-enriched cell preparations. Comparable percentages of cells were stained by 2D7 and J175-7D4 in serial sections (Fig 5, *E and F*). Using a sequential double-staining approach, the same cells were stained by J175-7D4 (Fig 5, *H*) and then by 2D7 (Fig 5, *G*). If J175-7D4 and 2D7 were staining different cell populations, then additional cells other than those stained by J175-7D4 should have stained in the 2D7 double-staining step. Furthermore, no residual fluorescent staining was detectable after 2D7 double-staining, and in separate experiments, double-staining performed with and without inclusion of the 2D7 primary antibody resulted in identical staining and no chromogen-based staining, respectively, of the cells previously stained by J175-7D4 (data not shown).

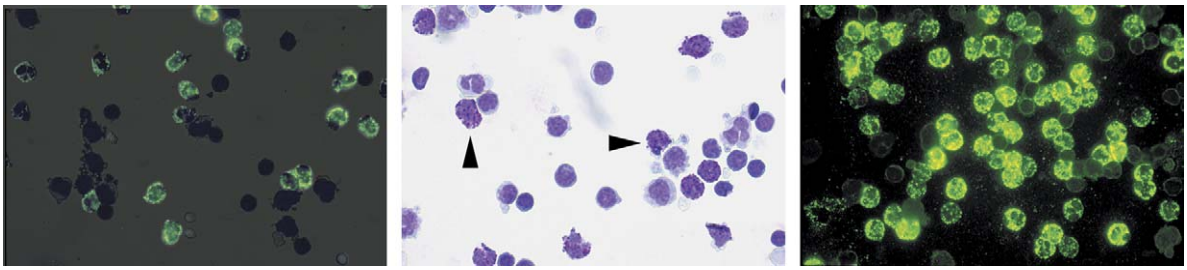

FIG 4. J175-7D4 specifically stains peripheral blood basophils. *Left,* To indicate the percentage of cells stained by J175-7D4, this photomicrograph shows the indirect immunofluorescence using J175-7D4 (100 μg/mL) that overlays the Wright-Giemsa counterstain of a cytospin slide containing a 36% basophil sample (see Table I). Note that basophils cannot be readily identified after counterstaining (not shown); however, none of the 7 counterstained cells that appeared to be monocytes were stained by J175-7D4. Immunostaining using an irrelevant isotype-matched mAb was negative (not shown). *Middle,* Photomicrograph shows a Wright-Giemsa stain of a second cytospin slide of the same 36% basophil preparation. Arrowheads indicate 2 of 9 intact basophils. *Right,* Photomicrograph shows indirect immunofluorescence using J175-7D4 of a 74% basophil preparation (see Table I).

Skin specimens from several patients with dermatologic diseases, some suspected to contain infiltrating basophils, were also tested by using J175-7D4. Two examples of positively staining specimens are shown in Fig 6.

DISCUSSION

After developing a mAb directed against recombinant human proMBP1, we recognized that this antibody, J175-7D4, stained cells in formalin-fixed, paraffin-embedded tissues that appeared to be basophils. Because of the developmental similarities between eosinophils and basophils, the ability of J175-7D4 to stain basophils was tested. From these studies, we conclude that J175-7D4 specifically recognizes basophils in peripheral blood and in formalin-fixed tissue specimens by binding an epitope at least partially contained within the 28 N-terminus amino acids of human intermediate proMBP1.

The J175-7D4 mAb was generated by using purified rproMBP1. Radioimmunoprecipitation (Fig 2, *A*), Western blotting (Fig 2, *B*), and indirect immunofluorescence (eg, Fig 3) all indicated that J175-7D4 specifically recognized proMBP1. Better performance of J175-7D4, compared with a second anti-proMBP1 mAb, J163-15E10, in radioimmunoprecipitation and indirect immunofluorescence experiments suggests that J175-7D4 preferentially recognizes folded (ie, nondenatured) proMBP1. The poor sensitivity of J175-7D4 compared with J163-15E10 in Western blotting under denaturing conditions (Fig 2, *C*) is consistent with its preferential recognition of folded proMBP1. Western blotting performed with comparable loaded quantities of intermediate rproMBP1 and MBP (Fig 2, *C*) further showed that J175-7D4 could detect intermediate rproMBP1, but not MBP. Thus, J175-7D4 recognizes an epitope at least partially contained within the 28 N-terminus amino acids of intermediate proMBP1 (Fig 1).

Immunofluorescence staining of partially purified basophils and other cells showed that J175-7D4 stained the anticipated proportion of basophils, on the basis of a differential cell count, metachromatic cytologic counterstaining of basophil-enriched preparations (Table I and Fig 4), and staining for IgE (data not shown). In contrast, contaminating cell populations (monocytes and lymphocytes; Table I and Fig 4) or enriched mature eosinophils or neutrophils (Table I) did not stain with J175-7D4. Similarly, in eosinophil-rich nasal polyp tissue, only a few infiltrating cells were stained by J175-7D4 (Fig 5). Serial section and double-staining comparisons with the previously characterized basophil-specific 2D7 mAb were consistent with basophil-specific staining by J175-7D4 within formalin-fixed, paraffin-embedded tissue as well (Fig 5). Furthermore, basophils have been previously shown (by immunoassays that do not fully discriminate MBP from proMBP) to contain some forms of MBP.[31] Overall, the highly restricted expression of proMBP1 (breast, placenta, and developing eosinophil)[33,34] and the ability to use routinely processed tissue specimens suggest that the J175-7D4 mAb will be particularly useful in the study of basophil-associated disease.

One caveat is the potential for identification of developing, immature eosinophils in combination with immature or mature basophils in peripheral tissues. For example, as anticipated from the work by Popken-Harris et al,[35] a portion of umbilical cord blood mononuclear cells cultured with IL-5 for 8 days was positively stained with J175-7D4 (data not shown). However, this well-defined potential cross-reactivity should not preclude its usefulness. Moreover, whether immature eosinophils infiltrate peripheral tissues is not known.[47] Therefore, detection of developing eosinophils in peripheral tissues may not be an issue, or such recognition may provide valuable insights into a disease process.

A second concern was whether J175-7D4 also recognized tissue mast cells. In 1 report, human mast cells expressed MBP at both the transcript and protein levels.[48] In most analyses, they used mast cells derived from cultured adult peripheral blood cells or umbilical cord blood cells. They also used more direct analyses that involved

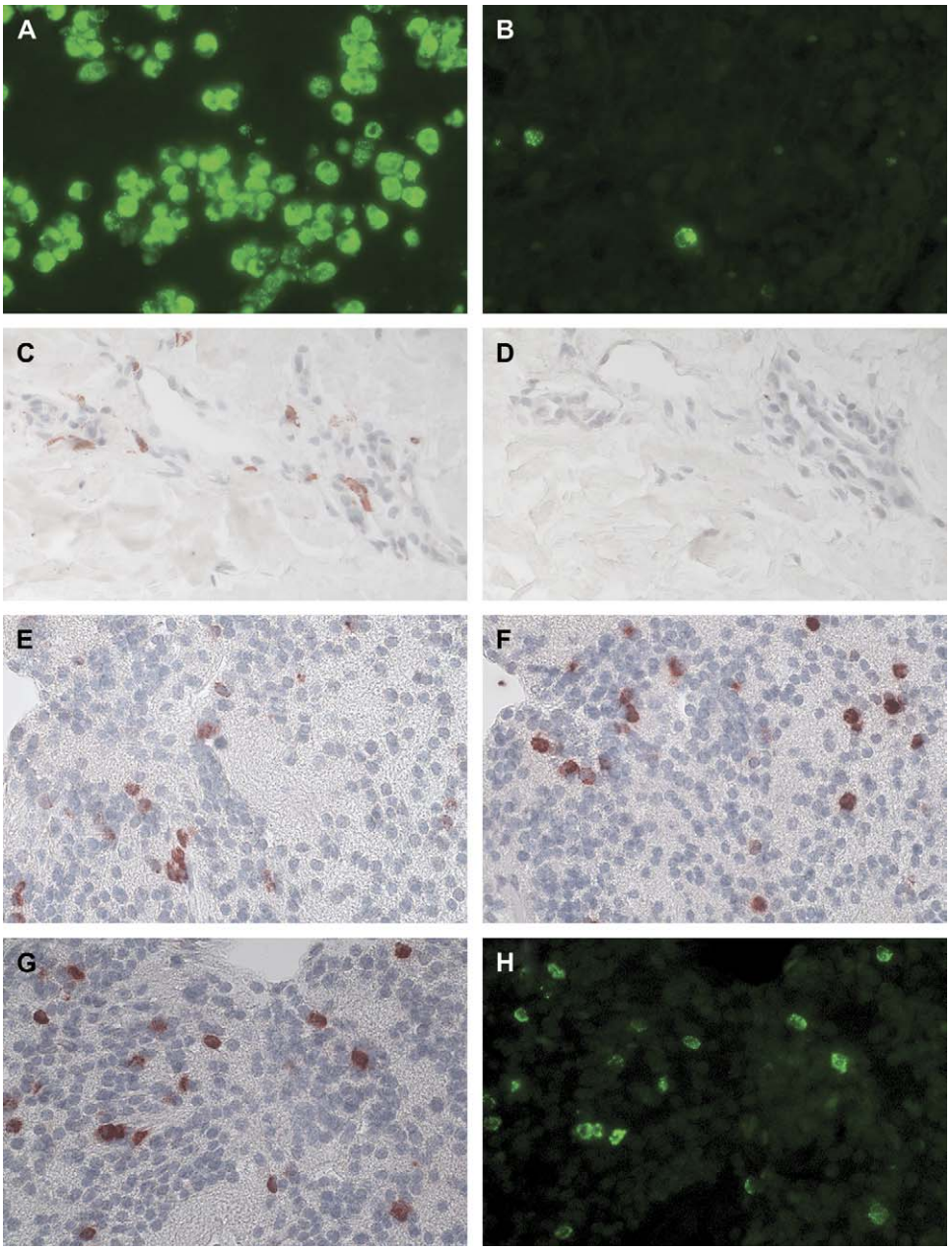

FIG 5. J175-7D4 does not stain nasal polyp eosinophils or skin mast cells, and it recognizes the same cells as 2D7 in a fibrin clot of a basophil-enriched cell preparation. Serial sections of formalin-fixed nasal polyp **(A and B)**, normal skin **(C and D)**, and a basophil-enriched fibrin clot **(E and F)** were stained for eosinophils with polyclonal anti-MBP *(A)*, for mast cells with anti-chymase *(C)*, for basophils with 2D7 *(E)*, and with J175-7D4 *(B, D, F)*. A section of the basophil-enriched fibrin clot was also stained sequentially with J175-7D4 *(H)* and then with 2D7 *(G)*. No residual fluorescent staining was detectable after 2D7 double-staining, and in separate experiments, sequential double staining performed with and without inclusion of the 2D7 primary antibody resulted in identical staining and no chromogen-based staining, respectively, of the cells previously stained by J175-7D4.

immunofluorescence staining for MBP in lung-derived mast cells from a patient with multiple lung cysts and double staining for MBP and tryptase in atopic dermatitis skin and, apparently, normal control skin. All of these data supported the presence of MBP in mast cells. However, these results are in stark contrast to previous work from our laboratory[49] and still may not exclude some level of MBP sequestration by mast cells.[50] Regardless, the lack of staining by J175-7D4 in normal skin indicates that J175-7D4 is not detecting dermal mast cells (Fig 5). If skin mast cells are producing MBP, the lack of mast cell staining may be analogous to the lack of mature eosinophil

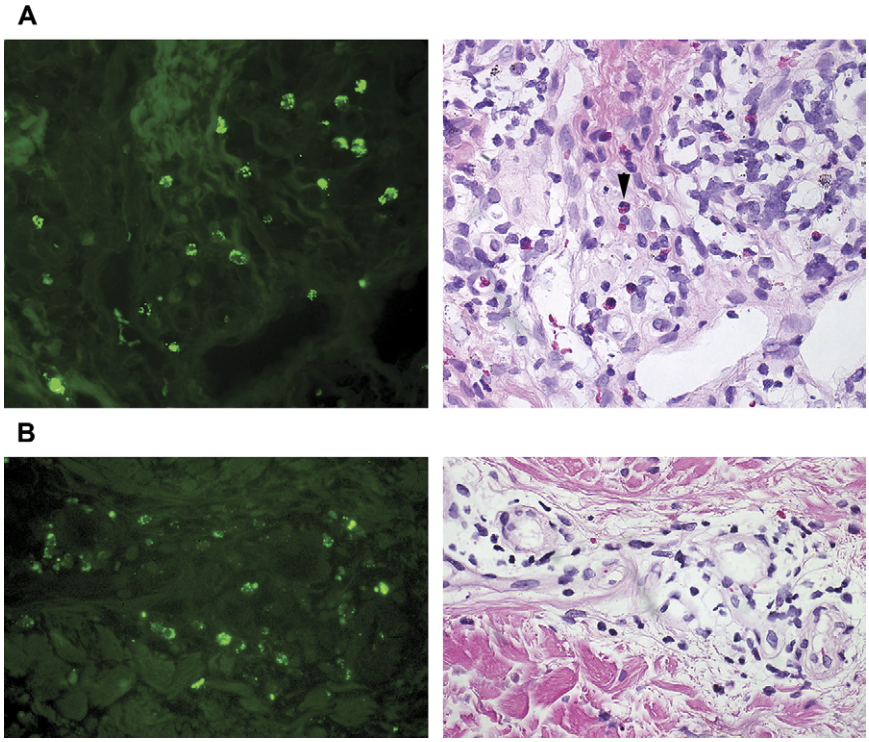

FIG 6. J175-7D4 staining of basophils in formalin-fixed skin tissue. Formalin-fixed lesional skin specimens from a patient with atopic dermatitis **(A)** and a patient with delayed pressure urticaria **(B)** were stained using J175-7D4 (100 μg/mL) and counterstained with hematoxylin and eosin. The *black arrowhead* points out 2 eosinophils in *A, right panel*.

staining. That is, the MBP may be present only in the fully processed 13.8-kd form, which is not recognized by J175-7D4. Alternatively, if skin mast cells contain proMBP1, a smaller quantity or unique packaging of proMBP1 compared with that in basophils might account for the lack of J175-7D4 staining.

In summary, the J175-7D4 mAb is directed against a well-defined antigen epitope at least partially contained within the 28 N-terminus amino acids of human intermediate proMBP1. J175-7D4 specifically detects peripheral blood basophils, and staining of selected tissues further suggests that J175-7D4 detects basophils within formalin-fixed, paraffin-embedded specimens. This novel basophil-specific marker, proMBP1, and the J175-7D4 mAb directed against it should prove useful for the characterization of basophil involvement in human health and disease.

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