ACTIVATION OF BASOPHIL AND MAST CELL HISTAMINE RELEASE BY EOSINOPHIL GRANULE MAJOR BASIC PROTEIN*

By MARTHA CRAMER O'DONNELL, STEVEN J. ACKERMAN, GERALD J. GLEICH, and LARRY L. THOMAS‡

From the Department of Immunology/Microbiology, Rush Medical College, Chicago, Illinois 60612; and the Departments of Medicine and Immunology, Mayo Medical School and Mayo Clinic and Foundation, Rochester, Minnesota 55901

A frequent characteristic of immediate hypersensitivity reactions is systemic or localized tissue eosinophilia (1). This response is attributed to mast cell and basophil release of acidic tetrapeptides (2–4) and other products (5) preferentially chemotactic for eosinophils. Because of this association and eosinophil release of histaminase (6) and other mediators (7, 8) that may dampen allergic reactions, eosinophils are postulated to play an important role in the maintenance of local homeostasis during immediate hypersensitivity reactions (7, 8). Eosinophils and their products, however, are also recognized to have effector roles in various host defense mechanisms. In addition to their well-described participation in anti-parasitic events (8–10), eosinophil participation in bactericidal (11, 12) and tumoricidal (13) mechanisms is suggested.

Major basic protein (MBP)[1] is a primary constituent of eosinophil granules. MBP, with a molecular weight of 9,300 in humans (14) and 11,000 in guinea pigs (15), is localized in the crystalline core of the eosinophil granule and comprises >50% of granule protein (16, 17). Release of MBP is implicated as a mechanism in eosinophil-mediated killing of parasites (18, 19) and elevated levels of MBP have been observed in serum of individuals with eosinophilia (20) and at eosinophil-rich sites of inflammation (21). A characteristic feature of MBP is its high arginine content, ~11% for human MBP (14) and 13% for guinea pig MBP (15). It has previously been shown (22) that large molecular weight polymers of basic amino acids can induce histamine release from human leukocytes, with polymers of L-arginine being the most potent. Because of its significant arginine content, we postulated that human MBP might possess similar activity. In this report, we show that purified human MBP induces noncytolytic release of histamine from human basophils. We confirmed that the release is due to a direct effect of MBP on basophils using preparations of pure (92–96%) human basophils. Purified rat peritoneal mast cells display a similar response to MBP.

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‡ To whom reprint requests should be addressed at Department of Immunology/Microbiology, Rush Medical College, 1753 West Congress Parkway, Chicago, IL 60612.

[1] *Abbreviations used in this paper:* HEGA, Hank's balanced salt solution, pH 7.35, containing 5 mM EDTA, 5.4 mM glucose, and 0.1% bovine serum albumin; MBP, eosinophil granule major basic protein; PA, PIPES-albumin buffer, pH 7.4; PIPES, piperazine-*N,N'-bis*-(2-ethane sulfonic acid).

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Materials and Methods

Leukocyte Isolation. Peripheral blood leukocytes were isolated from the venous blood of normal human volunteers by dextran sedimentation (23). The leukocytes were washed twice at 4°C before use in buffer containing 25 mM piperazine-*N,N'-bis*-(2-ethane sulfonic acid) (PIPES) (Sigma Chemical Co., St. Louis, MO), 150 mM NaCl, 5 mM KCl, 40 mM NaOH, and 0.003% human serum albumin (Calbiochem-Behring Corp., La Jolla, CA), pH 7.4 (PA). Cells were pelleted between washes by centrifugation at 200 *g* for 10 min at 4°C.

Mononuclear Cell Isolation. The basophil-containing mononuclear cell fraction was isolated from venous blood of normal human volunteers by Ficoll-Hypaque density gradient centrifugation (24). 10-ml aliquots of blood (containing 5 mM EDTA as anticoagulant) were diluted with 30 ml of Hanks' balanced salt solution (Gibco Laboratories, Grand Island, NY), pH 7.35, containing 5 mM EDTA, 5.4 mM glucose, and 0.1% bovine serum albumin (HEGA). The diluted blood was underlayed with 10 ml of Histopaque (Sigma Chemical Co.) and centrifuged at 400 *g* for 30 min at 22°C. Cells at the plasma/Histopaque interface were removed, diluted 1:1 with cold HEGA, and collected by centrifugation at 400 *g* for 10 min at 4°C. The cell pellet was washed with PA as described above. This procedure yielded cell preparations containing ~1–2% basophils by staining with alcian blue (25).

Human Basophil Purification. Basophils were purified by flow microfluorometry according to the method of Weil et al. (26) as described previously (27). Mononuclear cells isolated as described above were incubated for 30 min at 4°C with a 1:50 dilution of fluorescein isothiocyanate-conjugated sheep IgG anti-human IgE (Cappel Laboratories, Cochranville, PA). After washing three times at 4°C in HEGA, the cells were sorted using a fluorescence-activated cell sorter (FACS IV; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA). The cells were first sorted at a rate of ~10,000 cells/s and then re-sorted at a rate of ~1,000 cells/s; ice-cold HEGA served as the sheath and diluting fluid during sorting. The resultant cell preparation contained 92–96% basophils by alcian blue staining (25) and by differential cell counts of smears fixed with Mota's fixative and stained with toluidine blue (28). The mean histamine contents of basophils in the mononuclear and purified basophil preparations were not significantly different, being 1.47 ± 0.14 (mean ± SEM) and 1.59 ± 0.10 pg/basophil, respectively. The purified basophils were washed once at 4°C in PA before use.

Isolation of Rat Peritoneal Mast Cells. Male, 250–300-gm Sprague-Dawley rats (Locke-Erickson Laboratory, Oak Park, IL) were killed by ether inhalation and mast cells were collected by rinsing the peritoneal cavities with 20 ml of PA containing 10 U/ml of heparin (Lympho-Med Inc., Chicago, IL). Each experiment normally used cells pooled from two rats. Mast cells were purified by centrifugation through 38% (wt/vol) bovine serum albumin (Sigma Chemical Co.) as described by Sullivan et al. (29). The cell pellet was washed three times at 4°C in PA before use; cells were collected between washes by centrifugation at 50 *g* for 7 min at 4°C. The resultant cell preparations routinely contained >90% mast cells by toluidine blue staining, with cell viability >90% by trypan blue exclusion.

Purification of MBP. Native MBP was purified from eosinophils of patients with marked peripheral blood eosinophilia as described in detail elsewhere (14). Sephadex G-50 fractions containing native MBP, in 0.025 M sodium acetate column buffer, pH 4.3, were pooled and stored in aliquots at −70°C. The native MBP was homogenous as judged by the presence of a single protein band after electrophoresis on sodium dodecyl sulfate polyacrylamide gels as described previously (14). Aliquots were thawed before use, and repeated freezing and thawing were avoided. For some experiments, reduction and alkylation of native MBP were performed using a 60-fold molar excess of dithiothreitol and a 120-fold molar excess of iodoacetamide as described elsewhere (20). Reduction and alkylation prevents the tendency of the molecule to form disulfide-linked aggregates upon standing at neutral pH in the presence of oxygen (14, 15). The reduced and alkylated MBP was dialyzed against water, lyophilized, and stored at −70°C. Reduced and alkylated MBP was reconstituted in PA buffer as needed and stored in aliquots at −70°C. Concentrations of MBP were determined by an E_{277} of 26.3 (14).

Conditions for Basophil Histamine Release. Aliquots of leukocytes or purified basophils were added in PA to 12- × 75-mm Falcon plastic tubes (Falcon Labware, Oxnard, CA) to give 2×10^6 or 2×10^4 cells per tube, respectively. The MBP or a 13,900-dalton polymer of poly-L-arginine (Miles Laboratories Inc., Elkhart, IN) was added in volumes not exceeding 0.06 ml

and the reactions were initiated by addition in 0.01 ml of $CaCl_2$ and $MgCl_2$ to final concentrations of 0.6 and 1.0 mM, respectively. The final incubation volume was 0.25 ml. Spontaneous histamine release was determined with cells incubated in the absence of stimulus. The influence of the 0.025 M sodium acetate column buffer, pH 4.3, on spontaneous release was evaluated in experiments using native MBP. In some experiments as indicated, cells were preincubated with 4 mM EDTA, pH 7.35, or 3 mM 2-deoxy-D-glucose (Sigma Chemical Co.) for 10 min at 37°C before addition of the stimulus. Cells were incubated for 45 min, or for times indicated, at 37°C in an oscillating water bath. Reactions were stopped by addition of 0.25 ml cold PA and centrifuged for 2 min at 1,000 *g*. The histamine content of the cell-free supernatants was determined by the automated fluorometric procedure of Siraganian (30), with the total cellular histamine content determined in supernatants of cells lysed with 2% perchloric acid. Results are expressed as the net percent histamine release. Spontaneous release was always <10% for peripheral leukocytes and mononuclear cells and <15% for purified basophils. Because of the limited quantities of MBP, initial experiments using MBP were done in duplicate and subsequent experiments were done using single determinations; experiments using purified basophils were also done using single determinations. All other experimental determinations were done in duplicate, with <5% variation in the values for duplicate histamine determinations.

Conditions for Histamine Release from Rat Mast Cells. Aliquots containing 1.5×10^4 mast cells in PA were added to 12- × 75-mm Falcon plastic tubes, and MBP, poly-L-arginine, 0.025 M sodium acetate buffer, pH 4.3, or PA were added as described above to a final volume of 0.25 ml. Compound 48/80 (Sigma Chemical Co.) was used as a positive control stimulus with rat mast cells. After the addition of 0.6 mM $CaCl_2$ and 1 mM $MgCl_2$ to the incubation contents as above, the cells were incubated for 10 min, or as indicated, at 37°C. The remainder of the protocol is as described for the human cell preparations.

Results

Concentration Requirements and Kinetics of MBP-stimulated Histamine Release. Incubation of peripheral leukocytes with 5×10^{-7} to 7.5×10^{-6} M native MBP produced a marked and concentration-dependent histamine release. Results of four experiments, each using leukocytes of a different donor, are shown in Fig. 1. Release by leukocytes incubated with volumes of 0.025 M sodium acetate buffer, pH 4.3, equal to those required for MBP addition did not exceed spontaneous release by >4% in any experiment. Leukocyte sensitivity to MBP differed for individual donors, but the response of cells from the same donor remained similar in repeated experiments. Histamine release values of 58, 63, 72, and 61% were obtained in four separate experiments using leukocytes of the same donor and 3.7×10^{-6} M native MBP. An

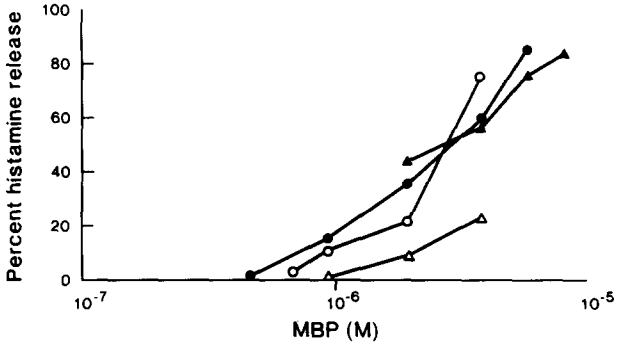

FIG. 1. Stimulation of histamine release from peripheral leukocytes by native MBP. Results obtained with leukocytes of four donors in four separate experiments are shown. Histamine release values for the donor denoted by the open circles are the means of duplicate measurements. All other values represent a single experimental determination.

examination of the kinetics for release caused by 3.7×10^{-6} M native MBP indicated that release increased with time for ~40 min (Fig. 2); half-maximal secretion occurred at ~17 min. Similar kinetics were obtained with leukocytes of an additional donor.

Comparison of Native MBP with Reduced and Alkylated MBP and Poly-L-Arginine. Concentrations of native MBP required for release were compared with those for reduced and alkylated MBP and the 13,900-dalton poly-L-arginine. Representative results obtained with leukocytes of two donors are shown in Fig. 3. Optimal concentrations for native MBP were within the concentration range of 10^{-6} to 5.7×10^{-6} M, whereas poly-L-arginine induced comparable levels of release at concentrations of 6×10^{-8} to 2×10^{-7} M. After reduction and alkylation, the concentration curve for MBP shifted ~1 log to the right and the maximum histamine release obtained seldom surpassed 40%. In some instances, release caused by higher concentrations of reduced and alkylated MBP plateaued or even decreased (data not shown). Similar release profiles were noted with at least five additional donors each for poly-L-arginine and the reduced and alkylated MBP, although leukocytes of individual donors also differed in their sensitivity to these stimuli. In those experiments, release

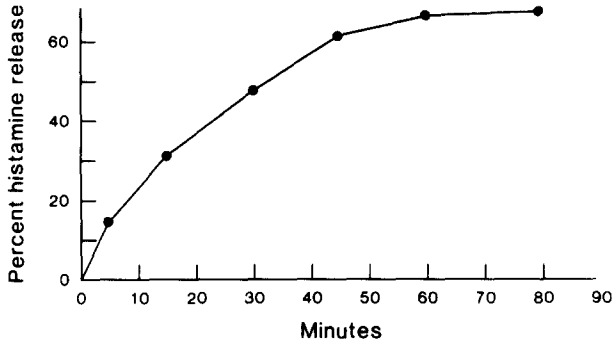

FIG. 2. Kinetics of native MBP-stimulated histamine release from peripheral leukocytes. The reaction was initiated by addition of 3.7×10^{-6} M native MBP at time zero and stopped at the times indicated by addition of 4 mM EDTA and removal of the cells by centrifugation. Each point represents a single experimental value.

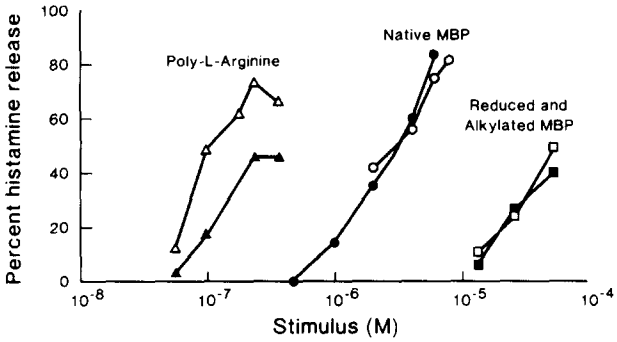

FIG. 3. Concentration requirements for stimulation of histamine release from peripheral leukocytes by native MBP, reduced and alkylated MBP, and poly-L-arginine. Leukocytes were incubated with native MBP (circles), reduced and alkylated MBP (squares), or poly-L-arginine (triangles) in the same experiment. Open and filled symbols indicate results obtained with leukocytes of two donors in separate experiments. Values depicted for poly-L-arginine as well as the values denoted by the open symbols for the MBP preparations are the means of duplicate determinations; all other points represent a single experimental value. The results presented for native MBP are included in Fig. 1.

caused by 2×10^{-7} M poly-L-arginine ranged from 29 to 64% ($n = 7$), whereas release with 4.6×10^{-5} M reduced and alkylated MBP ranged from 13 to 29% ($n = 5$).

Evaluation of Release Mechanism. Because reduced and alkylated MBP has been shown to be cytotoxic for some mammalian cells (18, 31), the calcium and energy requirements for native MBP-induced histamine release from human leukocytes were determined to assess a possible lytic effect of MBP on basophils (Fig. 4). Omission of calcium from the incubation buffer, together with addition of 4 mM EDTA, blocked the concentration-dependent increases in histamine release caused by native MBP. Release was similarly inhibited by preincubation of the leukocytes with 3 mM 2-deoxy-D-glucose. Temperature dependence was also demonstrated by the failure of native MBP to stimulate histamine release when incubated with the cells at 4°C. Calcium depletion or preincubation with 2-deoxy-D-glucose similarly inhibited (>88%) release from leukocytes of two additional donors and one additional donor, respectively, during incubation with 3.7×10^{-6} M native MBP. Similar calcium and energy dependence was also noted with reduced and alkylated MBP in two separate experiments.

Histamine Release from Purified Basophils. To establish that the histamine release was due to a direct effect of MBP on basophils, three experiments were performed using preparations of pure (92–96%) human basophils. Incubation with 1.9×10^{-6} and 3.7×10^{-6} M native MBP resulted in 7–71% histamine release in the three experiments (Table I). Comparable release was obtained with aliquots of the mononuclear cell preparations from which the basophils had been purified; the control mononuclear cells were held at 4°C in HEGA during purification of the remaining cells. Release with 0.025 M sodium acetate buffer, pH 4.3, never exceeded spontaneous release by >6% for either cell preparation. In results not shown, incubation at 4°C completely abrogated the response of each cell preparation to 3.7×10^{-6} M native MBP in each experiment. The results presented in Table I also demonstrate that reduced and alkylated MBP and poly-L-arginine each initiate histamine release by a direct influence on basophils. The relative concentration requirements of the three stimuli with the purified basophil preparations were similar to those observed with peripheral

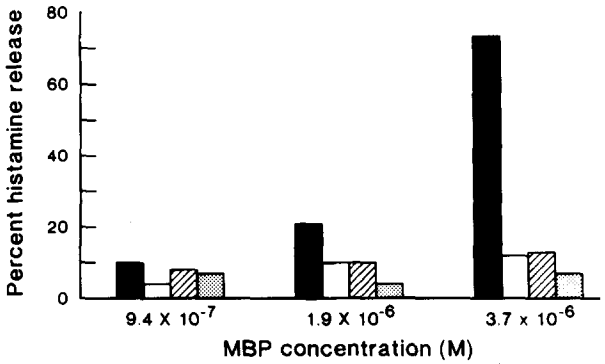

FIG. 4. Calcium, energy, and temperature dependence of native MBP-induced histamine release from peripheral leukocytes. Cells were preincubated with 4 mM EDTA or 2-deoxy-D-glucose for 10 min at 37°C before addition of the indicated concentrations of native MBP. Solid bars indicate MBP alone. Open bars indicate MBP plus 4 mM EDTA and no Ca^{2+}. Hatched bars show MBP plus 3 mM 2-deoxy-D-glucose. Stipled bars show cells incubated with MBP for 45 min at 4°C. Each value represents a single experimental determination.

TABLE I

Stimulation of Histamine Release from Purified Human Basophils by MBP and Poly-L-Arginine

Stimulus	Percent histamine release					
	Experiment 1		Experiment 2		Experiment 3	
	Mononuclear cells	Basophils	Mononuclear cells	Basophils	Mononuclear cells	Basophils
Native MBP						
1.9×10^{-6} M	28	7	45	55	58	46
3.7×10^{-6} M	32	15	47	71	63	32
Reduced and alkylated MBP						
2.4×10^{-5} M	ND	ND	ND	20	ND	12
4.6×10^{-5} M	ND	ND	ND	30	ND	24
Poly-L-Arginine						
1×10^{-7} M	14	63	45	64	65	56
2×10^{-7} M	45	72	61	67	75	60

Human basophils purified by flow microfluorometry and control aliquots of the mononuclear cells from which the basophils had been purified were incubated for 45 min at 37°C with the indicated concentrations of native MBP, reduced and alkylated MBP, or poly-L-arginine. Each value represents a single determination. Experiments 2 and 3 were done using cells isolated from the same donor. Spontaneous histamine release values in the three experiments were 15, 14, and 12% for the basophil preparations and 5, 6, and 8% for the mononuclear cell preparations. ND, not determined.

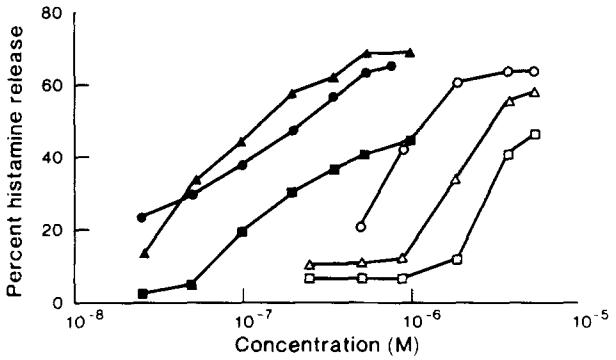

FIG. 5. Histamine release from purified rat mast cells. Rat mast cells were incubated with poly-L-arginine (filled symbols) or native human MBP (open symbols) for 10 min at 37°C. Results of three experiments are shown. Values shown for poly-L-arginine represent the means of duplicate determinations.

leukocytes (cf. Fig. 3).

Histamine Release from Purified Rat Mast Cells. Polycation-induced histamine secretion from rat mast cells is complete within 10 min at 37°C and does not require exogenous phosphatidyl serine (32–34). Incubating rat mast cells (>90% purity) with native human MBP and poly-L-arginine under these conditions resulted in a concentration-dependent histamine release similar to that observed with human leukocytes. Results of three experiments are shown in Fig. 5. The concentrations that caused release from rat mast cells closely approximated the concentrations that induced release from human leukocytes. Spontaneous release in the three experiments was 9, 8, and 14%; release caused by 0.1 μg/ml of compound 48/80 in the three experiments

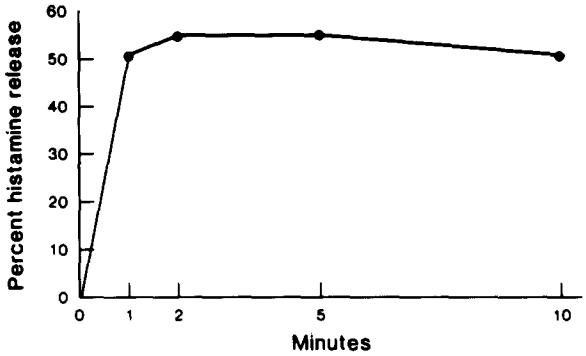

FIG. 6. Kinetics of MBP-stimulated histamine release from purified rat mast cells. Reactions were initiated by addition of 3.7×10^{-6} M native human MBP at time zero. Reactions were stopped by addition of ice-cold PA and centrifugation for 2 min at 1,000 *g*. Each point represents a single experimental value.

was 71, 75, and 27%. The apparently depressed response to native MBP and poly-L-arginine in the experiment denoted by the square symbols in Fig. 5 correlated with a higher spontaneous release and reduced response to 48/80. The concentration curve for poly-L-arginine obtained with rat mast cells was somewhat flatter than that observed with human leukocytes, whereas native MBP gave a rather steep concentration curve with either cell preparation.

A closer examination of the time course for release demonstrated that histamine release caused by native MBP was essentially complete after incubation for 1 min at 37°C (Fig. 6). Similar kinetics were obtained in a second experiment with native MBP and in two additional experiments with poly-L-arginine. Cell viability, as measured by trypan blue (0.05%) exclusion, was not altered significantly after incubation with 1–2 $\times 10^{-6}$ M native MBP and release of >70% of the histamine content. Although extensive degranulation was evident, cell viability remained 80 ± 6% (mean ± SD; n = 6); the viability of cells incubated in buffer only (<10% histamine release) was 86 ± 5% in the same experiments. In results not shown, the addition of 40 μg/ml of phosphatidyl serine did not enhance the response to poly-L-arginine, a finding similar to that reported for compound 48/80 (33).

Discussion

These results demonstrate that native MBP purified from human eosinophil granules initiates a concentration-dependent, noncytolytic histamine release from human leukocytes and purified rat mast cells. Furthermore, the results obtained using purified basophils indicate that the release from human leukocytes is due to a direct influence of MBP on basophils. Previous studies have demonstrated that reduced and alkylated MBP is cytotoxic for schistosomula and larvae of helminths (18, 19) and also mammalian cells (18, 31) during prolonged (>18 or >4 h, respectively) incubation. The release of histamine from human basophils by similar concentrations of reduced and alkylated MBP and also by native MBP, however, occurs within 45 min of incubation. More importantly, release with both MBP preparations exhibited the calcium and metabolic energy requirements as well as temperature dependence characteristic of secretory processes (35). The absence of a significant increase in trypan blue staining after stimulation of rat mast cells with native MBP provides

further evidence for a noncytolytic release mechanism. Of particular significance is the finding that native MBP was ~10-fold more potent than reduced and alkylated MBP as a stimulus for histamine release. This is the first occasion in which native MBP has been observed to be more potent than the reduced and alkylated form of MBP.

Although the mechanism for MBP-stimulated histamine release from human basophils has yet to be determined, the similarities to poly-L-arginine-stimulated release suggest that it is related to the polycationic nature of the molecule. In addition to sharing a noncytotoxic mechanism, the kinetics for release by optimal concentrations of native MBP closely approximate those reported for poly-L-arginine (22) and are slower than the rate of IgE-mediated release (23). The mechanism of poly-L-arginine-stimulated release has previously been shown to be independent of IgE antibody (22). Stimulation of histamine release by MBP from basophils purified under conditions known to desensitize basophils to IgE-mediated stimuli (36) is consistent with this finding. Although native MBP is less potent than poly-L-arginine molecules of similar molecular weight, this difference may reflect the lower arginine content (11%) of human MBP (14). Foreman and Lichtenstein (22) demonstrated that the potency of polymer amino acids is influenced by the chain length of the polymer, a finding they interpreted to indicate the importance of the number of free amino groups, or effective positive charge, of the polymer. We found in other experiments[2] that the 13,900-dalton polymer of L-arginine is approximately one-fifth less potent than the 60,000-dalton polymer used in the earlier study (22), which is consistent with the interpretation of Foreman and Lichtenstein. A polycationic basis for the release was further reinforced by the findings that native MBP also induced histamine secretion from purified rat mast cells. Both the kinetics of release and the absence of a phosphatidyl serine requirement are characteristic of polycation-stimulated secretion (32–34). The much-reduced potency of low molecular weight polymers of L-lysine[2] (30,000 daltons) and the inability of other polycations such as histones, spermine, and protamine to stimulate histamine release from leukocytes (22) indicate, however, that release by native MBP cannot be solely attributed to the net positive charge of the molecule. The findings presented here that reduction and alkylation of MBP reduce both the potency and efficacy of the molecule suggest a conformational requirement.

Based on the frequent association of eosinophilia and IgE-mediated hypersensitivity reactions (1) and the cellular distribution of specific mediators (2–6) described previously, a mast cell/basophil-eosinophil regulatory axis has been constructed in which a primary role of eosinophils is to dampen immediate hypersensitivity reactions (7, 8). That eosinophils may, however, also potentiate basophil and mast cell functions is shown by findings that relatively low concentrations of eosinophil peroxidase in the presence of hydrogen peroxide and a halide induce noncytotoxic degranulation of rat peritoneal mast cells (37) and also enhance mast cell tumoricidal activity (38). The results presented here extend possible activation by eosinophils to include the effect of MBP on human basophils and also rat mast cells. Importantly, concentrations of native MBP that initiated histamine release were similar to concentrations measured in serum of atopic patients with eosinophilia (20) and in sputum of asthmatic patients (39). With an influx of eosinophils into sites of immediate hypersensitivity reactions,

[2] O'Donnell, M. C., L. Potempa, H. Gewurz, and L. L. Thomas. Manuscript in preparation.

the release of MBP from eosinophils could prolong the basophil-mediated release events. It is not known whether the findings presented here can be extended to human mast cells. It has recently been reported (40) that human lung mast cells are unresponsive to a 60,000-dalton polymer of L-arginine in concentrations that were optimal for the 13,900-dalton polymer in the present study.

The implications for an effect of MBP on human basophils are not limited to allergic reactions. Infiltrates of eosinophils and basophils have recently been described in lesions of bullous pemphigoid (41). Basophil degranulation was observed that is consistent with the kinetics of MBP-stimulated histamine release reported here; the presence of MBP in lesions of bullous pemphigoid has also been documented (42). Basophil activation by MBP may also have important implications for eosinophil-mediated host defense roles. Mast cell products including histamine and the acidic tetrapeptides have been reported to enhance both antibody-dependent and complement-dependent killing of schistosomula (43, 44). A requirement for circulating basophils in the protection of guinea pigs against tick infection has also been described recently (45). The ability of native MBP to stimulate basophil mediator release may facilitate the destruction and elimination of parasitic organisms by eosinophils.

Thus, it is becoming apparent that the interaction of eosinophils with basophils and mast cells is bidirectional and that interaction in either direction is important to the maintenance of local homeostasis.

Summary

Major basic protein (MBP) is a primary constituent of eosinophil granules. In this report, we demonstrate that MBP from human eosinophil granules initiates a nonlytic histamine release from human leukocytes. A direct effect of MBP on basophils was confirmed using purified human basophils. The kinetics of release were similar to those reported for poly-L-arginine, although MBP was less potent than poly-L-arginine of similar molecular weight. Reduction and alkylation of MBP diminished both the potency and efficacy of the molecule. Native MBP also stimulated histamine secretion from purified rat peritoneal mast cells in a manner characteristic of other polycations. These results emphasize the bidirectional nature of the basophil/mast cell-eosinophil regulatory axis.

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