

Major Basic Protein Homolog (MBP2): A Specific Human Eosinophil Marker¹

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Human eosinophil granule major basic protein (MBP1) is an exceedingly basic (isoelectric point >11) 14-kDa protein, comprising the core of the secondary eosinophil granule. Recently, a less cationic homolog of MBP, termed MBPH or simply, MBP2, has been discovered. We prepared a panel of mAbs to MBP2 and used these Abs to localize and quantitate this molecule in leukocytes and biological fluids. Specific mAbs for MBP2 were selected using slot-blot analyses and used in a two-site immunoassay, Western blotting, and immunofluorescence microscopy. The sensitivity of the immunoassay was markedly improved by reduction and alkylation of MBP2. MBP1 is more abundant than MBP2 in lysates of eosinophils and their granules, as judged by immunoassay and Western blotting. By immunofluorescence, MBP1 is present in eosinophils, basophils, and a human mast cell line (HMC1), whereas MBP2 is only detected in eosinophils. Neither MBP1 nor MBP2 could be detected in any other peripheral blood leukocyte. MBP2 levels measured in plasma and serum were essentially identical. In contrast to past measurements for MBP1, MBP2 was not detected above normal levels in sera from pregnant donors. However, measurement of serum MBP2 discriminated patients with elevated eosinophils from normal subjects, and MBP2 was also detectable in other biological specimens, such as bronchoalveolar lavage, sputum, and stool. These results indicate that MBP2 is present only in eosinophils and that it may be a useful biomarker for eosinophil-associated diseases. *The Journal of Immunology*, 2006, 177: 7340–7345.

Eosinophils are implicated in allergic diseases and in resistance to helminthic parasites (1). The secondary granules of human eosinophils contain several proteins, including the major basic protein (MBP1),³ MBP homolog (here termed MBP2, previously denoted hMBPH), eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase (1, 2). Individually and collectively, these proteins likely damage tissues in diseases, such as asthma and atopic dermatitis, and also damage large multicellular parasites, such as microfilaria (3–5).

Eosinophils from guinea pigs, rats, and mice contain proteins orthologous to MBP1; furthermore, eosinophils from mice and guinea pigs contain proteins orthologous to MBP2 (6–8). Phylogenetic comparisons of the MBP1 and MBP2 amino acid sequences reveal similarities (66% sequence identity) between human and murine MBP2 (a genetic clade) and between rodent and human MBP1 proteins (8). Guinea pig MBP1 and MBP2 show the most striking similarities compared with the other homologous proteins (6, 7). Comparisons of these proteins' cationicity also reveal distinctions. The isoelectric point (pI) of human MBP2, pI = 8.7, differs considerably from that of human MBP1, pI = 11.4 (2). In contrast, the isoelectric points of guinea pig MBP1, pI = 11.7, and guinea pig MBP2, pI = 11.3 (6, 7), are quite similar, as are the

isoelectric points of murine MBP1, pI = 10.5, and MBP2, pI = 9.95 (8). Despite their different isoelectric points, the in vitro biological effects of human MBP1 and MBP2 appear similar, e.g., cell killing, inducing superoxide anion production, and IL-8 release from neutrophils, and inducing histamine and leukotriene C4 release from basophils, but human MBP1 appears to be more potent than MBP2 in these activities (2).

In this study, we describe preparation of mAbs to MBP2 and their use to identify MBP2 from eosinophil granules, to quantify MBP2 in eosinophils and in human biological fluids, and to localize MBP2 in human peripheral blood leukocytes. The results indicate that MBP2 is present only in eosinophils and may be a useful biomarker for human eosinophil-associated diseases.

Materials and Methods

Purification of MBP1 and MBP2

Eosinophils obtained by cytopheresis of patients with marked blood eosinophilia via a Mayo Institutional Review Board-approved protocol were processed to isolate the eosinophil granule proteins as described earlier (2, 9). After gel filtration over Sephadex G-50 equilibrated with 25 mM sodium acetate, 150 mM NaCl (pH 4.3), fractions enriched in MBP2 were pooled. In some instances, a pooled sample was fractionated twice with Sephadex G-50, and fractions containing MBP2 were identified by Western blotting (2). Alternatively, for improved separation of MBP1 and MBP2, pooled samples were further purified by ion exchange chromatography on carboxymethyl (CM)-Sepharose, equilibrated with 100 mM sodium acetate, 150 mM NaCl, 0.01% CHAPS (pH 4.3). MBP2 and MBP1 were eluted by stepwise elution with 0.5 and 1.0 M NaCl, respectively.

Monoclonal Abs production

Production of the anti-MBP2 mAb J191-12H11 has been described previously (2). To develop an immunoradiometric assay, a panel of mAbs to MBP2 was produced. MBP2 in RIBI adjuvant (RIBI Immunochemical Research) was injected i.p. into BALB/c mice (Charles River Laboratories) monthly for 3 mo, with a final injection of MBP2 in 0.15 M NaCl 3 days before isolating the spleens. Spleen cells were fused with FO myeloma cells using standard procedures. Culture supernatants from wells showing growth were screened for reactivity to MBP2 using the Falcon Assay

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³ Abbreviations used in this paper: MBP1, major basic protein; pI, isoelectric point; CM, carboxymethyl; ECP, eosinophil cationic protein; MCP, mast cell protease.

Screening Test system (FAST; BD Biosciences). Abs were tested by slot-blot analyses as described below. After these tests and subsequent subclonings, hybridomas were cultured in IMDM medium (Protide Pharmaceuticals) containing 10% bovine calf serum (HyClone), 0.5% Ex-cyte growth enhancement medium supplement (Bayer), and $1 \times$ hypoxanthine/aminopterin/thymidine (Sigma-Aldrich). The culture supernatants were purified using a PerSeptive Biosystems BioCAD Workstation and a POROS 20 G-Protein G column (PerSeptive Biosystems). Eluates were concentrated using a Centricon 10 filter (Millipore) by centrifugation at $1000 \times g$.

Western blotting

Samples of column fractions, whole eosinophil and eosinophil granule lysates, and purified MBP2 and MBP1, were denatured in SDS-Tris sample buffer by heating for 5 min at 75°C and electrophoresed on 16% precast Tris-glycine polyacrylamide gels (Invitrogen Life Technologies). After electrophoresis, gels were either stained with Gelcode Blue Stain Reagent (Pierce) or transblotted onto Immobilon-P polyvinylidene difluoride membranes (Millipore) at 120 mA for 1.5 h. Membranes were blocked in buffer containing 5% nonfat powdered milk for 30 min at 37°C and incubated overnight at room temperature with hybridoma-conditioned medium diluted 1/50 in 5% milk buffer or with protein G-purified anti-MBP1 or anti-MBP2 IgG at $1 \mu\text{g/ml}$. After washing with deionized water and ECL buffer (Amersham Biosciences), membranes were incubated for 40 min in secondary HRP-labeled rabbit anti-mouse Ab (DakoCytomation) diluted 1/4000 in ECL buffer. ECL Western blotting reagents (Amersham Biosciences) were used for detection, and the chemiluminescent signal was captured on Kodak Biomax MS film after a 1-min exposure.

For semiquantitative Western blotting analyses, 5-fold decreasing quantities of purified MBP1 or MBP2 per well (500 ng down to 4 ng) and four 5-fold stepwise dilutions of the test sample containing unknown quantities of MBP1 and MBP2 were loaded onto the same 16% Tris-glycine polyacrylamide gel. After transblotting and chemiluminescent detection using either anti-MBP1 (J6-8A4) or anti-MBP2 (J191-12H11), the resulting band intensities for the test sample dilutions were compared with those of the 5-fold titration of purified MBP1 or MBP2, and the quantities of test sample MBP1 and MBP2 were estimated.

Slot-blot analyses

Each mAb was tested against three different samples: MultiMark Multi-Colored Standard (Invitrogen Life Technologies) as a negative control for the MBP1 and MBP2 Abs; purified MBP1 ($350 \mu\text{g/ml}$) to test for cross-reactivity; and purified MBP2 ($350 \mu\text{g/ml}$). Samples were diluted 1/100 in PBS and $50 \mu\text{l}$ was applied to each slot of a slot-blotting apparatus. Immobilon-P polyvinylidene difluoride (Millipore) membranes were blocked with 5% milk buffer for 30 min at 37°C , and Ab binding was analyzed using a 1/50 dilution of hybridoma conditioned medium or $1 \mu\text{g/ml}$ purified J191-12H11, J195-1D4, or J196-1C8 as described above for Western blots.

Two-site immunoassay for MBP2

After preliminary screening, 10 new mAbs emerged as likely candidates for capture or detection of MBP2. As capture Abs, the mAbs to MBP2 were diluted to $5 \mu\text{g/ml}$ in PBS; $100 \mu\text{l}$ was added to wells of Immulon 4 HBX Removawell strips (Dynatech Laboratories) and incubated overnight at 4°C . Wells were washed three times and blocked with $200 \mu\text{l}$ of PPB-E (0.10 M phosphate, 0.1% protamine sulfate, 0.5% bovine calf serum, 0.1% NaN_3 , 0.01 M EDTA (pH 7.5)) for 1 h at room temperature. Wells were washed again, and standard curve dilutions of MBP2 (concentrations ranging from 1 to 500 ng/ml) and samples ($100 \mu\text{l/well}$) were added and incubated overnight at 4°C . In certain experiments, $20 \mu\text{g/ml}$ MBP2 in PPB-E containing 10 mg/ml BSA was reduced and alkylated by treatment with DTT and iodoacetamide as described earlier (10). Briefly, 0.1 ml of sample was diluted with 0.27 ml of Tris-EDTA buffer (0.33 M Tris, 0.12 M NaCl, 0.01 M EDTA (pH 8)) and $30 \mu\text{l}$ of 0.1 M DTT was added. After incubating for 1 h at room temperature, $30 \mu\text{l}$ 0.2 M iodoacetamide was added, followed by a 15-min incubation in the dark. Further dilutions were made in PPB-E as necessary. Wells were washed and $100 \mu\text{l}$ of ^{125}I -labeled detection mAb, diluted to 50 ng/ml in PPB-E, was added ($\sim 3 \times 10^5$ counts/well) and incubated for 2 h at room temperature. Finally, the wells were washed and counted on a gamma scintillation counter.

Two-site immunoassay for MBP1

MBP1 was detected as described earlier (11).

Eosinophil and eosinophil granule lysates

We determined the levels of MBP1 and MBP2 in lysates of whole eosinophils and eosinophil granules. To prepare whole eosinophil lysates, 10^6 cells/ml purified ($>99\%$) eosinophils (12, 13) from a normal individual were incubated for 30 min at room temperature with 0.5% Nonidet P-40 containing 10 mM HCl and Complete Protease Inhibitor Mixture (Roche). The lysate was centrifuged at $35,000 \times g$, and the supernatant was diluted in PPB-E containing 10 mg/ml BSA and reduced and alkylated for the two-site assay. Alternatively, a sample of the entire whole eosinophil lysate or samples of the lysate supernatant and sediment (after centrifugation at $35,000 \times g$) were treated with SDS-PAGE buffer plus DTT and tested by semiquantitative Western blot analysis as described above. Eosinophil granules were prepared as described earlier (9). Eosinophil granule protein samples for two-site immunoassays or for semiquantitative Western blot analyses were prepared by lysing an arbitrary quantity of granule slurry with 0.5% Nonidet P-40, 10 mM HCl, and Complete Protease Inhibitor solution and processing as described above for whole eosinophil samples.

Isolation of PBL

Specimens of fresh peripheral blood were obtained from human volunteers as approved by the Mayo Institutional Review Board. After anticoagulation with 40 U/ml heparin, 2 ml of hetastarch was added per 5 ml of blood, and this mixture was incubated for 15 min at 37°C . The resulting clear upper layer was collected and centrifuged at $200 \times g$ for 10 min. The supernatant was removed, and the remaining buffy coat cells were resuspended in 1 ml of PIPES buffer with 1% alpha calf serum. A small sample was removed for cell counting with Randolph's stain. Additional samples were used to prepare cytocentrifuge slides for immunofluorescence and for Wright-Giemsa staining.

Immunofluorescence

Cytocentrifuge slides of peripheral blood leukocytes and cells from a human mast cell line (HMC1) were prepared, fixed in 100% methanol at -20°C for 3 min, and incubated overnight at 4°C in PBS containing 10% normal goat serum to block nonspecific Ab binding. After washing in PBS, each slide was incubated in a humid chamber at 37°C for 30 min with $150 \mu\text{l}$ of primary Ab, either protein G-purified monoclonal IgG diluted to $100 \mu\text{g/ml}$ in 10% normal goat serum or hybridoma-conditioned medium with 10% normal goat serum. The slides were washed in PBS and stained in 1% Chromotrope 2R (J. T. Baker) for 30 min to eliminate nonspecific eosinophil staining (14). Slides were washed again and incubated for 30 min at 37°C in a humid chamber with affinity-purified FITC-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 1/50 in PBS. After washing, coverslips were mounted with a PBS and 10% glycerol solution containing 0.1% *p*-phenylenediamine and sealed with clear nail polish. Slides were examined at $\times 400$ using a Zeiss Axiophot fluorescence microscope, photographed with Kodak Ektachrome 200 film, and subsequently counterstained with Wright-Giemsa stain.

Patient samples

Following reduction and alkylation, MBP1 and MBP2 levels were measured in plasma and serum specimens from normal or pregnant donors and from patients with eosinophilia. MBP2 levels were also measured in urine, bronchoalveolar lavage fluid, sputum, and stool.

Results

Improved separation of MBP1 and MBP2

Separation of MBP1 and MBP2 had previously been achieved using sequential gel filtration (2). Fractions enriched in MBP2 from the first gel filtration column were pooled and analyzed to isolate MBP1 and MBP2. Here, after the first gel filtration column, we used ion exchange chromatography on CM-Sepharose (Fig. 1). Two peaks emerged, and clear separation of MBP2 and MBP1 was verified by Western blotting using previously characterized mAbs (Fig. 1) (2).

Specificity of mAbs for MBP2

Several MBP2-reactive hybridomas were identified and subcloned. Ten newly generated mAbs were purified and tested for cross-reactivity with MBP1 by slot-blot analysis. Four of the mAbs reacted with both MBP2 and MBP1, one mAb did not react with either, and five mAbs were specific for MBP2 (Fig. 2). Three

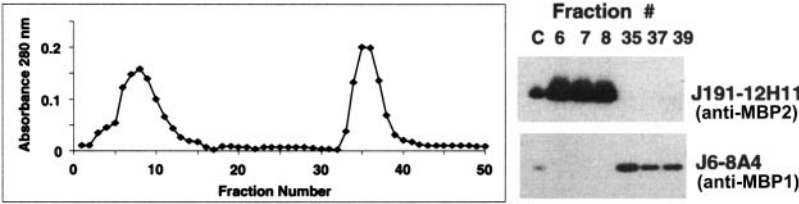


FIGURE 1. Separation of MBP1 and MBP2 on CM-Sepharose. Fractions rich in MBP2 from a Sephadex G-50 separation of eosinophil granule lysate were pooled and applied to a 5-ml CM-Sepharose column. After washing, MBP2 was eluted with 0.5 M NaCl (fractions 3–15), and MBP1 was eluted with 1.0 M NaCl (fractions 33–40) (*left*). Western blot analysis (*right*), using mAb J191-12H11 for MBP2 and J6-8A4 for MBP1, shows separation of these molecules. C is the positive control lane for MBP2 (*top*) and MBP1 (*bottom*).

mAbs specific for MBP2 showed intense binding: J197-5A3, J197-14E1, and J197-14D12. Interestingly, in these experiments J6-8A4, previously thought to be specific for MBP1, also was reactive with MBP2.

Two-site immunoassay for MBP2

The five newly generated mAbs specific for MBP2, along with J191-12H11 (2), were tested as capture and detection Abs in a two-site immunoassay. Three Ab pairs detected MBP2 with strong binding; the other combinations showed minimal binding (data not shown). When these three pairs were tested with MBP2 concentrations ranging from 1 to 500 ng/ml, only one pair (capture J196-1C8, detection J197-14D12) showed a striking concentration response (Fig. 3, untreated MBP2). Further tests with this Ab pair showed a plateau in the response above 1000 ng/ml MBP2. Next, MBP2 in PPB-E with 10 mg/ml BSA was reduced and alkylated to test whether this treatment improved MBP2 detection. Prior experiments with MBP1 had shown about a 10-fold increase in MBP1 reactivity after reduction and alkylation (10, 15). As shown in Fig. 3, reduction and alkylation increased the reactivity of MBP2 10- to 15-fold at the lowest detectable quantity (2 ng/ml), with a plateau above 60 ng/ml for the reduced and alkylated MBP2. Other experiments showed that addition of purified MBP2 to PBS containing 50 mg/ml human serum albumin or to normal human serum before reduction and alkylation enhanced the detec-

tion of MBP2 compared with reduction and alkylation in PPB-E only (data not shown). Overall, reduction and alkylation in PPB-E with 10 mg/ml BSA appeared to be equally effective for the most sensitive detection of purified MBP2.

Eosinophil content of MBP1 and MBP2

Prior studies showed that the ratio of MBP1 to MBP2 mRNA transcripts in developing eosinophils was ~8:1; the ratio of MBP1 to MBP2 protein content in eosinophil granules was ~16:1, based on 280 nm absorbance of fractions eluting from Sephadex G-50 columns (2). After increasing the number of HCl extractions of the starting eosinophil granule slurry to seven (instead of three), measurements of MBP1 and MBP2 in fractions from Sephadex G-50 gel filtration of an eosinophil granule lysate showed that the MBP1:MBP2 ratio was ~8:1 (Fig. 4). We also measured MBP1 and MBP2 in lysates of eosinophils and their granules. Table I compares the quantities of MBP1 and MBP2 in extracts prepared from whole eosinophils and eosinophil granules using two-site immunoassays as well as semiquantitative Western blotting for MBP1 and MBP2. Ratios varied from 1.3 to 7.0 with the lowest ratios obtained using whole eosinophil lysates. Thus, more MBP2 is likely present in eosinophils than shown by our prior analysis of Sephadex G-50 fractions.

Localization of MBP1 and MBP2 in eosinophils, basophils, and HMC1 cells

Among peripheral blood leukocytes, MBP1 is present in eosinophils and, to a lesser extent, in basophils (16). Using a new MBP2-specific mAb, J196-1C8, the presence of MBP2 in peripheral blood leukocytes was tested by immunofluorescence. Only eosinophils contain detectable MBP2 (Fig. 5, A and B), whereas MBP1



FIGURE 2. Slot-blot analysis of MBP2 mAbs. Ten Abs for MBP2 were tested with both MBP1 and MBP2 (175 ng of purified MBP1 or MBP2 per slot). As controls, mAbs J6-8A4 (reactive with MBP1) and J191-12H11 (reactive with MBP2), were also tested. Purified J191-12H11, J195-1D4, and J196-1C8 were used at 1 μ g/ml, and all other Abs were used as 1/50 dilutions of hybridoma conditioned medium.

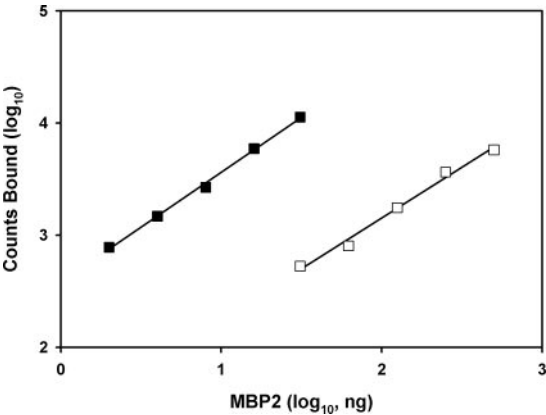


FIGURE 3. MBP2 detection by two-site immunoassay. Plates were coated with mAb J196-1C8 and bound MBP2 was detected with radiolabeled J197-14D12. ■, The concentration-response curve for reduced and alkylated MBP2; □, the results for untreated MBP2.

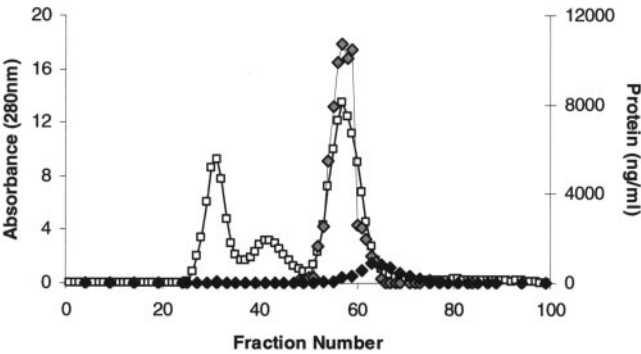


FIGURE 4. Fractionation of an eosinophil granule extract over Sephadex G-50. Seven extractions of an eosinophil granule sample with 0.01 M HCl were pooled and fractionated over a Sephadex G-50 column, and absorbance at 280 nm measured (□). Two-site immunoassays for MBP1 (gray diamonds) or MBP2 (♦) were performed on the collected fractions. A total of ~69 mg MBP1 and 9 mg MBP2 were detected in this experiment.

is detectable in eosinophils, basophils (Fig. 5, *C* and *D*), and HMC1 cells (Fig. 5, *E* and *F*). Although counterstaining with a histological stain (such as Wright-Giemsa) after indirect immunofluorescence typically suffers from degradation of cell morphology (Fig. 5, *B* and *D*), careful inspection of the entire cytospin slides for neutrophils (lightly staining with segmented, polymorphic nuclei) and for the few mononuclear cells (lightly staining with larger, nonsegmented nuclei) showed that these cells were not immunofluorescently stained using the anti-MBP Abs.

Quantitation of MBP2 in biological fluids

Using the two-site immunoassays, Fig. 6 shows the quantities of MBP1 and MBP2 in serum and plasma from normal or pregnant subjects and from patients with eosinophil-associated diseases. MBP2 levels in serum and plasma were similar. When compared with the quantities of MBP1, MBP2 levels are consistently lower, but they readily distinguish patients with eosinophil-associated diseases from normal controls. Interestingly, analyses of pregnancy sera showed MBP2 levels in the normal range. MBP2 was not detectable in 16 of 17 urine samples; the remaining specimen showed a level of 13 ng/ml. MBP2 was measurable in a random selection of stool extracts (*n* = 24, median = 18 ng/ml, mean = 18

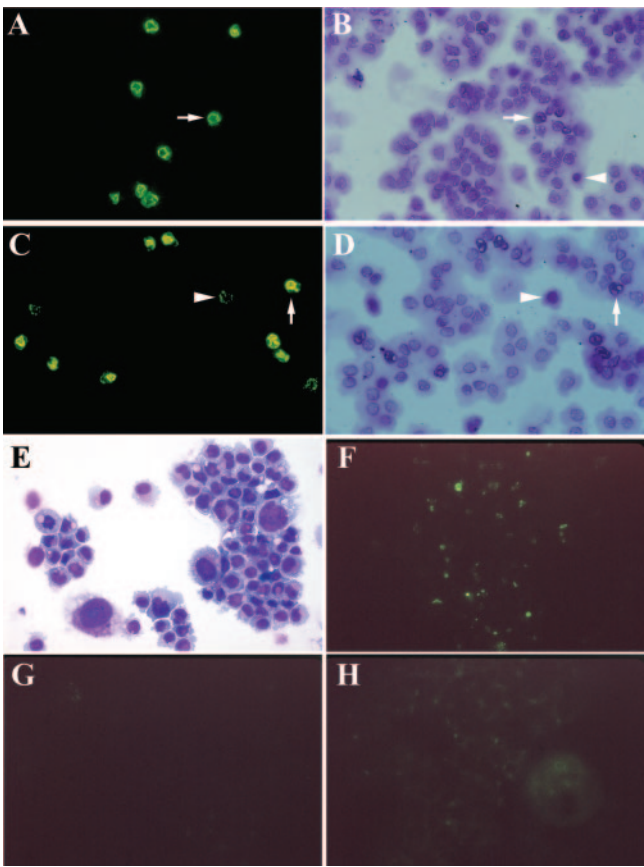


FIGURE 5. Photomicrographs of peripheral blood leukocytes and HMC1 cells stained with specific Abs for MBP1 and MBP2. *A*, Cells that stain with anti-MBP2 (J196-1C8) by immunofluorescence; *B*, Wright-Giemsa counterstain of the same area shown in *A*. *C*, Cells that stain with anti-MBP1 (J171-8B2); *D*, Wright-Giemsa counterstain of the same area shown in *C*. Arrowheads point to basophils (*B–D*) and arrows point to eosinophils (*A–D*). The basophil in *B* (white arrowhead) was identified on the basis of its nuclear and cytoplasmic morphology. Inspection of the entire cytospin stained with anti-MBP2 (J196-1C8) failed to show positively staining cells resembling basophils comparable to those seen in *C*. HMC1 cell cytospins stained with Wright-Giemsa (*E*), anti-MBP1 (J171-8B2) (*F*), anti-MBP2 (J196-1C8) (*G*), and anti-MOPC isotype control (*H*) are shown. Original magnifications were all at $\times 400$.

Table I. MBP1 and MBP2 in lysates of whole eosinophils and eosinophil granules^a

	Whole Eosinophils			Eosinophil Granules		
	MBP1 ($\mu\text{g}/10^6$ cells)	MBP2 ($\mu\text{g}/10^6$ cells)	Ratio of MBP1:MBP2	MBP1 ($\mu\text{g}/\text{ml}$)	MBP2 ($\mu\text{g}/\text{ml}$)	Ratio of MBP1:MBP2
Two-site immunoassay ^b						
Supernatant	6.8 (2.1)	5.4 (2.0)	1.3	31 (9.6)	10 (8.4)	3.1
Entire-mixture	10 20	4 10	2.5 2	1250 60	300 10	4 6
Semiquantitative Western ^c						
Supernatant	10 20	4 10	2.5 2	600 60–75	200 10	3 7
Sediment	20 0	0 0		500 0	150 0	3

^a Lysis procedures are described in *Materials and Methods*.

^b Results are means and (SD) from four assays using triplicate samples of the same preparation of whole eosinophils or granules. Sediment was not analyzed due to the requirement for nondenatured, monodisperse protein.

^c Semiquantitative Western estimates are within a factor of 2 (i.e., one-half to twice the value shown) and were determined in two separate assays using either whole eosinophils or eosinophil granules; values for assays 1 and 2 for each of the three sample types tested (entire mixture, supernatant, sediment) are shown in the upper and lower row, respectively. The values for the eosinophil granules used two different samples from an arbitrary quantity of slurry of granules; therefore, the marked assay-to-assay variability in absolute quantities of MBP1 and MBP2 is not surprising.

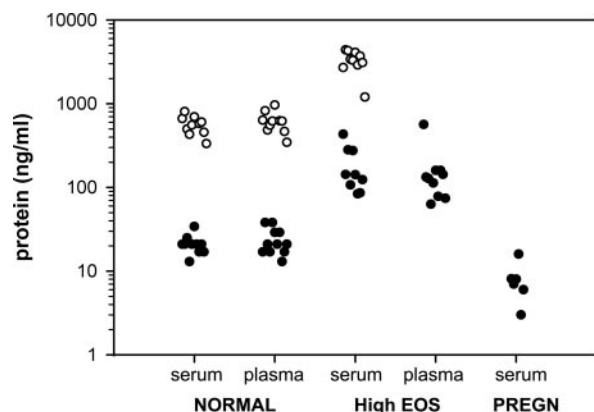


FIGURE 6. MBP1 or MBP2 in serum or plasma from normal or pregnant (PREGN) subjects or patients with eosinophilic disease (High EOS). Values were determined by the two-site immunoassays for MBP1 (○) and for MBP2 (●). All sera were reduced and alkylated as described in *Materials and Methods*.

ng/ml, range = 0–49 ng/ml) and in both sputum ($n = 52$, median = 22 ng/ml, mean = 65 ng/ml, range = 0–624 ng/ml) and bronchoalveolar lavage ($n = 47$, median = 8 ng/ml, mean = 28 ng/ml, range = 0–580 ng/ml) fluids.

Discussion

MBP1 and MBP2 are homologous molecules with similar molecular weights but different isoelectric points, whereas MBP1 is quite basic with a $pI > 11$, MBP2 is considerably less basic with a pI of 8.7 (2). Furthermore, these molecules differ antigenically, and a mAb to MBP2, J191-12H11, was produced and used to purify MBP2. Here, we have extended those results by producing a panel of mAbs specific for MBP2. These Abs have been used to localize MBP2 in peripheral blood leukocytes and to establish a two-site immunoassay for MBP2. It is necessary to reduce and alkylate samples for sensitive detection of MBP1 by immunoassay (10, 15), and the same treatments are needed for sensitive detection of MBP2. Thus, the MBP molecules apparently form disulfide bonds with themselves and other molecules, and these disulfide bonds interfere with detection of the MBP molecules.

Isolation of MBP2 free of MBP1 has been difficult. By sequential gel filtration chromatography, we obtained reasonable separation of these molecules (2), but the yield of MBP2 was poor. Because of the difference in isoelectric points between MBP1 and MBP2, we attempted to separate them by ion exchange chroma-

tography on CM-Sepharose. This procedure readily separated MBP1 and MBP2 to give homogeneous preparations (Fig. 1) and was reproducible on several occasions. However, exposure to the high concentrations of sodium chloride used to elute MBP1 and MBP2 from CM-Sepharose may be deleterious as subsequent dialysis of fractions against 25 mM sodium acetate and 150 mM NaCl (pH 4.3) resulted in varying precipitation. Therefore, although this procedure effectively isolated these molecules, obtaining high yields of soluble molecules remained a challenge. Recently, repetitive extractions of the eosinophil granule preparation, followed by gel filtration chromatography over a 200-cm Sephadex G-50 column, successfully isolated soluble MBP2 (17). MBP2 appears to be differentially extracted from the granule, with later extractions yielding increased amounts of MBP2. Furthermore, doubling the chromatographic bed height resolves the two MBP molecules, and a unique MBP2 peak elutes from the column. Although protein yields remain relatively low, this new procedure simplifies the purification and isolation of soluble MBP2.

With the two-site immunoassays, we estimated the quantities of MBP2 and MBP1 in Sephadex G-50 column fractions of eosinophil granule extracts and in whole eosinophil and eosinophil granule lysates. Our prior experiments suggested that eosinophils contain more MBP1 than MBP2, and the present results corroborate those early findings. Table I shows estimates of the ratios of MBP1 to MBP2 in whole eosinophil and eosinophil granule lysates, and these ratios range from 1.3 to 7.0. Interestingly, the experiments showing a greater proportion of MBP1, including the results shown for the Sephadex G-50 column in Fig. 4, were performed with eosinophil granules rather than with intact eosinophils. The reason for this difference is obscure; preliminary electron microscopy results detected MBP2 in the eosinophil granule and not in other eosinophil organelles. Overall, these results support the view that more MBP1 is present in the eosinophil than MBP2.

Immunofluorescent localization of MBP1 and MBP2 in human leukocytes has confirmed prior findings that MBP1 is present in both eosinophils and basophils (16). MBP1 is also detected in HMC1 cells. In contrast, the present results show that MBP2 is present only in eosinophils (Fig. 5). Public transcriptome data from human leukocytes with and without basophils and from mast cells derived from various tissues (including tonsil, lung, blood, and skin) also indicate that transcripts for MBP1 (i.e., *PRG2*), but not for MBP2 (i.e., *PRG3*), are detected in basophils and mast cells (Table II) (18). Thus, MBP2 appears to be a specific marker for the eosinophil.

Several reports have described the quantitation of another eosinophil granule protein, the eosinophil cationic protein (ECP), and

Table II. *Public basophil and mast cell transcriptome data^a*

Gene Symbol		Affymetrix Probe Set No.	Relative or Absolute Signal	
Basophils	<i>PRG2</i> (MBP1)	211743_s_at	With basophils ^b	Without basophils
	<i>PRG3</i> (MBP2)	220811_at	4.69	0.77
	<i>FceRIA</i> ^c	211734_s_at	1.78	1.78
Mast cells			226.20	16.59
			Minimum signal (P/A call) ^d	Maximum signal (P/A call)
	<i>PRG2</i> (MBP1)	211743_s_at	199.7 (P)	26,748.1 (P)
	<i>PRG3</i> (MBP2)	220811_at	30.0 (A)	314.9 (A)
	<i>FceRIA</i>	211734_s_at	1,265.4 (P)	13,174.3 (P)

^a Data from www.nch.go.jp/imal/GeneChip/public.htm (18) using Affymetrix Human U133A GeneChips.

^b Data generated from the analysis of RNA from human leukocytes with and without basophils present.

^c *FceRIA*, High-affinity IgE receptor; for comparison to *PRG2* and *PRG3*.

^d Minimum and maximum fluorescence signal among the 13 different mast cell samples analyzed, including human mast cells derived from skin, lung, tonsil, and cord and peripheral blood. P, "present" (the given transcript is probably present) and A, "absent" (the given transcript is probably absent; i.e., nonspecific signal) and these are assigned by an Affymetrix-derived algorithm that assesses the overall quality of the fluorescence signals associated with a given probe set.

its value as a biomarker in patients with eosinophil-associated diseases (19–21). However, both ECP (RNase 3) and eosinophil-derived neurotoxin (RNase 2) are present in human neutrophils (22–24). Therefore, in some instances the elevated levels of these proteins may be a consequence of their release from neutrophils. In addition, eosinophil-derived neurotoxin and ECP are released when blood clots, resulting in increased basal levels (25). Because it is apparently absent from mast cells and all other peripheral blood cells save eosinophils, MBP2 becomes a strong candidate as a marker for eosinophil-associated diseases. Other advantages in quantitating MBP2 over MBP1 are the observations that MBP2 mRNA is not expressed in placenta (2) and that, as described above, MBP2 levels are not elevated in sera of pregnant women (Fig. 6). Therefore, despite their similarities, only MBP1 is associated with reproduction. This finding implies the existence of specific regulatory functions governing the production of these molecules. The distinct gene structure and regulatory elements of the MBP2 gene compared with the MBP1 gene are consistent with this concept (26).

Fig. 6 shows the concentrations of MBP2 and MBP1 in serum and plasma and indicates relatively low levels for the former and higher levels for the latter. The relatively high basal levels of MBP1 may favor MBP2 as a biomarker of eosinophil-associated diseases. This follows because the low levels of MBP2 in these fluids constrain the normal range and thus should permit better discrimination between normal and abnormal fluids.

The biological function(s) and potential role(s) in disease of human MBP2, like those of MBP1, remain difficult to define precisely. We have previously shown that MBP2 has similar, but generally less potent, *in vitro* biological activity compared with that of MBP1 (2). Numerous other studies have implicated MBP1, and thus presumably MBP2, in host defense against parasites and in allergic disease pathology. A unique property of human MBP2 is its relatively low positive charge among the known MBPs; therefore, unique biological activity attributable to human MBP2 might relate to this reduced cationicity. A potentially instructive example is the increased diffusion of mouse mast cell protease (MCP)-7, and as a consequence its increased activity in blood, compared with the more cationic mouse MCP-6 after their release from connective tissue mast cells (27). Perhaps human MBP2 and MBP1 have a similar relationship to that of MCP-7 and MCP-6? Regardless, several molecular properties common to MBP2 and MBP1 (intact signal and prosequence amino acid sequences, two conserved disulfide linkages, and abundant expression in eosinophils (2)) suggest that MBP2 is more than a nonfunctional evolutionary remnant.

In summary, we have established a two-site immunoassay for MBP2 and have established the conditions for optimal measurement of this molecule. Human serum contains relatively low levels of MBP2, and MBP2 is present only in the eosinophil. This combination of low baseline levels as well as specificity of this molecule for the eosinophil strongly suggest MBP2's usefulness as a marker for eosinophil-associated diseases.

Disclosures

The authors have no financial conflict of interest.

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