

Mediators of Histamine Release from Human Platelets, Lymphocytes, and Granulocytes

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ABSTRACT Lysates of mixed human leukocyte suspensions released histamine from intact human leukocytes in vitro. Microgram quantities of leukocyte lysate protein released up to 90% of the total available histamine. The mixed leukocyte lysates were separated by differential centrifugation into nuclear (800 *g* pellet), lysosomal (25,000 *g* pellet), and postlysosomal supernatant (25,000 *g* supernatant) fractions. The degree of separation of the lysosomal from the other two fractions was assessed by measuring the relative activities of four lysosomal enzymes. The average distribution of enzyme activity was $11 \pm 2\%$ (mean ± 1 SD), $72 \pm 10\%$, and $17 \pm 8\%$ for the nuclear, lysosomal, and supernatant fractions respectively. The histamine-releasing activity was equally distributed between the lysosomal and supernatant fractions, each of which had 5-fold greater activity than the nuclear fraction.

Purified suspensions of platelets, lymphocytes, and granulocytes were prepared, and the lysates of these suspensions all had histamine-releasing activity. Centrifugation at 100,000 *g* for 18 hr sedimented the histamine-releasing activity from all three types of lysate. After 20% ethanol fractionation for the preparation of cationic protein, only the activity from the platelet lysates was found in the 20% ethanol insoluble fraction.

These mediators of histamine release from human platelets, lymphocytes, and granulocytes may play a role in the development of the vasodilation and increased vascular permeability which characterize the acute inflammatory response.

INTRODUCTION

The acute inflammatory response is characterized by certain vascular phenomena including vasodilation and increased vascular permeability. Various naturally oc-

curing cellular constituents have been implicated as mediators of these vascular responses. Among these, histamine has proven to be particularly important since it is responsible for the early stages of vasodilation and increased vascular permeability in the venules and capillaries of the microcirculatory apparatus. (1) Accordingly, there has been considerable interest in the mechanisms of histamine release in inflammation.

Extracts of various animal tissues have been found to release histamine from rat peritoneal mast cells, (2-4) and cationic proteins from rabbit and guinea pig leukocyte lysosomes have a similar activity (5). Cationic proteins from human platelet granules have also been shown to release histamine from rat mast cells (6). Histamine-releasing activity was detected in extracts of human white blood cells several years ago, (2) but recent attempts to demonstrate such activity have failed (5) in spite of the fact that large quantities of cationic protein have been found in human platelet and leukocyte lysosomes (7).

The present study was undertaken to determine whether preparations made from human platelets and leukocytes contain histamine-releasing activity. It was found that lysates of human platelets, lymphocytes, and granulocytes released histamine from human leukocytes in vitro.

METHODS

Preparation of leukocytes. The leukocytes used throughout this study were prepared as follows: blood was drawn through 18 gauge needles attached to plastic tubing and allowed to drain by gravity into polypropylene centrifuge tubes containing one part sedimentation fluid and two parts blood. The sedimentation fluid contained 3% Dextran (mol wt 188,000) in 2.5% glucose and normal saline with 20 units heparin/ml or 0.017 M-EDTA (ethylenediaminetetraacetic acid) as anticoagulant. After mixing, the erythrocytes were allowed to sediment $\frac{2}{3}$ the length of the tube at room temperature. The resulting platelet and leukocyte-rich supernatant was aspirated and prepared for use in the procedures outlined below.

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Separation of platelets, lymphocytes, and granulocytes. Leukocyte suspensions were prepared from 150–200 ml blood using heparin anticoagulated dextran sedimentation fluid. The leukocytes were centrifuged at 150 *g* for 10 min at room temperature (Sorvall centrifuge model SP/X, Ivan Sorvall, Inc, Norwalk, Conn.). Preliminary experiments revealed that most of the platelets sedimented with the leukocytes due to clumping or adherence to the leukocytes. To collect the remaining platelets and to prepare platelet-free plasma-dextran-heparin solution for subsequent use in the glass bead column fractionation, the 150 *g* supernatant plasma was centrifuged at 4300 *g* for 15 min at 2°C (Sorvall centrifuge model SS-34, Ivan Sorvall, Inc, Norwalk, Conn.). The 150 *g* pellets were resuspended in 20 ml of plasma-dextran-heparin solution since the presence of plasma was required for the glass bead column fractionation. A 5 ml portion of this suspension was made platelet rich by the addition of the 4300 *g* plasma pellets. This platelet-rich suspension was washed once in Tris A buffer (tris(hydroxymethyl)aminomethane plus albumin) (8) and resuspended in a final volume of 5 ml Tris A. Plasma was not included in this suspension since the gradient separation described below was more efficient without plasma.

Platelets were separated from the other formed elements by layering this suspension over two discontinuous sucrose gradients composed of 4-ml layers of 40, 35, 30, 25, 20, and 15% sucrose in Tris A. The gradients were centrifuged at 150 *g* for 6 min at 2°C in a swinging bucket rotor (IEC centrifuge model PR-2 or B-20, International Equipment Co., Needham Heights, Mass.). The leukocyte and erythrocyte-free platelet bands which formed at the tops of the gradients were aspirated and centrifuged at 2500 *g* for 30 min at 2°C. The platelet buttons were resuspended in 20 ml of cold Tris A.

Lymphocytes and granulocytes were separated from the remaining leukocyte suspension in plasma using a glass bead column technique essentially the same as that described by Rabinowitz (9) except that 35-ml plastic syringe barrels rather than glass tubing were used to prepare the glass bead columns, and Tris A was used for buffering throughout. In order to reduce erythrocyte and platelet contamination, the lymphocyte and granulocyte suspensions were each incubated at 37°C for 20 min in a solution containing one part pH 7.4 Tris buffer and nine parts 0.83% ammonium chloride. The cells were then collected by centrifugation at 150 *g* for 10 min and resuspended in Tris A at 1.0×10^6 cells/ml.

Using these procedures, platelet suspensions were obtained that were essentially free of other types of formed elements. Lymphocyte suspensions were 95–100% pure while granulocyte suspensions were 90–98% pure. Both had very little or no platelet and erythrocyte contamination.

Subcellular fractionation of leukocytes. Hypotonic sucrose lysates of leukocyte suspensions were prepared, and leukocyte lysis was 98–100% complete as monitored by microscopic examination and leukocyte counts. These lysates were separated by differential centrifugation into 800 *g* sedimented nuclear fractions, 25,000 *g* sedimented lysosomal fractions, and 25,000 *g* postgranular supernatant fractions according to the method of Chodirker, Bock, and Vaughan (10). All the fractions were treated by rapid freezing and thawing 6 times or by sonification to release any latent activity.

Preparation of leukocyte lysates. Mixed leukocyte suspensions, prepared by dextran sedimentation, as well as suspensions of purified platelets, lymphocytes, or granulocytes, were ruptured in most of the experiments by 5 min

of sonification using a Biosonic III ultrasonic disintegrator (Bronwill Scientific, Inc, Rochester, N. Y.) with care to avoid heating the suspensions. Early in the study, lysates were prepared by six cycles of rapid freezing and thawing. After final preparation, all lysates and extracts were either used immediately or quick-frozen and stored at –70°C until use. Since lysates began to lose histamine-releasing activity after 3 wk storage at –70°C, all assays were carried out within 1 wk of lysate preparation.

Preparation of cationic protein. Platelet, lymphocyte, and granulocyte lysates were prepared and centrifuged at 100,000 *g* for 18 hr (Beckman model L ultracentrifuge, Beckman Instruments, Inc., Fullerton, Calif.). The resulting pellets were then fractionated according to the method of Zeya and Spitznagel (11) for the preparation of cationic protein. This involved extraction for 30 min in dilute H₂SO₄ followed by fractionation of the acid extracts with 20% ethanol.

Protein and enzyme assays. The protein concentrations of the extracts and lysates used in these studies were determined by the technique of Lowry, Rosebrough, Farr, and Randall (12) using crystalline lysozyme as a standard. The activities of the lysosomal enzymes, muramidase, β -glucuronidase, acid phosphatase, and cathepsin were determined according to the methods described by Cohn and Hirsch (13).

Histamine release assay. The method developed by Lichtenstein and Osler (8) for the measurement of allergic histamine release *in vitro* was adapted to assess the histamine-releasing activity in these studies. Serial dilutions of cell extract were used in place of antigen.

RESULTS

Histamine release with mixed leukocyte lysates. The initial observations made in this study were that lysates of mixed leukocyte suspensions released histamine from intact human leukocytes in the *in vitro* system (Fig. 1). Leukocyte suspensions prepared by dextran sedimentation were found to contain appreciable numbers of platelets, lymphocytes, monocytes, and granulocytes as well as erythrocytes. Leukocyte suspensions were adjusted to contain 1×10^6 cells/ml. Lysates of these suspensions were prepared and their protein concentrations ranged from 600 to 1500 μ g protein/ml. These lysates were serially diluted over a range of 100- to 100,000-fold, and the histamine-releasing activity of each dilution was determined.

A large percentage of the total available histamine was released by small amounts of leukocyte lysate (Fig. 1). The shape of the dose-response curve presented in Fig. 1 is typical of the results obtained throughout these studies. Histamine release increased in a nearly linear fashion with increases in the log of the extract concentration until a maximum was reached. The amount of histamine available for assay then decreased linearly with further increases in extract concentration suggesting that the histamine release process was inhibited or the histamine was bound or destroyed after release.

Since the nature of this process is unknown, a method of expressing the histamine-releasing activity of differ-

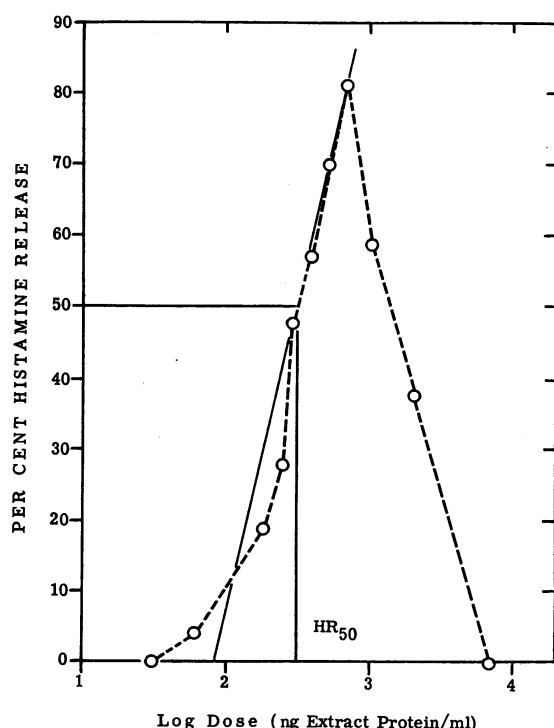


FIGURE 1 Dose-response curve of histamine release. Serial dilutions of leukocyte lysate were incubated with leukocytes, and histamine release was determined as described in the text. The results are plotted as the per cent of the total available histamine released *versus* the \log_{10} of the nanograms/milliliter of lysate protein in each serial dilution. The dose required for 50% histamine release (HR_{50}) was determined as described in the text.

ent lysates and extracts was developed based upon the increasing portion of the dose-response curve. The activity was expressed as the lowest concentration of extract which resulted in 50% histamine release (HR_{50}). The HR_{50} was determined for each extract by constructing the most probable line through the points on the increasing portion of the dose-response curve as presented in Fig. 1. If this line did not pass through the 50% release coordinate, it was extended to reach that point. The protein concentration corresponding to 50% histamine release was then determined by extrapolation, and the HR_{50} is expressed as ng (nanogram) extract protein/ml.

Histamine-releasing activity was not significantly different whether autologous or homologous human cells were used in the assay. This finding permitted the use of homologous leukocytes in the assays of certain extracts although the cells of a single donor were used for both the lysates and assays as much as possible. Experiments were done to insure that histamine-releasing activity was not induced by exposure of the leukocytes to dextran. Lysates prepared from buffy coat leukocytes

had comparable activity to those prepared from leukocytes after dextran sedimentation. Also Tris A buffer did not induce histamine-releasing activity since lysates of leukocytes in distilled water, normal saline, and 0.34 M-sucrose all had histamine-releasing activity. All mixed leukocyte lysates in approximately 100 separate experiments involving the leukocytes of 30 different individuals had histamine-releasing activity although the amount of histamine released varied from individual to individual.

Cellular localization of histamine-releasing activity. The mixed leukocyte preparations contained platelets, lymphocytes, granulocytes, and monocytes as well as erythrocytes. To determine which of the formed elements present in these suspensions contained the histamine-releasing activity, purified suspensions of platelets, lymphocytes, and granulocytes were prepared by sucrose gradient centrifugation and glass bead column fractionation. Lysates of these purified suspensions were prepared and assayed for histamine-releasing activity.

In 10 separate experiments, lysates of purified platelets, lymphocytes, and granulocytes all contained significant histamine-releasing activity (Fig. 2). Therefore, the histamine-releasing activity observed in the mixed leukocyte preparations was found in all three

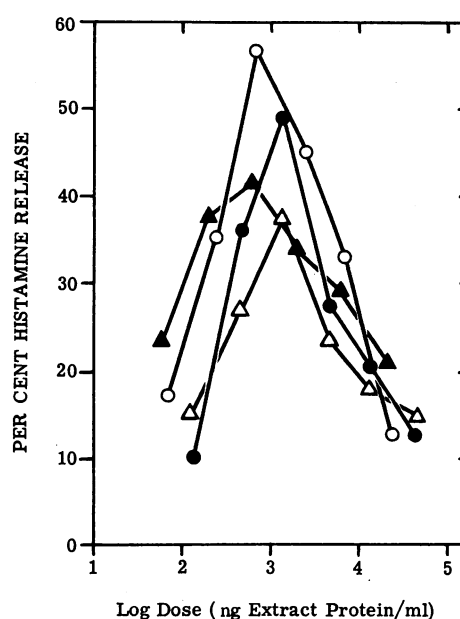


FIGURE 2 Histamine-releasing activity in lysates of human platelets, lymphocytes, and granulocytes. Serial dilutions of lysates of purified human platelet, lymphocyte, and granulocyte suspensions were incubated with intact leukocytes, and histamine release was determined as described in the text. (○) Mixed leukocyte lysate; (●) platelet lysate; (△) lymphocyte lysate; (▲) granulocyte lysate.

TABLE I
Sedimentation of Histamine-Releasing Activity at 100,000 g

Fraction	Histamine-releasing activity (HR ₅₀)*		
	Plate- let	Lympho- cyte	Granulo- cyte
100,000 g pellet	288	355	288
100,000 g supernatant	7,940	24,000	1,000,000

* HR₅₀, amount of protein required for 50% histamine release expressed as nanograms protein/milliliter.

types of formed elements which made up the bulk of these preparations.

Sedimentation of histamine-releasing activity at 100,000 g. To obtain preliminary information about the physical nature of the histamine-releasing activity in platelets, lymphocytes, and granulocytes, lysates of each of these elements were centrifuged at 100,000 g for 18 hr at 2°C (Beckman model L Ultracentrifuge, Beckman Instruments, Inc, Fullerton, Calif.). The resulting pellets were resuspended in 10 ml of Tris A and assayed for histamine-releasing activity along with the supernatants. Most of the histamine-releasing activity was found in the 100,000 g pellets with relatively little activity in the supernatant fractions for all three types of formed elements (Table I).

20 per cent ethanol fractionation of platelet, lymphocyte, and granulocyte extracts. In view of the previous observations that lysosomal cationic proteins from human platelets and rabbit granulocytes have histamine-releasing activity, (5, 6) attempts were made to isolate such proteins from the lysates used in these studies. 20 per cent ethanol precipitates were prepared from purified platelets, lymphocytes, and granulocytes and subsequently assayed for histamine-releasing activity.

Most of the histamine-releasing activity from purified platelets was retained in the 20% ethanol insoluble fraction, and there was an approximately 10-fold purification of the activity (Table II). On the other hand, the 20% ethanol precipitates of the lymphocyte and granulocyte lysates had less histamine-releasing activity than their corresponding unfractionated lysates.

Intracellular localization of histamine-releasing activity. To determine the intracellular localization of the histamine-releasing activity, mixed leukocyte lysates were separated by differential centrifugation into 800 g nuclear fractions, 25,000 g lysosomal fractions, and post-25,000 g supernatant fractions. The degree of separation of the lysosomal from the other two fractions was assessed by determining the specific activities of four lysosomal enzymes in each fraction. The histamine-releasing activities of each fraction were determined.

TABLE II
20% Ethanol Fractionation of Platelet, Lymphocyte, and Granulocyte Histamine-Releasing Activity

Preparation	Histamine releasing activity (HR ₅₀)*		
	Plate- let lysate	Lympho- cyte lysate	Granulo- cyte lysate
Unfractionated	1,900	11,800	4,300
20% Ethanol insoluble	200	105,000	110,000

* HR₅₀, amount of protein required for 50% histamine release expressed as nanograms protein/milliliter.

The lysosomal fraction contained most of the muramidase, acid phosphatase, β -glucuronidase, and cathepsin activity (Table III). In five experiments, the average distribution for these four enzymes, expressed as per cent of the total activity, was 11% nuclear, 72% lysosomal, and 17% supernatant, indicating good separation of the lysosomal fractions. In contrast, histamine-releasing activity, expressed as the average HR₅₀, was about equally distributed between the lysosomal and supernatant fractions with less activity in the nuclear fraction.

DISCUSSION

In the present studies, we have demonstrated that lysates of human leukocytes and platelets have in vitro histamine-releasing activity. Our studies are unique in that the system used to detect histamine-releasing activity made use of human leukocytes rather than rat or rabbit mast cells or skin as used in other systems. In this way we were able to obviate the difficulties encountered in crossing species barriers and test the effects of human cell extracts directly on human cells.

Our studies indicating that lysates of mixed leukocyte suspensions are capable of releasing histamine, are in general agreement with the work of Archer (4). In

TABLE III
Subcellular Localization of Histamine-Releasing Activity

Subcellular fraction	Lysosomal enzyme activity*	Histamine- releasing activity (HR ₅₀)*
Nuclear	11 \pm 2	11,588 \pm 8,506
Lysosomal	72 \pm 10	2,360 \pm 3,180
Postlysosomal supernatant	17 \pm 8	2,193 \pm 3,422

* Average per cent of the total activity of the lysosomal enzymes, acid phosphatase, β -glucuronidase, muramidase, and cathepsin, expressed as the mean \pm 1 SD for five experiments. HR₅₀, amount of protein required for 50% histamine release in nanograms protein/milliliter expressed as the mean \pm 1 SD for five experiments.

the latter studies, saline extracts of various rat and rabbit tissues and of human white blood cells were prepared, and they induced histamine release from rat peritoneal mast cells in vitro. In more recent studies, Scherer and Janoff (5) and Seegers and Janoff, (14) extracted cationic proteins from rabbit and guinea pig exudative granulocytes and found that these proteins caused mast cell degranulation. However, their attempts to isolate similar proteins from human circulating or exudative granulocytes were not successful, possibly due to their use of nonhuman cells or inappropriate extract concentrations in the histamine release assay (5). The present studies provide evidence that human lymphocytes and granulocytes do contain histamine-releasing activity. However, this activity is probably not lysosomal cationic protein.

Cationic proteins are characterized by their association with the leukocyte lysosomal fraction and by their precipitation in 20% ethanol (5, 11). The results of our subcellular fractionation studies (Table III) indicate that the histamine-releasing activity in human leukocytes is associated with both the lysosomal and post-granular supernatant fractions. It is possible that the histamine-releasing activity is located in the lysosomes and in the cytoplasm of the cells, or the histamine-releasing activity may leak out of the lysosomal granules while the lysosomal enzymes do not. In either case, the histamine-releasing activity from human leukocytes does not conform to the characteristics of cationic protein with regard to subcellular localization. The 20% ethanol fractionation studies (Table II) provide additional evidence that the histamine-releasing activity in human lymphocytes and granulocytes is not cationic protein since relatively little activity was found in the 20% ethanol insoluble fractions from these elements. Although these data do not exclude cationic proteins as agents of histamine release in human lymphocytes and granulocytes, they do suggest that other factors may play a more important role.

In a recent study published by Nachman, Weksler, and Ferris (6) cationic proteins were extracted from human platelet granules and shown to release histamine from rat mast cells. Our studies confirm these results since we found histamine-releasing activity in human platelet lysates, and this activity could be extracted by a method for the preparation of cationic protein. Our findings also extend the work of Nachman et al. (6) since we were able to demonstrate that platelet extracts induce histamine release from human cells.

Materials other than cationic proteins are known to induce histamine release. Arginine or lysine-rich histones and poly-L-lysine have been shown to release histamine from mast cells, (15) and rat eosinophil peroxidase has been implicated as a mediator of histamine

release (4). Although it is possible that some of these substances are active in our system, identification of the histamine-releasing agents in human lymphocytes and granulocytes must await purification of the active principles involved.

Several mechanisms of histamine release are known to exist. Uvnäs (16) suggested that histamine release induced by compound 48/80 involves two stages: granule extrusion and exchange of granule histamine for extracellular cations. Levy (17) summarized the available data on the mechanism of allergic histamine release. These data suggest that histamine release is a secretory process similar to the secretion of insulin or catecholamines. Release of histamine after mast cell injury was described by Uvnäs, (18) and Henson (19) described complement-dependent histamine release from rabbit platelets by a lytic process.

The mechanism of histamine release and the nature of the dose-response curve are currently under investigation in our laboratory. Data available at present suggest that our system is unlike any of those mentioned above. It resembles the allergic histamine release system in certain respects, but it differs in that thermally-injured leukocytes have enhanced rather than abolished histamine-releasing activity in our system. Preliminary observations of leukocyte viability, using trypan blue exclusion, suggest that the inhibition of histamine release at high lysate concentrations in the dose-response curve (Fig. 1) is not due to a cytotoxic effect (unpublished observations). It appears that histamine release is actually inhibited at high lysate concentrations since the histamine remains in association with the leukocytes after incubation (unpublished observations). The mechanism of histamine release, the nature of the dose-response curve, and the identity of the histamine-releasing factor(s) are important areas for future investigation.

The present studies indicate that human peripheral blood platelets, lymphocytes, and granulocytes harbor mediators of histamine release. Considerable evidence has been accumulated to support the role of histamine as a mediator of the changes in the microcirculation which are characteristic of the acute inflammatory response (1). While many details need to be supplied, the progression from vasodilatation and altered vascular permeability through infiltration of the inflamed area by leukocytes has been outlined in broad detail by sequential morphological studies (20). To understand the sequence of events from the first stimulus to the release of vasoactive peptides which cause changes in the microcirculation requires new approaches which investigate the biochemical interactions involved.

Most of the histamine in the body is sequestered in storage granules in tissue mast cells and circulating basophils. Mast cells are particularly abundant in peri-

vascular tissues, where they might be affected by chemical mediators from formed elements in the blood. Similarly, circulating basophils would be a position for intimate contact with these chemical mediators.

A variety of diverse stimuli may be followed by histamine release. Anaphylactic histamine release occurs when an antigen reacts with specific cell-associated antibody to initiate the release mechanism. In the case of inflammation accompanying bacterial infections, the interaction of bacterial antigens, such as staphylococcal protein A, with cells containing histamine may be one means of initiating the release process (21).

The mediators of histamine release that we have investigated may be important in the intermediary steps between a number of diverse stimuli and the release of histamine. Leukocytes and platelets are distributed to all tissues by the circulation, and some lymphocytes and polymorphonuclear leukocytes are normally present in the extravascular spaces of tissues. The leukocyte and platelet factors in the present studies are active in very low concentrations *in vitro*. It is probable that release of these factors from a very small number of leukocytes or platelets *in vivo* could achieve local concentrations that would mobilize histamine from nearby stores in mast cells or blood basophils, initiating the characteristic sequence of acute inflammation.

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