Elevated Serum Levels of the Eosinophil Granule Major Basic Protein in Patients with Eosinophilia

DONALD L. WASSOM, DAVID A. LOEGERING, GRAHAM O. SOLLEY, S. BREANNDAN MOORE, ROBERT T. SCHOOLEY, ANTHONY S. FAUCI, and GERALD J. GLEICH, *Departments of Medicine, Laboratory Medicine and Immunology, Mayo Medical School, Mayo Clinic and Foundation, Rochester, Minnesota 55905; Laboratories of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205*

ABSTRACT A radioimmunoassay was established for the human eosinophil granule major basic protein (MBP). The mean level of MBP in sera from 105 normal control patients was 454 ng/ml, whereas in a sample of 188 patients with various forms of disease, including the hypereosinophilic syndrome, levels as high as 14,000 ng/ml were measured. Serum levels of MBP did not correlate with eosinophil counts in normal subjects, but a positive correlation was seen in patients with eosinophilia; the patients with eosinophil counts > 350/mm^3 generally showed increased levels of MBP. Many patients with skin disease and normal eosinophil counts had elevated levels of serum MBP. Monomer MBP has a molecular weight of 9,300, but in sera of patients with eosinophilia, the MBP activity was of high molecular weight, >50,000. Analyses of serum by Sephadex G-200 and by electrofocusing suggest that MBP is not simply polymerized, but rather is bound to a larger carrier molecule. Monomeric MBP can be isolated from serum by reduction of serum with dithiothreitol, alkylation with iodoacetamide, and acidification to pH 2 followed by fractionation on Sephadex G-50 at pH 2. Under these conditions, up to 80% of the MBP emerges in monomeric form. The results indicate that eosinophil granule proteins circulate in blood covalently bound to serum proteins, and that elevated concentrations of serum MBP are present in some diseases associated with eosinophilia.

INTRODUCTION

Eosinophilia is associated with many diseases, and it is characteristic of allergic and parasitic diseases (1, 2). It seems likely that eosinophils are protective in parasitic diseases, especially those due to helminths (3–5), but its role in allergic diseases is speculative. We have studied a protein unique to eosinophils, the major basic protein (MBP)[1] (6–8), which constitutes 50% of the total granule protein of the eosinophil and accounts for ~25% of all protein within the cell (6, 9). The MBP is a strongly basic molecule with a molecular weight of 9,300 in the human (8) and 11,000 in the guinea pig (7). The MBP exists within the crystalloid core of the eosinophil granule (10), and it is released to the exterior of the cell during degranulation (11–14). MBP differs from Charcot-Leyden crystal protein in its molecular weight and amino acid composition (8). Purified MBP damages schistosomules of *Schistosoma mansoni* (11) and newborn larvae of *Trichinella spiralis* (15), and recently we have shown that it is toxic to a wide variety of mammalian tissue cells including tracheal epithelium (11, 16, 17). Because the MBP has the ability to damage cells in vitro, and thus might cause damage in disease, we have developed a sensitive radioimmunoassay (RIA) to measure levels of human MBP in blood and other body fluids.

METHODS

Patient samples and normal controls. Patient sera used in these studies were obtained from individuals seen for medical treatment at either the Mayo Clinic or at the National Institutes of Health. Sera from normal individuals were obtained from volunteers donating blood to the Mayo Clinic Blood Bank. In the case of the normal controls, total eosinophil counts were performed using EDTA anticoagulated whole blood stained with Randolph's stain, and the cells were counted using standard cell counting chambers.

Dr. Wassom's present address is Diagnostic Laboratory, New York State School of Veterinary Medicine, Cornell University, Ithaca, N. Y. Address reprint requests to Dr. Gerald Gleich, Allergic Diseases Research Laboratory, Mayo Clinic, Rochester, Minn. 55905.

Received for publication 11 February 1980 and in revised form 27 October 1 1980.

[1] *Abbreviations used in this paper*: DTT, dithiothreitol; ECP, eosinophil cationic protein; FCS, fetal calf serum; MBP, major basic protein; PPF buffer, 0.1 M Na_2HPO_4-KH_2PO_4, pH 7.4, containing 1 mg/ml protamine sulfate and 0.5% FCS; RIA, radioimmunoassay.

Purification of human eosinophil MBP. Human eosinophils were purified from leukocytes from a patient with eosinophilia, using a Haemonetics cell separator (Haemonetics Corp., Natwick, Mass.); 7×10^{10} leukocytes, 53% eosinophils, were present in the starting material. Erythrocytes were allowed to sediment in hydroxyethyl starch (Volex, McGaw Inc., Santa Ana, Calif.). Cells in the supernate were centrifuged at 200 *g* for 5 min, and the residual erythrocytes were lysed by treatment of the pellet with 4 ml of NH_4Cl lysing buffer (8.29 g/liter NH_4Cl, 1.0 g/liter K_2CO_3, and 0.0372 g/liter Na_2EDTA) for each 1 ml of packed cells. After 2 min, the cells were centrifuged at 200 *g* for 5 min and resuspended in 100 ml RPMI-fetal calf serum (RPMI-1640, catalogue No. 380-2400, Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y., containing 10% heat-inactivated fetal calf serum [FCS]). The method of Parrillo and Fauci (18) was used to purify eosinophils from this suspension. Sucrose was dissolved in Hanks' balanced salt solution (catalogue No. 310-4065, Grand Island Biological Co.) containing 20% FCS to give solutions of 40, 35, 30, 25, 20, and 15%. A discontinuous 15–40% gradient was prepared by layering 6 ml of the various sucrose concentrations in a 50-ml round-bottom tube. 10 ml of cell suspension containing 7×10^8 leukocytes/ml was layered over the top of the gradients and the tubes were centrifuged at 200 *g* for 5 min at room temperature. Cells in the bottom half of the sucrose gradient were recovered. From a total of nine sucrose gradients, prepared as described above, the overall yield was 4.6×10^{10} cells, 55% eosinophils. After washing in RPMI-FCS, cells were applied to nylon wool columns containing 3 g scrubbled nylon wool (Fenwal Laboratories, Deerfield, Ill.) equilibrated wilth RPMI-FCS in a 30-ml syringe. 10 ml of cell suspension containing 4.2×10^8 cells/ml, 55% eosinophils, was applied to each column, incubated at 37°C for 15 min, and eluted with 60 ml of RPMI-FCS at 37°C; 10 columns were employed. A total of 5.6×10^9 cells, 99% eosinophils, was recovered, for an overall eosinophil recovery of 15%.

Methods for the purification of MBP from eosinophils are described in detail elsewhere (6–8). Briefly, cells were washed in 0.25 M sucrose, disrupted by homogenization using six passes of the pestle in a Tenbroeck homogenizer (Kontes Glass Co., Evanston, Ill.), and centrifuged at 400 *g* to remove unbroken cells and larger particles. In these experiments, heparin was not added to the 0.25 M sucrose in the cell homogenization step, because in preliminary experiments we had found it could be eliminated in most cases. The supernate was centrifuged at 13,000 *g* for 20 min; the sediment was dissolved in 0.01 M HCl and fractionated on a column of Sephadex G-50 equilibrated with 0.025 M CH_3CO_2H-CH_3CO_2Na, pH 4.3. After purification, the MBP was stabilized by alkylation of its two sulfhydryl groups. EDTA at a final concentration of 0.002 M and dithiothreitol (DTT) at a 60-fold molar excess above available sulfhydryl groups were added to the MBP at pH 8.0 and the mixture was incubated at room temperature for 40 min. Iodoacetamide at a 120-fold molar excess was then added, and the mixture was incubated in the dark for 20 min. The reduced and alkylated sample was transferred to a 3,500-dalton cutoff dialysis casing (Spectrum Medical Industries Inc., Los Angeles, Calif.) and dialyzed overnight against 0.15 M NaCl. The concentration of human MBP was determined by absorbance at 277 nm, $E^{1\%}_{1\,cm} = 26.3$ (8).

Antiserum to the human MBP. Freshly isolated MBP was mixed with an equal amount of crystalline rabbit serum albumin (Mann Research Laboratories, New York), and the resulting precipitate emulsified in complete Freund's adjuvant. Material containing 1 mg MBP was injected intradermally into rabbits at six lateral sites. 16 d and 6 wk later, the animals each received intradermally 1 mg MBP emulsified in complete Freund's adjuvant. 17 wk after the initial immunization, the animals were given three injections of 0.5 mg MBP in saline, subcutaneously on day one and intravenously on days two and three. 13 wk later the animals received another series of three injections identical to those above. The animals were exsanguinated 2 wk after the final immunization, and the antiserum was stored at −20°C.

RIA for human MBP. Reduced and alkylated human MBP was radioiodinated with ^{131}I by a modification of the procedure described by McConahey and Dixon (19). Approximately 600 μCi of ^{131}I, diluted in 200 μl of 0.5 M Na_2HPO_4-KH_2PO_4, pH 7.5, was added to a plastic tube on ice followed by 10 μg of reduced and alkylated MBP in 25 μl PPF buffer (0.1 M Na_2HPO_4-KH_2PO_4, pH 7.4, containing 1 mg/ml protamine sulfate and 0.5% FCS). Chloramine T, 25 μl of 1 mg/ml in 0.5 M phosphate buffer, was added and mixed intermittently for 3 min. Sodium metabisulfite, 25 μl of 1 mg/ml, was added, and the mixture was incubated for another 2 min, after which 50 μl of 1% KI was added and the contents of the tube thoroughly mixed. Next, 3 ml of PPF buffer was added, and the contents transferred to a 3,500-dalton cutoff dialysis casing and dialyzed against four changes of 0.15 M NaCl. Aliquots of ^{131}I-MBP were frozen, and 1 μg/ml working stock was stored at 4°C. Specific activity ranged from 25 to 30 μCi/μg and >98% of counts were precipitated by 10% tungstic acid. Details for performing the RIA are similar to those described elsewhere for measurement of guinea pig MBP (20). Briefly, 0.1 ml of a 1:10,000 dilution of rabbit anti-human eosinophil MBP in PPF buffer, 0.5 ml of PPF buffer, and 0.1 ml of the sample to be tested were added to 10 × 75-mm glass tubes. The contents were thoroughly mixed, incubated at 37°C for 30 min and at 4°C for 15 min, and 1 ng ^{131}I-MBP diluted in 0.1 ml PPF buffer was added. The tubes were covered and incubated overnight at 4°C. Immune complexes were precipitated by addition of 0.1 ml of 1:20 dilution of normal rabbit serum in PPF buffer and 0.1 ml of burro anti-rabbit IgG. Tubes were mixed, incubated at 4°C for 2 h, and centrifuged for 20 min at 4°C and 2,500 *g*. The supernates were discarded, the tubes drained, and the precipitate counted in a Nuclear Chicago gamma scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.). Controls containing 100% of the ^{131}I-MBP added to each tube were counted for a sufficient time to accumulate 10,000 counts or more; assay tubes were counted for an equal time and expressed as a percentage of total counts bound. At a dilution of 1:10, anti-MBP bound 77.6% of ^{131}I-MBP; an antiserum titration curve is shown in Fig. 1.

Reduction and alkylation of serum. Before RIA, 0.1 ml of serum was diluted with 0.27 ml of a buffer consisting of 0.12 M NaCl, 0.01 M disodium EDTA, and 0.33 M Tris (hydroxymethyl) aminomethane (Trizma base, Sigma Chemical Co., St. Louis, Mo.), pH 8. DTT, 0.03 ml of 0.1 M solution (Sigma Chemical Co.), was added (final concentration 0.0075 M), and the solution was incubated at room temperature for 60 min. Iodoacetamide (Sigma Chemical Co.), 0.03 ml of 0.2 M solution, was added, final concentration 0.014 M, and the incubation continued for an additional 15 min in the dark. As will be discussed below, these conditions were optimal for detecting MBP activity in serum. Samples were further diluted in PPF buffer before assay as needed.

Stability of MBP in serum. To test whether prolonged storage or repeated freeze-thawing of serum samples resulted in significantly decreased MBP activity in serum, internal controls consisting of a serum from a patient with a high level of MBP and serum from a normal control were included in each assay. Additionally, a single sample was repeatedly freeze-thawed, an aliquot removed after each freeze-thaw cycle, and all aliquots tested for MBP activity in a single RIA. The mean values, standard deviations, and coefficients of variation for the internal controls over an 18-mo period along with

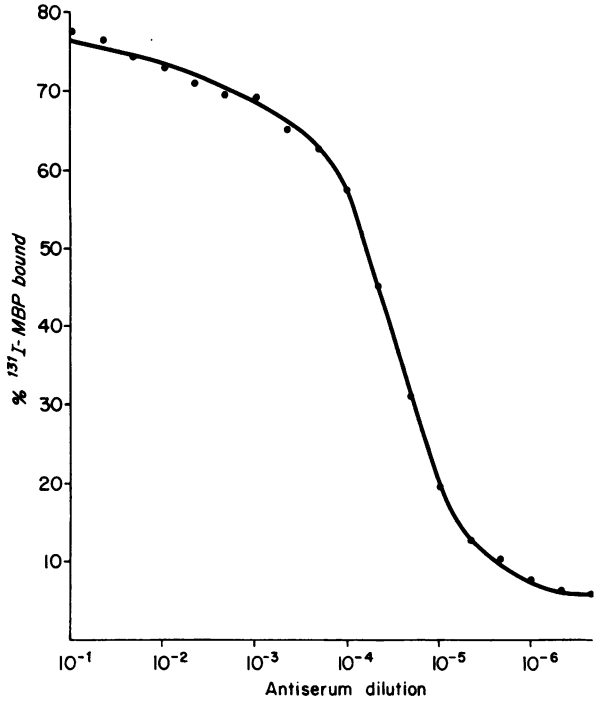

FIGURE 1 Antiserum titration curve of rabbit antiserum to human eosinophil MBP. The titration used 0.1 ng ^{131}I-MBP in 0.6 ml PPF buffer reacted with 0.1 ml of antiserum at increasing dilutions at 4°C. After overnight incubation, 0.1 ml of normal rabbit serum diluted 1:20 in PPF buffer and 0.1 ml of burro anti-rabbit IgG were added. The tubes were mixed and incubated for 2 h at 4°C. The percentage of counts in the precipitate is plotted on the ordinate, and the reciprocal of the antiserum dilution is plotted on the abscissa.

data from repetitive freeze-thaw cycles are shown in Table I.

Fractionation of serum by gel filtration. The molecular weight of MBP in blood was estimated by gel filtration of both untreated serum and serum that had been reduced and alkylated. In the case of untreated serum, ~125 mg of serum protein, as determined by biuret analysis, was diluted with 0.01 M Na_2HPO_4-KH_2PO_4 and 0.2M NaCl, pH 7.4, and applied to a 5 × 87-cm Sephadex G-200 column equilibrated with the diluent buffer. The column flow rate was ~21.3 ml/h. Fractions of 7.3 ml were collected and the absorbance at 277 nm was determined. Samples of these fractions were then reduced and alkylated as described above and assayed for MBP activity by RIA.

In other experiments, the serum sample was first diluted with two parts of PPF buffer and then reduced and alkylated as described above. The pH was lowered to pH 1.5 by addition of 2 M HCl, and the sample applied to a 1.2 × 45-cm Sephadex G-50 column equilibrated with 0.01 M HCl–0.15 M NaCl. The column flow rate was ~30 ml/h. Absorbance at 277 nm of 1-ml fractions was measured, and fractions were assayed by RIA for MBP activity.

RESULTS

Specificity and optimal conditions for the RIA

In initial RIA experiments the specificity and the optimal conditions for measurement of MBP were determined. In agreement with prior results with guinea pig MBP (20), we found it necessary to include FCS and a basic protein, such as protamine, in the RIA buffer to lower nonspecific binding and to obtain steep inhibition curves. Tests of specificity revealed that platelet factor IV, 60 μg/tube, Charcot-Leyden crystal protein, 47 μg/tube, and guinea pig MBP, 1 μg/tube, did not inhibit. Similarly, human serum albumin or human immunoglobulin (Ig)G, both 5 mg/tube, showed very slight or no inhibition. In contrast, purified MBP could be measured at concentrations as low as 2 ng/ml. MBP activity averaged 454 ng/ml in normal sera, and it was markedly elevated in the sera of certain patients with eosinophilia (*vide infra*, Table V).

TABLE I
Stability of MBP in Serum

Sample	Times tested	MBP	Coefficient of variation
		ng/ml	%
Internal control*			
High MBP serum	45*	6,748±1,521	23
Low MBP serum*	45*	380±89	23
Freeze-thaw six times			
High MBP serum	7‡	1,739±158	9
Purified MBP	7	19,442±2,292	12

* Internal controls were tested each time that an RIA was performed. Aliquots of a single high MBP serum and a single low MBP serum were stored over an 18-mo period at −20°C, and after reduction and alkylation they were assayed independently in 45 different assays. The possibility that the serum MBP levels changed during the 18-mo period was tested by regression analysis. The correlation coefficients for the relationship between time and level of MBP in the high and low sera, respectively, were +0.12 (NS) and −0.64 ($P < 0.001$). The mean±SD for the first 10 analyses of the lower MBP serum was 476±82, whereas the mean for the last 10 analyses was 320±79.

‡ A high MBP serum (different from that used as an internal control) was repeatedly freeze-thawed in dry ice-acetone within a 30-min period, and aliquots were removed for testing after each freeze-thaw cycle. The purified, reduced, and alkylated MBP standard was treated similarly. All samples, including that before freeze-thawing, for a total of seven, were tested in a single RIA.

Molecular properties of MBP activity

Sera with elevated MBP activity. To determine whether MBP activity in sera had the expected molecular weight of monomer MBP, 9,300, we analyzed sera with elevated MBP activities by passing them over a column of Sephadex G-50. All of the MBP activity, as measured by RIA, emerged in the void volume, suggesting that MBP was either bound to a larger carrier molecule or existed as a polymer. Because polymerized MBP can be rendered monomeric by reduction and

alkylation (7), we measured MBP activity in sera after reduction and alkylation to determine whether immunoreactivity was altered. The results in Table II show that levels of MBP in reduced and alkylated serum increased 8- to 10-fold. By testing two sera from normal subjects and six sera from patients with high levels of MBP activity, we established that concentrations of DTT between 5 and 10 mM, a reaction time of 1–2 h, a temperature of 22°C, and a pH of 8.0 were optimal for treating serum prior to assay by RIA. Using these conditions, we tested whether MBP activity could be detected by gel filtration in the molecular weight range expected for the monomeric form (7, 8). The reduced and alkylated sera were passed over a Sephadex G-50 column (1.2 × 45 cm), equilibrated with phosphate-buffered saline-0.15 M NaCl, pH 7.4, and 1-ml fractions tested for MBP activity by RIA. Under these conditions, all of the MBP activity remained associated with the void volume protein peak. However, if the serum was reduced and alkylated and then acidified to pH 2 by addition of 2 N HCl before gel filtration at pH 2.0, up to 80% of the MBP activity eluted from the column as monomer. Fig. 2 shows the results of a representative experiment where most of the serum MBP activity emerged from the column at the elution volume of radioiodinated MBP used as a marker. The peak MBP activity also coincided with the expected position for its molecular weight, 9,300, as determined by the protein calibration markers. The monomeric MBP emerging from the Sephadex G-50 column produced inhibition curves in the RIA which were indistinguishable from that of the reduced and alkylated MBP standard. We found that similar results

TABLE II
Effect of Reduction and Alkylation on MBP Activity in Sera

Serum	DTT concentration	MBP activity
	mM	*ng/ml*
Patient	0	1,010
	1	1,560
	10	8,280
	40*	2,520
Normal	0	30
	1	36
	10	309
	40	70

Samples of serum containing 20 mg/ml protein measured by biuret were adjusted to pH 8 with Tris; DTT was added and the solution was incubated at room temperature for 1 h. A twofold molar excess of iodoacetamide (over that of DTT) was added and reacted for 20 min at room temperature in the dark. The reduced and alkylated serum was diluted in PPF buffer for the RIA.

* Precipitation occurred during the incubation with DTT.

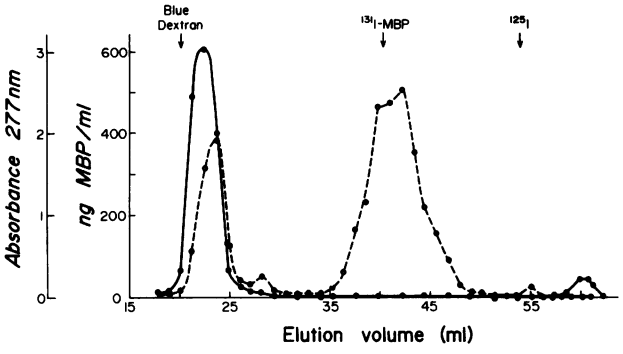

FIGURE 2 Separation of monomeric MBP from other serum proteins. Serum was reduced, alkylated, and acidified as described in Methods and then passed over a column of Sephadex G-50 equilibrated with 0.01 M HCl–0.15 M NaCl. The absorbance at 277 nm (●) and the MBP activity (○) were determined for each of the 1-ml fractions. Blue dextran and radiolabeled MBP eluted at the positions shown by the arrows.

were produced with each of the several patient sera tested. However, reduction and alkylation, as well as acidification to pH 2, were required to separate monomeric MBP from other serum proteins, and the amount of MBP separable from the void volume peak was highly sensitive to experimental conditions. An alteration in DTT concentration or failure to lower the pH to 2 resulted in lower amounts of total MBP activity present in the monomeric form. Acidification alone did not result in the release of monomeric MBP into the serum, in that all of the MBP activity eluted in the void volume. Finally, even when reduced, alkylated, and radiolabeled MBP is added to serum and passed over the Sephadex G-50 column at pH 6.6, 78% of the MBP activity elutes in the void volume peak and only 22% in the expected position for the MBP monomer.

The experiments described above established that sera must be reduced and alkylated before RIA to reveal their maximal MBP content. To determine the efficiency of this procedure, known amounts of reduced MBP were added to sera and the recoveries determined in two experiments. The results in Table III highlight the need for reduction and alkylation of sera for quantitative recovery of MBP. For example, without reduction and alkylation 2% or less of the added MBP was recovered. In contrast, 71% was recovered from normal human serum in experiment 1 and 97±4% (Mean ±SEM) in experiment 2. Also, as shown in experiment 2, when increasing amounts of MBP were added to serum, there was a dose-related recovery. Finally, when reduced and alkylated MBP was added to serum, recovery was essentially quantitative; in this experiment, sera were analyzed without reduction and alkylation (results not shown).

To determine the molecular weight of MBP activity in blood, untreated serum samples were fractionated on a Sephadex G-200 column; the fractions were then

TABLE III

Recovery of Known Amounts of MBP Added to Sera

Serum	No addition of MBP		MBP added	
	Not reduced and alkylated	Reduced and alkylated	Not reduced and alkylated	Reduced and alkylated
Experiment 1*				
Normal human				
1	12	255	84 (1)‡	7,128 (71)
Experiment 2§				
Normal human				
1	16	282	128 (1)	9,080 (88)
2	17	214	108 (1)	11,520 (113)
3	23	264	122 (1)	8,360 (81)
4	17	252	121 (1)	11,560 (113)
5	24	190	133 (1)	10,280 (101)
6	17	208	76 (1)	9,880 (97)
Normal human				
1	16	282	—	—
Plus 2,500 ng MBP	—	—	59 (2)	2,280 (80)
Plus 5,000 ng MBP	—	—	85 (1)	4,560 (86)
Plus 10,000 ng MBP	—	—	132 (1)	11,360 (111)
Plus 20,000 ng MBP	—	—	200 (1)	20,480 (101)

* For experiment 1, MBP was prepared from human eosinophil granules as described in Methods and stored at −20°C. Stored protein was reduced with DTT, a 60-fold molar excess for available MBP sulfhydryl groups, as described in Methods, and gel-filtered over Sephadex G-50 at pH 4.3. Reduced MBP, 9,700 ng, was added to sera and incubated at 37°C for 4 h.

‡ Percent recovery is in parentheses.

§ Experiment 2 differs from 1 in that MBP was freshly isolated and thus not reduced with DTT prior to addition to sera; 10,000 ng was added to sera 1 through 6. In both experiments the quantity of MBP added was based on absorbance at 280 nm using $E^{1\%}_{1\,cm} = 26.3$.

reduced and alkylated and assayed for MBP activity. As shown in Fig. 3, the MBP activity was associated with molecules having a molecular weight > 50,000 and 46% of the MBP activity was found in the IgG (7S) peak. Analysis of another serum on Sephadex G-200 revealed that MBP was found in fractions containing IgG and IgA proteins, but not in the albumin fractions (results not shown). These experiments revealed that MBP activity in blood was associated with macromolecules that have molecular weights > 50,000, but they did not provide insight into whether the MBP was simply polymerized, or if it were bound to a larger carrier. If MBP were present in polymerized form, presumably it would be extremely basic. Therefore, we analyzed serum containing an elevated level of MBP by electrofocusing. Peaks of MBP activity were found in fractions with pI values of 5.4–5.8 and 6.3–7.0; total recovery of MBP activity was 53%. This result discourages belief that the bulk of MBP exists as a simple polymer, and when taken together with the results in Fig. 3, suggests that MBP is bound to larger carrier molecules.

Sera with normal MBP activity. The experiments

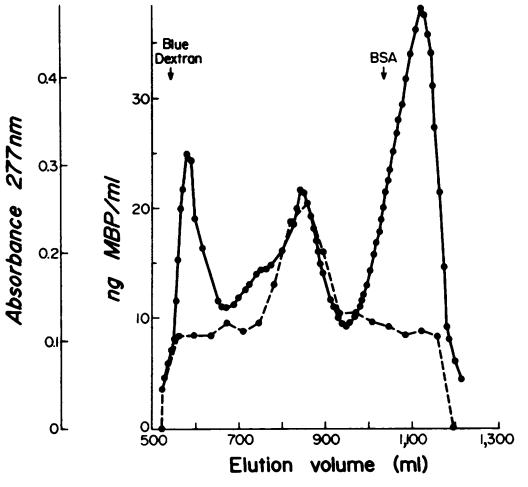

FIGURE 3 MBP activity in serum fractions from Sephadex G-200. Serum was passed over a column of Sephadex G-200 and fractions were analyzed for absorbance at 277 nm (●) and after reduction and alkylation for MBP activity by RIA (○). Blue dextran and bovine serum albumin (BSA) eluted at the positions shown by the arrows. The 7S immunoglobulin peak eluted from this column at 850 ml.

described above indicate that in sera from patients with eosinophilia, monomer MBP activity can be demonstrated by gel filtration after reduction, alkylation, and acidification. As shown in Table II, MBP activity found in normal serum also increased after reduction and alkylation; the MBP activity in normal sera produced inhibition curves identical in shape to those shown in Fig. 4. However, numerous attempts to demonstrate monomer MBP in normal sera by reduction, alkylation, and acidification and separation on Sephadex G-50 were unsuccessful. This finding raised the question of whether or not the MBP activity in normal serum represented specific MBP or resulted from nonspecific inhibition due to interference from other proteins in the RIA. To help answer this question, we tested whether sera from other species or high concentrations of serum proteins would produce inhibition in the RIA after reduction and alkylation. We found little or no inhibition in these samples, and if MBP activity was present, levels never exceeded 30 ng/ml. Additionally, we analyzed sera before and after heating. The results in Table IV indicate that the activity of the purified, reduced, and alkylated MBP standard is destroyed by heating, in keeping with similar results for guinea pig MBP (20). In serum with elevated MBP activities, about half of this activity is lost, provided that the serum is heated after reduction and alkylation; if patient serum is heated before reduction and alkylation, MBP activity is unaltered. In normal serum, MBP activity is unchanged by heating regardless of whether it is heated before or after reduction and alkylation. Therefore, although it appears that normal individuals show low levels of circulating MBP in their blood, we cannot eliminate the possibility that the levels of activity measured in such samples might be due to nonspecific factors.

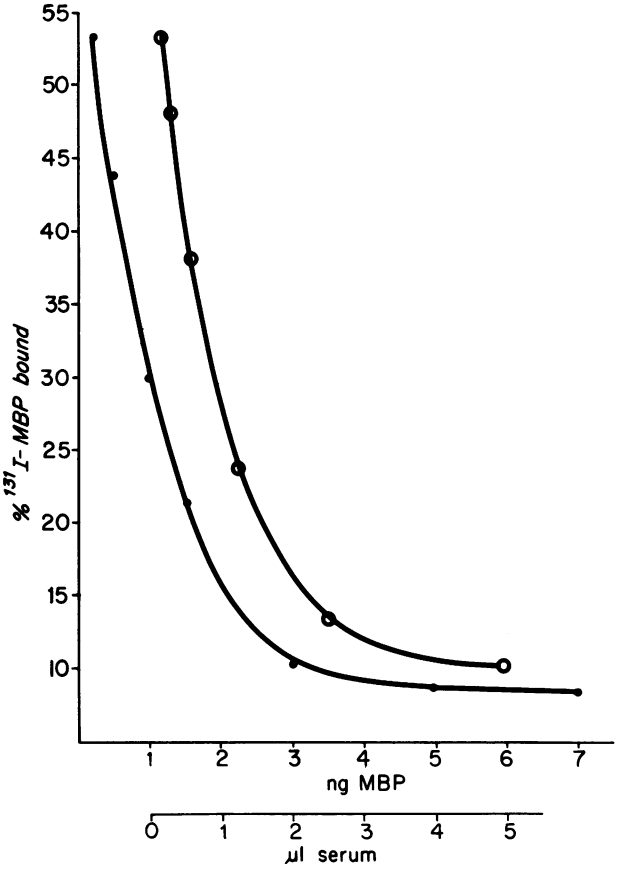

FIGURE 4 Inhibition curves in the RIA of MBP standard and serum after reduction and alkylation. MBP activity was measured in reduced and alkylated human serum (●) and compared with the inhibition curve given by reduced and alkylated MBP standard (○). The human serum was obtained from a patient with the hypereosinophilic syndrome and was reduced and alkylated before assay as described in Methods. Statistical analysis of the logit-log transformed data revealed that the null hypothesis of a common slope could not be rejected ($F_{1,9} = 1.844$; NS).

MBP levels in patient and normal control sera

Because reduction and alkylation are necessary for optimal recovery of MBP activity both in normal and patient serum, we reduced and alkylated sera before analysis in the RIA. The shapes of inhibition curves given by serum MBP and by the MBP standard are essentially identical, as shown in Fig. 4. The coefficient of variation for the RIA is 23% and the high MBP serum showed no decline in activity. However, the low MBP serum sample showed a significant loss of activity with time ($P < 0.001$) (Table I). Repetitive freeze-thawing of patient sera resulted in no decline of MBP activity.

Serum levels of MBP activity and total eosinophil counts were performed on samples from 105 healthy

TABLE IV
Effect of Heating on MBP Activity in Sera

Test substance	Treatment	MBP
		ng/ml
MBP (1 µg)	None	1,000
	Δ56° × 1 h	0
	Δ56° × 2 h	0
Patient serum	None	480
	R & A*	4,472
	R & A, then Δ56° × 2 h	2,382
	Δ56° × 2 h, then R & A	4,730
Normal serum	None	82
	R & A	244
	R & A, then Δ56° × 3 h	252
	Δ56° × 3 h, then R & A	285

* Patient and normal sera were reduced and alkylated (R & A) as described in Methods, and samples were analyzed for MBP activity in the RIA. Δ, heating.

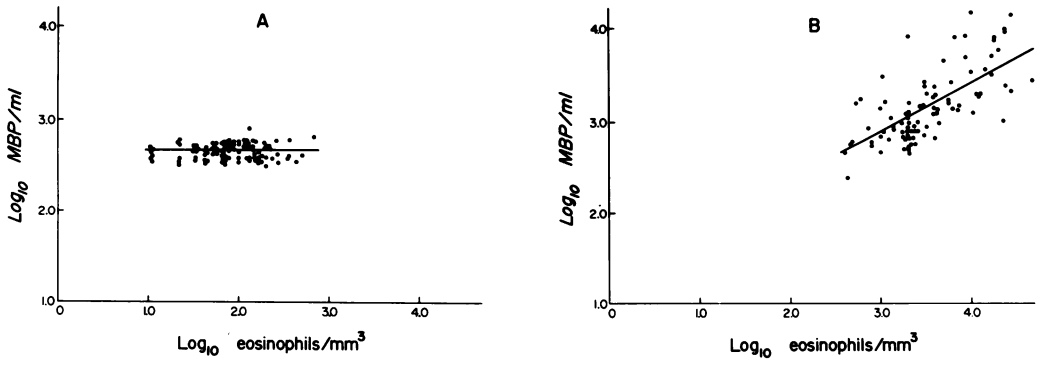

FIGURE 5 Relationship between peripheral blood eosinophilia and serum MBP activity. Eosinophil counts and serum MBP activity were determined in (A) normal subjects and (B) patients with diseases characterized by eosinophilia; the data were compared by linear regression analysis. All sera were reduced and alkylated before testing as described in Methods. Normal subjects (A) showed no correlation between eosinophil counts and MBP activity ($r = 0.058$), whereas patients with eosinophilia (B) showed a highly significant correlation between these two variables ($P < 0.001$, $r = 0.71$).

blood bank volunteers and compared with values obtained from 95 patients with increased peripheral blood eosinophil counts. The results of the analysis of these sera are shown in Fig. 5. When values for eosinophil counts are plotted on the ordinate, and nanograms MBP per milliliter on the abscissa, no correlation is seen when samples from healthy subjects are analyzed (Fig. 5A). However, a significant correlation exists between eosinophil counts and serum MBP levels in patients with eosinophilia (Fig. 5B). Values of serum MBP activity in normal patients ranged from 320 to 800 ng MBP/ml (mean, 454 ng MBP/ml), whereas values from patients with eosinophilia were usually elevated and ranged as high as 14,400 ng MBP/ml (Table V). Thus, patients with elevated levels of blood eosinophils tend to have parallel elevations of MBP. Normal values for eosinophil counts (<350/mm^3) and serum MBP levels (<600 ng/ml) are based on the values obtained from a sample of 105 such controls; 95% of these normal individuals had values below figures given.

Serum levels of MBP and peripheral blood eosinophil counts for 188 patients and 105 normal controls are summarized in Table V. Patients were grouped by diagnosis into categories based on major organ involvement or generalized disease process. Although the categories chosen are somewhat arbitrary, examination of the data in this form permits comparison of the relationships among major disease categories. Great variation exists among patients' eosinophil counts and levels of serum MBP, even when they share a common diagnosis. For example, patients with asthma showed eosinophil counts ranging from 0 to 13,000/mm^3 and MBP activities ranging from normal (<600 ng/ml) to 2,560 ng MBP/ml serum. The highest eosinophil counts and highest levels of serum MBP were seen in patients with the hypereosinophilic syndrome (21). These patients averaged almost 5,000 ng MBP/ml serum with levels as high as 14,400 ng/ml. Several patients with hypereosinophilic syndrome were receiving steroid therapy and their disease was under control. These patients showed slightly elevated eosinophil counts and normal or only slightly elevated levels of serum MBP. In addition, normal levels of MBP activity were found in renal transplant patients receiving immunosuppressive drugs, including prednisone, whose bloods were devoid of eosinophils.

Remarkably, a significant proportion of patients with skin disease showed elevated levels of MBP in the absence of peripheral blood eosinophilia. Of 16 patients with chronic urticaria, none showed eosinophil counts > 280/mm^3, yet 6 of these patients (38%) showed elevated levels of MBP (>600 ng/ml). Of all 88 patients with skin disease tested, 20 (22%) showed normal eosinophil counts but elevated levels of MBP. This contrasts sharply with patients in all other groups, where, of the remaining 100 patients tested, only 1 patient with asthma showed this relationship. Although a strong correlation exists between peripheral blood eosinophilia and elevated serum MBP, 13% of the 188 patients tested showed normal concentrations of serum MBP in spite of elevated eosinophil counts.

DISCUSSION

Measurement of a basic protein such as the eosinophil MBP presents a variety of problems due to the tendency of the protein to bind to surfaces and to other molecules. Additionally, MBP possesses two sulfhydryl groups, which must be stabilized by alkylation before the purified molecule can be stored. In experiments with guinea pig MBP, we found that the addition of protamine and FCS to the assay buffer resulted in

TABLE V
Levels of Eosinophil MBP in Patient Sera

Disease or major organ involvement	Number patients	Eosinophil level	MBP level	Percent patients*			
				A	B	C	D
		eosinophils/mm³	*ng MBP/ml*				
Normal controls‡	105	115±107 (11–678)	454±90 (312–800)	94	3	1	2
Lung							
Asthma	23	1,967±2,593 (0–13,000)	951±589 (424–2,560)	13	22	4	61
Other§	13	4,265±6,806 (90–23,100)	1,192±851 (496–3,584)	0	23	0	77
Total	36	2,797±4,622 (0–23,100)	1,038±693 (424–3,584)	8	22	3	67
Skin							
Chronic urticaria	16	119±80 (0–280)	607±189 (320–960)	62	0	38	0
Atopic dermatitis	16	709±1,540 (0–6,370)	687±299 (312–1,360)	37	12	13	38
Psoriasis	10	624±735 (50–2,200)	572±163 (376–880)	60	0	0	40
Other‖	46	3,289±5,877 (50–24,070)	1,155±1,166 (368–5,680)	24	11	26	39
Total	88	1,914±4,467 (0–24,000)	865±849 (312–5,680)	37	10	22	31
Heart							
Rheumatic disease-rheumatoid arthritis	7	7,426±7,261 (200–16,800)	1,742±1,633 (459–4,864)	29	0	0	71
Other¶	5	4,026±4,403 (80–8,800)	2,819±3,566 (727–8,192)	20	40	0	40
Total	12	6,009±6,235 (80–16,800)	2,191±2,527 (272–8,192)	25	17	0	58
Hypereosinophilic syndrome	14	12,563±13,421 (790–48,400)	4,973±4,746 (496–14,400)	0	14	0	86
Gastrointestinal**	10	1,974±1,088 (400–4,000)	1,239±566 (456–2,278)	0	10	0	90
Malignancies‡‡	13	5,732±7,245 (160–27,300)	1,638±1,928 (240–7,680)	23	8	0	69
Miscellaneous§§	15	1,539±1,776 (20–7,070)	845±466 (239–1,971)	20	13	0	67
Total, all patients‖‖	188	—	—	24	13	11	52

* A, eosinophil count < 350/mm³, MBP < 600 ng/ml (normal); B, eosinophil count > 350/mm³, MBP < 600 ng/ml (elevated eos, normal MBP); C, eosinophil count < 350/mm³, MBP > 600 ng/ml (normal eos, elevated MBP); D, eosinophil count > 350/mm³, MBP > 600 ng/ml (elevated eos, elevated MBP).

‡ Normal subjects donating blood to Mayo Clinic Blood Bank.

§ Includes eosinophilic pneumonia, chronic obstructive pulmonary disease, Loeffler's pneumonitis, idiopathic pulmonary fibrosis, and Churg-Strauss syndrome.

greatly reduced nonspecific binding and a sensitive inhibition curve (20); these conditions are also important in the RIA for human MBP. The MBP RIA is rather variable, as shown by the coefficient of variation of 23% in Table I. In the case of the low MBP serum standard, some of the variability is due to loss of MBP activity, which dropped significantly after serum storage for 18 mo. In the high MBP serum standard the values did not change with time. As shown in Table I, freeze-thawed samples assayed in the same experiment had coefficients of variation of 9–12%. The difference between the intraassay coefficient of variation of 9–12% (using the results from the freeze-thaw experiment) and the interassay value of 23% presumably is due to the reduction and alkylation procedure, which must be employed to recover MBP activity (as shown by the results in Table III).

In the guinea pig we found that normal sera and sera from animals infected with *T. spiralis* contained <5 ng/ml MBP. In humans, both normal sera and sera from patients with eosinophilia contain considerably higher levels of MBP activity. Whether the MBP activity in normal human serum represents specific MBP is ambiguous; however, reduction and alkylation increased the MBP activity in normal sera and these inhibition curves parallel that of the MBP standard. Furthermore, if the inhibition produced by normal serum were nonspecific and due to competition between antibody and serum protein for radioiodinated MBP, one would expect the sera of other species such as guinea pig and newborn calf to give comparable inhibition. Contrary to this prediction, these controls produced little inhibition. Thus, the present results support but do not prove that MBP is present in normal sera.

In sera from patients with eosinophilia, MBP is bound to serum proteins in the 4S, 7S, and 19S peaks from Sephadex G-200 gel filtration. To release MBP from these carrier proteins, serum must be reduced and alkylated and acidified with HCl to a pH of 2. Under these conditions, as much as 80% of the MBP activity can be isolated from the major serum proteins by passage over Sephadex G-50 at pH 2.0. Gel filtration and preparative electrofocusing experiments indicate that the bulk of the MBP in serum is not a simple polymer of the molecule itself, but rather is bound to a larger carrier. Because reduction and alkylation are required to release the MBP, presumably it is covalently bound to carrier protein molecules. The experiments testing the recovery of reduced MBP from serum highlight the need for reduction and alkylation prior to assay (Table III).

Serum levels of MBP show no correlation with eosinophil counts in normal subjects, but show a significant positive correlation in patients with eosinophilia. Patients who had received renal transplants and who were treated with glucocorticoids and immunosuppressive drugs showed no detectable eosinophils in the peripheral blood, yet still possessed MBP activity in the serum. The marked increase in MBP activity present in serum from patients with eosinophilia is specific, as we have separated MBP from such samples. Eosinophil counts from the 188 patients in our sample (exclusive of normal controls) varied widely and ranged from 0 to 48,400/mm^3, whereas serum levels of MBP ranged from 239 to 14,400 ng/ml. Patients with normal eosinophil counts (<350/mm^3) generally had levels of serum MBP in the normal range (<600 ng/ml), and patients with peripheral blood eosinophilia generally showed parallel increases in serum MBP. For example, 80% of all patients with eosinophilia (>350/mm^3) showed MBP levels in excess of 600 ng/ml. Of the patients with normal eosinophil counts, 68% showed levels of MBP in the normal range; of the remaining 32% of these patients, who showed increased MBP levels in spite of normal eosinophil levels, all but one individual were represented by patients with vari-

‖ Includes neurodermatitis, recurrent angioedema, eczema, dermal reticulosis, bullous pemphigoid, systemic vasculitis, erythema multiformis, subcutaneous nodules with eosinophilia, scleroderma, dermatitis (chronic, contact, etc.), alopecia areata, urticaria pigmentosa, verruca vulgaris, dermographism, cholinergic urticaria, seborrheic dermatitis, lichenified chronic dermatitis, systemic mastrocytosis, mycosis fungoides, prurigo nodularis, necrotizing vasculitis, eosinophilic vasculitis.

¶ Includes systemic lupus erythematosus, Raynaud's disease, synovitis, undifferentiated connective tissue disorders.

** Includes eosinophilic gastroenteritis, chronic ulcerative colitis, colecystitis, allergic gastroenteropathy, and chronic acute liver disease.

‡‡ Includes cancer of bladder, reticulum cell sarcoma, cancer of rectum or colon (see gastrointestinal also), Hodgkin's disease, early lymphoproliferative disease, basal cell carcinoma, lymphatic lymphoma, leukemia, acute leukemia, and basophilic leukemia.

§§ Includes endolymphatic hyperplasia, methicillin nephritis, methicillin allergy, Paget's disease, necrotizing glomerulonephritis, chronic rhinitis, otosclerosis, hyperparathyroidism, acromegaly, transient eosinophilia, eosinophilic hepatitis, myositis with eosinophilia, central and peripheral neuropathy, polyneuropathy with eosinophilia, and indeterminant neurological disorder.

‖‖ All patients tested, excluding normal controls.

ous forms of skin disease. This tendency for patients with skin disease to show elevated levels of MBP in the absence of peripheral blood eosinophilia was unexpected, and the reason for the MBP elevation is obscure. MBP could be derived from eosinophils in the bone marrow, circulating in the peripheral blood, or in the peripheral tissues. If blood eosinophils degranulate, then one would expect a lower content in them, but we do not know whether patients in the various disease categories differ in the content of MBP within their blood eosinophils. Thus the source of the serum MBP is not known and requires further investigation.

Researchers in Sweden (22–25) have also described eosinophil cationic proteins (ECP) and have measured levels of these proteins in the serum of normal subjects and in patients with asthma. The ECP described by Olsson et al. (22, 23) is about twofold larger in molecular weight than the MBP and differs from the MBP in amino acid composition. They estimate that the ECP accounts for ~30% of the granule protein (23). In contrast, the MBP has been localized to the crystalloid core of the granule of the guinea pig and accounts for >50% of the granule protein (6, 10). Although it seems unlikely that the two proteins are structurally related, their relationship has not been defined. Venge et al. (24, 25) measured levels of ECP in the serum of healthy individuals and found a direct correlation between the eosinophil count and the serum levels of ECP. They further found unexpectedly low levels of ECP in serum from patients with asthma. These findings contrast with those described in our studies, where no correlation was found of eosinophil counts with serum MBP in healthy subjects and elevated levels of MBP in the serum of most asthmatics with eosinophilia. The conditions used by Venge and his colleagues (24) to isolate ECP from serum were not effective in our laboratory when applied to isolation of MBP from serum.

Monomer MBP is cytotoxic to parasites (11, 15) and mammalian cells (11, 16, 17), and the findings of elevated levels of serum MBP indicate that eosinophils are releasing granule contents into the circulation. The highest concentrations of serum MBP were found in patients with the hypereosinophilic syndrome. If eosinophils degranulate in the vicinity of the endothelium of blood vessels, it is possible that certain of the cardiovascular manifestations of the hypereosinophilic syndrome (26) could be due to damage to tissue by the release of eosinophil granule constituents.

ACKNOWLEDGMENTS

We thank Ms. Gail M. Olson and Ms. Laurie Lindor for assistance in these experiments, Dr. K. G. Mann for a gift of the platelet factor IV, and Dr. Sylvester Sterioff for providing us with sera from the renal transplant patients.

This work was supported by grants from the National Institutes of Health, AI 15231, AI 9728, and AI 07047, and from the Mayo Foundation.

REFERENCES

1. Beeson, P. B., and D. A. Bass. 1977. The eosinophil. *In* Major Problems in Internal Medicine. W. B. Saunders Co., Philadelphia, Pa. Volume XIV.
2. Ottesen, E. A., and S. G. Cohen. 1978. The eosinophil, eosinophilia, and eosinophil-related disorders. *In* Allergy, Principles and Practice. E. Middleton, Jr., C. E. Reed, and E. F. Ellis, editors. C. V. Mosby Co., St. Louis, Mo. 584–632.
3. Mahmoud, A. A. F., K. S. Warren, and P. A. Peters. 1975. A role for the eosinophil in acquired resistance to *Schistosoma mansoni* infection as determined by antieosinophil serum. *J. Exp. Med.* **142:** 805–813.
4. Grove, D. I., A. A. F. Mahmoud, and K. S. Warren. 1977. Eosinophils and resistance to *Trichinella spiralis*. *J. Exp. Med.* **145:** 755–759.
5. Gleich, G. J., G. M. Olson, and H. Herlich. 1979. The effect of antiserum to eosinophils on susceptibility and acquired immunity of the guinea pig to *Trichostrongylus colubriformis*. *Immunology.* **37:** 873–880.
6. Gleich, G. J., D. A. Loegering, and J. E. Maldonado. 1973. Identification of a major basic protein in guinea pig eosinophil granules. *J. Exp. Med.* **137:** 1459–1471.
7. Gleich, G. J., D. A. Loegering, F. Kueppers, S. P. Bajaj, and K. G. Mann. 1974. Physiochemical and biological properties of the major basic protein from guinea pig eosinophil granules. *J. Exp. Med.* **140:** 313–332.
8. Gleich, G. J., D. A. Loegering, K. G. Mann, and J. E. Maldonado. 1976. Comparative properties of the Charcot-Leyden crystal protein and the major basic protein from human eosinophils. *J. Clin. Invest.* **57:** 633–640.
9. Archer, G. T., and J. G. Hirsch. 1963. Isolation of granules from eosinophil leukocytes and study of their enzyme content. *J. Exp. Med.* **118:** 277–286.
10. Lewis, D. M., J. C. Lewis, D. A. Loegering, and G. J. Gleich. 1978. Localization of the guinea pig eosinophil major basic protein to the core of the granule. *J. Cell Biol.* **77:** 702–713.
11. Butterworth, A. E., D. L. Wassom, G. J. Gleich, D. A. Loegering, and J. R. David. 1979. Damage to schistosomula of *Schistosoma mansoni* induced directly by eosinophil major basic protein. *J. Immunol.* **122:** 221–229.
12. Butterworth, A. E., M. A. Vadas, D. L. Wassom, A. Dessein, M. Hogan, B. Sherry, G. J. Gleich, and J. R. David. 1979. Interactions between human eosinophils and schistosomula of *Schistosoma mansoni*. II. The mechanism of irreversible eosinophil adherence. *J. Exp. Med.* **150:** 1456–1471.
13. McLaren, D. J., C. D. Mackenzie, and F. J. Ramalho-Pinto. 1977. Ultrastructural observations on the in vitro interaction between rat eosinophils and some parasitic helminths (*Schistosoma mansoni, Trichinella spiralis* and *Nippostrongylus brasiliensis*). *Clin. Exp. Immunol.* **30:** 105–118.
14. Perrudet-Badoux, A., A. Anteunis, S. M. Dumitrescu, and R. A. Binaghi. 1978. Ultrastructural study of the immune interaction between peritoneal cells and larvae of *Trichinella spiralis*. *J. Reticulendothel. Soc.* **24:** 311–314.
15. Wassom, D. L., and G. J. Gleich. 1979. Damage to *Trichinella spiralis* newborn larvae by eosinophil major basic protein. *Am. J. Trop. Med. Hyg.* **28:** 860–863.

16. Gleich, G. J., E. Frigas, D. A. Loegering, D. L. Wassom, and D. Steinmuller. 1979. Cytotoxic properties of the eosinophil major basic protein. *J. Immunol.* **123:** 2925–2927.
17. Frigas, E., D. A. Loegering, and G. J. Gleich. 1980. Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium. *Lab. Invest.* **42:** 35–43.
18. Parrillo, J. E., and A. S. Fauci. 1978. Human eosinophils, purification, and cytotoxic capability of eosinophils from patients with the hypereosinophilic syndrome. *Blood.* **51:** 457–473.
19. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29:** 185–189.
20. Wassom, D. L., D. A. Loegering, and G. J. Gleich. 1979. Measurement of guinea pig eosinophil major basic protein by radioimmunoassay. *Mol. Immunol.* **16:** 711–719.
21. Chusid, M. J., D. C. Dale, B. C. West, and S. M. Wolff. 1975. The hypereosinophilic syndrome: analysis of fourteen cases with review of the literature. *Medicine (Baltimore).* **54:** 1–27.
22. Olsson, I., and P. Venge. 1974. Cationic proteins of human granulocytes. II. Separation of cationic proteins of the granules of leukemic myeloid cells. *Blood.* **44:** 235–246.
23. Olsson, I., P. Venge, J. K. Spitznagel, and R. I. Lehrer. 1977. Arginine-rich cationic proteins of human eosinophil granules. Comparison of the constituents of eosinophilic and neutrophilic leukocytes. *Lab. Invest.* **36:** 493–500.
24. Venge, P., L. E. Roxin, and I. Olsson. 1977. Radioimmunoassay of human eosinophil cationic protein. *Br. J. Haemat.* **37:** 331–335.
25. Venge, P., O. Zettastrom, R. Dahl, L. E. Roxin, and I. Olsson. 1977. Low levels of eosinophil cationic proteins in patients with asthma. *Lancet.* **II:** 373–375.
26. Parrillo, J. E., J. S. Borer, W. L. Henry, S. M. Wolff, A. S. Fauci. 1979. The cardiovascular manifestations of the hypereosinophilic syndrome: prospective study of 26 patients with review of the literature. *Am. J. Med.* **67:** 572–582.