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Isolation and Partial Characterization of a Major Basic Protein from Rat Eosinophil Granules[1] (39429)

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The eosinophil major basic protein (MBP) is a low molecular weight, highly basic material which constitutes over 50% of the protein content of the eosinophil granule (1). MBP has been isolated from the eosinophils of guinea pigs and humans (1, 2). The physiochemical analyses of the guinea pig MBP indicate that it has a molecular weight of 11,000, is rich in arginine (13%), and contains two free sulfhydryl groups, as well as two intrachain disulfide bonds (3). On standing, MBP readily aggregates presumably by oxidation of its two free sulfhydryl groups. As yet, no specific biological activity has been found for this protein.

The rat is a potentially useful species for the study of eosinophil function. For example, parasitic infections, most notably *Nippostrongylus brasiliensis*, have been well studied in the rat and evidence that the eosinophil plays a protective role in immunity to parasitic infections has been presented (4, 5). Further, an immunoglobulin E (IgE)-producing tumor has been found in the rat (6). Thus, the rat could serve as a useful model for the study of the relationship between IgE and eosinophils in immunity to parasitic infections and in allergic diseases. Because of these considerations we determined whether rat eosinophils possess a protein like the MBP found in the guinea pig and human, and here we report the isolation and characterization of such a protein.

Materials and methods. Eosinophil granules were prepared essentially as described previously for the guinea pig (7, 8). Briefly, the peritoneal cavities of rats were repeatedly lavaged at weekly intervals with 50 ml of sterile saline until an eosinophil rich population of cells was obtained (7, 8). These cells were pooled and washed once with 0.34 *M* sucrose, and a granule-rich fraction was prepared by extraction with 0.34 *M* sucrose. In this procedure, 2-5 × 10^8 cells were suspended in approximately 10 ml of sucrose, mixed vigorously by repetitively pipetting with a 10-ml serologic pipet, and centrifuged at 400*g* for 10 min to remove unbroken cells and nuclei. The sediment from the first extraction was extracted three more times in the same manner, and the supernates from these extractions were pooled and centrifuged at 15,000*g* for 20 min. The resulting pellet was extracted with 1.5 ml of 0.01 *N* HCl and chromatographed on a Sephadex G-50 column equilibrated with 0.01 *N* HCl. Eosinophil granule proteins were analyzed by polyacrylamide gel using an 8% gel containing 1% sodium dodecyl sulfate (SDS) according to the method of Fairbanks *et al.* (9). Gels were stained for protein with Coomassie blue (9). Isoelectrofocusing was performed using an LKB multiphor electrophoresis apparatus according to manufacturer's recommendations with a 2-10 pH gradient. Amino acid composition was performed as described by Spackman *et al.* (10), using a Beckman Model 119 amino acid analyzer. Samples of MBP were hydrolyzed for 24 and 48 hr in 6 *M* HCl. Cysteine content was determined after oxidizing the protein with performic acid as described by Moore (11). Eosinophil granules were fixed in 3% glutaraldehyde, embedded in Epon 812, and processed for electron microscopy according to standard procedures.

Results. Although rats tolerated repeated peritoneal lavaging and responded with an increased number of eosinophils in the lavage fluids, their response was quantita-

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tively different than that obtained with guinea pigs (7). In spite of frequent lavaging, the number of cells obtained from the rats was significantly less than we could obtain from the guinea pig. For example, on the average a single lavage in the guinea pig yielded about 6×10^7 total leukocytes of which approximately 45% were eosinophils. In the rat, we normally obtained 1×10^7 cells of which 40% were eosinophils. Thus on a per animal basis there was a sixfold difference in the number of cells obtained by peritoneal lavage. Because the small number of eosinophils obtained from a single lavage yielded insufficient quantities of protein for analysis, the rats were lavaged three times a week instead of once a week. The animals tolerated the more frequent lavaging well, and similar numbers of eosinophils were obtained. However, extraction of the mixed preparation of cells from the peritoneal cavity (40% eosinophils) yielded few granules and vanishingly small quantities of protein. We attempted to alter the extraction protocol by adding heparin (250 units/ml) to the sucrose solution because heparin has been shown to be important for the extraction of human eosinophil granules (2). We also enriched the rat eosinophils by sedimentation through a sodium ditrizoate cushion (final purity 80% eosinophils), but neither modification nor their combination improved the yield of granules and protein from the cells. Therefore, we increased the number of animals from 20 to 50 and by using this larger population and by lavaging at weekly intervals, we were able to obtain sufficient quantities of granules for study.

Cells contained in the peritoneal lavage fluid were osmotically disrupted with 0.34 *M* sucrose, and the granules were pelleted by centrifugation at 15,000*g* for 20 min. When examined under the electron microscope, this pellet contained free granules with some cytoplasmic membranes but no apparent nuclear debris. The granules in this pellet were not in as high a state of purity as had been obtained with the guinea pig eosinophils, but the contaminating cellular debris evidently did not affect subsequent extraction procedures. The pelleted granules from approximately 2×10^8 eosinophils were solubilized in 1.5 ml of 0.01 *N* HCl and analyzed by SDS polyacrylamide gel electrophoresis. The SDS gels showed a number of bands with a major band in a position which corresponded to the guinea pig major basic protein, i.e., with a molecular weight of approximately 11,000 (Fig. 1). The granule extract was chromatographed on a Sephadex G-50 column equilibrated with 0.01 *N* HCl and two major peaks were found, a void volume peak and a second peak eluting just after the cytochrome *c* marker indicating a molecular weight of around 11,000 (Fig. 2). When the material

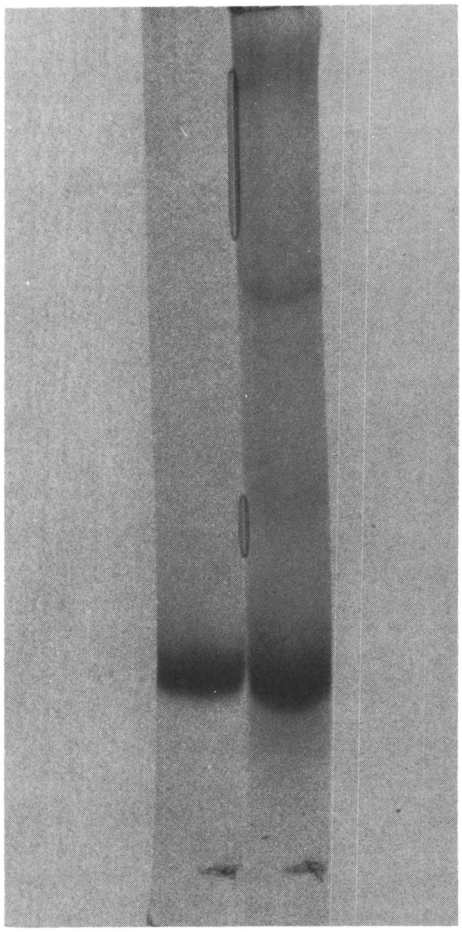

FIG. 1. SDS polyacrylamide gel electrophoresis of rat eosinophil granule extracts. Eosinophil granules solubilized in 0.01 *N* HCl show a number of bands with a major, low molecular weight band (right). Analysis of a concentrated pool of the second peak of a Sephadex G-50 column equilibrated with 0.01 *N* HCl reveals a single band at the same position as the major band (left). The india ink mark at the bottom of the gels indicates the position of the pyronin Y marker at the conclusion of electrophoresis.

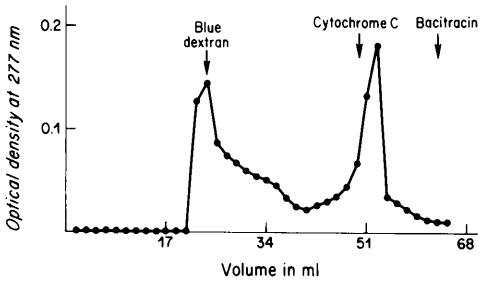

FIG. 2. Analysis of solubilized eosinophil granules on Sephadex G-50. Granules were solubilized in 0.01 *N* HCl and chromatographed on a 1.2 × 50 cm column equilibrated with 0.01 *N* HCl. The second peak fractions (elution volumes between 48–54 ml) were pooled, concentrated, and analyzed in the SDS-PAGE experiment shown in Fig. 1.

in the second peak was analyzed by SDS gel electrophoresis, it yielded a single band migrating in the position of the major band of the acid extract (Fig. 1). The protein from the second peak of the Sephadex G-50 column was analyzed by isoelectric focusing and a single band was seen at the cathodal wick indicating that the protein has an isoelectric point greater than 10. Amino acid composition of the eosinophil major basic protein was performed and the results are shown in Table I. The amino acid compositions of guinea pig and human MBP are shown for comparison. The amino acid compositions of the three proteins are quite similar for a number of amino acid residues and most significantly in their arginine content.

Discussion. Rats responded to repeated peritoneal lavage with sterile saline by developing a peritoneal eosinophilia. While the number of leukocytes obtained from the rat was less than the number obtained from guinea pigs by similar procedures, the yield was comparable to that reported by Bosworth and Archer (8). Surprisingly, we were unable to extract granules from cells obtained by frequent lavaging (three times per week) while granules could be obtained if the rats were only lavaged once a week. It is possible that the frequent lavaging selected a more immature population of eosinophils and the immature cells did not behave in the extraction protocol as expected from our prior studies.

The rat eosinophil appears to contain a protein analogous to the MBP found in guinea pig and human eosinophil granules.

TABLE I. AMINO ACID COMPOSITION OF MBP ISOLATED FROM RAT, GUINEA PIG, AND HUMAN

Amino acid	Rat	Guinea pig[d]	Human[e]
Aspartic	7[a]	6	7
Threonine	5	4	4
Serine	7	4	4
Glutamic	7	8	8
Proline	4	5	1
Glycine	14	12	8
Alanine	7	8	5
Valine	6	9	5
Methionine	3	1	0
Isoleucine	5	2	3
Leucine	5	4	6
Tyrosine	1	4	4
Phenylalanine	4	5	5
Histidine	2	3	2
Lysine	3	2	3
Arginine	13	13	11
Tryptophan	—[b]	4	8
Cysteine	5[c]	6	6

[a] Number of residues.
[b] Not done.
[c] As cysteic acid.
[d] From Ref. 3.
[e] From Ref. 2.

This conclusion is based on three lines of evidence: (i) SDS polyacrylamide electrophoresis and gel filtration on Sephadex G-50 show a major band with a molecular weight of 10–12,000. (ii) Isoelectric focusing reveals that this protein has an isoelectric point greater than pH 10 indicating that it is highly basic. (iii) The amino acid composition data show that this is an arginine-rich protein. We concluded, therefore, that the rat eosinophil contains an MBP very similar to that isolated from the guinea pig and human. Although we did not obtain rat eosinophil granules in a high state of purity, the similarity of this protein to guinea pig MBP, which we have localized in the eosinophil granule core by immunoperoxidase electron microscopy (12), leads us to believe the rat MBP is derived from the eosinophil granule. The demonstration of MBP in rat eosinophil granules as well as our prior findings in the guinea pig and human suggests that this protein is common to most, if not all, mammalian eosinophils.

Summary. The rat eosinophil granule possesses a major basic protein (MBP) similar to that previously isolated from human and guinea pig eosinophils. This conclusion is based on demonstration that the major protein of the rat eosinophil granule is a low

molecular weight, highly basic material with an amino acid composition similar to that of the human and guinea pig MBP.

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1. Gleich, G. J., Loegering, D. A., and Maldonado, J. E., J. Exp. Med. **137,** 1459, 1973.
2. Gleich, G. J., Loegering, D. A., Mann, K. G., and Maldonado, J. E., J. Clin. Invest. **57,** 633, 1976.
3. Gleich, G. J., Loegering, D. A., Kueppers, F., Bajaj, S. P., and Mann, K. G., J. Exp. Med. **140,** 313, 1974.
4. Ogilvie, B. M., and Jones, V. E., Exp. Parasit. **29,** 138, 1971.
5. Mahmoud, A. A. F., Warren, K. S., and Peters, P. A., J. Exp. Med. **142,** 805, 1975.
6. Bazin, H., Querinjean, P., Becker, A., Heremans, J. F., and Dessy, F., Immunol. **26,** 713, 1974.
7. Gleich, G. J., and Loegering, D. A., J. Lab. Clin. Med. **82,** 522, 1973.
8. Bosworth, N., and Archer, G. T., Aust. J. Exp. Biol. **39,** 165, 1961.
9. Fairbanks, G., Steck, T. L., and Wallach, D. F. H., Biochemistry **10,** 6206, 1971.
10. Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem.**30,** 1190, 1958.
11. Moore, S., J. Biol. Chem. **238,** 235, 1963.
12. Lewis, D. M., Lewis, J. C., and Gleich, G. J., Fed. Proc. **35,** 515, 1971.

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