LOCALIZATION OF THE GUINEA PIG EOSINOPHIL MAJOR BASIC PROTEIN TO THE CORE OF THE GRANULE

DANIEL M. LEWIS, JON C. LEWIS, DAVID A. LOEGERING, and GERALD J. GLEICH

From the Departments of Immunology, Internal Medicine, Pathology and Anatomy, Mayo Clinic and Mayo Foundation, and Mayo Medical School, Rochester, Minnesota 55901. Dr. D. M. Lewis' present address is the Department of Otolaryngology, Ohio State University Hospitals, Columbus, Ohio 43210, and Dr. J. C. Lewis' present address is the Department of Pathology, Bowman-Gray School of Medicine, Winston-Salem, North Carolina 27103.

ABSTRACT

The localization of the guinea pig eosinophil major basic protein (MBP) within the cell was investigated by the use of immunoelectron microscopy and by isolation of the granule crystalloids. First, by immunoperoxidase electron microscopy, we found that the MBP of eosinophil granules is contained within the crystalloid core of the granule. Specific staining of cores was present when rabbit antiserum to MBP was used as the first stage antibody in a double antibody staining procedure, whereas staining was not seen when normal rabbit serum was used as the first stage antibody. Second, crystalloids were isolated from eosinophil granules by disruption in 0.1% Triton X-100 and centrifugation through a cushion of 50% sucrose. Highly purified core preparations yielded essentially a single band when analyzed by electrophoresis on polyacrylamide gels containing 1% sodium dodecyl sulfate (SDS). The $E^{1\%}_{1cm}$ of the core protein was 26.8 ± 1.0 ($\bar{X}$ ± SEM); the $E^{1\%}_{1cm}$ for the MBP was 26.3. The core protein could not be distinguished from the MBP by radioimmunoassay (RIA) and essentially all of the protein in the core preparations could be accounted for as MBP. The results indicate that the MBP is contained in the core of the guinea pig eosinophil granule and that it is probably the only protein present in the core.

KEY WORDS eosinophilia · basic protein · eosinophil granule · crystalloid · guinea pig · localization

When examined under the electron microscope, the granules of mature eosinophils possess a characteristic morphology consisting of an electron-dense crystalloid core surrounded by a less dense matrix (21). Numerous proteins have been isolated from eosinophil granules (1, 9), and some of these have been localized by histochemical techniques. For example, eosinophil peroxidase and acid phosphatase are located in the matrix of the primary granule (5, 7, 29), and in human eosinophils arylsulfatase and acid phosphatase are found chiefly in small granules (4, 26). The nature of the material contained within the core of the primary granule has not been identified although several authors have postulated that the Charcot-Leyden crystal could originate from the core of human eosinophils (6, 30).

 J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/78/0601-0702$1.00

We have isolated a major basis protein (MBP)[1] from eosinophil granules of guinea pigs (12, 13), humans (14), and rats (20), and in the guinea pig this material comprises over 50% of the protein content of the granule. The MBP of the guinea pig has a mol wt of 11,000, a high arginine content (13%), and readily aggregates through oxidation of its two free sulfhydryl groups. We have produced an antiserum in rabbits to guinea pig MBP and have characterized the reactivity of this antiserum by quantitative microcomplement fixation (19). In this report, we present results obtained by immunoelectron microscopy and by analyses of isolated cores which indicate that the MBP is contained in the core of the eosinophil granule and that it is probably the only protein present in the core.

MATERIALS AND METHODS

Materials

Horseradish peroxidase (HRP) (type II), *o*-dianisidine, diaminobenzidine, crystalline human serum albumin, protamine sulfate, and bovine serum albumin fraction V were obtained from Sigma Chemical Co. (St. Louis, Mo.). Hydrogen peroxide, acetic acid, and sucrose were obtained from Fisher Scientific Co. (Pittsburgh, Pa.). Triton X-100 and sodium dodecyl sulfate (SDS) were purchased from Schwarz-Mann (Orangeburg, N. J.). Sepharose 2B, DEAE-Sephadex, and Sephadex G-50 were obtained from Pharmacia Fine Chemicals Inc. (Piscataway, N. J.).

Antiserum to MBP

The production and characterization of the antiserum to guinea pig MBP have been described elsewhere (19). Normal rabbit sera (NRS) used in the immunoelectron microscopy experiments were preimmunization bleedings from the same rabbits injected with MBP. These NRS lacked antibody to MBP as judged by complement fixation assay (19).

Preparation of the HRP-Anti-Rabbit IgG Conjugate

Purified rabbit immunoglobulin G (IgG) used for immunization and as an immunoabsorbent was prepared by mixing equal volumes of NRS (Pelfreeze Inc., Rogers, Ariz.) with 28% Na_2SO_4. The resulting precipitate was washed twice with 14% Na_2SO_4, dissolved in 0.01 M K_2HPO_4-KH_2PO_4, pH 7.4, dialyzed, and chromatographed on a DEAE-Sephadex column equilibrated with the phosphate buffer. The protein fraction which did not absorb to the column was concentrated by ultrafiltration to 12 mg/ml. Immunoelectrophoretic analysis using a potent goat anti-rabbit whole serum and goat anti-rabbit IgG revealed only a single band in the slow gamma region. The rabbit IgG was stored at −20°C until used for either immunization or immunoabsorption procedures.

Antiserum to rabbit IgG was produced in a burro by subcutaneous injection of 1.0 mg of rabbit IgG emulsified in Freund's complete adjuvant. A second injection of 2.0 mg of rabbit IgG in complete adjuvant was given subcutaneously 2 wk after the first injection. 3 wk later, the burro was serially bled and the serum was collected, pooled, and stored at −20°C.

For preparation of Fab-peroxidase conjugates, the procedure suggested by Kraehenbuhl and Jamieson (18) was modified as follows: Burro anti-rabbit IgG was fractionated by mixing serum with an equal volume of 28% Na_2SO_4. The resulting precipitate was washed twice with 14% Na_2SO_4, dissolved in 0.01 M K_2HPO_4-KH_2PO_4, 0.13 M NaCl, pH 7.5 (phosphate-buffered saline [PBS]), and dialyzed against PBS. Burro gamma globulin was digested with papain according to the method of Porter (28). Briefly, 1 mg of papain was added to 100 mg of burro gamma globulin (assumed $E^{1\%}_{1cm}$ = 14.5) in PBS containing 0.01 M cysteine and 0.002 M EDTA and incubated overnight at 37°C. Fab fragments possessing antibody activity to rabbit IgG were isolated by passing the digest over an immunoabsorbent column of purified rabbit IgG coupled to Sepharose 2B by a modification of the method of Axen et al. (3, 32). The column was eluted with 0.05 M glycine-HCl, pH 2.8, and the purified Fab fraction was pooled. The purified Fab fragments were conjugated with HRP by glutaraldehyde (2) at a ratio of 2 mg of HRP/mg Fab protein. The HRP was dissolved in the Fab pool, and 25% glutaraldehyde was added slowly with constant stirring to a final concentration of 0.45%. The mixture was allowed to react for 2 h at 23°C. After dialysis to remove glutaraldehyde, the conjugate was passed over the rabbit IgG immunoabsorption column a second time to remove free HRP and Fab fragments which had lost antibody activity during the conjugation procedure. The immune Fab-HRP conjugate was eluted with 0.05 M glycine HCl, pH 2.8; the pH was adjusted with twice-concentrated PBS; and the conjugate was stored at −70°C. To demonstrate that the Fab-HRP conjugate possessed antibody activity, dilutions of the conjugate were mixed with an equal volume of a 1/20 dilution of normal rabbit serum and incubated for 1 h at room temperature. The mixture, presumably containing complexes of rabbit IgG and the univalent Fab-HRP

[1] *Abbreviations used in this paper:* HRP, horseradish peroxidase; MBP, major basic protein; NRS, normal rabbit serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Phos-BSA-P, phosphate buffer containing bovine serum albumin and protamine sulfate; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate.

conjugate, was tested by Ouchterlony analysis using goat anti-rabbit IgG to precipitate the rabbit IgG-Fab HRP complexes. The resulting precipitin lines were stained for peroxidase with a saturated solution of diaminobenzidine in a pH 7.5 Tris-HCl buffer containing 0.05% H_2O_2. The Fab-HRP conjugate had an antibody titer of 1/16 by this method. Peroxidase activity associated with the Fab-HRP was measured by the rate of decomposition of hydrogen peroxide with *o*-dianisidine as the hydrogen donor, and the conjugate possessed 2.57 U/mg protein of enzyme activity (31). By comparison of the absorbance at 277 and 400 nm of fresh HRP to the HRP Fab conjugate, we estimated the molar ratio of HRP to Fab protein to be 1:7 (assuming $E^{1\%}_{1cm}$ = 15 for the Fab protein). The protein concentration of the conjugate was 1.6 mg/ml.

Localization of the MBP by Immunoelectron Microscopy

Eosinophils were obtained from guinea pigs by peritoneal lavage with saline, and granules were prepared as previously described (11, 13). The lavage fluids containing ~45% eosinophils or the purified eosinophil granules were fixed in 4% formalin in 0.1 M KH_2PO_4-Na_2HPO_4, pH 7.4, for 2 or 4 h at 23°C. After fixation, the suspensions were centrifuged and the pellets were suspended in 0.15 M NaCl containing 10% acetic acid and incubated overnight at 23°C. The samples were washed thrice with PBS, divided into two aliquots, and exposed to either NRS or rabbit anti-MBP, each diluted 1/10, for 1 h at 23°C. After three additional washes with PBS, the samples were incubated with undiluted Fab HRP conjugate for 1 h at room temperature. The samples were washed twice with PBS, twice with 0.1 M Tris-HCl, pH 7.4, and stained for peroxidase using saturated diaminobenzidine in Tris-HCl containing 0.5% H_2O_2 for 4 min (15). The samples were then washed twice with Tris-HCl, and postfixed with 2% osmium tetroxide. After dehydration though graded alcohols, they were embedded in Epon 812. Thin sections were examined in a Hitachi HU-12 electron microscope (Hitachi American Ltd., Indianapolis, Ind.) without further staining. This study was conducted as a single blind study in that the electron microscope examination was performed on coded samples and the code was not broken until all specimens of an experiment were examined and the results recorded.

Isolation of Cores from Eosinophil Granules

Eosinophil granules were prepared from guinea pig eosinophils essentially as described previously (12, 13). Briefly, peritoneal cells were subjected to hypotonic lysis by suspension in 0.046 M sodium chloride for 1 min, after which the osmolality was reconstituted by addition of 10-fold concentrated PBS. After centrifugation at 400 *g* for 5 min, the sedimented cells were suspended in ~5 ml of 0.25 M sucrose and washed twice. The cells were resuspended in 0.25 M sucrose and disrupted in a Tenbroeck tissue grinder (Kontes Glass Co., Evanston, Ill.) by 10 passes of the pestle. The disrupted cells were centrifuged at 400 *g* for 5 min and the supernate was placed in a Tenbroeck tissue grinder, and 10% Triton X-100 was added to achieve a final concentration of 0.1%. The granules were disrupted by 10 passes of the pestle and the suspension was centrifuged at 400 *g* for 20 min. The supernate was layered over a cushion of 50% sucrose and centrifuged at 20,000 *g* for 30 min in a Beckman Instruments model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) equipped with an SW 50.1 rotor. The supernatant solution above the sucrose layer, the material at the interface, and the sucrose layer were aspirated. The tube was gently rinsed with 0.25 M sucrose, and the pellet was suspended in 0.4 ml of 0.25 M sucrose. Portions of the pellet suspension as well as the layers above were fixed in 3% glutaraldehyde in 0.1 M KH_2PO_4-Na_2HPO_4, pH 7.4, postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate before examination with the electron microscope.

Physicochemical Analysis of Solubilized Core Proteins

Eosinophil cores were dissolved in 0.01 M HCl, 0.15 M NaCl, and centrifuged at 20,000 *g* for 10 min. The resulting solutions were analyzed for their content of peroxidase by the rate of decomposition of hydrogen peroxide with *o*-dianisidine as hydrogen donor (13, 31), for the molecular weights and heterogeneity of proteins by electrophoresis in polyacrylamide gels containing 1% SDS (SDS-PAGE) (8), for the dye staining associated with the MBP band by scanning of SDS-PAGE gels stained with Coomassie brilliant blue in a Beckman model 25 spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.), for their protein content by a scaled down biuret procedure (16), for their absorbance at 277 nm using a Gilford model 252 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio), and for their content of MBP by radioimmunoassay (RIA) as described below. In the RIA experiments, either freshly prepared core proteins were compared to freshly prepared MBP or alkylated core proteins were compared to alkylated MPB. Although no difference was found between the reactivity of freshly isolated MBP and that of alkylated MBP in the RIA, nonalkylated MBP readily polymerizes (12), and we found that stored nonalkylated MBP which had polymerized was a less potent inhibitor in the RIA than alkylated MBP. Therefore, comparisons of solubilized core proteins to MBP were always performed either using nonalkylated preparations freshly prepared on the day of the RIA experiment or using alkylated preparations. MBP or core protein was alkylated at pH 8 by treatment with iodoacetamide, 1.7 μM, in the presence of 0.002 M ethyl-

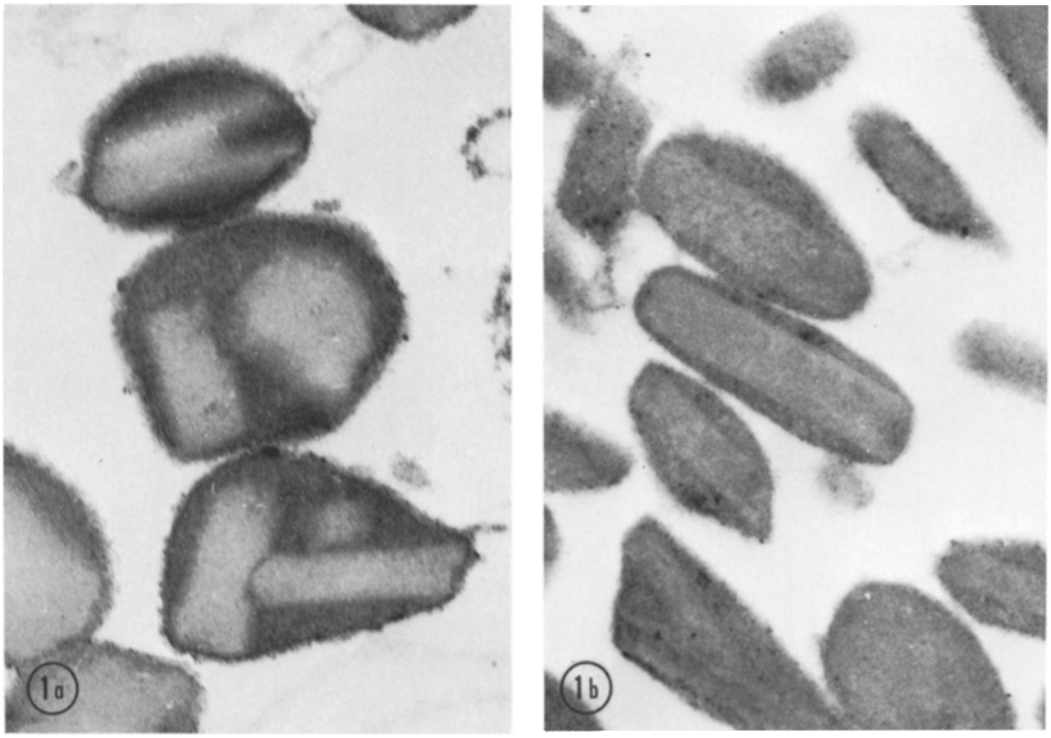

FIGURE 1 Purified eosinophil granules stained for peroxidase activity with diaminobenzidine. (*a*) (left) Granules fixed in formalin show peroxidase activity in the matrix portion of the granule, (*b*) (right) while granules that had been fixed in formalin and exposed to 10% acetic acid show a marked reduction in peroxidase activity. × 46,500.

enediamine tetraacetate in the dark for 20 min at 20°C (17).

Radioimmunoassay for MBP

This procedure will be described in detail in a subsequent separate report.[2] Briefly, alkylated MBP was radioiodinated with ^{131}I by a modification of the procedure described by McConahey and Dixon (23). After completion of the iodination reaction, a solution of 0.1 M K_2HPO_4-KH_2PO_4, pH 7.4, containing 1% bovine serum albumin and 0.1% protamine sulfate (Phos-BSA-P), was added to the vial, and the contents were transferred to a 3,500 dalton cut-off dialysis casing (Spectrum Medical Industries, Inc., Los Angeles, Calif.) and dialyzed overnight. The specific activity of the ^{131}I-MBP averaged 33 $\mu Ci/\mu g$, and ~95% of counts was precipitated by 10% tungstic acid. The RIA was performed by additions of 0.1 ml of a 1:6,000 dilution of rabbit anti-MBP in Phos-BSA-P buffer, 0.3 ml of Phos-BSA-P buffer, and 0.1 ml of inhibitor to a 10 × 75 glass tube. After incubation for 30 min at 37°C and for 15 min at 4°C, ~2 ng of ^{131}I-MBP was added to each tube and the solution was incubated at 4°C overnight. The resulting immune complexes were precipitated by addition of 0.1 ml of a 1:20 dilution of normal rabbit serum in 0.1 M K_2HPO_4-KH_2PO_4 containing 1% bovine serum albumin and 0.1 ml of burro anti-rabbit IgG. All solutions except the burro anti-IgG contained 0.1% sodium azide. The tubes were mixed, incubated at room temperature for 2 h, and centrifuged for 20 min at 4°C and 2,500 *g*. The supernates were decanted and the sediment was suspended in 0.8 ml of Phos-BSA-P buffer and transferred to a fresh tube. After centrifugation and decantation of the supernate, the precipitates were counted in Nuclear-Chicago gamma scintillation counter (G. D. Searle & Co., Des Plaines, Ill).

Statistical Analyses

The results of analysis of MBP and core protein by RIA were tested by logit-log transformation of the inhibition curves and by comparison of the slopes of the resulting regression lines using analysis of covariance with the aid of a programmable Hewlett-Packard

[2] Wassom, D. L., D. A. Loegering, and G. J. Gleich. Measurement of the guinea pig eosinophil major basic protein by radioimmunoassay. Manuscript in preparation.

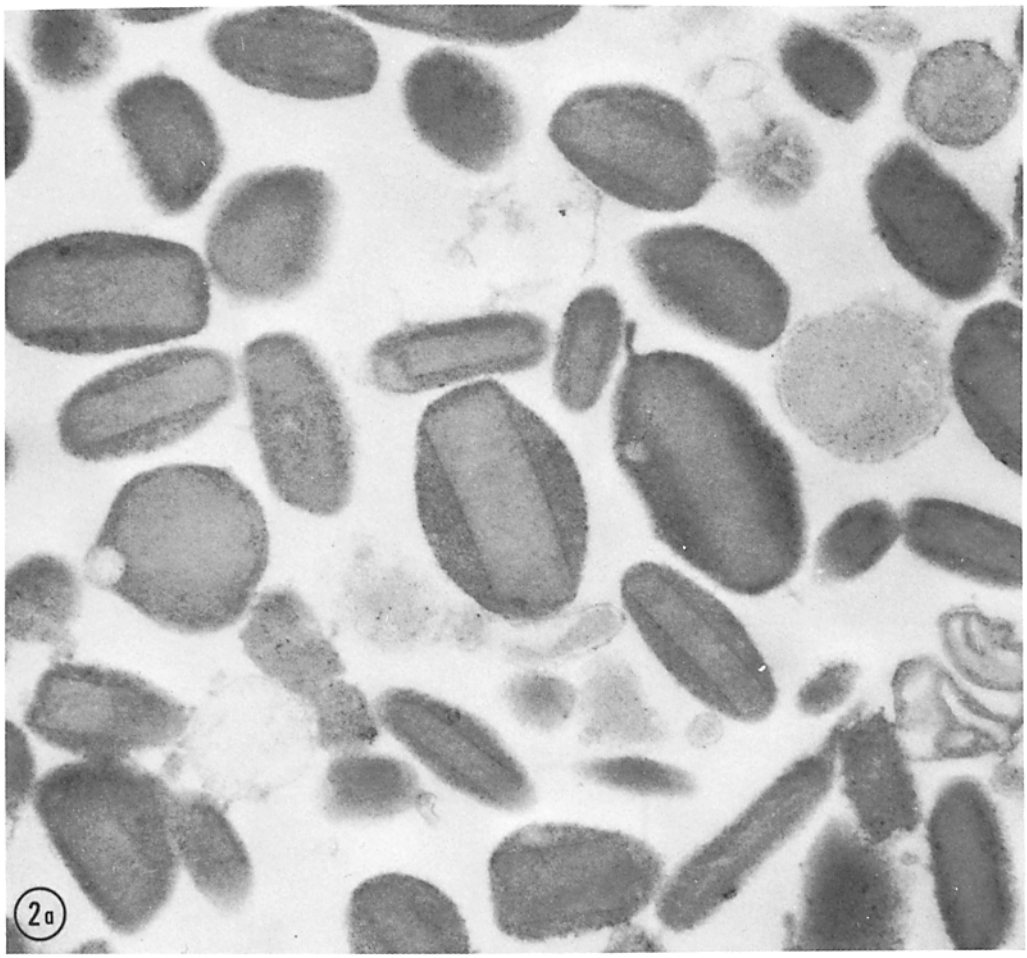

FIGURE 2*a* Purified eosinophil granules after immunoperoxidase staining. On the left are granules that had been exposed to NRS as the first-stage antibody. × 28,000.

9810 A calculator (Hewlett-Packard Co., Palo Alto, Calif.).

RESULTS

Localization of MBP by Immunoelectron Microscopy

In initial experiments, we attempted to localize the MBP within the eosinophil by immunoelectron microscopy. However, before immunoperoxidase staining could be attempted, it was necessary to devise a fixation procedure which would reduce the native peroxidase activity while maintaining cellular architecture and protein antigenicity. Earlier studies had suggested that, at low pH, eosinophil peroxidase activity was labile while MBP was stable (13). Therefore, we exposed formalin-fixed cells to various concentrations of acetic acid and monitored their peroxidase activity by staining with diaminobenzidine and by examination under the light microscope. The peroxidase activity of the cells was diminished by 3 h of incubation in 5% acetic acid in 0.15 M NaCl, while overnight incubation in 10% acetic acid almost totally abolished the peroxidase activity. In control experiments, we found that MBP was not extracted from formalin-fixed granules by acetic acid as judged by analyses using SDS-PAGE and that its antigenicity was not reduced by exposure to 10% acetic acid as measured by RIA. Fig. 1 shows the comparison of purified granules stained for peroxidase with and without the acetic acid treatment. In the untreated granules, peroxidase activity is apparent in the matrix portion of the granule, but after acetic acid treatment this peroxidase activity is

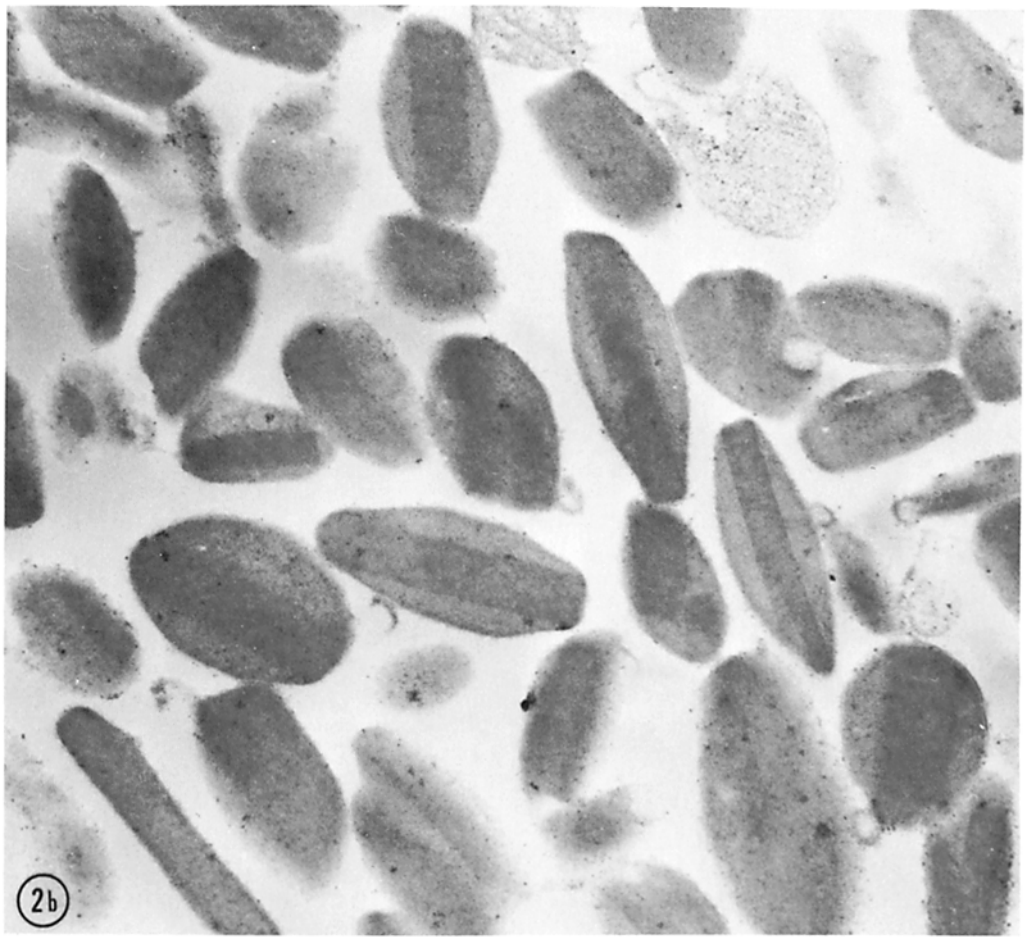

FIGURE 2*b* On the right, anti-MBP was used as the first-stage antibody. Note the deposition of peroxidase reaction product in the core of the granules on the right. × 28,000.

virtually abolished.

To localize the MBP, isolated granules and intact eosinophils were fixed in formalin, treated with 10% acetic acid, and examined by the immunoperoxidase staining procedure. In initial experiments with whole cells, reaction product was present over the granule core and also at the interface between the core and the matrix; no deposition of peroxidase reaction product was seen in other areas of the eosinophil cytoplasm or in other cell types that were present. As shown in Fig. 2, electron microscope examination of isolated granules revealed that the electron-dense peroxidase reaction product was deposited throughout the crystalline core of samples which had been exposed to anti-MBP as the first-stage antibody. In contrast, granules exposed to NRS as the first-stage antibody showed a slight deposition of peroxidase reaction product within the matrix of the granule. Controls included granules treated with NRS as the first-stage antibody, granules not exposed to either antibody, and granules that were exposed to Fab-HRP and not washed before reaction with diaminobenzidine and H_2O_2. In the latter control, the peroxidase reaction product did not nonspecifically adhere to the cores, strengthening our conclusion that the staining of the core protein with anti-MBP was specific.

To obtain a less subjective interpretation of the results, the negatives of the electron micrographs were examined in a spot densitometer (Macbeth TD504 Densitometer, Macbeth Co., Newburgh, N. Y.). The optical density of the matrix portion and the core portion of a number of granules was determined and the relative

TABLE I

Comparison of the Optical Densities of the Matrix and Core of Granules after Immunoperoxidase Staining

	Optical Density		
	Core	Matrix	Difference§
NRS (12)*	0.926 ± 0.047‡	0.853 ± 0.036	±0.073 ± 0.033
Anti-MBP (10)	0.831 ± 0.017	0.886 ± 0.02	−0.055 ± 0.015

The optical densities were performed on photographic negatives so that areas of high electron density show lower values than do areas of low electron density.

* No. of granules examined.

‡ Mean ± SD.

§ $P < 0.001$ by t test.

difference in density between the two portions of the granule was calculated. These results are summarized in Table I and indicate that the core of granules stained with anti-MBP is more dense relative to the matrix, while in the case of granules stained with the NRS control the core is less dense than the matrix ($P < 0.001$).

In another series of experiments, we measured the peroxidase activity in isolated granules after exposure to antibody and Fab HRP. For these experiments, the granules were carried through the staining protocol up to the reaction step with diaminobenzidine, at which point *o*-dianisidine was added as a hydrogen donor instead of diaminobenzidine. Because of the low level of enzyme activity, the reaction was allowed to proceed for 1 h at room temperature. The granule preparations were solubilized by the addition of SDS, final concentration 0.1%, and after centrifugation to remove particulate matter, the absorbance of the supernates at 460 nm was determined. In two experiments, granules which had been exposed to anti-MBP as the first-stage antibody showed approximately three times as much enzyme activity as granules exposed to NRS.

Isolation of Granule Cores

The experiments employing immunoelectron microscropy were consistent with the view that the MBP is localized in the core of the granules. To obtain further support for this conclusion and to determine whether the MBP was the only protein present in the core, we attempted to purify cores from eosinophil granules. In initial experiments, we used the approach taken by Gessner et al. (10) in which purified eosinophils are disrupted in 0.25 M sucrose containing 0.1% Triton X-100 and the cores are obtained by sedimentation through a cushion of 50% sucrose. After centrifugation, the pellet and supernate layers were analyzed by electron microscopy, by SDS-PAGE, and for peroxidase activity. The results indicated that the supernatant material was free of cores and contained 98% of the peroxidase activity present in the starting preparation, and little MBP was found by SDS-PAGE. In contrast, the pellets contained many cores, were devoid of peroxidase activity, and were enriched in MBP as judged by SDS-PAGE. However, the pellets always contained organelles other than crystalloids, and the electrophoretic analyses showed numerous bands in addition to the MBP. Therefore, we attempted to purify cores using isolated eosinophil granules as the starting material. Fig. 3*a* shows a preparation of cores obtained by centrifugation of granules after treatment with 0.1% Triton X-100 in 0.25 M sucrose. Numerous blunt rod-shaped bodies were recovered which resembled granule crystalloids; in many areas, these bodies are surrounded by granular debris. In Fig. 3*b*, the core preparation is shown at higher magnification, and it appears that much of the granular debris might be derived from the cores. In particular, one core has rounded edges and is surrounded by clumps of irregular material. These experiments were repeated on several occasions, with essentially the same results.

Physicochemical and Immunochemical Analyses of Core Proteins

Cores were dissolved in 0.1 M HCl, 0.15 M NaCl and analyzed for their content of MBP. In the first experiment, samples from the isolation of cores shown in Fig. 3 were analyzed by SDS-PAGE. As shown in Fig. 4, a band in the expected position for the MBP is present in all

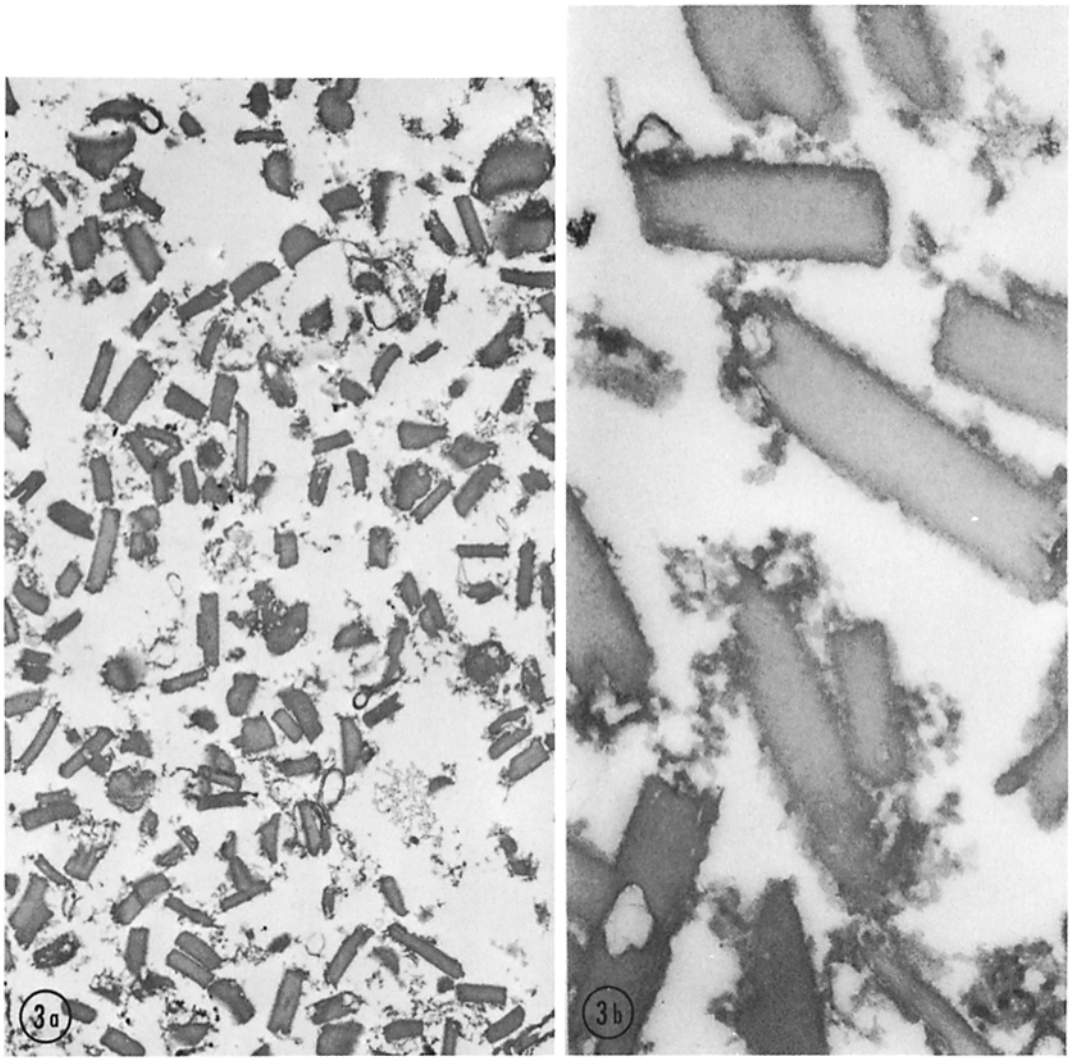

FIGURE 3 Electron micrographs of eosinophil granule cores. After centrifugation of disrupted granules through a cushion of 50% sucrose, the pellets were fixed with glutaraldehyde and osmium tetroxide and sections were stained with lead citrate and uranyl acetate. (*a*) Portion of a field showing numerous roughly rectangular bodies resembling eosinophil cores. × 10,120. (*b*) Higher magnification of eosinophil cores showing some with irregular edges associated with irregular material around these sites. × 51,415.

samples, and it is essentially the only band in the sample of the pellet. This experiment was repeated several times, and in every case the MBP was the principal band. Quantitative scanning of SDS-PAGE gels loaded with 5–20 μg of core protein revealed that between 79 and 91% (83.7 $\pm$ 5.1; $\bar{X} \pm$ SD) of the dye binding was associated with the band in the position of the MBP.

To determine whether the protein derived from the core preparations was the MBP, a double antibody RIA was established to identify and quantitate the MBP. Utilizing this procedure, we compared the inhibition produced by solubilized core protein to that produced by the MBP. Alkylated MBP was used as the standard, and its inhibitory ability was compared to that of alkylated core protein. As shown in Fig. 5, the inhibiton curves produced by these materials were superimposable, and statistical analysis of the logit-log regressions did not reveal a differ-

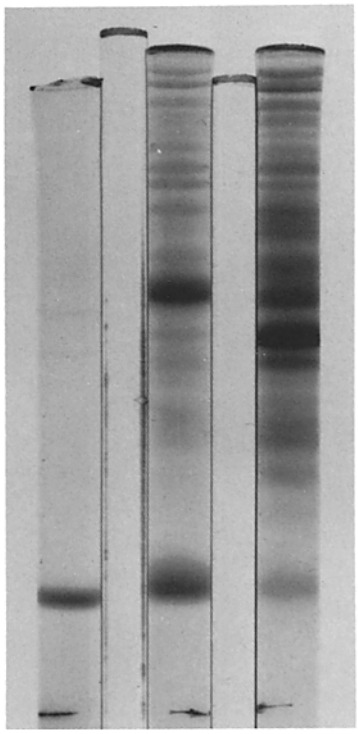

FIGURE 4 Analysis of eosinophil cores by SDS-PAGE. Eosinophil granules were disrupted by homogenization in 0.25 M sucrose containing 0.1% Triton X-100, and cores were purified by centrifugation through a layer of 50% sucrose. The pellet (left), interface (middle), and supernate (right) were analyzed by SDS-PAGE. These preparations were derived from the experiment shown in Fig. 3.

ence. Thus, the core protein could not be distinguished immunologically from the MBP, and essentially all of the protein in the alkylated core preparation could be accounted for as MBP. Furthermore, alkylated core protein radiolabeled with ^{131}I was reactive with antibody to the MBP, and the binding of the alkylated core protein was inhibited equally well by the MBP and the core protein (results not shown). In another series of experiments, MBP and core protein were freshly prepared and immediately analyzed by RIA. The results of a typical experiment are shown in Fig. 6. The inhibition curves produced by MBP and core protein were virtually identical, and statistical analysis of the logit-log regressions did not reveal a difference. Again, essentially all of the protein in the solubilized core preparations could be accounted for as MBP.

Finally, if the core protein is the MBP, then the extinction coefficient at 277 nm of core protein should be similar to that of the MBP. The extinction coefficient, $E^{1\%}_{1cm}$, of the MBP is 26.3 by biuret analysis using human serum albumin as a standard (12). Using the same analytical conditions in eight experiments, the extinction coefficient of the core protein was 26.8 ± 1.0 ($\bar{X}$ ± SEM).

DISCUSSION

Miller and his associates observed that the core of the eosinophil granule is a crystal which has a cubic lattice with a repeat of ~30 Å in rodents and ~40 Å in man (24). On the basis of their observations as well as the observation by Cotran and Litt that the granule core does not possess peroxidase activity (5), we hypothesized that the MBP was derived from the granule core (13). The results presented here support this hypothesis. In experiments employing rabbit antibody to MBP, we found that more antibody activity was associated with granules exposed to antibody than granules exposed to NRS. This result is expected, in that our prior work had shown that the MBP was derived from the eosinophil granule (12, 13). Evidence for the localization of the MBP within the granule was provided by immunoelectron microscopy. No deposition of peroxidase reaction product was seen in other organelles in the eosinophil cytoplasm or in other cells, and as shown in Fig. 2, antibody to MBP was localized to the core of the granule. The conclusion that the staining of the core protein was specific is further supported by the absence of staining with the NRS control and the failure of the peroxidase reaction product to nonspecifically localize in the core when excess Fab-HRP was not washed away before the diaminobenzidine reaction step.

In another approach to the localization of the MBP, we isolated cores from granules and determined the characteristics of the proteins derived from the core. Early experiments with whole eosinophils indicated that eosinophil cores were concentrated in the pellet after centrifugation through a cushion of 50% sucrose and that the interface layer did not contain crystalloids. However, in these early experiments, the cores were contaminated by considerable debris, and analysis of the proteins by SDS-PAGE revealed the presence of numerous proteins in addition to the MBP. Therefore, we used granules as the starting material for the preparation of cores and were

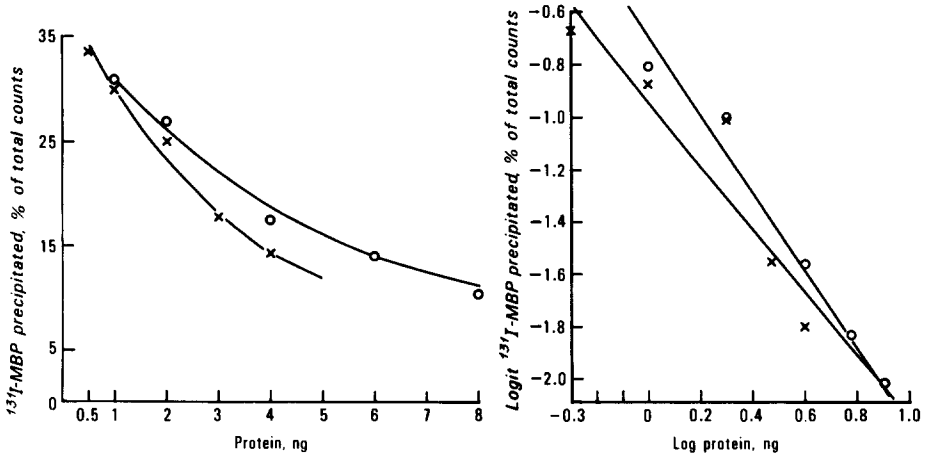

FIGURE 5 Comparison of alkylated MBP with alkylated core protein by RIA. Rabbit anti-MBP was reacted with ^{131}I-MBP and the inhibitory potencies of alkylated MBP and alkylated core protein were tested. In the absence of inhibitor, 36% of the counts bound to antibody, whereas in the absence of antibody 4.8% of counts were precipitated. The concentration of MBP was determined by absorbance at 277 nm, and the concentration of core protein was determined by biuret analysis using human serum albumin as a standard. The results with the MBP are shown by O and with the core protein by x. On the left the binding curves are shown, and on the right the logit-log regressions are shown. The correlation coefficients for the MBP inhibition regression line was −0.98 and for the core protein −0.96. Comparison of the logit-log regression lines revealed that the null hypothesis of a common line could not be rejected ($F_{2,6}$ = 1.46; NS).

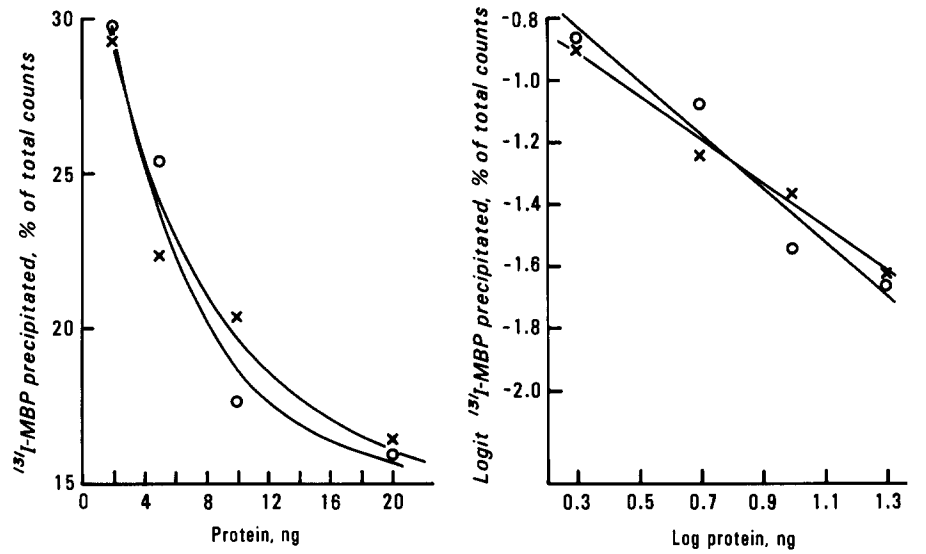

FIGURE 6 Comparison of freshly prepared MBP with freshly prepared core protein by RIA. The ability of MBP and core protein, both freshly prepared, to inhibit the binding of ^{131}I-MBP to antibody was tested. In the absence of inhibitor, 33% of counts bound to antibody, whereas in the absence of antibody, 7.4% of counts were precipitated. The concentrations of MBP and core protein were determined by absorbance at 277 nm and by biuret analysis, respectively. On the left the binding curves are shown, and on the right, the logit-log regressions. The results with the MBP are shown by O and with the core protein by x. The correlation coefficients for the logit-log regressions of MBP and core protein were −0.97 and −0.99, respectively. Analysis of these regression lines revealed that the null hypothesis of a common line could not be rejected ($F_{2,4}$ = 0.61; NS).

able to obtain highly purified preparations as shown in Fig. 3. Even the apparent debris among the crystalloids may be core protein, as inspection of Fig. 3*b* indicates that this irregular material is found around the edges of cores which are losing their characteristic shape. Numerous analyses of the core protein by SDS-PAGE showed a band in the expected position for the MBP, and this accounted for up to 90% of the dye binding in the gel. More conclusive evidence that the crystalloid protein is the MBP was provided by the RIA experiments in which we could not show a difference between the inhibitory properties of the core protein and the MBP. Moreover, we could account for virtually all of the protein in the solubilized core as MBP. The results of the two sets of experiments employing immunoelectron microscopy and analysis of isolated crystalloids indicate that the MBP is contained within the core of the eosinophil granule, and furthermore, that it appears to be the only protein in the crystalloid.

Our results do not exclude the possibility suggested by certain authors (6, 30) that the Charcot-Leyden crystal protein is contained in the human eosinophil granule core. Although human MBP and Charcot-Leyden crystal protein differ in their molecular weights and amino acid compositions and are distinct proteins (14), it is possible that the human and guinea pig eosinophil crystalloids differ. Therefore, if the Charcot-Leyden crystal protein is derived from the core of the human eosinophil granule, then it presumably coexists there with the MBP.

Finally, Okun et al. (25) reported that the crystalloid core of human eosinophil granules contained melanin. Their conclusion was based on results obtained with histochemical staining techniques, and in particular with the Fontana-Masson argentaffin stain. Because we have not seen pigment in any protein preparation made from purified granules, we do not believe that melanin is present in guinea pig eosinophil granules. Moreover, MacRae and Spitznagel (22) have presented evidence that the ammoniacal silver reaction, which is the basis of the Fontana-Mason argentaffin stain (27), can be used as a cytochemical marker for the detection of cationic proteins by electron microscopy. Thus, the observations of Okun and his associates might be viewed by supportive evidence for the presence of the cationic MBP in the granule core.

We thank Dr. Harold L. Moses and Dr. Robert E. Scott for helpful discussions of these results. Dr. Jorge E. Maldonado participated in some of the early experiments to purify cores from disrupted eosinophils.

This work was supported by grants from the National Institute of Allergy and Infectious Diseases, A1 9728 and A1 11483, and from the Mayo Foundation.

Received for publication 20 September 1976, and in revised form 24 January 1978.

REFERENCES

1. ARCHER, G. T., and J. G. HIRSCH. 1963. Isolation of granules from eosinophil leucocytes and study of their enzyme content. *J. Exp. Med.* **118:**277-286.
2. AVRAMEAS, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugate for the detection of antigens and antibodies. *Immunochemistry*. **6:**43-52.
3. AXEN, R., J. PORATH, and S. ERNBACK. 1967. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature (Lond.)*. **214:**1302-1304.
4. BAINTON, D. F., and M. G. FARQUHAR. 1970. Segregation and packaging of granule enzymes in eosinophilic leukocytes. *J. Cell. Biol.* **45:**54-73.
5. COTRAN, R. S., and M. LITT. 1969. The entry of granule-associated peroxidase into phagocytic vacuoles of eosinophils. *J. Exp. Med.* **129:**1291-1306.
6. EL-HASHIMI, W. 1971. Charcot-Leyden crystals. Formation from primate and lack of formation from non-primate eosinophils. *Am. J. Path.* **65:**311.
7. ENOMOTO, T., and T. KITANI. 1966. Electron microscopic studies on peroxidase and acid phosphatase reaction in human leukocytes. *Acta Haematol. Japn.* **29:**554-570.
8. FAIRBANKS, G., T. L. STECK, and D. F. H. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10:**2606-2617.
9. GESSNER, T. P., S. R. HIMMELHOCH, and E. SHELTON. 1973. Partial characterization of the protein components of eosinophil granules isolated from guinea pig exudates. *Arch. Biochem. Biophys.* **156:**383-389.
10. GESSNER, T. P., E. SHELTON, and S. R. HIMMELHOCH. 1972. Liberation of crystalloid from eosinophil granules. *Fed. Proc.* **31:**253 (Abstr.).
11. GLEICH, G. J., and D. A. LOEGERING. 1973. Selective stimulation and purification of eosinophils and neutrophils from guinea pig peritoneal fluids. *J. Lab. Clin. Med.* **82:**522-528.
12. GLEICH, G. J., D. A. LOEGERING, F. KUEPPERS, S. P. BAJAJ, and K. G. MANN. 1974. Physi-

ochemical and biological properties of the major basic protein from guinea pig eosinophil granules. *J. Exp. Med.* **140:**313–332.

13. 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.
14. 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.
15. GRAHAM, R. C., JR., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14:**291–302.
16. KABAT, E. A., and M. N. MAYER. 1961. Estimation of protein with the biuret and ninhydrin reactions. *In* Experimental Immunochemistry. Charles C Thomas, Publisher, Springfield, Ill. 2nd edition. 559.
17. KONIGSBERG, W. 1972. Reduction of disulfide bonds in proteins with dithiothreitol. *Methods Enzymol.* **25:**185–188.
18. KRAEHENBUHL, J. P., and J. D. JAMIESON. 1974. Localization of intracellular antigens by immunoelectron microscopy. *Int. Rev. Exp. Path.* **13:**1–53.
19. LEWIS, D. M., D. A. LOEGERING, and G. J. GLEICH. 1976. Antiserum to the major basic protein of eosinophil granules. *Immunochemistry.* **13:**743–746.
20. LEWIS, D. M., D. A. LOEGERING, and G. J. GLEICH. 1976. Isolation and partial characterization of a major basic protein from rat eosinophil granules. *Proc. Soc. Exp. Biol. Med.* **152:**512–515.
21. LOW, F. N., and J. A. FREEMAN. 1958. Electron Microscopic Atlas of Normal and Leukemic Human Blood. McGraw-Hill Book Co., New York.
22. MACRAE, E. K., and J. K. SPITZNAGEL. 1975. Ultrastructural localization of cationic proteins in cytoplasmic granules of chicken and rabbit polymorphonuclear leukocytes. *J. Cell Sci.* **17:**79–94.
23. 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.
24. MILLER, F., E. DEHARVEN, and G. E. PALADE. 1966. The structure of the eosinophil leukocyte granules in rodents and in man. *J. Cell Biol.* **31:**349–362.
25. OKUN, M. R., B. DONNELLAN, S. H. PERASON, and L. M. EDELSTEIN. 1974. Melanin: A normal component of human eosinophils. *Lab. Invest.* **30:**681–685.
26. PARMLEY, R. T., and S. S. SPICER. 1974. Cytochemical and ultrastructural identification of a small type granule in human late eosinophils. *Lab. Invest.* **30:**557–567.
27. PEARSE, A. G. E. 1972. Histochemistry, Theoretical and Applied. Vol 2. Williams & Wilkins Co., Baltimore. 3rd edition. 1485.
28. PORTER, R. R. 1959. The hydrolysis of rabbit gamma globulin and antibodies with crystalline papain. *Biochem. J.* **73:**119–126.
29. SEEMAN, P. M., and G. E. PALADE. 1967. Acid phosphatase localization in rabbit eosinophils. *J. Cell Biol.* **34:**745–756.
30. WELSH, R. A. 1959. The genesis of the Charcot-Leyden crystal in the eosinophilic leukocyte of man. *Am. J. Pathol.* **35:**1091–1104.
31. WORTHINGTON, Enzyme manual. 1972. Worthington Biochemical Corporation, Freehold, N. J. 43.
32. YUNGINGER, J. W., and G. J. GLEICH. 1972. Comparison of the protein binding capacities of cyanogen bromide-activated polysaccharides. *J. Allergy Clin. Immunol.* **50:**109–116.