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MEDIATORS OF INFLAMMATION IN LEUKOCYTE LYSOSOMES

II. Mechanism of Action of Lysosomal Cationic Protein Upon Vascular Permeability in the Rat*

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Considerable emphasis has been placed on the role of acid-proteases within lysosomes of polymorphonuclear leukocytes (PMNL) in the pathogenesis of allergic and other forms of tissue injury (1, 2). We reported previously (3) that a cationic protein-containing fraction of PMNL lysosomes, extracted by weak mineral acid and precipitated by ethanol at a 20 per cent (v/v) concentration, produced inflammatory changes in the microcirculation of rat and rabbit mesentery. However, such cationic protein fractions of leukocyte granules are reportedly free of acid-proteases (4) and other hydrolases (3) normally associated with intact leukocyte lysosomes. This apparent discrepancy led us to suggest alternative mechanisms of action of lysosomal cationic protein fractions in local tissue injury reactions and to propose that a part, at least, of the inflammatory changes induced by whole granule preparations might be mediated independently of their protease content.

In the course of these earlier investigations it was found that the cationic protein fraction, in addition to causing adhesion and emigration of leukocytes, also produced a marked increase in vascular permeability to circulating Evan's blue dye when the protein was applied topically onto rat mesentery or when it was injected intracutaneously into rat skin. However, *in vitro* tests using isolated smooth muscle preparations failed to reveal any kinin-like, serotonin, or histamine activity in this extract (3). Further studies have now been carried out on the mechanism of action of crude extracts of PMNL lysosomes, and

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also of the cationic protein isolated from these granules, upon vascular permeability in skin and cremasteric muscle of the rat. The present report will present evidence which suggests that the early increase in vascular permeability produced in rat tissues by PMNL lysosomes is caused by vasotropic agents discharged from tissue mast cells and not by protease activity of the granules. This effect is due to a mastocytolytic agent present in the cationic protein fraction of PMNL lysosomes.

Materials and Methods

Preparation of Leukocyte Lysosome Fractions.—Rabbit peritoneal exudate polymorphonuclear leukocytes (PMNL) were collected and their granules isolated by the technique of Cohn and Hirsch (5). Supernates of frozen-thawed granules and cationic protein-containing extracts of the granules were prepared by previously described techniques (3) except that in the present studies dialysis of the fractions was carried out against sodium bicarbonate-buffered, mammalian-Ringer's balanced salt solution at pH 7.2. Protein content of the various fractions was determined by the method of Lowry (6), with crystalline bovine serum albumin as a standard.

General Method of Permeability Tests in Rat Skin.—The method employed to evaluate permeability effects in rat skin was essentially similar to that described by Miles and Wilhelm (7). Materials to be tested were injected in a volume of 0.05 ml, intracutaneously, into the shaved abdominal skin of rats (males, Sprague-Dawley strain, 250 to 350 gm body weight) anesthetized with sodium pentobarbital (nembutal, Abbott Laboratories, North Chicago, Ill.). In each of the test animals at least two separate sites were prepared at each dose of test agent. Either immediately or at varying times after the intracutaneous administration of test substances, Evan's blue dye was injected intravenously as a 1 per cent solution in isotonic saline and at a dose of 0.4 ml per 100 gm of body weight. Animals were regularly sacrificed 20 minutes after dye injection and the abdominal skin was reflected for examination of the undersurface. The grade of edema in the injected skin site was estimated according to the diameter and color intensity (visual observation) of the dye accumulated in the tissues.

General Method of Permeability Tests in Cremaster Muscle of the Rat.—Permeability effects in rat cremasteric muscle were studied using the carbon-labelling technique described by Majno (8). Aliquots of 0.05 ml of the materials to be tested were injected subcutaneously into the scrotal sac just over the mid-ventral surface of the testis. Either immediately or at varying times after the injection of test substances, carbon suspension (pelikan C11-1431a, Gunther Wagner, Germany) was injected intravenously (0.1 ml per 100 gm body weight). Animals were sacrificed 1 hour after injecting the carbon suspension, and the testes were placed briefly in 10 per cent formalin. The muscle was then dissected free of other structures, stretched, and pinned on dental wax, fixed in formalin for an additional 24 hours, cleared in xylene, and mounted in glycerine-jelly for low power light microscopic observation. In this test, vessels whose permeability has been abnormally increased by the subcutaneous injection of test material appear blackened by carbon particles which have passed between endothelial cells. Furthermore, the characteristic topography of the microcirculation in rat cremasteric muscle (8) readily permits identification of the type of vessel (venule, capillary, or arteriole) most affected by the test agent.

Assay of Mastocytolytic Activity in Vitro.—The effect of leukocyte granule fractions upon rat mast cells *in vitro* was assayed according to the technique described by Norton (9). Briefly, this involved incubation of pieces of rat mesentery in Ringer-Locke glucose solution buffered with bicarbonate to pH 7.45 and containing varying concentrations of either leukocyte frac-

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tions or known mastocytolytic agents such as compound 48/80 (Burroughs Wellcome and Co., Inc., Tuckahoe, New York). Thirty minutes of incubation at room temperature was routinely employed in all tests; and, incubation in Ringer-Locke alone was always included as a control for spontaneous mast cell rupture. Following the period of incubation, the tissues were fixed in 10 per cent formalin containing 0.1 per cent toluidine blue, dehydrated in acetone, cleared in xylene, mounted in permount (Fisher Scientific Co., Fairlawn, New Jersey), and examined microscopically for the enumeration of intact *vs.* disrupted mast cells.

Details of methodology which were varied according to the needs of each experiment are described in the appropriate sections of the Results.

TABLE I

Time Course of Vascular Permeability Change Produced by PMNL Lysosomes in Rats

Test agent	Test site/dose (μg protein)	Reaction/No. of rats in test				
		0 min.*	30 min.*	60 min.*	120 min.*	180 min.*
Lysosomal cationic protein (20 per cent ethanol fraction)	Skin	++++	trace	0	0	0
	(10)	(10)	(6)	(6)	(6)	(6)
	Cremaster	++			0	
	(20)	(6)			(4)	
Extract of frozen-thawed granules	Skin	++++	+	0	0	0
	(10)	(10)	(6)	(6)	(6)	(6)
	Cremaster	++			0	
	(30)	(4)			(4)	

Scale of response:

++++ (skin), raised, intensely blue wheal, 12 to 15 mm diameter.

+ (skin), flat, pale-blue, uneven reaction, 5 mm or less in diameter.

++ (cremaster), postcapillary collecting venules show carbon deposits over area 10 to 15 mm in diameter.

0 (no reaction), no blue dye grossly visible (skin) or carbon deposits microscopically visible (cremaster).

* Elapsed time between intracutaneous (skin) or subcutaneous (cremaster) injection of test agent and intravenous injection of Evan's blue (skin) or carbon suspension (cremaster).

RESULTS

Time Course of the Permeability Change Produced in Skin and Cremaster Muscle of Rats by PMNL Lysosomes.—Table I shows that the permeability changes induced in skin and cremaster muscle of rats by single injections of PMNL lysosomes (both extracts of frozen-thawed granules and cationic protein fractions) are acute, transient changes which disappear rapidly.

When animals were injected intravenously with Evan's blue either immediately before or after intracutaneous application of lysosomal materials, an intense exudation of plasma-bound dye developed, rapidly reaching a maximum diameter of 12 to 15 mm in 15 or 20 minutes. However, when dye was

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injected 30 or 60 minutes after the lysosomal fractions, only trace reactions occurred (pale, incomplete staining); and, at 2 and 3 hours after injection of materials into the skin, no discernible exudation of dye was observed except at the finely circumscribed point of passage of the needle into the skin (see Table I). The absence of dye exudation at later intervals was not exclusively the result of mechanical interference with local blood flow following engorgement of tissue spaces with fluid during the early phase of the reaction. This was shown by the fact that injection of 2 μg of histamine into the same skin sites previously treated with lysosome fractions produced typical histamine blueing reactions at these sites in the later time-groups (2 and 3 hours).

When animals received an intravenous injection of carbon suspension immediately after subcutaneous application of lysosomal materials over the cremaster muscle (frozen-thawed granules or cationic protein), a widespread blackening of venules developed in the muscle, in a pattern identical with that produced in this tissue by similar injections of histamine. However, if carbon suspension was administered 2 hours after injection of lysosomal materials over the muscle, no labelling of vessels could be discerned (see Table I). Again, it could be shown that potential vascular reaction was still present in the cremaster at this time, since reinjection of the scrotal sac with the standard dose of histamine just before the administration of carbon produced a normal venular-labelling pattern in animals that had been treated with lysosomal materials 2 hours earlier.

Although the doses of lysed granule protein and cationic protein shown in Table I were the ones most frequently employed in permeability tests, other experiments have shown that strong permeability changes (2+ and 3+ reactions, see Table I) can be produced by as little as 2 μg of protein in the skin and 10 μg in cremaster muscle.

Mastocytolytic Activity of PMNL Lysosomes in Vitro.—In view of the acute, transient nature of the vascular permeability change produced in rat tissues by PMNL lysosomal fractions, it seemed reasonable to suppose that the reaction might be mediated by vasotropic agents released from tissue mast cells which had been disrupted by contact with the injected lysosomal material. It was therefore decided to investigate the effect of PMNL lysosomes upon rat mast cells *in vitro*, in order to determine whether the lysosomal fractions employed in our permeability tests were, in fact, capable of rupturing these cells.

Table II shows the results of these experiments. Both lysosomal cationic protein (20 per cent ethanol fraction) and extracts of frozen-thawed lysosomes were compared with a known mast cell–rupturing agent, compound 48/80 (Burroughs Wellcome and Co., Inc., Batch No. NS280). It can be seen that both of the lysosomal preparations produced a marked mastocytolytic effect in these tests, although the activity of the lysosome fractions was less than one-tenth that of compound 48/80 when these agents were compared on a

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simple weight per weight basis. However, if the lysosomal cationic protein was compared with compound 48/80 on a molar weight basis (see Discussion), then the two agents appeared to be more nearly equal in mastocytolytic potency.

TABLE II

Effects of PMNL Lysosomes on Rat Mast Cells In Vitro

Test material	Percentage disruption of mast cells*								ED_{50}§
	Concentration (μg)‡								
	0	0.2	0.3	0.4	0.5	0.6	0.8	1.0	*μg per ml*
Compound 48/80	3 4 5	32	63	66 72		87	97 99		0.27
C.P. (lysosomal cationic protein, 20 per cent ethanol fraction)	2 4 4 4	21	49	68		66	92 94	100	3.4
C.P. in presence of trasylol‖	28							100	
F.T. (extract of frozen-thawed lysosomes)	3 3 3		41		45	69		72 89	5.6
F.T. in presence of trasylol‖	13							65	

C.P. = cationic protein fraction (20 per cent ethanol); F.T. = extract of frozen-thawed granules.

* Percentage disruption is based on the number of mast cells showing granule extrusion out of 1500 cells examined (×640).

‡ μg are per ml in the case of compound 48/80 and per 0.1 ml in the case of all other test agents.

§ Concentration of test agent calculated to produce 50 per cent disruption of mast cells (estimated from log dose/response plot).

‖ Trasylol = zymofren (Societe Parisienne d'Expansion Chimique—S.A., Lot No. ES 1402) present in a concentration of 10 kallikrein-inhibiting units per ml.

Preliminary evidence was also obtained which suggested that the action of PMNL lysosomal cationic protein upon mast cells was not simply due to the basic groups on these protein molecules, but rather may have been associated with some as yet unidentified, specific component of the granules, present only in the 20 per cent ethanol-precipitated fraction of acid extracts of the lysosomes (see Discussion).

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In view of Archer's recent report (10) that a basic protein fraction derived from rat eosinophil granules is capable of rupturing rat mast cells *in vitro* and is also especially rich in peroxidase activity, the mastocytolytic fraction of PMNL lysosomal cationic protein was also tested for peroxidase activity in our experiments. Using an assay system for this enzyme based on the colorimetric measurement of the rate of oxidation of *o*-dianisidine, and possessing a sensitivity sufficient to detect 0.01 μg of crystalline horseradish peroxidase (Sigma Chemical Company, St. Louis), it nevertheless proved impossible to detect any activity of this enzyme in as much as 100 μg of the cationic protein fraction.

Finally, it can also be seen from the data in Table II that the mast cell–rupturing activity of each of the PMNL lysosomal extracts was not inhibited in the presence of salivary kallikrein-inhibitor, a broad-spectrum proteolytic enzyme-inhibiting drug (zymofren, trasylol, S.A., Lot No. ES 1402, Societe Parisienne d'Expansion Chimique, Paris). At the concentration of the drug employed in these tests (see Table II) the protease-inhibitor produced, by itself, a slight increase in the percentage disruption of mast cells over control values.

Effect of PMNL Lysosomes Upon Vascular Permeability in Rats Pretreated With a Mast Cell–Depleting Agent or an Antihistamine Drug.—Having made the observation that PMNL lysosome fractions possessed mastocytolytic activity *in vitro* and also produced vascular permeability changes in rat tissues which closely resembled the known vascular responses to vasotropic agents contained in rat mast cells, it seemed appropriate to investigate the effect of prior reduction in numbers of mast cells or prior treatment with an antihistaminic upon the vascular reactions of rats to these lysosomal materials.

In order to reduce the mast cell population in the skin, animals were given repeated injections of compound 48/80 according to the scheme proposed by Riley (11). The agent was administered intraperitoneally twice daily in the following doses: 100 μg on the 1st day, 200 μg on the 2nd, 300 μg on the 3rd, and 400 μg on the 4th and last day. Animals were then tested 24 hours after the final injection of the compound. Other rats were injected intravenously with the antihistamine drug chlorpheniramine maleate (Schering Corporation, Bloomfield, New Jersey), 6 mg per 100 gm body weight, immediately before carrying out the permeability tests. The results of these experiments are shown in Table III. It can be seen that chronic pretreatment with compound 48/80 almost completely suppressed the vascular permeability changes normally induced in skin by 10 μg of lysosomal cationic protein or by 10 μg of protein from frozen-thawed granules. In addition, the effects of the cationic protein (20 μg quantities) in the microcirculation of cremaster muscle were also blocked by this pretreatment. Injection of an antihistaminic prior to the test also inhibited the effects of frozen-thawed granule supernate and cationic protein

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upon vessel permeability in rat skin, although the degree of suppression was slightly less pronounced than in the 48/80 pretreated group. Cremaster tests were not performed in antihistamine-pretreated animals. Although not shown in the table, pretreatment with this antihistaminic also markedly reduced the permeability response in rat skin to doses of lysosomal cationic protein as large as 50 and 100 μg per test site.

In several of the antihistamine-pretreated rats, representative skin sites were excised 40 minutes after intracutaneous injection of lysosomal cationic

TABLE III

The Effect of Mast Cell Depletion and an Antihistamine Drug upon Vascular Permeability Changes in Rats Induced by PMNL Lysosomes

Pretreatment	Lysosome fraction* μg protein per test site	Test site	No. of rats	Permeability response‡
None	C.P./10	Skin	20	++++
	F.T./10	Skin	12	++++
	C.P./20	Cremaster	6	++
Chronic 48/80§	C.P./10	Skin	10	0 to ±
	F.T./10	Skin	4	0 to ±
	C.P./20	Cremaster	4	0 to ±
Single injection of chlorpheniramine maleate ‖	C.P./10	Skin	10	± to +
	F.T./10	Skin	4	± to +

* C.P. = cationic protein fraction (20 per cent ethanol); F.T. = extract of frozen-thawed granules.

‡ Permeability changes were evaluated in all animals during the period of maximal response (injection of dye or carbon suspension at zero minutes).

§ Intraperitoneal injections twice daily (doses are stated in accompanying text); animals tested 24 hours after the last dose.

‖ 6 mg per 100 gm body weight administered intravenously immediately prior to test.

protein and were formalin-fixed and stained with Harris' hematoxylin-eosin. This was done in order to compare the leukocyte emigration occurring in response to the lysosomal cationic protein (3) in normal rats with that occurring in animals whose permeability responses had been inhibited by the antihistamine drug. Although the semiquantitative techniques used in this study did not permit a meaningful comparison of the degrees of inhibition of the two reactions (permeability response *vs.* leukotactic response), it was clear that the latter reaction was also partly inhibited in the skin of antihistamine-pretreated rats. However, in the rat mesentery, chlorpheniramine maleate did not sup-

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press margination of leukocytes caused by the cationic protein. This question is currently undergoing further study.

DISCUSSION

The foregoing observations indicate the presence of a mastocytolytic agent in the lysosomes of rabbit exudate polymorphonuclear leukocytes. This mastocytolytic agent is specifically contained in PMNL granules and is not an unrelated cytoplasmic substance contaminating the granule preparation. This is clearly shown by the fact that the granule-free cytoplasmic fraction of homogenized leukocytes possesses no demonstrable mast cell–rupturing activity, even when tested at protein concentrations 10 times greater than that which produces 50 per cent rupture of mast cells in the case of granule protein (12).

The active principle can be extracted from PMNL granules by weak mineral acid and is retained in the cationic protein fraction precipitated by ethanol at a 20 per cent concentration. The mastocytolytic activity present in this fraction of lysosomal basic protein does not appear to be due to non-specific effects of positive charges. This is shown by the fact that a second basic protein fraction of the granules, precipitated from the acidified extract at 45 per cent concentration of ethanol and containing more positively charged species of protein (as judged from electrophoretic data), is inactive against mast cells *in vitro* at concentrations which produce significant disruption of cells in the case of the 20 per cent ethanol fraction (12). Furthermore, neither arginine-rich nor lysine-rich preparations of calf-thymus histones produce significant mastocytolysis *in vitro* at concentrations 10 times greater than the ED_{100} concentration of the active PMNL basic protein fraction (12). These observations suggest that the activity present in the 20 per cent ethanol fraction of PMNL lysosomal cationic protein may be due to a highly specific component of this fraction acting through a mechanism independent of simple charge effects. Studies currently in progress, designed to further fractionate the active mixture of cationic proteins, may provide additional information concerning this aspect of the problem.

Previous work (3) has already shown that the active cationic protein fraction is free of a number of the hydrolytic enzymes present in crude extracts of PMNL lysosomes; and, in the experience of other workers (4) such fractions are also devoid of catheptic activity. Moreover, the mastocytolytic effect of this material *in vitro* is not suppressed by a broad-spectrum protease inhibitor (trasylol). These tests were carried out at the highest concentration of the antiprotease drug which can be employed without causing damage to the mast cells sufficient to interfere with the assay. Unlike the experience of Archer (10) with mastocytolytic basic protein obtained from eosinophil granules, the active PMNL fraction does not contain detectable levels of peroxidase. Earlier work (3) has also shown that the active PMNL fraction is non-pyrogenic and, on

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this basis, the mastocytolytic agent present in PMNL granules would not appear to be related to the leukocyte permeability factor ("granulocytic substance") described by Moses and coworkers (13).

Frimmer has recently extended his studies on the general mechanism of action of nuclear histones and basic polypeptides upon vascular permeability to include investigations of artificial membrane systems (14). He suggests a mechanism of altered permeability in his membrane system based on physicochemical changes in acid-mucopolysaccharides caused by their interaction with basic protein. Thus, there may be additional modes of action of PMNL lysosomal cationic proteins upon vessel wall permeability *in vivo* in addition to the indirect effect mediated through mast cell degranulation. It should be noted that the permeability-enhancing lysosomal cationic protein fraction was tested by us for acid-mucopolysaccharases and was found to be free of measurable chondroitin sulfatase and hyaluronidase activity (turbidity-reduction assay method of Tolksdorf *et al.* (15), modified after Kass and Seastone (16)).

The data presented in this paper show that the cationic protein is less active than compound 48/80 when the two are compared on a weight per weight basis. Until the PMNL factor is identified and characterized it will not be possible to accurately compare its potency with that of compound 48/80 on a molar weight basis, but reasonable estimates can be made on the grounds of presently available facts. Since the mastocytolytic principle of PMNL granules is not dialyzable (see Materials and Methods) it can be assumed to possess a molecular weight of at least 10,000. On the other hand, the molecular weight of the active form of compound 48/80 is known. It has been reported (17) that the trimer (mol wt = about 500) and tetramer (mol wt = about 650) forms of the compound are active, while polymers of the pentamer class or higher are inactive. Thus, if the two mastocytolytic principles are compared on the basis of estimated molar weights, the PMNL principle is highly active with reference to compound 48/80.

In conclusion, while these studies do not exclude the possible role of lysosomal acid proteases in terminal phases of necrotizing tissue injury reactions such as the Arthus reaction (1, 2) or the dermal Shwartzman reaction (18, 19), the present studies do show that the early phase of vascular injury induced in rat tissues by extracts of frozen-thawed PMNL lysosomes, or by certain of the cationic proteins isolated from these granules, is mediated by vasotropic agents released from disrupted mast cells rather than by proteases present in the leukocyte granules themselves. It therefore seems reasonable to suggest that PMN leukocytes entering perivascular tissue spaces during naturally-occurring tissue injury reactions, release their lysosomal granules and that these, in turn, may affect local mast cells in a manner analogous to that described in the *in vitro* studies reported here. Such a reaction would constitute

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a distinct mechanism by which leukocyte lysosomes play a part in the pathogenesis of inflammation.

SUMMARY

The vascular permeability-increasing action of rabbit PMNL lysosomes has been studied in skin and cremaster muscle of the rat.

Both an extract of frozen-thawed granules and a cathepsin-free cationic protein fraction of the granules (which had previously been demonstrated to cause leukocyte adhesion and emigration *in vivo*) induce increased vascular permeability in skin and muscle which resembles that produced by histamine or histamine-liberators with respect to the timing of the response and the predominant type of microvessel affected.

Extracts of frozen-thawed lysosomes and the inflammatory lysosomal cationic protein both cause disruption of rat mesenteric mast cells *in vitro*, whereas a granule-free cytoplasmic fraction of PMN leukocytes and a non-inflammatory cationic protein fraction of the granules do not do so under identical test conditions. The mastocytolytic action of lysosomal materials *in vitro* is not inhibited in the presence of 10 kallikrein-inhibiting units of trasylol per ml. The mast cell rupturing fraction of PMNL granules (cationic protein) possesses no detectable peroxidase activity or acid-mucopolysaccharase activity. When compared with compound 48/80 on the basis of estimated molecular weight, the lysosomal cationic protein appears to be at least as active as the latter compound with respect to *in vitro* mastocytolytic potency.

Chronic pretreatment of rats with an agent known to reduce tissue mast cell numbers causes marked suppression of the vascular permeability change normally induced in skin and muscle by lysosomal extracts and cationic protein. Similar results are obtained if lysosomal materials are tested in rats pretreated with an antihistaminic.

These observations are discussed with respect to the mode of action of PMNL lysosomes in the early and late phases of local tissue-injury reactions.

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