PHYSIOCHEMICAL AND BIOLOGICAL PROPERTIES OF THE MAJOR BASIC PROTEIN FROM GUINEA PIG EOSINOPHIL GRANULES*

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The functions of eosinophils not shared by neutrophils remain obscure in spite of numerous investigations and an enormous amount of literature dealing with observations of laboratory animals and patients (1–3). It seems likely that the specific functions of the eosinophil are related to the distinctive granules associated with this cell. In the past, a variety of investigators have purified eosinophil granules and investigated their properties. Vercauteren isolated eosinophil granules from horse eosinophils and analyzed the granule material (4, 5). His studies suggested the presence of arginine-rich proteins in the granules which functioned as an antihistamine. Archer and Hirsch purified rat and horse eosinophils and isolated granules from these cells. Their studies showed that eosinophil granules differed from neutrophil granules in that eosinophil granules contained a high content of peroxidase and did not contain lysozyme and phagocytin (6). More recently Gessner and his associates analyzed the proteins in guinea pig eosinophil granules and identified four major and several minor proteins (7).

We have described methods for the purification of eosinophils and neutrophils from the peritoneal cavity of the guinea pig (8) and have identified a major basic protein (MBP)[1] present in eosinophil granules (9). This protein does not possess peroxidase activity and accounts for approximately 55% of the total granule protein. In this report we have extended our earlier observations on the eosinophil MBP and have shown that it has a mol wt of about 11,000, that it is characterized by a high content of arginine, that it possesses two free sulfhydryl groups per molecule, and that it readily polymerizes and becomes insoluble. The MBP does not increase vascular permeability nor does it contract ileal muscle. It has only weak, if any, antihistaminic activity and is considerably less

[1] *Abbreviations used in this paper:* ATEE, acetyltyrosine-ethyl ester; BAEE, benzoyl-L-arginine ethyl ester; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; MBP, major basic protein; TAME, tosyl-arginine methyl ester.

potent than histones as bactericidal agents. The MBP precipitates with DNA and neutralizes heparin. Finally, it readily activates mercuripapain and is more active in this respect than cysteine.

Materials and Methods

Materials.—Sucrose, EDTA, and hydrogen peroxide were obtained from Fisher Scientific Co., Pittsburgh, Pa. Dithiothreitol (DTT), diisopropylfluorophosphate, benzoyl-L-arginine ethyl ester (BAEE), iodoacetamide, diphenylamine, histone (type IV, arginine rich), histamine, bradykinin, Compound 48/80, tosyl-arginine methyl ester (TAME), acetyltyrosine-ethyl ester (ATEE), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and protamine were purchased from Sigma Chemical Co., St. Louis, Mo. Paratoluene sulfonic acid and formic acid were obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. DNA and papain were purchased from Worthington Biochemical Corporation, Freehold, N. J. Heparin and D-ribose were obtained from Calbiochem, La Jolla, Calif. Orcinol was purchased from K & K Laboratories, Inc., Plainview, N. Y., and Bayol from the Exxon Corporation, New York. Bovine trypsin was purchased from Boehringer & Soehne, Mannheim, Germany. Bio-Gel Agarose A-0.5 M was purchased from Bio-Rad Laboratories, Richmond, Calif.

Preparation of Eosinophil Granules.—Eosinophil leukocytes were isolated from peritoneal fluids obtained from the guinea pig and granules were liberated from purified eosinophils essentially as described previously (9). Briefly, suspensions of cells in 0.34 M sucrose were repeatedly pipetted with a Pasteur pipette. After centrifugation at 400 g for 10 min at 4°C to remove unbroken cells the opalescent supernate was transferred to another tube and centrifuged at 5,000 g for 20 min at 4°C. The granules were solubilized in 0.025 M pH 4.3 acetate buffer or in 0.01 M HCl and analyzed by electrophoresis on polyacrylamide gels or by gel filtration on columns of Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) 1.2 × 45 cm. As shown previously (9) two principal fractions emerge from the column, one at the void volume containing many proteins, including peroxidase, and another, at about twice the void volume, containing the MBP. In these experiments we noted that the use of freshly prepared 0.34 M sucrose was important to obtain high yields of granule proteins.

Electrophoresis on Polyacrylamide Gels.—Solubilized eosinophil granules were analyzed by electrophoresis in 15% polyacrylamide gels at pH 3 in the presence of 6.25 M urea (10). Eosinophil granule proteins were also analyzed by electrophoresis in 8% polyacrylamide gels containing 1% SDS using the procedure described by Fairbanks et al. (11). Gels were stained as described by Fairbanks et al. (11).

Physiochemical Analyses of MBP and Solubilized Eosinophil Granules.—The MBP eluting in the second peak was analyzed for its protein content by the biuret procedure (12) and for its content of carbohydrate by the phenol-sulfuric acid procedure (13). Solubilized eosinophil granules were analyzed for their content of ribose by the orcinol reaction (12) and for deoxyribose by the diphenylamine procedure (12). The number of free sulfhydryl groups in the MBP was determined by reaction with DTNB (Ellman's reagent) (14, 15). Amino acid analysis was performed as described by Spackman et al. (16) using a Beckman model 119 automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.). Samples of MBP were hydrolyzed for 24, 48, and 72 h in 6 M HCl at 110°C. For the determination of cysteine content, the protein was oxidized with performic acid as described by Moore (17) and Hirs (18) and then hydrolyzed as described above. For determinations of tryptophan content, MBP was hydrolyzed using *p*-toluene sulfonic acid as described by Liu (19, 20). The extinction coefficient, $E_{1cm}^{1\%}$, of MBP was determined by the method of Babul and Stellwagen using the model E analytical ultracentrifuge (21).

The apparent molecular weight of MBP was determined by gel filtration of the reduced carboxymethylated protein on a calibrated 10% agarose column (Bio-Gel A-0.5 M, Bio-Rad Laboratories) equilibrated with 6 M guanidinium chloride (22, 23). The column was calibrated

with a series of polypeptides of known amino acid sequence in terms of N, the number of amino acid residues in the protein. The value of N determined experimentally by the elution position of the sample, when combined with $\overline{M}_o$, the mean residue weight determined from composition studies, provides a measure of M, the molecular weight of the protein: $M = N\overline{M}_o$. The determination of M by this technique is accurate to about $\pm$ 5% and depends upon the assumption that the protein behaves as a linear random coil in 6 M guanidinium chloride, an assumption valid for most polypeptides and proteins in this solvent.

Biological Analyses of MBP.—Antibacterial activity of the MBP was tested using a modification of the procedures described by Muschel and Treffers (24) and by Zeya and Spitznagel (25). Single colonies were removed from blood agar plates and after overnight growth in trypticase soy broth the bacteria were sedimented at 1,200 g for 20 min. After resuspension in 5 ml of broth a sample of 0.25–0.5 ml was incubated in 10-ml broth until the bacteria were in the log phase of growth. At this point they were diluted to an absorbance of 0.15 at 650 nm and suspended in 0.05 M pH 7 citrate-phosphate buffer. In these experiments eosinophil granules were solubilized in 0.01 M HCl and fractionated on columns of Sephadex G-50 equilibrated with 0.02 M pH 4.3 citrate buffer. Before testing, fractions containing the MBP were adjusted to pH 7 with 1 M NaOH and 0.05 M citrate-phosphate buffer. Varying concentrations of MBP or histone were incubated with test bacteria in citrate-phosphate buffer for 1 h, and 5 ml of trypticase soy broth was added. Incubation was continued until control tubes had an absorbance at 650 nm between 0.3 and 0.6. The test cultures were analyzed at the completion of the experiment to ensure that the same populations of bacteria were present as at the beginning and that overgrowth by a contaminant had not occurred. The concentration of protein needed for 50% killing was taken as the end point.

The ability of the MBP to alter vascular permeability was tested in 400 g guinea pigs as described by Miles and Miles (26). Guinea pigs were injected intravenously with 0.5 ml of 1% Evans blue in saline after the skin of the back had been shaved. Various materials were injected and the size and intensity of blueing were measured 15 min after intracutaneous injection of the test materials, using the grading scheme suggested by Levine (27).

The contractibility of the isolated guinea pig ileum in the presence of histamine, bradykinin, and MBP was tested using the methods described by Code and McIntire (28) and Austen (29). A 5-ml bath and recording device obtained from Adaps, Inc., Dedham, Mass., were used in these experiments. Fractions containing MBP were adjusted to pH 8 with 1 M Tris-HCl and no more than 0.2 ml was added to the gut bath.

The effect of the MBP on synthetic substrates and on the activity of enzymes was measured with a Radiometer pH-stat equipped with an autoburette and an automatic recorder (Radiometer Co., Copenhagen, Denmark). For determination of the activity of the MBP on synthetic substrates the assay mixture contained 165 μg of MBP in 0.5 ml of 0.03 M pH 7.4 phosphate buffer; 0.5 ml of 0.03 M TAME or ATEE; and 0.5 ml of 0.03 M pH 7.4 phosphate buffer. The reaction was carried out at 37°C in a thermostatically controlled vessel and the reaction velocity was measured by the addition of 10 mM NaOH. The assay for measuring trypsin inhibitory activity was the same except that 4 or 10 μg of trypsin in 0.1 ml of phosphate buffer was added. With this procedure less than 1 μg of trypsin inhibition can be detected.

The ability of MBP to neutralize heparin was tested and compared to protamine using a modification of the procedure described by Bowie et al. (30). Heparin and basic protein were mixed and then whole guinea pig blood obtained by cardiac puncture was added to a 10 $\times$ 75 mm glass tube. The time required for clotting to occur was recorded.

RESULTS

Preparation of Eosinophil Granules.—In all of our earlier experiments we had prepared granules from eosinophils purified from peritoneal washings by sedimentation through a cushion of sodium diatrizoate. This procedure limits the

yield of granule protein because of the loss of eosinophils during purification
(8). We determined whether eosinophil granules could be prepared from the
peritoneal cell populations containing eosinophils, about 40–50%, and mono-
nuclear cells, lymphocytes, and macrophages, without prior purification of
eosinophils. We found that the homogeneity of granule preparations as judged
by electron microscopy was essentially the same as when the granules were pre-
pared from purified eosinophils. In fact, granules isolated from mixed cell
preparations appeared to retain their structural integrity better than those pre-
pared from purified eosinophils. Also, analyses by electrophoresis in poly-
acrylamide gels of solubilized granules prepared from purified eosinophils or
from mixed peritoneal cells did not reveal a difference in the patterns and both
contained the MBP. Finally, injection of 15 ml of Bayol into the peritoneal
cavity of the guinea pig yielded an exudate containing almost entirely mono-
nuclear cells and virtually devoid of eosinophils (< 0.1%). When these mono-
nuclear cell preparations were carried through the procedures for preparation
and analysis of granules, no MBP component was seen. These results indicated
that eosinophil granules could be separated from the cytoplasmic constituents of
eosinophils and other contaminating cells. In all of the experiments in this
report, except as noted, we prepared eosinophil granules from peritoneal wash-
ings without prior purification of eosinophils. By eliminating this purification
step our yield of granule protein was increased about fourfold.

The presence of a basic protein derived from eosinophil granules raised the
question whether this might be a histone. Although we had not noted nuclei
when granule preparations were examined by electron microscopy, we mea-
sured the content of deoxyribose in solubilized whole granules from purified
eosinophils. No deoxyribose was detected. Analyses of the granule solutions
for ribose revealed a content of 5.2%. This degree of contamination with ribose
is likely explained by the presence of ribosomes in the 5,000 g sediment (9).

Purification of the MBP.—Previously we had shown that fractionation of
solubilized eosinophil granules on Sephadex G-50 yielded three peaks, the bulk
of the 280 nm absorbance being associated with the second peak. As shown in
Fig. 1, analyses of these fractions by electrophoresis on urea-acetic acid poly-
acrylamide gels sometimes showed a single component, but in other experiments
a series of bands were seen or alternatively a smear above the MBP was noted.
Also when MBP containing fractions were concentrated or frozen at −70°C,
usually several bands were noted. We performed a variety of studies to deter-
mine whether these findings represented another component in the second peak
fractions or whether they were a consequence of polymerization or proteolysis.
First, a variety of different conditions for disruption of granules were tested
including freezing and thawing in distilled water or in the presence of 0.15 M
NaCl or in 0.05 M pH 6.5 phosphate buffer. Also granules were disrupted by
exposure to distilled water, or 0.01 M HCl, or by exposure to various detergents,
including Tween 80 (Atlas Chemical Industries Inc., Wilmington, Del.), Brij
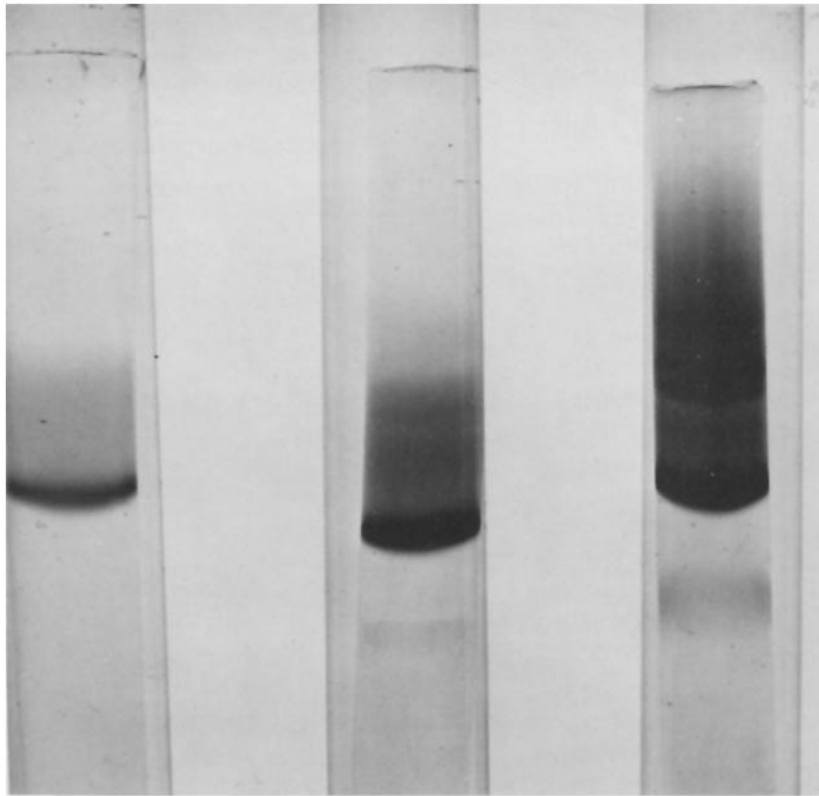
FIG. 1. Analysis of MBP on urea-acetic acid polyacrylamide gels. MBP containing fractions from the second peak of a Sephadex G-50 column were pooled, concentrated, and the concentrate frozen at −70°C for 24 h. The MBP peak and the concentrate of the peak were analyzed by electrophoresis on the same day and the frozen concentrate was analyzed a day later. The patterns produced by the pool are on the left, by the concentrate in the middle, and by the frozen concentrate on the right. The pH 4.3 acetate buffer used in this particular Sephadex G-50 separation contained 0.1% hexadecyltrimethylammonium bromide and the faint band below the MBP especially evident in the concentrates represents the presence of the detergent.

35 (Atlas Chemical Industries Inc.), or Triton X (Rohm and Haas Co., Philadelphia, Pa.), all at 1%. However, the same variability in the appearance of the MBP on the polyacrylamide gels was seen in all these experiments. Second, banding patterns were not altered by using purified eosinophils as opposed to whole peritoneal cells as starting material. Third, inclusion of 0.1% hexadecyltrimethylammonium bromide (7) in the 0.05 M pH 4.3 acetate buffer used for disruption and fractionation of granules did not alter the appearance of the MBP. Finally, disruption of granules in the presence of diisopropylfluorophosphate and EDTA did not change the banding pattern.

In none of the above experiments did we observe bands migrating faster than the MBP, and we suspected that the presence of multiple bands might be due to polymerization of the MBP. To investigate this possibility, we performed electrophoresis in 8 % polyacrylamide gels containing 1 % SDS with and without reduction with DTT. The results of a typical experiment are shown in Fig. 2. Fractions containing MBP were concentrated and stored at −70°C. Analysis without reduction revealed that the bulk of the protein did not enter the gel and a "cap" was prominent. Reduction with DTT abolished the cap and the protein had the expected position in the gel. The same result was seen when MBP was frozen in the pH 4.3 acetate buffer, indicating that the lower pH did not appreciably interfere with the polymerization process. In other experiments, we observed the presence of multiple bands above the MBP band, as shown in Fig. 3. Analysis revealed that these additional bands had molecular weights consistent with the formation of a series of polymers from a monomer with a mol wt of approximately 11,000. Furthermore, we found that a single band was produced by the MBP regardless of manipulations, providing it was reduced at pH 8 with DTT and alkylated with iodoacetamide before electrophoresis on the SDS polyacrylamide gels. These experiments indicated that a single component was present and that polymer formation was a consequence of the formation of disulfide bonds.

Physiochemical Properties of the MBP.—The above experiments indicated that the MBP had a mol wt of approximately 11,000 and likely contained at least two free sulfhydryl groups. The molecular weight of the reduced and carboxymethylated protein was measured by gel filtration chromatography on columns of 10 % agarose equilibrated with 6 M guanidinium chloride. As shown in Fig. 4 a single peak was identified with a mol wt of 11,000. The peak was symmetrical and there was no evidence of any other peaks, indicating that the MBP was composed of a single polypeptide chain.

The amino acid content of the MBP is shown in Table I. The protein is remarkable in that it contains 13 % arginine. The presence of tryptophan indicates that the MBP is not a histone (31). In Table II, the molecular weights of the MBP determined by three independent methods are listed. The MBP was also analyzed for carbohydrate and less than 1 % carbohydrate was present. The extinction coefficient obtained in the analytical ultracentrifuge was $E_{1cm}^{1\%} = 25.9$, a value in remarkably good agreement with the result from biuret analysis $E_{1cm}^{1\%} = 26.3$ using human serum albumin as a standard. Analyses to determine the content of available sulfhydryl groups are shown in Table III and indicate that two available sulfhydryl groups are present. Because a total of six half-cystine residues are present, it is clear that four of these are joined to form two disulfide bonds and that the remaining two are present in the reduced form.

Biological Properties of the MBP.—A variety of experiments were performed to define biological activities associated with the MBP. Initially we tested the

FIG. 2. Analysis of MBP on SDS polyacrylamide gels. Fractions from the second peak of a Sephadex G-50 column equilibrated with 0.05 M pH 4.3 acetate buffer were pooled and the pH adjusted to 8 with 1 M Tris-HCl. The MBP was concentrated (602 μg/ml) and stored at $-70°$C. Samples, 12 μg, were analyzed, either reduced with DTT (right), or not reduced (left) (11). The india ink mark at the bottom of the gels indicates the position of the pyronin Y marker at the conclusion of electrophoresis.
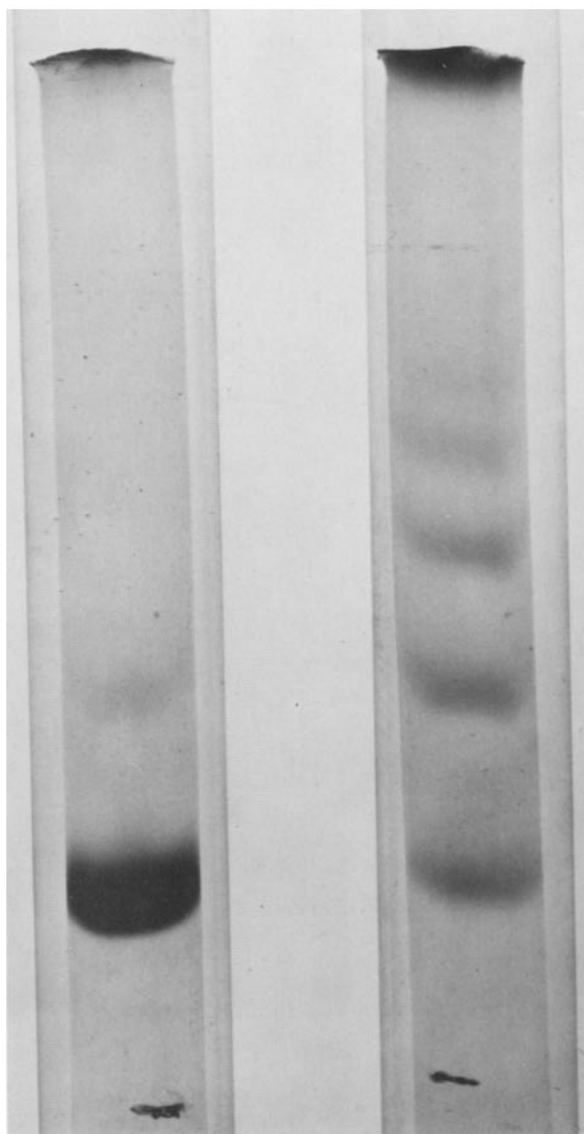
FIG. 3. Analysis of MBP on SDS polyacrylamide gels. MBP containing fractions in pH 4.3 acetate buffer were adjusted to pH 8 with 1 M Tris-HCl and DTT, final concentration 0.01 M, was added. The MBP was concentrated using an Amicon UM-2 membrane (Amicon Corp., Lexington, Mass.) and the concentrate was left at 20°C for 3 days. Before analysis additional DTT was added to the sample on the left. Note that both gels have "caps", but the gel on the right reveals a series of prominent bands above the MBP band.
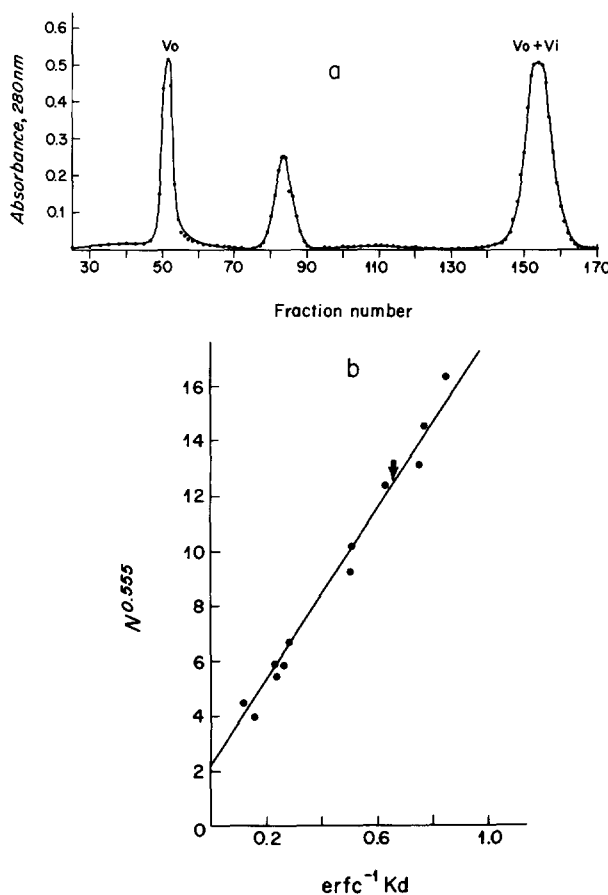
FIG. 4. Determination of the number of amino acid residues in MBP. Reduced and carboxymethylated MBP was analyzed by gel filtration on a calibrated 10% agarose column equilibrated with 6 M guanidinium chloride. Briefly, MBP in 0.2 M acetic acid was lyophilized, the resulting powder taken up in pH 8.6 6 M guanidinium chloride, reduced in 0.1 M mercaptoethanol for 18 h at 20°C, and carboxymethylated with an excess of iodoacetic acid (22). In 4 a, Vo (void volume) is the elution position of the blue dextran and Vo + Vi is the elution position of DNP-alanine (Vi is the column internal volume). The elution position of MBP is intermediate between these markers. In 4 b, the calibration data for 10% agarose column are shown. N refers to the number of residues in the polypeptide and $erfc^{-1}$ Kd is the inverse error function complement of Kd. Kd is the distribution coefficient for the column. Closed circles represent the standards of known sequence and thus the number of residues (23). The arrow indicates the $erfc^{-1}$ Kd for MBP.

effect of the MBP on vascular permeability in the skin and the results are shown in Table IV. Injection of various amounts of MBP up to. 11 μg did not increase vascular permeability. Injection of the first peak from the Sephadex G-50 gel filtration columns increased vascular permeability slightly and the

TABLE I

Amino Acid Composition of MBP

Amino acid	No. of residues
Aspartic	6
Threonine	4
Serine	4
Glutamic	8
Proline	5
Glycine	12
Alanine	8
Valine	9
Methionine	1
Isoleucine	2
Leucine	4
Tyrosine	4
Phenylalanine	5
Histidine	3
Lysine	2
Arginine	13
Tryptophan*	4
Cysteine‡	6
Total	100

* After hydrolysis in 3N *p*-toluene sulfonic acid (19, 20).
‡ As cysteic acid.

TABLE II

Molecular Weight of MBP

SDS polyacrylamide	11,102
Amino acid composition	11,350
Gel filtration	10,800

The value for SDS polyacrylamide represents the mean of 16 individual determinations and for gel filtration, two determinations. The accuracy of the molecular weight determined by SDS polyacrylamide is $\pm 10\%$ and by gel filtration, $\pm 5\%$. In the case of the SDS polyacrylamide determinations the values used for calculation of molecular weight of MBP were those of dimers, trimers, and tetramers, the mobilities of which were bracketed by the standards (9, 11). The amino acid composition value represents the minimal molecular weight based on one residue of methionine.

controls using histamine, bradykinin, and the histamine releasor 48/80 were clearly positive. To determine whether MBP might neutralize histamine or bradykinin we mixed these materials with MBP and then injected them into guinea pig skin. No inhibition of the activity of the histamine on the permeability of cutaneous vessels was found nor was there any inhibition of the reactivity of bradykinin or 48/80.

We also examined the activity of the MBP on the guinea pig ileum using a

TABLE III

Available Sulfhydryl Groups in MBP*

Material	Free SH mol/mol
MBP	
Exp. 1	2.14
Exp. 2	2.13
Dithiothreitol	2.05

* MBP, 2.08×10^{-2} μM in a vol of 0.6 ml of 0.05 M pH 4.3 acetate buffer, was added to 2.4 ml of 2% SDS, 0.08 M pH 8 sodium phosphate containing EDTA 0.5 mg/ml, and incubated at 50°C for 20 min. The resultant pH was 7.8. DTNB, 0.1 ml of 4 mg/ml in 0.1 M pH 8 phosphate, was added and the color developed for 15 min at room temperature before reading at 410 nm (15).

Schultz-Dale gut bath and the results of certain of these experiments are shown in Table V. Addition of MBP to the bath solution did not contract the gut either immediately or in a delayed fashion and this was so regardless of whether or not atropine was added to reduce muscle irritability. The amounts added were comparable, on a molar basis, to the quantities of histamine which are regularly active on the gut. Three experiments were performed to determine whether MBP could block the action of histamine on the gut. In one of these (Table V, tests 2 and 3) there were modest reductions, 26% and 35%, in the amplitude of contraction when the gut was preincubated with MBP. In the second experiment (Table V, test 4) there was a slight increase in the amplitude of contraction. In the third experiment (not shown), reductions, 26% and 38%, in the amplitude of contraction were noted in two of five tests, but reductions of the same magnitude, 33% and 35%, were noted after incubation with neutralized column buffer only. In none of these experiments did we observe a stepwise increase in the response of the gut to histamine (5). Preincubation with fraction I from the Sephadex G-50 column did not alter the contraction of the gut by histamine. Mixing MBP with histamine or with bradykinin did not alter the reactivity of the gut to these materials.

Because of prior reports that histones and neutrophil basic proteins are bactericidal, we investigated the bactericidal properties of the MBP with several organisms including *Staphylococcus epidermidis*, *Proteus vulgaris*, *S. aureus*, *Streptococcus pyogenes*, and two strains of *Escherichia coli*. Whereas histone was very active in suppressing the growth of *S. epidermidis*, *P. vulgaris*, and *E. coli*, MBP did not reduce the growth of any of the organisms save for *E. coli* 182 (25 μg for 50% inhibition).

The MBP was tested for esterolytic activity using the synthetic esters TAME and ATEE. No hydrolysis of either substrate was detected. Also the MBP had no trypsin inhibitory activity using both TAME and ATEE as substrates of trypsin. In these assays the molar ratio of MBP to trypsin was 34

TABLE IV

*Effect of the MBP on Cutaneous Vascular Permeability**

Test material	Exp. 1	Exp. 2	Exp. 3	Exp. 4
MBP				
11 μg	Neg‡	3 Tr		
1 μg	Neg	3 Tr		
5.4 μg			5 Tr	5 MP
Peak I§	6 P	6 MP		
Histamine				
1 μg	9 MP	10 M	12 M	13 M
0.5 μg	9 P	6 P		
Bradykinin				
1 μg	9 M	10 MS		
0.5 μg	8 MP	10 MS	9 S	8 S
48/80				
1 μg	8 MP	7 M	6 M	7 MS
2 μg	9 M	5 MP		
NaCl	2 Tr	2 Tr	2 Tr	5 MP
	3 Tr	2 Tr		
Histamine, 1.0 μg‖ + MBP, 5.4 μg			11 M	13 M
Bradykinin, 0.5 μg‖ + MBP, 5.4 μg			10 S	9 S
48/80, 1 μg‖ + MBP, 5.4 μg			9 MP	6 MP

* Guinea pigs weighing from 470 to 680 g were injected intravenously with 0.5 ml of 1% Evans blue dye and immediately thereafter the test materials were injected intracutaneously. The animals were sacrificed after 15 min and the reactions graded by the average diameter of the blue spot and by the intensity of color: trace (Tr), pale (P), moderate-pale (MP), moderate (M), moderate-strong (MS), and strong (S) (27).

‡ Less than 0.15 M NaCl control.

§ Absorbance at 280 nm was 0.73 which corresponds roughly to 28 μg/0.1 ml injection site, using the same extinction coefficient as used for MBP.

‖ Histamine, bradykinin, and 48/80 were mixed with freshly prepared MBP in glass tubes at room temperature about 15–20 min before injection.

(10 μg trypsin) and 85 (4 μg trypsin), respectively. Because of the presence of free sulfhydryl groups in the MBP we determined whether it might alter the activity of papain, a sulfhydryl dependent protease (32). In the first experiment, papain was activated with cysteine and separated from excess cysteine by gel filtration on Sephadex G-25. The activated papain was then incubated with MBP and BAEE. Rather than inhibiting the activity of papain, we found that MBP increased its activity by 72%. This result suggested that the enzyme was

TABLE V

Reactivity of Ileal Muscle to MBP, Histamine, and Bradykinin

Test	Material tested	Contraction, units
1	MBP, alkylated, 10 µg	0
	MBP, 23 µg	0
	18.7 µg	0
	5.6 µg	0
	Fraction I from G-50 column, 14 µg	0
2	MBP, 5.6 µg, incubated with gut for 23 min followed by histamine, 100 ng	52
	Histamine, 100 ng	70
3	MBP, 18.7 µg, incubated with gut for 17 min followed by histamine, 50 ng	17
	Histamine, 50 ng	26
4	MBP, 18.7 µg, incubated with gut for 30 min followed by histamine, 100 ng	40
	Histamine, 100 ng	36
5	Fraction I, 14 µg, followed after 17 min by histamine, 50 µg	49
	Histamine, 50 µg	54
6	Histamine, 25 ng	54
	Histamine, 25 ng + MBP, 7.7 µg	52
7	Bradykinin, 12.5 ng	48
	Bradykinin, 12.5 ng + MBP, 7.7 µg	47

MBP, histamine, bradykinin, or mixtures of these were added to the 5-ml gut bath chamber and the response noted. In experiments 2–4, testing the ability of MBP to alter the reactivity of gut to histamine, the protein was freshly prepared by gel filtration of granules solubilized in 0.01 M HCl and fractions from the second peak were neutralized by addition of 1 M Tris-HCl, pH 8. The protein was added to the gut bath and incubated, and histamine was added. The response of the gut to histamine alone was measured several times immediately before addition of MBP and also after the mixture of MBP and histamine had been washed from the chamber. In experiments 6 and 7, MBP was added to histamine or bradykinin and the mixture was added directly to the gut bath. Units are the number of millimeters of deflection on the recorder.

maintained in its active form by the MBP. We next determined whether the MBP could activate mercuripapain and the results of this experiment are shown in Fig. 5. Addition of MBP to mercuripapain resulted in a definite increase in the activity of the enzyme. Comparison of the ability of cysteine and MBP to activate mercuripapain showed that MBP was approximately 1.3 times as active as cysteine on a molar basis. These experiments indicate that the sulfhydryl groups in the MBP are readily available and are capable of activating an enzyme which requires a free sulfhydryl group for its proteolytic action.

Because of the basic character of the MBP we tested its ability to interact
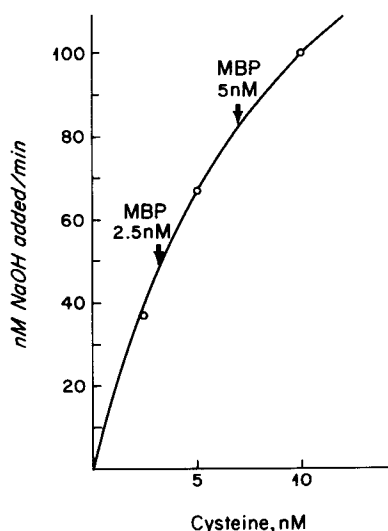
FIG. 5. Activation of papain by cysteine and MBP. Mercuripapain, 54 μg in pH 6.2 0.02 acetate-0.025 M phosphate buffer, was incubated with either cysteine or MBP for 5 min at 20°C, final vol 1 ml. The substrate was 0.041 M BAEE, containing 0.0004 M EDTA. A sample of MBP or cysteine and enzyme, 0.1 ml, was added to 1.0 ml of BAEE and 1.0 ml of 3 M NaCl. The rate of hydrolysis of BAEE was determined by addition of 0.01 M NaOH. Open circles denote the effect of cysteine.

with acidic molecules such as DNA. It is known that histones will precipitate with DNA (31), and we determined whether the MBP would also precipitate with DNA. Mixing of MBP and DNA resulted in immediate turbidity and within minutes a stringy precipitate formed.

Finally, the ability of the MBP to neutralize the effect of heparin on the clotting time of freshly drawn guinea pig blood was determined. Both protamine and MBP neutralized heparin and addition of either of these proteins to heparin resulted in the formation of opalescence. While protamine by itself did not alter the clotting time appreciably, MBP increased the clotting time from three to fivefold in two experiments.

DISCUSSION

In these experiments we have characterized a protein isolated from the granules of guinea pig eosinophils. This material accounts for the bulk of the protein in solubilized eosinophil granules, is of relatively low molecular weight, and has an isoelectric point of 10 or greater (9). In our prior experiments eosinophil granules prepared from purified eosinophils were solubilized in 0.01 M HCl and fractionated by gel filtration on Sephadex G-50 columns. Because purification of eosinophils from the peritoneal cell mixtures limits the yield of granules and thus of MBP, we determined whether granules could be directly

purified from the peritoneal cell mixtures. We found that the resulting granule preparations were as homogeneous as those from purified eosinophils and the yield of MBP was increased about fourfold. Analysis of the MBP-containing fractions from the Sephadex G-50 column by electrophoresis in urea-acetic acid polyacrylamide gels often revealed a single band but after concentration or freezing and thawing, a series of bands were found (Fig. 1). Even though we varied the conditions during disruption of granules, the finding of multiple bands after concentration or storage of MBP was repeatedly observed. When the MBP was analyzed by SDS polyacrylamide electrophoresis, we found that protein which was concentrated and frozen did not enter the gel unless it was reduced with DTT (Fig. 2). In contrast freshly prepared MBP usually yielded single major bands. Thus the apparent inhomogeneity of the MBP is due to a marked propensity of the protein to aggregate and this is particularly evident when the protein is concentrated or when it was stored before analysis. We found that the additional bands represented polymers of a monomer with a mol wt of 11,100. Reduction of the polymerized MBP before electrophoresis largely abolished the polymers and increased the intensity of staining of the major band (Fig. 3). Furthermore, we found that storage of the MBP in the presence of disulfide-reducing agents such as mercaptoethanol or DTT prevented or markedly reduced the formation of polymers. Alkylation of freshly prepared MBP with iodoacetamide prevented the formation of polymers. These results indicate that the MBP is a single component and that the apparent inhomogeneity is due to the formation of a series of polymers produced by the formation of disulfide bonds. Further evidence for the conclusion that the MBP is a unique homogeneous protein is the preliminary finding from amino acid sequence studies which show that single amino acids are found at residues 1–10.[2]

The mol wt of the MBP as determined by gel filtration on columns of 10% agarose equilibrated with 6 M guanidinium chloride was 10,800, a value in good agreement (a) with that derived from the studies on SDS polyacrylamide gels and (b) with the minimal molecular weight (based on one residue of methionine) from amino acid composition (Table II). Amino acid analyses of the MBP revealed that it contained 13% arginine, accounting for its high isoelectric point. In addition, four residues of tryptophan are present, a finding which established the protein as distinct from histones which are devoid of tryptophan (31). Finally, the MBP contains less than 1% carbohydrate.

We have conducted a variety of experiments to determine whether the MBP possesses biological properties comparable to the basic proteins from rabbit neutrophil granules (33–37). In contrast to basic proteins derived from neutrophil granules, our experiments indicate that the eosinophil MBP does not increase vascular permeability. In addition it does not contract the guinea pig

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[2] Fass, D. N., K. G. Mann, and G. J. Gleich. Unpublished observations.

ileum. The MBP does not inhibit histamine or bradykinin and did not interfere with the activity of the histamine releasing compound, 48/80. Incubation of guinea pig ileum with MBP did not consistently reduce the amplitude of contraction after addition of histamine. These results differ from those reported by Vercauteren and Peeters, but they used considerably larger amounts, up to 2.4 mg of crude extract of horse eosinophil granules (5). Also their extracts contracted the gut while the guinea pig MBP did not. Our results with bradykinin also differ from those of Archer and Broome who showed that aqueous extracts and their ultrafiltrates inhibited the production of edema in horse skin by bradykinin (38). However, the biological reaction in the horse may not be analogous to the tests of MBP on vascular permeability and gut contractibility we have performed. Finally in contrast to neutrophil basic proteins and histone (25, 35, 36, 39) which show striking antibacterial activity, the MBP displays little such activity, and in the one instance in which activity was detected it was considerably less than that found with histone.

The physiochemical structure of the MBP, namely, its marked basicity and the presence of two available sulfhydryl groups, suggested that the protein might interact with acidic molecules and with enzymes which depend on the presence of sulfhydryl groups for their activity. Mixing the MBP with DNA resulted in a marked increase in turbidity and the formation of a stringy precipitate. Also the MBP was found to activate papain and in this respect was more active on a molar basis than cysteine. We did not detect any enzymatic activity in the MBP when it was incubated with ATEE and TAME nor did it inhibit trypsin. The MBP neutralized the anticoagulant activity of heparin and its activity in this respect was comparable to that of protamine. When added to guinea pig blood, it prolonged the clotting time. Saba and his associates (40) have shown that the cationic protein fraction of rabbit polymorphonuclear leukocytes exerts a potent anticoagulant effect on human blood in vitro. Their results suggested that the cationic proteins interfered with the formation of intrinsic thromboplastin. Earlier, Archer had shown that extracts of whole eosinophils possess heparin-neutralizing activity, but these extracts were prepared from whole cells and therefore may have contained histones (41).

Our findings do not point to any unique biological activity associated with the MBP other than those which one would expect of a protein as basic as this and which possesses reactive sulfhydryl groups. However, the ability of the eosinophil to deposit this material in tissue when free granules are liberated could modify a variety of biological reactions. Bosworth and Archer have found that exposure of bacteria to lysed rat eosinophils promoted phagocytoses by monocytes and neutrophils (42). In preliminary experiments we have found that the MBP readily precipitates with proteins such as bovine serum albumin and rabbit IgG, and it binds to erythrocytes. Thus the MBP may alter biological reactions by adhering to acidic groups on cell membranes and altering the reactivity of receptors or other properties of membranes (43). Further, Chen

and Hirsch have shown that mercaptoethanol can substitute for macrophages in the Mishell-Dutton culture system (44, 45) and Broome and Jeng have reported that mouse lymphoid tumors showed growth promotion when thiols, such as cysteine and mercaptoethanol, were added to standard culture media (46). Eosinophils appear in lymph nodes after administration of antigen (47), presumably in response to chemotactic factors derived from lymphocytes (48, 49), and are prominent in human lymphoma tissue (2). Perhaps a function of eosinophils is the regulation of the growth of lymphoid cells through the activity of the thiol-rich MBP.

SUMMARY

Guinea pig eosinophil granules are characterized by the presence of a basic protein of low molecular weight which accounts for greater than 50% of granule protein. This protein, termed the major basic protein (MBP), readily aggregates and becomes insoluble, and the formation of aggregates is dependent on the establishment of disulfide bonds. Analysis of concentrated preparations of MBP often revealed a series of bands which were multiples of a monomeric unit with a mol wt of approximately 11,000. Analysis of reduced and alkylated MBP on a 10% agarose column equilibrated with 6 M guanidinium chloride revealed a single polypeptide chain with a mol wt of 10,800. Amino acid analysis of the protein revealed the presence of 13% arginine, consistent with the basic character of the molecule. Four residues of tryptophan, were present, indicating that MBP is not a histone. The MBP did not increase vascular permeability when injected into the skin of guinea pigs, nor did it antagonize the effect of histamine and bradykinin in the skin. MBP also did not contract the isolated guinea pig ileum and when mixed with histamine or bradykinin did not inhibit their activity on the gut. MBP had only weak, if any, antihistaminic activity. MBP possessed weak bactericidal activity when compared to histone and then only with one strain of E. coli. MBP precipitated DNA, neutralized heparin, and activated papain. On a molar basis MBP was more active than cysteine in activating papain. These results do not point to any unique biological activity associated with MBP other than those expected of a protein as basic as it is and one which possesses reactive sulfhydryl groups. Possible functions of eosinophils based on the properties of the MBP are discussed.

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