MEDIATORS OF INFLAMMATION IN LEUKOCYTE LYSOSOMES

VI. Partial Purification and Characterization of a Mast Cell–Rupturing Component*,‡

By WINNIFRED SEEGERS,§ M.D., and AARON JANOFF,‖ Ph.D.

(From the Departments of Pathology and Medicine, New York University School of Medicine, New York)

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Several recent investigations have led to renewed interest in polymorphonuclear leukocytes (PMN), and in particular the lysosomes of these cells, as sources of mediators involved in the development of inflammation and local vascular injury.

Among the components of PMN granules which have received attention in this regard are: (*a*) lysosomal cathepsins, implicated in the pathogenesis of Shwartzman lesions (1, 2), Arthus reactions (1, 3–5), and most recently in injury to vascular basement membrane during immunologic responses (6); (*b*) a delayed permeability factor with the properties of leukocytic pyrogen, which is released from PMN upon sodium chloride incubation (7); and (*c*) kinin-forming and kinin-degrading enzymes, the latter also localized to lysosomes (8). In addition, permeability factors have been identified in a specific fraction of protein extracted from the lysosomes of PMN by weak acid and concentrated by ethanol precipitation at 20% (v/v) concentration (ET20 fraction). This fraction is largely composed of cationic proteins (9, 10) and polypeptides. ET20 material has been shown to injure cutaneous vessels of rabbits causing delayed increases in vascular permeability and the development of Arthus-type lesions (11, 12). This fraction also produces febrile responses in rabbits (13), and has been reported to possess anticomplementary activity in this species (6).

Previous work from our laboratory has shown that the ET20 fraction extracted from lysosomes of exudative PMN of rabbits also contains an agent which causes rupture and degranulation of rat mast cells (14). We have suggested that this mast cell–rupturing factor (MCF) is responsible for the *immediate* increase in vascular

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§ Advanced Research Fellow, American Heart Association (supported in part by the Sullivan County, New York, Heart Chapter). Present address: Department of Pediatrics, Division of Medical Genetics, Mt. Sinai Hospital, New York.

‖ Career Development Award Fellow of the United States Public Health Service (GM-K3-6461).

permeability following the injection of lysed PMN granules or ET20 fraction into rat tissues, since such reactions are blocked by an antihistamine drug (14, 15) or by prior depletion of mast cell histamine (14). The ET20 fraction derived from rabbit peritoneal macrophage granules does not possess MCF, and lysed macrophage granules are not able to provoke comparable, immediate permeability responses in rats (16). Quantitative comparison of MCF activity with that of several other polycations upon mast cells in vitro readily attests to the marked effectiveness of the PMN agent (17). The presence of mastocytolytic activity in the ET20 fraction of PMN neutrophil lysosomes has recently been confirmed by several independent investigators (6, 18, 19) and extended to include mast cells of other species besides the rat.

The present study was undertaken to further purify and characterize the MCF derived from lysosomes of rabbit exudative PMN neutrophil leukocytes.

Materials and Methods

Preparation of the ET20 Fraction.—Harvests of cells containing 95 to 100% PMN neutrophil leukocytes were obtained from 4-hr-old, glycogen-induced, peritoneal infiltrates in adult, New Zealand rabbits. The cells were homogenized in hypertonic sucrose by the technique of Cohn and Hirsch (20). After discarding nuclei and unbroken cells, the 8500 × *g* granule pellet of the homogenate was extracted 3 times with 2-ml volumes of ice cold, 0.2 N sulfuric acid, and the combined acid extracts were clarified by a final centrifugation at 12,500 × *g*. Ethanol, prechilled to −20°C, was added dropwise to the acid extract until a concentration of 20% was reached, yielding a flocculent white precipitate (ET20 fraction). After several hours at −5°C, the precipitate was collected by low speed centrifugation and redissolved in a small volume of 0.01 N hydrochloric acid in 0.1 M sodium chloride. Protein-sulfate was converted to protein-chloride by addition of barium chloride to a final concentration of 0.025 M. After discarding the precipitated barium sulfate, excess barium ion was eliminated by dialysis of the sample for 4 ½ hr, in the cold, against 3 changes of 0.15 M saline (150 volumes) containing 15 mg/100 ml of sodium bicarbonate and adjusted to pH 7.45. Part of the material (15 to 20%) became insoluble during dialysis at this pH and was subsequently discarded. The remaining material constituted the ET20 fraction employed in these studies. After assay of protein content, it was stored in lyophilized form until used. In the course of this study, 11 separate preparations of the ET20 fraction were made and all showed similar behavior in chromatographic and electrophoretic procedures.

Gel Filtration Chromatography.—Gel filtration chromatography was carried out using Sephadex cross-linked dextran gels (Pharmacia Fine Chemicals Inc., New Market, New Jersey). In preliminary experiments, Sephadex G-15, G-50, and G-100 preparations were used; but, the majority of the analytical and preparative chromatographic procedures were carried out as follows: 8 g of Sephadex G-25 Fine (approximate fractionation range: 100 to 5000 mol wt) were swollen in acetate buffer (0.05 M, pH 4.0) containing 0.85% sodium chloride, and equilibrated for 24 to 48 hr. Columns were made from glass tubing, 0.7 cm (I.D.) by 100 to 110 cm long, plugged at the bottom with glass wool, and fitted with a rubber cap pierced by narrow bore (PE 60) polyethylene tubing. The gel, after being poured into the tubing through buffer and washed with additional buffer, packed to a height of 88 to 92 cm and provided a bed volume of approximately 40 ml. Flow rate through the column was between 9 and 12 ml per hour. Blue Dextran 2000 (mol wt = 2×10^6) (Pharmacia Fine Chemicals) was dissolved in buffer and filtered through the column to check packing and to determine void volume. Internal volume of the column was estimated by filtration of potassium bromide (mol wt = 119).

1 to 8 mg of the material to be fractionated were dissolved in 0.4 to 0.5 ml of distilled water, applied to the top of the column under a narrow zone of buffer, and washed in with additional small volumes of buffer. The level of the latter was thereafter maintained at 10 to 15 cm above the top of the gel bed by a siphon from a buffer reservoir. Fractions were collected at room temperature in an automatic fraction collector (Packard Instrument Company, Inc., Jamaica, New York) set to deliver 22 drops (0.5 ± 0.02 ml) per tube.

Protein Assays.—Concentrations of eluted proteins and polypeptides were routinely determined with the Folin-Ciocalteu reagent according to the method of Lowry (21), using crystalline bovine serum albumin as a reference standard. Poly-L-lysine was measured with the biuret reagent (22). For calculation of amino acid composition data, nitrogen determinations were carried out by micro-Kjeldahl techniques (23).

Concentration and Storage of Materials.—Unless otherwise specified, ET20 fractions and all eluted fractions from Sephadex columns were routinely concentrated by lyophilization and stored at 4°C. If prolonged storage was required, the lyophilized materials were kept under vacuum and desiccation.

Starch Gel Electrophoresis.—Horizontal starch gel electrophoresis was carried out basically as described by Smithies (24). Each gel contained 18 g of starch (Connaught Medical Laboratories, Toronto, Canada) in 150 ml of acetate buffer (0.05 M, pH 4.0). Trays were of internal dimensions 10 × 13.8 × 0.6 cm. Each sample to be electrophoresed (about 70 μl) was absorbed into a strip of filter paper (Whatman No. 17) measuring 0.6 × 1.0 cm, and inserted into a slot in the gel approximately ⅓ of the distance from the anodal end. Methylene blue was applied on Whatman 3 MM paper as a marker. Electrode vessels containing 0.1 M acetate buffer, pH 4.0, were connected to the ends of the gel with wicks made of 3 thicknesses of Whatman 3 MM filter paper. A constant voltage power source was set to deliver 2 ½ to 3 ½ v per cm through the length of the gel. After electrophoresis at room temperature for 3 to 5 hr, at which time the methylene blue spot had migrated approximately 6 cm toward the cathode, gels were chilled and sliced in preparation for staining. One half of the gel was stained with Amido Schwartz 10B for 1 min followed by destaining in several rinses of methanol–acetic acid–water (4 ½:1:4 ½). The other half of the gel was stained overnight in 0.005% nigrosine in methanol–acetic acid–water and then rinsed.

Similar gels, containing 8 M urea in the acetate buffer described above, were prepared according to Smithies (25), except that mercaptoethanol was not included. These gels were subjected to higher voltages than were gels without urea. After insertion of samples as before, urea gels were kept at room temperature for 2 to 3 hr to allow dissociation in the gel before electrophoresis (26).

Two-dimensional (starch, starch-urea) gels were run according to Poulik (26), except that gel dimensions, buffer and voltage were as described above for one-dimensional gels.

Assay of Mastocytolytic Activity.—MCF activity was expressed as per cent of mast cells (rat mesentery) which became degranulated during incubation with test fractions in vitro. The method of preparing tissue fragments and incubation media, and the histologic processing of experimental and control tissues were based on the method of Norton (27) and have been described in an earlier publication (14).

Vascular Permeability Tests in Rabbit Skin.—Materials eluted during gel filtration chromatography of ET20 were tested for permeability effects in rabbit skin. Concentrated lyophilates of the eluted fractions were first rendered isotonic and neutral in pH. Aliquots (0.1 ml) containing 30 μg of Lowry-assayed material were then injected into the shaved abdominal skin of unanesthetized rabbits, with pyrogen-free tuberculin syringes and 26 gauge hypodermic needles. Either immediately before or 2 hr after the intracutaneous administration of test materials, the rabbits were injected intravenously with 4 ml per kg of a 1% solution of Evan's blue in 0.85% sodium chloride. 30 min after dye injection, the animals were sacrificed and the

extent of local dye extravasation at sites of intracutaneous injection was measured through the transilluminated skin and expressed as the mean of the largest and smallest diameters of the blued area.

Analysis of Amino Acid Composition.—Hydrolysis and preparation of the sample were carried out according to the method of Tallan et al. (28). Compositional analysis was performed by the procedure of Piez and Morris (29), using an amino acid analyzer (Phoenix Precision Instrument Company, Philadelphia). The resin employed was prepared by Dr. Paul B. Hamilton of the Alfred I. Dupont Institute, Nemours Foundation, Wilmington, Delaware.

Guinea Pig Ileum Assay for Musculotropic Activity.—MCF was assayed for ability to contract guinea pig ileum in the following manner. Strips of ileum were obtained from male, Albino-Hartley guinea pigs weighing 450 to 600 g. Lengths of ileum measuring 30 to 35 mm were suspended vertically in a 20-ml bath containing atropinized (0.2 μg per liter) magnesium-free Tyrode's solution, and were maintained at 31°C and gassed continuously with 95% plus 5% oxygen–carbon dioxide mixture. 2 g of tension were applied to the muscle strips. Following introduction of test agents into the bath, isotonic contractions were measured with an isotonic Myograph and recorded on a Physiograph recording apparatus (E and M Instrument Company, Houston, Texas). Bradykinin and histamine were used as reference standards. Doses of histamine refer to the weight of the base.

Biologicals Employed.—Insulin, chain A, oxidized (glycyl), was purchased from Mann Research Labs., New York (lot L-2742).

Bradykinin (BRS-640) (batch 65236) and kallidin (KL-698) (batch 63068) were both kindly supplied by Sandoz Pharmaceuticals, Hanover, New Jersey. 10 ml of the kallidin preparation (containing 1 mg) were dialyzed for 30 min at 25°C against 2 liters of distilled water in order to reduce the concentration of salts and methylbutanol preservative present in the kallidin vehicle. The dialyzed solution was then concentrated by lyophilization prior to filtration through Sephadex.

Poly-L-lysine hydrobromide, type II, was purchased from Sigma Chemical Company, St. Louis (lot 124-B-0450).

RESULTS

Gel Filtration Chromatography of ET20.—Preliminary trials at pH 4.0, through Sephadex G-100, G-50, and G-25 gels, demonstrated that MCF activity was associated with the small mol wt components of ET20 which were retarded during gel filtration, while the large mol wt components (excluded fractions) were inactive when tested on mast cells. The retarded components, including MCF, eluted as a single peak from Sephadex G-50 and G-100 gels, but a greater degree of resolution of these components was achieved by filtration through Sephadex G-25. The latter was thereafter routinely employed. Fig. 1 represents a typical experiment using Sephadex G-25 and is characteristic of all fractionations that were eventually carried out. Fig. 1, *A* shows the protein concentration in the eluted fractions, while Fig. 1, *B* and *C* represent, respectively, the electrophoretic patterns and mast cell activities of these fractions, pooled according to the scheme shown. The results indicate the following: The component of ET20 with an elution volume corresponding to that of Dextran 2000 and composed of the slowest migrating component in starch gel electrophoresis possessed no MCF activity (pool I). The ET20 components

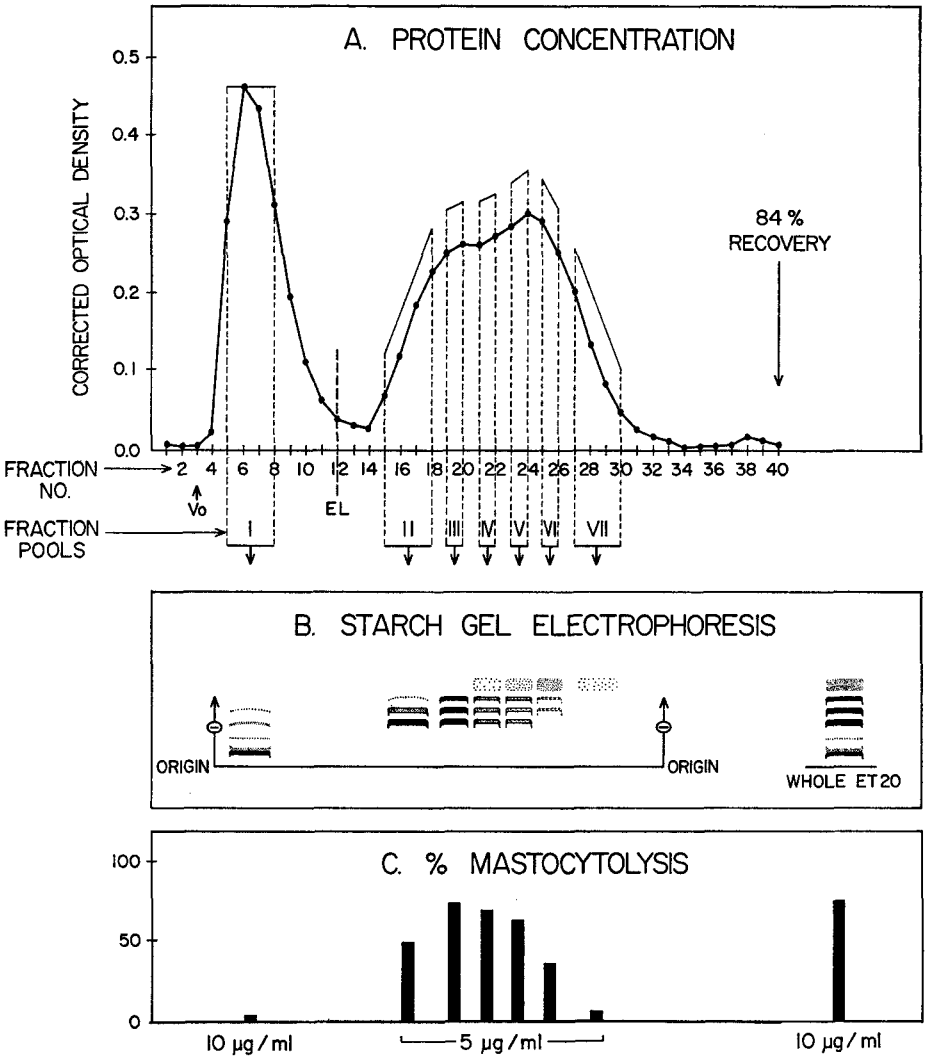

FIG. 1. Fractionation of ET20 by Sephadex G-25 gel filtration. (*A*) Protein concentration in eluted fractions. Vo = void volume, EL = end of elution of excluded material (Blue Dextran 2000). (*B*) Electrophoretic patterns of pooled fractions. The densities of the electrophoretic bands correspond to the intensity of nigrosine staining in starch gel. Staining of gels with Amido Schwartz 10B gave similar results except that the electrophoretically fastest components of the pattern, including the first of the intermediate bands, rapidly faded during destaining. (*C*) Mast cell-rupturing activity of same pooled fractions. Unfractionated ET20 is shown at the right of the figure.

which were retarded during gel filtration (smaller mol wt materials) were resolved into (in the order of their appearance in the eluate) material of intermediate electrophoretic mobility (pools II and III), intermediate material combined with rapidly migrating components (pools IV, V, and VI), and finally the latter components alone (pool VII). Eluted fractions containing only electrophoretically fast material also possessed no MCF activity. Only those aliquots of the fractionated ET20, which contained "intermediate" material, possessed this activity. Clearly, MCF is associated with that part of the ET20 mixture which possesses intermediate electrophoretic mobility in starch gel at pH 4.0. In the following sections of this report MCF-fraction refers to Sephadex eluates containing only material of intermediate electrophoretic mobility.

Comparison of MCF and Other Polypeptides During Gel Filtration.—The observed retardation of the MCF-fraction of ET20 in Sephadex G-25, which suggested a relatively small mol wt, led to its comparison with other polypeptides of known mol wt. MCF, insulin-chain A (mol wt = 2400), and kallidin (mol wt = 1188) were successively subjected to gel filtration in a single column maintained under uniform conditions. Because exposed cationic groups on MCF molecules might have retarded its passage through the gel,[1] another highly charged polycation, poly-L-lysine hydrobromide, type II, with a reported mol wt of 2600, was also filtered through the column and its elution volume compared with that of the marker polypeptides. The results of these procedures are shown in Fig. 2. From the superimposed plots of the protein concentration curves it can be seen that the elution volume of MCF is between that of insulin-chain A and kallidin. The elution volume of the poly-L-lysine was somewhat less than that of insulin-chain A, suggesting that, under the conditions of the experiment, the former was not significantly retarded in its passage by interactions between its charged groups and the Sephadex gel.

The estimated molecular weight of MCF, based on gel filtration chromatography under the described conditions, is thus between 1200 and 2400.

Starch Gel Electrophoresis of the MCF-Containing Fraction.—The MCF-rich fractions eluted from Sephadex columns appeared to be heterogeneous in conventional starch gel electrophoresis, with 3 bands of intermediate mobility associated with activity (see Fig. 1). As this heterogeneity might have been due to aggregation of a single species of polypeptide, rather than the presence of different species, electrophoresis of other samples was carried out in the presence of 8 M urea. Two bands appeared in urea gels, as opposed to 3 in regular starch gels. In order to identify the source of that electrophoretic heterogeneity still apparent in the presence of urea, two-dimensional electrophoresis

[1] Precautions taken to minimize this possibility included use of elution buffer of ionic strength greater than 0.01, and of moderately acid pH (4.0) to suppress ionization of carboxyl groups of the dextran.

was performed. In this procedure, electrophoresis of the MCF fraction was first carried out in a conventional starch gel and a narrow block of gel cut from the sample-track was then transferred to another gel containing 8 M urea (see Fig. 3). After an interval of 2 hr, electrophoresis in the second gel was carried out in a direction at right angles to the original axis of migration. As shown in Fig. 3, only the slowest of the 3 bands which were separated in the first dimen-

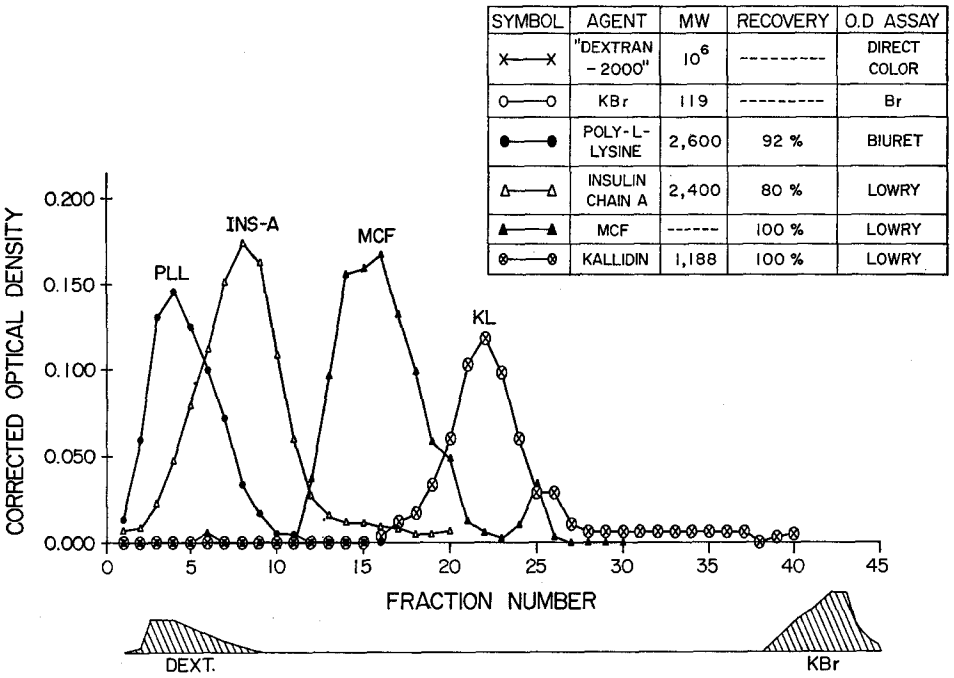

FIG. 2. Comparison of elution of MCF and small mol wt polypeptides (Sephadex G-25 gel filtration at pH 4.0). All materials are identified within the figure. For convenience, the elution of Blue Dextran (*Dext.*) 2000 and potassium bromide is shown below the base line.

sion contributed to the heterogeneity persisting in urea gel. The bulk of the material was electrophoretically homogeneous in the presence of urea. The minor component may either be a different species of polypeptide or an aggregated residue undissociated under the conditions of the experiment.

Amino Acid Composition of the MCF Fraction.—An analysis of the amino acid composition of the MCF-rich fraction was carried out, despite its possible heterogeneity, for comparison with data obtained using guinea pig whole ET20 fraction (30). The results are shown in Table I. 86% of the total moles recovered in this analysis were contributed by the 8 amino acids listed first in the table, while the remaining 14% were represented by trace amounts of 9 other

amino acids (second group). Assuming that the first group of amino acids are constituents of the major component of the MCF fraction (in two-dimensional electrophoresis), it would seem likely that MCF is composed of these amino acids. This interpretation is based on the fact that the major component in two-dimensional electrophoresis is constituted mainly of the first 2 bands of MCF in regular starch gel electrophoresis (see Fig. 3), and these in turn are associated with the highest mast cell activity (see Fig. 1).

Another feature of the amino acid data is the relatively large proportion of arginine present in this fraction. This basic amino acid constituted 30% of

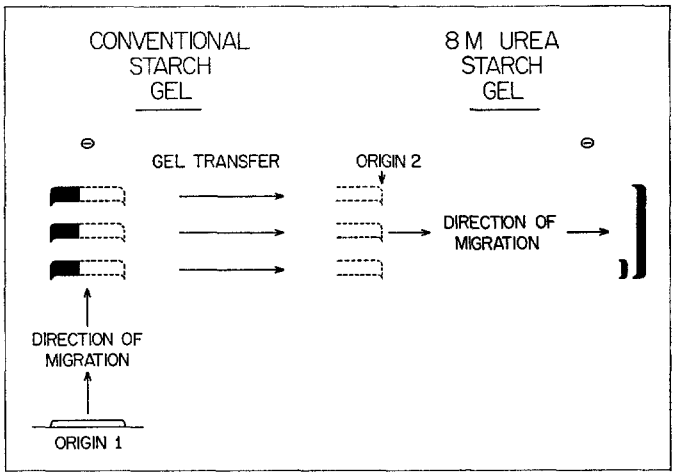

FIG. 3. Two-dimensional starch gel electrophoresis of MCF. Blackened portions in the first gel represent stained material; dotted portions represent material transferred to the second gel.

the total moles of amino acids recovered in the analysis, whereas the other cationic amino acids together accounted for only 4% of the total. Thus, the cationicity of the MCF fraction is almost exclusively due to its arginine content. In contrast, the arginine content of guinea pig whole ET20 extract (30) appears to be lower than that of the MCF-enriched fraction from rabbit PMN.

Thermostability and Dialyzability of MCF.—Solutions containing 20 μg per ml of MCF, dissolved in normal sodium chloride buffered to pH 7.4 with bicarbonate, were heated to 37°C, 56°C, and 80°C for varying lengths of time. MCF activity was not diminished by any of these treatments (see Table II).

The dialyzability of MCF was tested by assaying for mast cell activity in the external compartment following prolonged dialysis of the material. 200 μg of MCF were dissolved in 1 ml of normal sodium chloride and dialyzed for 48 hr in the cold against 5 ml of bicarbonate-buffered mammalian Ringer-

Locke salt solution at pH 7.4. The vessel containing the dialyzing solution was well siliconized to prevent adsorption of dialyzed MCF to glass surfaces. A second dialysis bag filled with sodium chloride alone and dialyzed in an adjacent vessel served as a control. After 48 hr, the bags were removed and the external solutions were assayed for MCF activity by direct incubation of rat mesentery fragments in the solutions. The results of this experiment are also given in Table II. Significant mast cell activity was detected in the external solution which had formerly held the dialyzing bag containing MCF. In

TABLE I

*Amino Acid Composition of MCF Fraction**

Alanine	10.4	
Arginine	30.0	
Glutamic acid	4.5	
Glycine	9.4	
Isoleucine	4.8	
Leucine	12.9	
Phenylalanine	5.3	
Proline	9.0	
		86.3
Aspartic acid	1.3	
Cysteic acid	2.2	
Histidine	1.3	
Lysine	0.7	
Ornithine	2.1	
Serine	1.8	
Threonine	0.7	
Tyrosine	0.6	
Valine	3.0	
		13.7

* Expressed as per cent of total moles of amino acids recovered.

addition, samples of the external solution, concentrated by lyophilization and subjected to regular starch gel electrophoresis, showed the characteristic 3 bands of MCF. As a check against mechanical leakage of MCF through defects in the dialysis membrane, crystalline horse-radish peroxidase (Sigma Chemical Company) (mol wt = 44,000) was added to the bag containing MCF. Only 0.002% of the added enzyme was detected in the external solution at the end of the experiment, the balance being recovered from within the bag. It is thus clear that the great bulk of the MCF present in the external solution had dialyzed through the membrane. Although this experiment shows that MCF activity is dialyzable, other experiments with MCF fractions and with cationic polypeptides such as kallidin and low mol wt poly-L-lysine have suggested that the rate of diffusion of these agents through dialyzing membranes

is quite slow. The retardation may be due to interactions between cationic groups of the basic polypeptides and the dialyzing membrane itself, which bears a slight negative charge.

Effect of Trypsin on MCF.—A sample containing 100 μg of MCF was incubated for 19 hr at 37°C with 10 μg of crystallized-lyophilized trypsin (Worthington Biochemical Corporation, Freehold, New Jersey). The solution also contained calcium chloride at 0.002 M concentration and was adjusted with bicarbonate to pH 8.3. Following incubation with trypsin, aliquots were tested for mast cell–rupturing activity and were also subjected to electrophoresis in

TABLE II

Effects of Heating and Prolonged Dialysis on MCF Activity

Test solution	Treatment	MCF activity recovered*
MCF in Ringer-Locke (20 μg/ml)	No treatment	99 ± 2
" " " " "	37°C × 2 hr	98 ± 4
" " " " "	56°C × 30 min	82 ± 25
" " " " "	80°C × 10 min	94 ± 6
Ringer-Locke	No treatment	1 ± 2
Ringer-Locke	48/80‡	94 ± 5
Ringer-Locke	No treatment	5 ± 5
Ringer-Locke	48/80‡	60 ± 30
Ringer-Locke dialyzing solution	MCF dialysis	94 ± 16
Ringer-Locke dialyzing solution	Saline-dialysis (control)	4 ± 4

* Expressed as per cent degranulation of mast cells ± SE (750 cells counted).

‡ Compound 48/80 (Burroughs Wellcome and Company, Inc., Tuckahoe, New York), a known mastocytolytic agent present in a concentration of 1 μg/ml.

starch gel. Treated MCF was devoid of mast cell activity. Furthermore this material no longer showed the characteristic electrophoretic mobility or sharply defined staining pattern of untreated MCF, but instead produced a broad, undefined pattern of very rapidly migrating material. MCF, incubated under identical conditions but without added trypsin, showed undiminished mast cell activity and characteristic behavior during gel electrophoresis. Trypsin itself, at the concentration employed in the experiment, did not interfere with standard mast cell responses to compound 48/80. These results show that digestion by trypsin destroys mast cell–rupturing activity of MCF.

Effect of MCF on Isolated Guinea Pig Ileum.—Strips of atropinized guinea pig ileum (see Materials and Methods) which responded to as little as 0.008 μg per ml of histamine and bradykinin, gave no contractions (either immediate or delayed) when incubated with over 1 μg per ml of MCF (see Table III). This result is not surprising in view of earlier observations of a similar lack of

smooth muscle stimulating activity in the whole ET20 fraction (12, 31). In the present tests, responses of the ileum strip to control agonists (histamine and bradykinin) were measured both before and after incubation with the test agent (MCF). In all experiments reported here, contractions produced by control agonists after MCF incubation were equal in magnitude to those elicited at the start of the experiment. The results clearly show that the arginine-rich MCF agent is different in its behavior on smooth muscle (ileum) from other arginine-containing vasoactive polypeptides such as bradykinin and kallidin, and that MCF is also different from anaphylatoxin in this respect (see Discussion).

TABLE III

Effect of MCF on Isolated Guinea Pig Ileum Strips

Guinea pig no.	Test agent	Concentration	Height of contraction*
		10^{-9} g/ml	mm
1	Histamine	8	40 ± 1.0
	Bradykinin	8	16 ± 0.6
	MCF	1250	0 (immediate)
	MCF	1250	0 (delayed −30 min)
2	Histamine	8	34 ± 2.0
	Bradykinin	8	13 ± 0.8
	MCF	1250	0 (immediate)
	MCF	1250	0 (delayed −30 min)

* Average of 4 trials ± SE.

Presence of MCF in Frozen-Thawed Lysosomes of Exudate PMN.—Although previous work had already shown mast cell–rupturing activity in lysed granules of rabbit exudate PMN (14), it was desirable to demonstrate the same electrophoretic components in lysate of whole PMN granules that had been detected in MCF-rich fractions eluted from Sephadex. Accordingly, rabbit exudate PMN cells were homogenized and fractionated by centrifugation as before and the granule pellets obtained from the cells were suspended in bicarbonate-buffered sodium chloride (pH 7.4) and disrupted by freeze-thawing. Supernate from the frozen-thawed granules was then immediately subjected to electrophoresis in parallel with a sample of the MCF fraction. Other aliquots of the disrupted granules were tested for mast cell activity in vitro. The results again confirmed the presence of mast cell rupturing activity in PMN granule lysate and also showed that this lysate contained material with electrophoretic mobility and staining properties identical to that in the MCF fraction.

Effects of ET20 Sephadex Fractions on Vascular Permeability in Rabbit Skin.—Since other investigators had shown that the ET20 fraction of rabbit PMN

granules provoked *delayed* permeability changes in rabbit skin (11, 12), unlike the *immediate*, transient permeability responses to ET20 in rat tissues, it seemed possible that a second permeability factor might be present in the ET20 extract of PMN lysosomes in addition to MCF. To examine this question, the major chromatographic fractions obtained by gel filtration of ET20 were tested for production of delayed permeability response in rabbits (see Materials and Methods). The following fractions were tested: excluded fractions (pool I in Fig. 1), MCF-rich fractions, and post-MCF eluted fractions (pool VII in Fig. 1). 30 μg of these materials were injected in each of 2 skin sites in 6 animals. Only pool I (excluded, large mol wt material) gave moderate bluing reactions in rabbit skin at 2 hr (8 to 12 mm diameter blue spots in 4 of 6 rabbits). The MCF fraction was completely inactive. None of the 3 fractions caused immediate bluing responses in the rabbit (dye administered intravenously immediately before intracutaneous injection of test samples). These results support the view that the slow acting permeability factor described by other workers in the ET20 extract of rabbit PMN lysosomes is not related to the MCF component of the granules. Rather, the results suggest that a second, larger mol wt permeability factor may be present in these particles. Further work on this second agent is in progress.

DISCUSSION

On the basis of the foregoing results, the mast cell–rupturing factor (MCF) present in lysosomes of rabbit exudate PMN neutrophil leukocytes appears to be an arginine-rich polypeptide with a molecular weight of 1200 to 2400.[2] That the biological activity of this agent is associated with peptide linkages has been established by our observation that mast cell–rupturing activity in vitro is destroyed by trypsin and by Cochrane's finding that permeability effects in vivo are likewise destroyed by this enzyme (6). However, the latter author reported that the permeability factor in the cationic protein fraction of PMN lysosomes is between 6000 and 8000 in molecular weight (6). This disparity between Cochrane's and our own findings with respect to molecular weight of the acutely inflammatory component of rabbit PMN lysosomes remains to be resolved. Whether the anticomplementary activity reported to be present in the ET20 extract (6) and the fever-producing activity identified in this same extract (13) are properties of the large or small molecular weight components also remains to be determined.

With respect to the molecular weight of MCF, one additional point requires clarification. In an earlier publication (31), we estimated the molecular weight

[2] Preliminary peptide-mapping of the material after digestion with trypsin has revealed a small number of basic peptide units. This result combined with information obtained from amino acid analysis (30% arginine, see Table I) lends additional support to the view that MCF is of low mol. wt.

of this agent to be at least 10,000. Our original impressions were based on the observation that permeability activity of the crude ET20 extract was not lost after dialysis of the material. A similar observation has been reported by Golub (12) after dialysis of PMN granules lysed by freezing-thawing. However, the slow rate of diffusion of these highly charged molecules through dialyzing membranes (see Results) can cause an impression of apparent nondialyzability. Furthermore, the presence of a large molecular weight substance with independent permeability effects in the same extract adds to the confusion. Finally, our original impressions and those of Golub were indirectly based on an apparently undiminished inflammatory activity of the dialyzed material, and these estimates, in turn, depended on semiquantitative assay techniques such as microcirculatory changes in mesenteric vessels (31), and bluing responses of injected skin (12). On the other hand, the observation reported in the present paper on passage of MCF through dialyzing membranes is based on its direct detection in the dialysate, using a more quantitative assay method (in vitro mast cell degranulation), and starch gel electrophoresis techniques.

The observation that purified MCF fractions were without activity when tested on guinea pig ileum clearly distinguishes this factor from the known vasoactive kinins and from anaphylatoxin. However, since the latter substance causes contraction of ileum strips by releasing histamine from mast cells in the intestinal preparation (32), the failure of MCF to act in a similar fashion upon guinea pig ileum may seem contradictory. The explanation lies in the fact that guinea pig mast cells are unusually resistant to the degranulating action of histamine liberators of the type exemplified by compound 48/80 (33), to which class MCF most probably also belongs. Unlike these agents, anaphylatoxin is very active on mast cells of the guinea pig, but is less active on mast cells of other species which are more sensitive to the action of 48/80 and MCF (e.g. rat, mouse, hamster). On these grounds, a clear distinction can be made between the two agents.

Clark and Higginbotham (18) have recently described the probable fate of MCF following its injection into tissues rich in mast cells. These authors report that mastocytolytic cationic "protein" (ET20 extract) labeled with fluorescein and injected subcutaneously into mice becomes bound to mast cell granules liberated as a result of the action of the lysosomal material. These granules labeled with fluorescein are eventually ingested by adjacent fibroblasts. The suggestion of the authors is that "heparin-containing granules released from mast cells in response to leukocytic secretions may sequester extracellular lysosomal substances and enhance their removal by fibroblasts."

In preliminary experiments, we have attempted to recover MCF activity from circulating leukocytes of the rabbit in order to determine if it is present in the lysosomes of peripheral PMN as well as those present in exudate fluids. These trials have so far failed to detect this agent in granules obtained from

circulating white cells. This has been the case in leukocyte preparations obtained following dextran sedimentation of erythrocytes and in preparations of buffy coat. Negative findings also resulted when peripheral leukocytes were obtained from rabbits in which glycogen-induced peritoneal exudates were present at the time of bleeding as well as from animals that had not been treated with glycogen. These data at least raise the possibility that MCF is not present in a preformed, active state in circulating PMN; but, instead, requires an activation step provided by the exudative environment. Studies are planned to determine the effect of glycogen, and of trace amounts of bacterial endotoxin (which may be present as a contaminant in the glycogen used) upon the formation of active MCF in peripheral leukocytes. In addition, separation of lymphocytes from PMN neutrophil leukocytes in buffy coat preparations will also be done, in order to then properly compare the latter cells to their counterparts in exudate fluids.

Additional questions bearing on the potential significance of MCF in inflammatory reactions remain to be answered. For example, it is not known whether MCF can release histamine and other vasoactive substances from storage sites such as platelets and basophil leukocytes in addition to the tissue mast cells. Nor is it clear whether MCF, or a related agent, is present in leukocyte lysosomes of other species besides the rabbit. Some evidence is available to suggest that this is the case. Archer (34) has detected mast cell–rupturing activity in a cationic protein fraction obtained from rat eosinophil granules. Basic polypeptides with permeability-increasing properties have also been recovered from the lysosome fraction of bovine leukocytes by Hegner (35). In future studies of this question, eosinophil as well as neutrophil leukocytes should be considered as potential sources of MCF in view of the large amount of arginine-rich cationic protein present in the cytoplasmic granules of the former cells (36).

In conclusion, many investigators have shown that the lysosomes of PMN leukocytes contain a number of potential phlogistens with widely differing modes of action. These substances probably contribute to the development of tissue injury reactions in which PMN are a causal factor. The partial purification and characterization of one of these phlogistens, a mast cell–rupturing polypeptide, has been described in this report.

SUMMARY

The mast cell–rupturing component present in the lysosomes of rabbit exudate PMN neutrophil leukocytes has been identified and some of its physical and chemical properties have been described.

The active agent is a low molecular weight (1200 to 2400) polypeptide containing a relatively large proportion of the basic amino acid, arginine. It is thermostable and dialyzable, and does not cause contraction of the isolated

guinea pig ileum. The mast cell–rupturing activity of the agent is destroyed by trypsin.

A second permeability factor with a larger molecular weight is present in crude extracts of PMN granules. Although this substance does not lyse mast cells, it is capable of evoking delayed permeability responses in rabbit skin.

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