LOCALIZATION OF EOSINOPHIL GRANULE
MAJOR BASIC PROTEIN IN HUMAN BASOPHILS*

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The large specific granule of the human eosinophil contains a number of low molecular weight, highly basic proteins that have been purified to homogeneity, including the major basic protein (MBP) (1–3),[1] the eosinophil cationic protein (4, 5), and the eosinophil-derived neurotoxin (6, 7). The comparative physicochemical and immunochemical characteristics of these proteins have been examined in detail, and they clearly represent distinctive cationic moieties of the granule.[2] MBP, the most abundant of the three proteins, is a small polypeptide of 9,300 D (3) that has been localized by subcellular fractionation and immunoelectron microscopy to the electron-dense crystalloid core of the guinea pig (8) and human (S. J. Ackerman, D. A. Loegering, and G. J. Gleich, unpublished observations) eosinophil granule. MBP has been regarded as a unique eosinophil protein and its release both in vitro and in vivo has been used as a specific marker for eosinophil localization, degranulation, and function (9–18).

Using a sensitive double-antibody immunofluorescent method (19), MBP has been localized extracellularly in a number of tissues and organs whose dysfunction in disease is generally associated with eosinophil infiltration (11, 20, 21).[3] In these experiments, a small number of cells stained for MBP by immunofluorescence that subsequently could not be clearly identified as eosinophils. Because we recently demonstrated that the Charcot-Leyden crystal (CLC) protein (lysophospholipase), another eosinophil protein, was also present in and crystallized from human basophils (22), we postulated that MBP might also be a constituent

[1] *Abbreviations used in this paper:* ACP anti-MBP, affinity chromatography-purified antibody to MBP; CLC, Charcot-Leyden crystal; FACS, fluorescence-activated cell sorter; HEGA, Hank's balanced salt solution containing 5 mM EDTA, 5.4 mM glucose, and 0.1% bovine serum albumin; HSA, human serum albumin; MBP, eosinophil granule major basic protein; NRIgG, normal rabbit IgG; PBS, Dulbecco's phosphate-buffered saline; PPF, 0.1 M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.4) containing 0.1% protamine sulfate, 0.5% newborn calf serum, and 0.1% sodium azide; RIA, radioimmunoassay; TSIRA, two-site immunoradiometric assay.

[2] Ackerman S. J., D. A. Loegering, P. Venge, I. Olsson, J. B. Harley, A. S. Fauci, and G. J. Gleich. Distinctive cationic proteins of the human eosinophil granule: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. Manuscript submitted for publication.

[3] Kephart, G. M., G. J. Gleich, D. H. Connor, D. W. Gibson, and S. J. Ackerman. Deposition of eosinophil granule major basic protein onto microfilariae of *Onchocerca volvulus* in the skin of patients treated with diethylcarbamazine. Manuscript submitted for publication.

of the blood basophil. We tested this hypothesis using highly purified suspensions of normal human basophils (23), a sensitive radioimmunoassay (RIA) (15), and an immunofluorescence procedure (11, 19) for the detection of MBP. We now report that normal human blood basophils as well as basophils from a patient with basophil leukemia contain a protein that is immunochemically indistinguishable from eosinophil MBP.

Materials and Methods

Preparation of Mononuclear Cells. Basophil-containing mononuclear cell fractions were prepared from venous blood of normal donors by Ficoll-Hypaque density gradient centrifugation (24). 10-ml aliquots of blood anticoagulated with buffered EDTA (pH 7.35, 5 mM final concentration) were diluted with 30 ml of Hank's balanced salt solution (pH 7.35, calcium and magnesium free; Gibco Laboratories, Grand Island, NY) containing 5 mM EDTA, 5.4 mM glucose, and 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) (HEGA). Diluted blood was underlayered with 10 ml Histopaque (Sigma Chemical Co.) and centrifuged at 400 *g* for 30 min at room temperature. Cells at the plasma-Histopaque interface were removed, diluted 1:1 with ice-cold HEGA, and centrifuged at 400 *g* at 4°C for 10 min. Cell pellets were washed twice with HEGA. This procedure yielded cell suspensions containing primarily lymphocytes and monocytes with 0.5-2.0% basophils as determined by alcian blue staining (25), and no eosinophils or neutrophils as determined by differential cell counts of Wright's-stained cytocentrifuge slides.

Basophil Purification. Highly enriched suspensions of blood basophils were prepared by flow microfluorometry using a fluorescence-activated cell sorter (FACS IV; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) according to the method of Weil et al. (23) as previously described (22, 26). Briefly, mononuclear cells isolated as described above were labeled for 30 min on ice with a 1:50 dilution of fluorescein isothiocyanate-conjugated IgG fraction sheep anti-human IgE (Cappel Laboratories, Cochranville, PA) in HEGA buffer. After three washings at 4°C in HEGA, the cells were sorted using the FACS IV. Bright fluorescent cells were sorted first at a rate of ∼10,000 cells/s without constraints and then re-sorted with contraints at a rate of ∼1,000 cells/s; ice-cold HEGA was used as the sheath and diluting fluid during sorting. The resultant cell preparations contained 87-97% basophils, as determined by alcian blue staining (25) and by differential cell counts of cytocentrifuge slides fixed with absolute methanol and stained with Wright's stain, or fixed with Mota's fixative and stained with toluidine blue (27). FACS-purified basophil preparations did not contain any eosinophils or neutrophils and the contaminating cells consisted of small lymphocytes and monocytes. Basophils in the FACS-purified preparations contained normal amounts of histamine (1.59 ± 0.1 pg/basophil) as previously reported (26).

Eosinophil Purification. Eosinophils were purified from venous blood of normal donors using the method described by Vadas et al. (28). Briefly, blood from healthy normal donors was collected in buffered EDTA, pH 7.35 (final concentration 5 mM). Leukocytes and erythrocytes were separated by sedimentation of a mixture of 5 vol blood with 1 vol of 6% dextran T70 (Pharmacia Fine Chemicals, Piscataway, NJ) in Dulbecco's phosphate-buffered saline (PBS) (Gibco Laboratories) at 37°C for 30-60 min. Eosinophils were purified further by discontinuous gradient centrifugation on a series of six metrizamide solutions (Nyegaard, Oslo, Norway) of 18, 20, 22, 23, 24, and 25% (wt/vol) as previously described (28). The discontinuous gradient interfaces containing 85-95% eosinophils were collected and washed three times by centrifugation at 400 *g* with HEGA buffer before use. Eosinophils were counted in a hemocytometer after staining with Randolph's phloxine-methylene blue (29).

Eosinophil-rich leukocyte suspensions were obtained from two patients with the hypereosinophilic syndrome by cytapheresis on a semicontinuous flow cell processor (Haemonetics Corp., Natwick, MA) as previously described (30). The anticoagulant used was 2% sodium citrate added to 6% hydroxyethyl starch. After sedimentation of erythrocytes,

cells in the supernatants were centrifuged at 400 g for 10 min, washed twice with RPMI 1640 containing 10% fetal calf serum (Gibco Laboratories), resuspended in ice-cold RPMI 1640 containing 40% fetal calf serum and 20% dimethylsulfoxide (Sigma Chemical Co.), aliquoted at 1.0 × 10^7 eosinophils per vial into Nunc vials, frozen slowly to −70°C (approximately −1 to −2°C/min) and stored under liquid nitrogen (31). Eosinophils were recovered from liquid nitrogen storage according to the method of Holden et al. (31) with quick thawing of frozen aliquots at 37°C and controlled dilution of the dimethylsulf-oxide-containing freezing medium to maintain maximum cell viability. Cryopreserved eosinophils were washed twice by gentle centrifugation at 200 g for 10 min in HEGA before use. The final cell preparations from the two patients contained 94 and 98% eosinophils.

Immunofluorescent Localization of MBP and CLC Protein in Basophils.   Cytocentrifuge slides of mononuclear cells (see Preparation of Mononuclear Cells) or FACS-purified preparations of basophils were stained for MBP and CLC protein by a double-antibody indirect immunofluorescence procedure as previously described (19, 22) with minor modifications. Briefly, cytocentrifuge slides were fixed for 5 min in absolute methanol, washed three times with PBS, and incubated overnight in 10% heat-inactivated normal goat serum in PBS at 4°C. Slides were washed three times in PBS and placed in a humidified chamber. To stain for MBP, slides were overlaid with either a protein A-purified normal rabbit IgG (NRIgG) as negative control or an affinity chromatography-purified rabbit antibody to human MBP (ACP anti-MBP) used at equal IgG concentrations. The preparation of these reagents and the specificity for MBP localization in tissues and cells have been described in detail previously (20, 21). To stain for CLC protein, slides were overlaid with a 1:40 dilution of either the preimmunization serum from the CLC-immunized rabbit as negative control or the postimmunization rabbit anti-CLC antiserum as previously described (22). After incubation overnight at 4°C, slides were washed three times with PBS, stained for 30 min with a 1% solution of chromotrope 2R (16570; Harleco, Gibbstown, NJ) at room temperature, and washed again with PBS. Slides were then overlaid with a 1:40 dilution of fluorescein-conjugated IgG fraction goat antirabbit IgG (13823; Cappel Laboratories), incubated for 30 min at 37°C, washed three times with PBS, mounted with a 10% PBS/90% glycerol solution containing p-phenylenedi-amine (P2396-2; Aldrich Chemical Co., Milwaukee, WI) (32), coverslipped, and sealed with clear nail polish.

Slides were examined with a standard microscope (Carl Zeiss Inc., Oberkochen, Federal Republic of Germany) equipped with Zeiss IV FL vertical illumination for epifluorescence and a fluorescein filter system (487710; Carl Zeiss Inc.). Addition of p-phenylenediamine to the mounting medium significantly reduced fluorescent quenching (32), allowing for prolonged examination and multiple fluorescence photomicrographs of individual fields. Immunofluorescence was photographed using Kodak Ektachrome 400 ASA slide film with 30–60 s exposure time. Background fluorescence resulting from the treatment of mononuclear cell fractions with fluoresceinated sheep anti-human IgE for cell sorting on the FACS was minimal and did not interfere with the bright specific staining for either MBP or CLC protein. In some cases, fluorescent cells were photographed, the coverslips removed, the slides counterstained with Wright's stain, and the identical fields rephoto-graphed under normal transmitted light using ASA 64 Kodak Ektachrome slide film.

Specificity Controls for Immunofluorescence Staining of MBP in Basophils.   Three types of specificity controls were used for immunofluorescence staining of MBP in basophils. First, immunofluorescence using preimmunization sera from individual rabbits was compared with immunofluorescence obtained using sera from the same rabbits after immunization with MBP. Second, immunofluorescence obtained with rabbit anti-MBP antisera absorbed with unrelated Sepharose 4B-conjugated proteins, including histone and human serum albumin (HSA), or unconjugated Sepharose, was compared with that obtained using antisera absorbed with the homologous solid-phase antigen, MBP-Sepharose. Sepharose 4B was activated by cyanogen bromide and 2 mg of histone, HSA, or MBP was coupled to 1 ml of Sepharose 4B. Uncoupled sites were blocked with 0.1 M lysine monohydro-chloride. Protein-Sepharose conjugates were washed with 0.05 M glycine-HCl buffer, pH

3.0, and used to absorb sera (1 ml conjugate to 2 ml of a 1:10 serum dilution). Third, immunofluorescent staining of basophils by ACP anti-MBP was compared with staining obtained using a protein A-purified NRIgG at equivalent concentrations of rabbit IgG (see Immunofluorescent Localization of MBP and CLC Protein in Basophils).

Preparation of Cell Extracts. Basophil-, eosinophil-, and mononuclear cell-enriched suspensions were centrifuged at 400 g and the cell pellets resuspended in small volumes (0.25–1.0 ml) of 0.1 M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.4) containing 0.1% protamine sulfate, 0.5% newborn calf serum, and 0.1% sodium azide (PPF buffer) with 1.0% Triton X-100 (Sigma Chemical Co.). These suspensions were subjected to 10 successive freeze-thaw cycles using dry ice-acetone and a 37°C water bath, with vortexing between each cycle. Extracts were centrifuged at 12,000 g for 10 min. The resulting clear supernatants were stored on ice, and MBP levels were measured immediately by RIA.

Measurement of MBP Levels in Cell Extracts by Radioimmunoassay. MBP concentrations in the cell extracts were measured by a double-antibody radioimmunoassay (RIA) as previously described (15). Measurement of maximal MBP concentrations requires reduction and alkylation of samples before RIA (15, 33). Therefore, aliquots of each cell extract were diluted 1:1 with a buffer consisting of 0.12 M NaCl, 0.01 M EDTA, and 0.33 M Tris hydroxymethyl aminomethane (TRIZMA base; Sigma Chemical Co.), pH 8.0. A 0.1 M solution of dithiothreitol (Sigma Chemical Co.) was added to achieve a final concentration of 0.0075 M, and the sample was incubated at room temperature for 60 min. Next, a 0.2 M solution of iodoacetamide (Sigma Chemical Co.) was added to achieve a final concentration of 0.015 M and the incubation continued for an additional 20 min at room temperature. Samples were diluted further in PPF buffer as required for RIA. The RIA results were expressed as a percentage of the maximum ^{131}I-MBP bound, and data were analyzed by unweighted logit-log transformation using a computer (9845B; Hewlett-Packard Co., Palo Alto, CA) with RIA program No. 09845-14254 and regression analysis methods program No. 09845-15014.

In one experiment (see Fig. 4), MBP levels in basophil extracts were measured using a two-site immunoradiometric assay (TSIRA) (34) as described in detail elsewhere (35). Briefly, protein A-purified rabbit IgG anti-MBP antibody was bound to plastic wells of a Microelisa 96-well flexible plastic plate (Dynatech Laboratories, Inc., Freehold, NJ), incubated with dilutions of reduced and alkylated basophil extract or MBP standard, and, after extensive washing, the resulting bound MBP was detected by adding radiolabeled ^{125}I-ACP anti-MBP. After further washing, the wells were cut out and counted as described above.

Preparation of Human Basophil Leukemia Cells. Basophil leukemia cells were obtained from a Mayo Clinic patient with basophil leukemia (T. M. Habermann, S. J. Ackerman, G. J. Gleich, and P. R. Greipp, manuscript in preparation). Cells for immunofluorescence and cell extraction were obtained from heparinized blood. For immunofluorescence, 10 ml heparinized blood was diluted with 30 ml of HEGA buffer and underlayered with 10 ml of Histopaque. After centrifugation at 400 g for 30 min at room temperature, the cells at the plasma-Histopaque interface were removed and washed three times in HEGA with centrifugation at 400 g before preparation of cytocentrifuge slides. The final cell suspensions contained 80% basophils as determined by alcian blue staining (26) and differential counts of toluidine blue-stained cytocentrifuge slides (27). The remainder of the cells were primarily mononuclear (lymphocytes and monocytes) with no eosinophils. Cells for extraction and MBP RIA were prepared in the identical fashion. Cell pellets containing 1.3 × 10^7 basophils were resuspended in 0.5 ml of PPF buffer with 1% Triton X-100 and soluble extracts prepared as described above.

Results

Detection of MBP in Basophils by Immunofluorescence. To determine whether basophils contained immunoreactive MBP, we stained cytocentrifuge slides of basophil-containing mononuclear cell preparations and FACS-purified preparations of basophils for MBP by immunofluorescence. When mononuclear cell

preparations were stained with ACP anti-MBP antibody, a small percentage of cells fluoresced brightly with a speckled or granular staining pattern (Fig. 1 a and c). The mononuclear cell preparation contained primarily lymphocytes and monocytes, and was slightly enriched for basophils (~2%) as determined by alcian blue staining. The mononuclear cell preparations did not contain any eosinophils or neutrophils as determined by differential cell counts of duplicate Wright's stained slides. When slides stained by immunofluorescence for MBP were counterstained with Wright's stain and the identical microscopic fields examined, the morphology of the cells showing granular fluorescence was not distinctive (Fig. 1 b and d). Although the cells possessed polymorphic nuclei characteristic of basophils, their granular morphology was not preserved by this procedure.

To determine whether an enrichment for surface IgE-positive cells, i.e., basophils, would also enrich for cells staining positively by immunofluorescence for MBP, immunofluorescent staining was performed on cytocentrifuge slides of FACS-purified basophils. Slides were stained by immunofluorescence for both MBP and CLC protein, and by standard histochemical stains for the identification of basophils by their characteristic granule metachromasia (Fig. 2). FACS-purified, surface IgE-positive cells stained with toluidine blue showed the typical
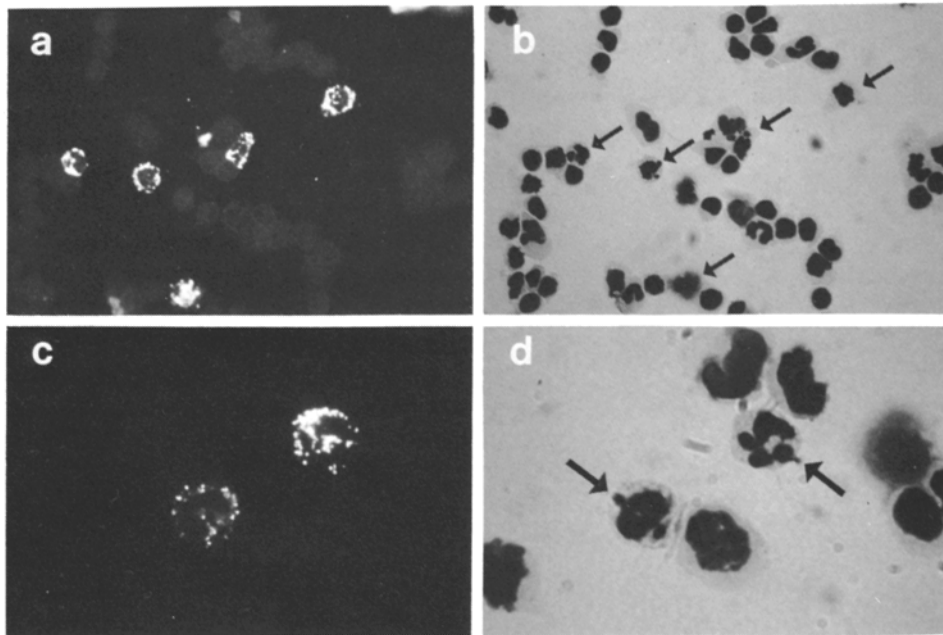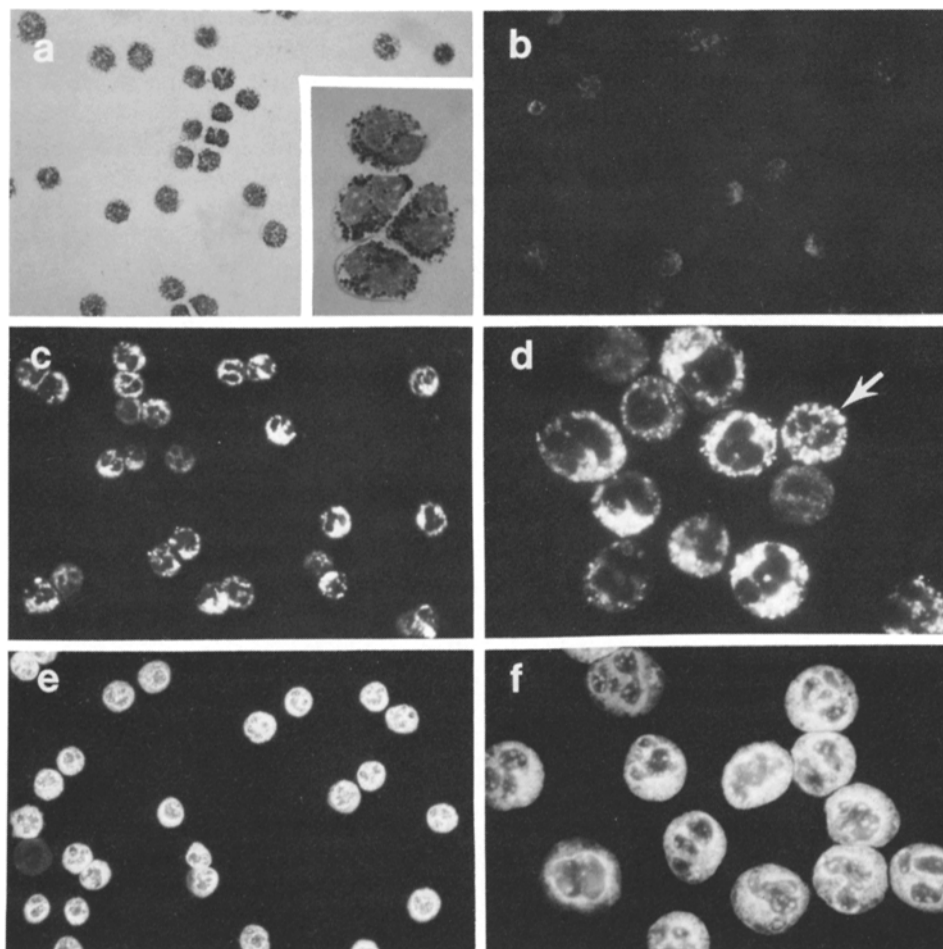

FIGURE 1.   Immunofluorescent staining of basophil-containing mononuclear cell preparations for MBP. (a and c) Fluorescence photomicrographs (× 400, × 1,000, respectively) of a cytocentrifuge slide of a mononuclear cell preparation containing basophils, lymphocytes, and monocytes, but no eosinophils. Note the bright fluorescent cells with distinctive granular staining. (b and d) Identical fields to a and c, counterstained with Wright's stain, showing the polymorphonuclear appearance of the cells that stained by immunofluorescence (arrows), presumably basophils, the granular morphology of which has been lost before counterstaining with Wright's stain. Note that the mononuclear cells do not stain for MBP by immunofluorescence.

FIGURE 2. Immunofluorescent staining of FACS-purified basophils for MBP and CLC protein. (a) FACS-purified basophils (>95% basophils) stained with toluidine blue (× 400, inset × 1,000). Note the typical polymorphic nuclei and granularity of the cytoplasm typical of blood basophils. (b) Cells stained by immunofluorescence using NRIgG as negative control (× 400). Note the absence of any fluorescent staining. (c and d) Cells stained by immunofluorescence for MBP using ACP anti-MBP (× 400, × 1,000, respectively). Note the bright distinctive granular fluorescence (arrow). (e and f) Cells stained by immunofluorescence for CLC protein (× 400, × 1,000, respectively). In comparison with the granular fluorescence of cells stained for MBP (c and d), staining for CLC protein produces a much brighter but diffuse cytoplasmic and perinuclear staining (e and f).

granule metachromasia of blood basophils (Fig. 2 a). Slides stained with ACP anti-MBP (Fig. 2 c and d) showed a bright granular staining pattern that was not observed when these cells were stained with NRIgG as negative control for immunofluorescence (Fig. 2 b). For comparative purposes, we also stained these cells for CLC protein, another eosinophil protein recently localized to human basophils (22). The bright diffuse cytoplasmic and perinuclear staining pattern for CLC protein (Fig. 2 e and f) differed markedly from that of the distinctive granular pattern observed for MBP (Fig. 2 c and d).

As shown in Table I, >95% of the surface IgE-positive cell populations in these experiments were basophils, as assessed by staining with alcian blue or by differential cell counts performed on cytocentrifuge slides stained with either toluidine blue or Wright's stain. Likewise, differential cell counts performed on duplicate slides stained for MBP and CLC protein by immunofluorescence showed that >95% of the cells in these preparations also fluoresced brightly with the granular fluorescent pattern for MBP or diffuse cytoplasmic and perinuclear pattern for CLC protein. These results show clearly that FACS enrichment for surface IgE-positive cells, primarily basophils, also enriched for cells staining positively for MBP by immunofluorescence (Table I).

TABLE I

Analyses of Purified Basophil Preparations by Histochemical Staining
and by Immunofluorescence for MBP and CLC Protein*

	Percent basophils			Percent positive cells	
	Staining method			Immunofluorescence for:	
	Alcian blue‡	Toluidine blue§	Wright's stain§	CLC Protein§	MBP§
Experiment 1	95.2	97.6	97.7	96.1	98.5
Experiment 2	97.4	95.2	95.2	96.4	97.2

* Differential cell counts of FACS-purified basophils by alcian blue staining of cells in suspension or staining of cytocentrifuge slides with toluidine blue, Wright's stain, or by immunofluorescence for CLC protein and MBP. There were no eosinophils in these preparations and the few contaminating cells consisted of small lymphocytes and monocytes.
‡ Hemocytometer counts.
§ Differential counts on 1,000 cells.

TABLE II

Specificity of Immunofluorescent Staining for MBP in Human Basophils*

Reagent tested	Absorption‡	Basophil fluorescence§	
		Rabbit 2	Rabbit 4
Rabbit anti-MBP serum	None	+	+
	Unconjugated Sepharose	+	+
	Histone-Sepharose	+	+
	HSA-Sepharose	+	+
	MBP-Sepharose	−	−
Preimmunization serum	None	NT	−
ACP rabbit anti-MBP	None	NT	+
NRIgG‖	None	NT	−

* The specificity of immunofluorescence for MBP in basophils was tested by staining cytocentrifuge slides of basophil-containing mononuclear cell preparations (primarily mononuclear cells with 1–2% basophils and no eosinophils).
‡ Rabbit anti-MBP sera from two rabbits were absorbed with various solid-phase immunosorbents before use as a first-stage reagent for immunofluorescence.
§ +, bright fluorescent staining; −, absence of fluorescent staining; NT, not tested.
‖ Protein A-purified NRIgG from a normal rabbit serum pool.

The specificity of immunofluorescence staining for MBP in basophils was assessed in three different ways (Table II). First, two different rabbit antisera to MBP were absorbed with various protein-Sepharose conjugates, and the absorbed antisera were used in the first stage of the immunofluorescence procedure. Absorption with the unrelated cationic protein histone coupled to Sepharose, HSA-Sepharose, or unconjugated Sepharose did not abrogate the bright granular staining of the basophils. In contrast, absorption with the homologous solid-phase antigen, MBP-Sepharose, completely eliminated fluorescence staining. Second, immunofluorescent staining was not produced using the preimmunization serum from the MBP-immunized rabbits. Third, ACP anti-MBP stained basophils brightly whereas a protein A-purified NRIgG (negative control), tested at the same IgG concentration, did not stain basophils.

Measurement of MBP in Freeze-Thaw Detergent Extracts of FACS-purified Basophils by RIA. To test whether basophils and eosinophils contained similar quantities of MBP, we measured MBP concentrations in the supernatants of freeze-thaw detergent extracts of various cell suspensions enriched for basophils by FACS, for eosinophils by discontinuous gradient centrifugation of normal peripheral leukocytes and by eosinapheresis of patients with the hypereosinophilic syndrome, and for mononuclear cells (basophil enriched, eosinophil depleted) by centrifugation over Histopaque (Table III). MBP was detected in all cell extracts that contained eosinophils, as well as in extracts prepared from FACS-purified basophils (92 and 89% purity in experiments 2 and 4, respectively, with no contaminating eosinophils) and from mononuclear cell suspensions that contained 0.54 and 1.9% basophils in experiments 2 and 4, respectively. When extracts were compared on the basis of MBP content per 10^6 cells, the basophil and eosinophil extracts contained more MBP than did the unseparated mononuclear cell extracts (Table III). In addition, when the MBP content per 10^6 basophils or 10^6 eosinophils was calculated, eosinophils contained considerably more MBP than did basophils. A summary of the comparative MBP content of

TABLE III

MBP in Freeze-Thaw Detergent Extracts of Human Leukocyte Preparations*

Cell extract analyzed	Percent basophil	Percent eosinophil	Total cells extracted $(\times 10^{-5})$‡	ng MBP/ extract	ng MBP/10^6 cells	ng MBP/ 10^6 baso-phils	ng MBP/10^6 eosinophils
Experiment 2							
Basophil	92	—	5.4	36	67	72	—
Mononuclear	1.9	—	200.0	37	2	97	—
Eosinophil (patient 1)	—	98	100.0	11,246	1,125	—	1,148
(patient 2)	—	94	100.0	8,923	892	—	949
Experiment 4							
Basophil	89.1	—	1.5	6	40	43	—
Mononuclear	0.54	—	200.0	9	<1	86	—
Eosinophil (patient 1)	—	98	100.0	8,253	825	—	842
(patient 2)	—	94	100.0	8,008	801	—	852
Eosinophil (normal donor 1)	—	95.5	65.0	17,756	2,732	—	2,860
(normal donor 2)	—	84.7	15.0	9,447	6,298	—	7,440

* MBP levels in soluble extracts of purified basophils, mononuclear cells, and eosinophils were determined by RIA. Cell counts performed as described in Materials and Methods.

‡ Cells in experiment 2 from the basophil, mononuclear, and eosinophil patients 1 and 2 preparations were extracted in volumes of 0.256, 0.54, 1.0, and 1.01 ml; and cells in experiment 4 from the basophil, mononuclear, eosinophil patients 1 and 2, and eosinophil normal donors 1 and 2 preparations were extracted in volumes of 0.225, 0.255, 0.51, 0.53, 1.01, and 1.02 ml PPF with Triton X-100, respectively.

normal basophils from four different donors, normal eosinophils from two different donors, and eosinophils from two patients with the hypereosinophilic syndrome, for four separate experiments, is shown in Table IV. Normal basophils averaged 140 ± 128 ng MBP/10^6 cells ($\bar{x}$ ± 1 SD, n = 7), whereas purified eosinophils from patients with the hypereosinophilic syndrome and from normal donors averaged 824 ± 173 (n = 8) and 4,979 ± 2,383 (n = 4) ng MBP/10^6 cells, respectively. Therefore, normal basophils contained, on the average, 17% of the MBP detectable in eosinophils from patients with the hypereosinophilic syndrome and 2.8% of the MBP detectable in eosinophils from normal healthy donors.

The RIA measures MBP concentrations via the inhibition of the binding of radiolabeled ^{131}I-MBP to specific antibody. Fig. 3 a and b shows the RIA inhibition curves obtained when increasing quantities of reduced and alkylated extracts of the various purified cell suspensions were reacted with specific antibody to MBP and 1 ng ^{131}I-MBP. The slopes of the inhibition curves were very similar within each experiment, and basophil extracts inhibited the binding of radiolabeled MBP as well as the MBP primary standard or the eosinophil extracts. Fig. 3 c and d shows the regression analysis for a logit-log transformation of these data. Statistical comparison of the regression lines by analysis of covariance showed no significant differences in their slopes. The finding that the slopes of the inhibition curves for the basophil extracts were not significantly different from those of the MBP primary standard or the other cell extracts prepared from eosinophils indicates that basophils contain a protein that is immunochemically indistinguishable from that of the eosinophil granule MBP.

In addition to analyzing the basophil extracts by double-antibody RIA, we

TABLE IV

Comparative MBP Content of Basophils and Eosinophils as Determined by RIA of Freeze-Thaw Detergent Extracts of Various Leukocyte Preparations

Cell extract analyzed		Expt. 1	Expt. 2	Expt. 3	Expt. 4
		ng MBP/10^6 basophils*			
Basophil[‡]		406	72	ND	43
Mononuclear[§]		204	97	72	86
		ng MBP/10^6 eosinophils			
Eosinophil[∥]	(patient 1)	734	1,148	559	842
	(patient 2)	766	949	745	852
Eosinophil[¶]	(normal donor 1)	ND	ND	3,015	2,860
	(normal donor 2)	ND	ND	6,600	7,440

* MBP levels in basophils obtained from a different normal donor for each experiment. ND, not done.

[‡] Purity of FACS-purified basophil preparations for expts. 1, 2, and 4 was 87.3, 92.0, and 89.1%, respectively.

[§] Mononuclear cell preparations for expts. 1-4 contained 0.57, 1.9, 1.63, and 0.54% basophils, respectively, and no eosinophils.

[∥] Eosinophil preparations from hypereosinophilic syndrome patients 1 and 2 contained 98 and 94% eosinophils, respectively.

[¶] Eosinophil preparations from normal donors 1 and 2 contained 95.5 and 84.7% eosinophils, respectively.
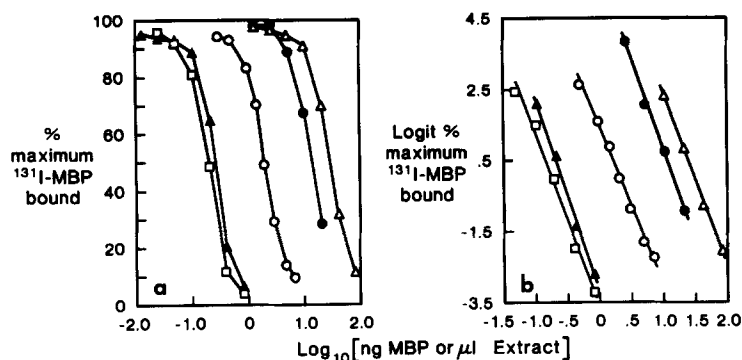
FIGURE 3. Inhibition of the binding of ^{131}I-MBP to specific antibody by MBP primary standard and freeze-thaw detergent extracts of purified human basophils, eosinophils, and mixed mononuclear leukocytes containing a small percentage of basophils. (a) Inhibition curves produced in experiment 2 when increasing quantities of MBP standard (O) or increasing amounts of freeze-thaw extracts of purified human basophils (●), unseparated mononuclear leukocytes containing 1.9% basophils (△), or eosinophils from hypereosinophilic patient 1 (▲) or 2 (□) were analyzed in the double-antibody RIA. (b) Regression lines for the logit-log–transformed data in a. The coefficient of determination (r^2) for the MBP standard inhibition regression line was 0.992 and for cell extracts, an average of 0.995 ± 0.004. Comparison of these logit-log regression lines by analysis of covariance showed that the null hypothesis of common slope could not be rejected ($F_{4,14}$ = 2.26; not significant).
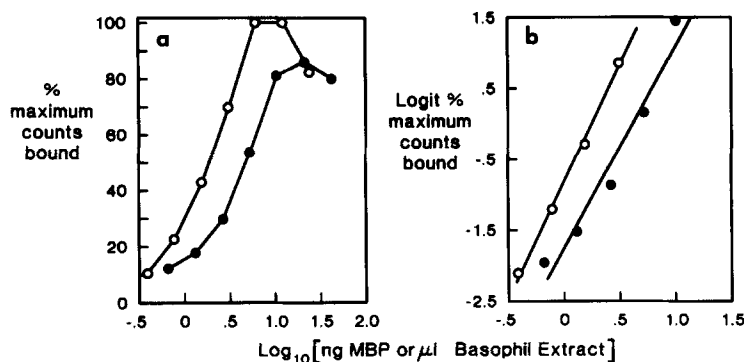

FIGURE 4. Comparative binding curves for MBP standard and a freeze-thaw detergent extract of purified human basophils (93.2%) in the TSIRA. (a) Binding curves produced when increasing amounts of either MBP standard (O) or basophil extract (●) were analyzed by the TSIRA. (b) Regression analysis for the logit-log–transformed data in a. The coefficient of determination (r^2) for the MBP standard was 0.997 and for the basophil extract, 0.960. Comparison of these regression lines by analysis of covariance showed that a null hypothesis of common slope could not be rejected ($F_{1,5}$ = 0.91; not significant).

performed a TSIRA involving the direct binding of MBP to specific antibody in the form of an antibody-antigen-antibody sandwich. The binding curves comparing a titration of MBP primary standard with that of a reduced and alkylated basophil extract in the TSIRA are shown in Fig. 4 a. As with the double-antibody RIA, a comparison of the regression lines for a logit-log transformation of these data did not show any significant differences in the slopes of their binding curves.

Detection of MBP in Basophil Leukemia Cells. Leukemic cells obtained from the peripheral blood of a patient with basophil leukemia were analyzed for their MBP content by immunofluorescence and by RIA of freeze-thaw detergent cell

extracts. These preparations contained 80% basophils with typical granule met-achromasia. Immunofluorescence staining of cytocentrifuge slides for MBP and CLC protein revealed the identical staining patterns observed for each of these proteins in normal basophils, with granular staining for MBP and brighter but more diffuse cytoplasmic staining for CLC protein (Fig. 5). In general, the staining of basophil leukemia cells for MBP was more variable and less intense (Fig. 5 a) than that of normal basophils (Fig. 2 c and d); by comparison, the intensity of staining for CLC protein was roughly equivalent for both normal (Fig. 2 e and f) and leukemic cells (Fig. 5 c). As with normal basophils, the leukemic cells did not stain by immunofluorescence when NRIgG or a preimmunization serum was substituted for specific antibody (Fig. 5 b and d).

Analysis of soluble extracts of the basophil leukemia cells by RIA revealed a significantly lower content of MBP: 9 and 18 ng MBP per 10^6 basophils in two experiments as compared with an average of 140 ng MBP per 10^6 normal basophils (see Table IV).
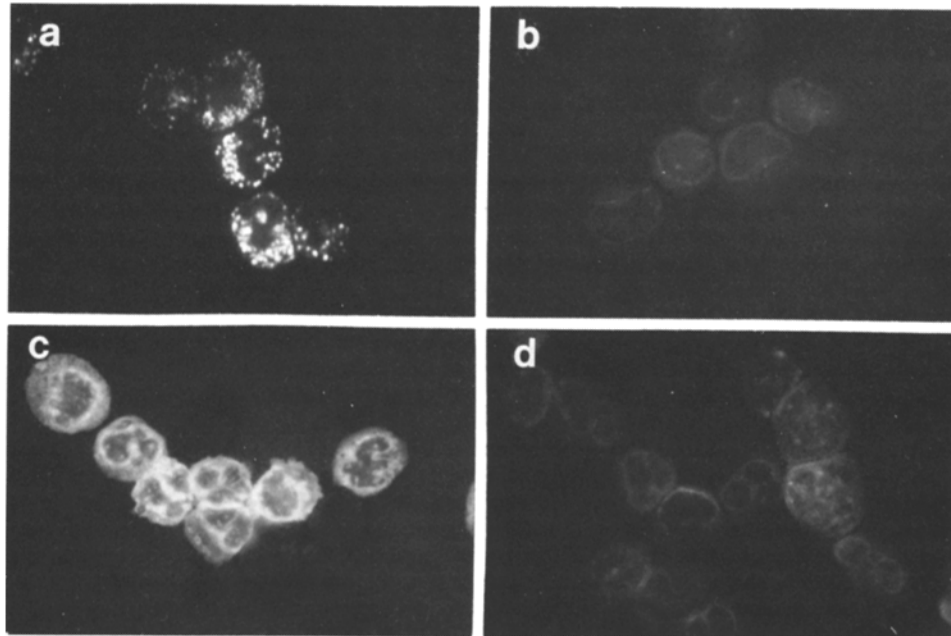

FIGURE 5. Comparative staining of basophil leukemia cells for MBP and CLC protein by immunofluorescence. (a) Immunofluorescent staining of cells for MBP using ACP anti-MBP. (b) Negative control for a stained by immunofluorescence using NRIgG. (c) Immunofluorescent staining of cells for CLC protein. (d) Negative control for c stained by immunofluorescence using the preimmunization normal rabbit serum. Note that the basophil leukemia cells (~80% basophils) stained with the same granular fluorescence for MBP and with more intense cytoplasmic and perinuclear fluorescence for CLC protein. Comparison of the immunofluorescence staining for MBP in a with that in Figs. 1 and 2 shows that basophil leukemia cells stained less brightly for MBP than did normal basophils, consistent with their lower MBP content as determined by RIA.

Discussion

MBP, the sole cationic constituent of the distinctive crystalloid core of the eosinophil's large specific granule (8) (S. J. Ackerman, D. A. Loegering, and G. J. Gleich, unpublished observations), is currently considered unique to the eosinophil. As such, it has been used as a marker for studies of eosinophil function in health and disease (13, 36) through its cellular and extracellular localization in tissues (11, 19–21),[3] its presence in body fluids such as serum (14, 15) or sputum (10), and in vitro as a marker of eosinophil degranulation in studies of the eosinophil's function as an effector cell for parasite killing (9, 12). Using an immunofluorescence procedure (19), MBP has been localized in a number of tissues and organs whose dysfunction in certain diseases is associated with either peripheral blood eosinophilia and/or obvious eosinophil infiltration (11, 20, 21).[3] During the course of these investigations, a very small number of cells have been observed that stained for MBP by immunofluorescence, but upon counterstaining could not be clearly identified as eosinophils. In certain cases these cells may have been partially degranulated eosinophils; in others, MBP may have been ingested by other cells such as alveolar macrophages (11). Examination of peripheral blood smears clearly showed cells staining for MBP that were not eosinophilic. Because we had already demonstrated that another prominent eosinophil protein, the Charcot-Leyden crystal protein (lysophospholipase), was also a blood basophil constituent (22), we tested the hypothesis that MBP might be present in basophils.

For this purpose, we stained slides of eosinophil-free, basophil-containing mononuclear cell preparations (1–2% basophils) (Fig. 1), highly purified basophil preparations (>95%) (Fig. 2), and cells from a basophil leukemia patient (>80% basophils) (Fig. 5) for MBP by a sensitive and specific (Table II) immunofluorescence procedure (11, 19–21). In all cases, cells with the distinctive nuclear morphology and granule metachromasia typical of basophilic leukocytes stained brightly for MBP, with a granular staining pattern consistent with localization in the histamine-, heparin-containing granule (Figs. 1, 2, and 5). The fluorescent localization of MBP in these cells was distinctly different from the staining pattern observed for CLC protein (22), which was considerably brighter, with a diffuse cytoplasmic and perinuclear localization (Figs. 2 and 5). Basophils possess considerable quantities of cell surface IgE, and we enriched for surface IgE-positive cells using the FACS, thereby obtaining cell suspensions containing up to 97% basophils, without any contaminating eosinophils (Tables I, III, and IV). When these cell preparations were stained by three different histochemical procedures, >95% of the cells were basophils and >95% also fluoresced brightly when stained for either MBP or CLC protein (Table I, Fig. 2). Likewise, cells obtained with considerably fewer in vitro manipulations from a patient with basophil leukemia contained MBP and CLC protein when stained by immunofluorescence (Fig. 5); when stained with toluidine blue, >80% of these cells showed the metachromatic granular staining typical of blood basophils in various stages of maturity. Finally, we demonstrated the presence of MBP in basophils by preparing freeze-thaw detergent extracts of highly enriched suspensions of basophils and basophil-containing mononuclear cells from four different normal donors, and eosinophils from two normal donors and two patients with the

hypereosinophilic syndrome. We compared the ability of these soluble extracts to inhibit the binding of radiolabeled MBP to specific antibody in an RIA or to bind antibody to MBP in a TSIRA. The results of these experiments (Figs. 3 and 4) indicate that basophils contain a protein that is immunochemically indistinguishable from either purified MBP or the MBP present in soluble extracts of normal eosinophils or eosinophils from patients with the hypereosinophilic syndrome. We calculated the MBP content of normal basophils and they contained considerably less MBP than did normal eosinophils or eosinophils from patients with the hypereosinophilic syndrome (2.8 and 17%, respectively) (Tables III and IV). In addition, basophils from the patient with basophil leukemia contained even less MBP ($9-18$ ng/10^6 cells) than did normal basophils (140 ng/10^6 cells).

Based upon these results, MBP can no longer be considered unique to the eosinophil, and the basophil's potential contribution to observed serum or other body fluid levels or tissue localization of MBP in health or disease must be taken into account. Although the MBP in the basophil appears by immunofluorescence to be localized to the histamine-, heparin-containing granule, this localization needs to be confirmed by appropriate studies using subcellular fractionation and/or immunoelectron microscopy. The role of MBP in basophil function is a matter of speculation. Exogenously added native or reduced and alkylated MBP was recently shown to stimulate basophil histamine secretion in vitro by an IgE-independent, noncytolytic mechanism (24) similar to that induced by polycations such as poly-L-arginine (37). However, the mechanism by which MBP induces basophil histamine secretion and the possibility that the secretion of the endogenous MBP from the basophil granule has a similar histamine-releasing function remain to be determined.

The current finding that two proteins, MBP and CLC, heretofore considered unique to the eosinophil, are also constituents of the blood basophil indicates biochemical similarities between these cells and suggests the existence of a common hemopoietic progenitor for basophils and eosinophils. In fact, results of recent studies of eosinophil and basophil colony growth in vitro (38, 39) are consistent with the hypothesis that these cells share a common committed hemopoietic progenitor cell in bone marrow and peripheral blood. If this were true, one might also expect to find other common eosinophil and basophil proteins, such as the eosinophil cationic protein (4, 5), the eosinophil-derived neurotoxin (6), or the eosinophil peroxidase (40). Indeed, previous studies have shown that basophils contain peroxidase activity (41, 42).

Summary

In experiments using an immunofluorescent method to localize human eosinophil granule major basic protein (MBP) in cells and tissues, a small number of cells stained for MBP that subsequently could not be identified as eosinophils. Because the Charcot-Leyden crystal protein, another eosinophil protein, was recently identified in basophils, we tested whether MBP might also be a constituent of blood basophils. Highly purified, eosinophil-free basophil suspensions were prepared using the fluorescence-activated cell sorter (FACS) to sort basophil-containing mononuclear cell preparations stained with fluorescein-conjugated sheep IgG anti-human IgE antibody. Using these FACS-purified basophils,

we found that: (a) enrichment for surface IgE-positive cells (>95% basophils) by FACS also enriched for cells staining for MBP by immunofluorescence; (b) MBP appeared to be localized in the histamine-, heparin-containing granules; (c) significant quantities of MBP were measurable by radioimmunoassay (RIA) in freeze-thaw detergent extracts of purified basophils; and (d) RIA dose-response curves for extracts of purified eosinophils and basophils had identical slopes. The MBP content of basophils from normal individuals averaged 140 ng/10^6 cells, whereas purified eosinophils from normal donors and patients with the hypereosinophilic syndrome averaged 4,979 and 824 ng/10^6 cells, respectively. MBP was also detected by immunofluorescence and RIA in cells obtained from a patient with basophil leukemia, although the MBP content for basophil leukemia cells was lower than that for normal basophils. We conclude that basophils contain a protein that is immunochemically indistinguishable from eosinophil granule MBP.

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