Characterization of Human Platelet
Vascular Permeability-Enhancing Activity

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ABSTRACT Human platelet acid extract obtained from both whole platelets and from isolated subcellular granules was partially purified by DEAE-cellulose chromatography and Sephadex gel filtration. The heat-stable, nondialyzable cationic protein fraction with a mol wt of approximately 30,000 produced a biphasic increase in vascular permeability in rabbit skin and also had antihistaminic activity. The acute (15 min) increase in vascular permeability was blocked by prior treatment of the animal with antihistamine and was characterized histologically by edema of perivascular tissues and dilation of capillaries and veins. The delayed (3 hr) permeability effect was not blocked by an antihistamine and was characterized histologically by leukocytic infiltration into the skin. The experiments described suggest that human platelet lysosomal release of cationic proteins may increase vascular permeability by several mechanisms including endogenous histamine release as well as delayed chemotaxis.

INTRODUCTION

Recent studies suggest that platelets may contribute to the inflammatory response accompanying tissue injury by the release during such interactions of intracellular constituents which increase vascular permeability (1, 2). We have previously demonstrated that a cationic protein extract obtained from human platelet granules increased vascular permeability in both mouse and rabbit skin presumably by inducing histamine release from tissue mast cells (3). The cationic protein fraction itself did not contain histamine, serotonin, kallikrein, plasmin, Cl or PF/dil. The present studies were undertaken to analyze in greater detail the cationic permeability protein(s) of human platelets. Human platelet acid extract was partially purified by DEAE-cellulose chromatography and Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) gel filtration. The heat-stable, non-dialyzable platelet permeability factor with a mol wt of approximately 30,000 produced a biphasic permeability response in rabbits. The acute (15 min) permeability effect was blocked by prior treatment of the animal with antihistamine while the delayed (3 hr) permeability effect was not blocked by an antihistamine and was characterized by leukocytic infiltration into the skin.

METHODS

Preparation of platelet fractions

Human platelet concentrates prepared from 10-20 U of freshly collected whole blood in acid citrate dextrose anticoagulant were generously supplied by the New York Blood Center. The concentrates were processed within 24 hr of the day of collection. Platelets were separated as previously described (3). Contamination of platelets by leukocytes in multiple samples was less than one cell per 10,000 platelets. The isolated platelets were washed (3) and extracted three times with 2 ml volumes of ice-cold 0.2 N H2SO4. The combined acid extracts were cleared by centrifugation at 12,000 g, pooled, neutralized by dropwise addition of 1.0 N NaOH and lyophilized. Before experimental use the human platelet acid extracts (HPAE)1 were dialyzed for 4 hr at 10°C against three changes of buffered saline pH 7.4 (0.15 M NaCl, 0.0175 M phosphate). It was noted that biologic activity diminished with storage in solution at 4°C. Thus in the experiments described below studies were performed with freshly dialyzed material obtained from stored lyophilized extract.

Isolated platelet granules were obtained and processed as previously described (4). Acid extract was prepared from pooled lyophilized platelet granules as described above for the whole platelet preparation.

Vascular permeability tests

Permeability-inducing activity of the test samples was evaluated by intradermal injection of 0.1 ml of the sample into the skin of a rabbit which had previously received an

1 Abbreviations used in this paper: HPAE, human platelet acid extracts; PRP, platelet-rich plasma; WBC, white blood cells.
intraocular injection of Evans blue. New Zealand white rabbits weighing 3–5 kg were injected intravenously with Evans blue (1.0 ml/kg of a 0.5% solution). Beginning 5 min later unless otherwise noted, 0.1 ml of test material was injected intracutaneously into the shaved depilated dorsal skin using No. 26 gauge disposable needles. Permeability-enhancing activity was expressed in terms of the average diameter of duplicate lesions in two or three rabbits. The permeability reactions were read 15 min and 3 hr after the challenging intradermal injection of the test sample.

The effect of the antihistamine, chlorpheniramine maleate, upon the permeability-enhancing properties of the HPAE or the isolated protein fractions was tested by intradermal injection of chlorpheniramine maleate 0.1 mg 30 min before the Evans blue injection. The test agents were then injected into the pretreated intradermal sites. In some experiments the antihistamine was given intraperitoneally (10 mg) 1 hr before the Evans blue injection.

### Table I

<table>
<thead>
<tr>
<th>Mixture tested</th>
<th>Permeability activity 15 min</th>
<th>Permeability activity 180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Buffer 30 min after intradermal</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Chlorpheniramine 0.1 mg</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>HPAE†</td>
<td>11.2</td>
<td>19.0</td>
</tr>
<tr>
<td>HPAE‡ 30 min after intradermal</td>
<td>4.2</td>
<td>14.4</td>
</tr>
<tr>
<td>Chlorpheniramine 0.1 mg</td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

*mm, diameter in millimeters of bluing.
‡HAE, human platelet acid extract 224 µg/ml.

**Histamine release from rabbit WBC and platelets**

**WBC preparation.** New Zealand white adult rabbits were injected intraperitoneally with 500 ml normal saline containing 0.15% oyster glycogen (Calbiochem, Los Angeles, Calif.). 15 hr later the rabbits were killed and the peritoneal fluid drained into 5 cc 0.25% EDTA saline. Leukocytes were sedimented by centrifugation at 77 g for 5 min at room temperature and were washed three times in a Tris buffer (5) and resuspended in the same buffer containing Ca**++ 6 × 10^-4 M and Mg**++ 1 × 10^-4 M, at a concentration of 9 × 10^7 polymorphonuclear leukocytes/ml.

**Platelet preparation.** Arterial blood from rabbits was drawn by cannula (Medicut, A. S. Aloe Co., St. Louis, Mo.) into ½ volume 3.8% trisodium citrate and centrifuged at 100 g for 30 min at room temperature to yield platelet rich plasma (PRP). The platelet count was 800,000/mm³. PRP was centrifuged at 225 g for 25 min at room temperature to sediment platelets. They were washed three times in Tyrode’s solution lacking Ca or Mg and resuspended in Tyrode’s solution containing 2 × 10^-4 M of Ca**++ and Mg**++ at a concentration of 855,000/mm³.

Samples of WBC suspension, PRP, and washed platelets (1.5–2 ml) were placed in siliconized glass tubes and saline or HPAE 100 to 200 µl/ml of cells was added. After gentle mixing, the tubes were incubated at 37°C with shaking on a Lab Tek (Ames Laboratories, Westmont, Ill.) aliquot mixer for 15 min and then centrifuged at 1000 g at room temperature for 5 min. Supernate (0.5 ml for platelets, 1 ml for WBC) was extracted for histamine according to the fluorimetric method of Shore, Burkhalter, and Cohn (6) as modified by Gocke and Osler (7). Per cent histamine release was calculated from comparison with total histamine extracted from whole WBC or platelet suspensions.

**Platelet factor 4 assay**

Antihistaminic activity (platelet factor 4) of the platelet fractions was measured using the thrombin time system of Poplawski and Niewiarowski (8). The mixture included 0.3 ml human platelet poor plasma, 0.1 ml saline or test platelet extract, 0.1 ml heparin (0.3 U/ml). ½ ml of Parke, Davis (Parke, Davis & Co., Detroit, Mich.) bovine thrombin (10 U/ml) was added and the clotting time recorded with mixing at 37°C.

**Separation of the acid extract on diethylaminoethyl-cellulose (DEAE)**

The lyophilized acid extract was dissolved in 2 ml distilled water. This was centrifuged at 10,000 g at 4°C for 10 min. The small precipitate was washed with 1 ml water and once with 0.5 ml acetate buffer (0.01 M pH 4.0). The pooled supernate (3.5 ml) was applied by gravity to a Whatman DEAE-cellulose column (1 × 27.5 cm) equilibrated with phosphate buffer 0.01 M pH 8.0. After the first protein peak was eluted with the equilibrating buffer, a gradient of sodium chloride was established using 0.5 M NaCl in 0.01 M NaHPO₄ as the terminal buffer. The fractions collected were tested for vascular permeability-inducing activity in the skin of rabbits after adjustment to physiological pH and salt concentration.

**Separation of the permeability-inducing factor(s) by gel filtration on a column of Sephadex G-75**

The permeability-inducing protein fractions from the DEAE column were pooled, lyophilized, and redissolved in 2 ml distilled water. The fraction was dialyzed over 90 min against 4 liters of distilled water and then reconstituted to buffered saline 0.05 M acetate 0.1 M NaCl pH 4.0. This was applied to a Sephadex (Pharmacia Fine Chemicals, Piscataway, N. J.) G-75 column (1 × 89 cm) and eluted with 0.05 M acetate 0.1 M NaCl pH 4.0 buffer. The fractions collected were tested for the vascular permeability-inducing activity in the skin of rabbits after adjustment to physiological pH and salt concentration.

**Sucrose density gradient ultracentrifugation of the permeability-inducing factor(s)**

The permeability-inducing protein fractions from the Sephadex G-75 column were pooled, lyophilized, and redissolved in distilled water. The fraction was dialyzed over 90 min against 4 liters of distilled water and then reconstituted to buffered saline 0.15 M NaCl, 0.0175 M phosphate pH 7.4. The concentrated extract was applied to a 10–40% sucrose gradient in buffered saline and centrifuged at 50,000 rpm for 24 hr in a model L Spinco (Beckman Instruments, Inc., Fullerton, Calif.) ultracentrifuge using a SW65 rotor. Molecular weight markers used in these studies included trypsin 23,800 and egg albumin 44,300 (Mann Research Labs., New York). The fractions collected were tested for their vascular permeability-inducing activity in the skin of...
rabbits after adjustment to physiological pH and salt concentration.

Partial purification of the permeability activity derived from the acid extracted human platelet granules was carried out using DEAE-cellulose chromatography and Sephadex G-75 gel filtration.

Analytical polyacrylamide gel electrophoresis.

This was carried out as described by Reisfeld, Lewis, and Williams (9) using β-alanine buffer pH 4.5 at 5 mA per tube for 60 min. Gels were stained with Coomassie blue.

RESULTS

Biphasic permeability-enhancing activity of human platelet acid extract. The acid extract obtained from the human platelets increased vascular permeability in rabbit skin. A biphasic permeability effect was observed. Direct injection of HPAE into the skin of an Evans blue-treated rabbit produced an acute permeability effect clearly visible in 15 min (Table I). The secondary or delayed permeability effect was characterized by the slow but progressive increase in permeability at the intradermal challenge site reaching a maximum in approximately 3 hr. The acute (15 min) increase in vascular permeability was blocked by prior intradermal injection of chlorpheniramine. Administration of antihistamine, however, did not block the delayed (180 min) permeability effect (Table I). Systemic antihistamine given as chlorpheniramine 10 mg intraperitoneally 1 hr before the intradermal challenge with HPAE also blocked the acute permeability effect but not the delayed response. It was not possible to dissociate the acute and delayed permeability-inducing effect by heating or dialysis. The acute and delayed permeability-enhancing activities of HPAE were equally inhibited by boiling for 30 min and equally resistant to extensive dialysis or heating to 80°C for 30 min.

Histologic changes. Histological changes in the skin site 15 min after the HPAE injection consisted of slight edema of the perivascular tissue with some dilation of the capillaries and venules. In contrast, marked polymorphonuclear infiltration in the tissues was observed in the skin of an animal 2 hr after HPAE injection into an antihistamine pretreated site (Fig. 1). Thus, the acute permeability effect was characterized by edema and vasodilation without any evidence of polymorphonuclear accumulation while the delayed permeability effect was characterized by leukocytic infiltration.

Duration of the intradermal antihistamine blocking effect. In view of the biphasic permeability-enhancing effect of HPAE, the possibility was considered that the 2–3 hr delayed response in antihistamine pretreated animals represented clearance or inactivation of antihistamine in the skin. The effect of varying time intervals between the intradermal antihistamine injection and the subsequent challenge with intradermal HPAE was tested.
(Table II). Pretreatment of the skin challenge site with antihistamine for as long as 2 hr before HPAE injection led to inhibition of the acute permeability response.

**Duration of activity of HPAE in the skin.** The duration of the permeability-enhancing effect of HPAE was also demonstrated by injecting the platelet material at different time intervals before the administration of intravenous Evans blue (Table III). In these studies the permeability response in the skin was measured 15 min after dye injection. Full permeability-inducing activity was detected at injection sites for up to 2 hr after intradermal injection of permeability factor. When intradermal injection preceded Evans blue administration by more than 2 hr significant diminution of activity was noted.

**Effect of platelet extract on rabbit platelet and leukocyte histamine.** In order to determine whether the acute permeability effect was due to the action of the HPAE on rabbit platelets, the extract was incubated with washed rabbit platelets in buffer and the supernate tested for the presence of histamine. Histamine was not released from washed platelets in buffer or from platelet rich plasma after incubation with HPAE (Table IV). For comparison, thrombin released 100% of the histamine present in washed rabbit platelets in buffer, while collagen released 83% of the histamine present in rabbit platelets in plasma. The HPAE also did not release significant amounts of histamine from rabbit exudative polymorphonuclear leukocytes.

**Partial purification of the HPAE.** Separation of the acid-extracted material on a column of DEAE-cellulose is shown in Fig. 2. The permeability-inducing activity was localized to the cationic protein fraction which passed through the column without binding. The more anionic platelet proteins including cathepsin A present in peak 4 (10) failed to induce increased vascular permeability upon intradermal injection. Peak 1 of the
TABLE III
Duration of Activity of Human Platelet Acid Extract in the Skin

<table>
<thead>
<tr>
<th>Time of injection of HPAE* before Evans blue</th>
<th>Permeability activity †</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hr</td>
<td>1.0</td>
</tr>
<tr>
<td>3 hr</td>
<td>4.6</td>
</tr>
<tr>
<td>2 hr</td>
<td>11.2</td>
</tr>
<tr>
<td>1 hr</td>
<td>12.4</td>
</tr>
<tr>
<td>½ hr</td>
<td>10.1</td>
</tr>
<tr>
<td>5 min</td>
<td>11.4</td>
</tr>
</tbody>
</table>

* HPAE, human platelet acid extract, 224 mg/ml.
† Acute reaction determined in 15 min.
§ mm, diameter in millimeters of bluing.

DEAE column which led to the acute as well as the delayed permeability-enhancing effect in rabbit skin showed multiple bands on cationic gel electrophoresis (Fig. 2).

The fractions containing permeability-inducing activity obtained from the DEAE column were pooled, concentrated by lyophilization, and applied to a Sephadex G-75 column. The second protein peak obtained from the Sephadex column which had an estimated mol wt of less than 50,000 contained the acute and delayed permeability enhancing activity (Fig. 3). Cationic gel electrophoresis of this peak revealed 2–3 protein bands.

The fractions containing the permeability-inducing activity obtained from the Sephadex column were pooled, concentrated by lyophilization, and subjected to sucrose density gradient ultracentrifugation. The major protein band sedimenting with a mol wt range of approximately 30,000 contained the acute and delayed permeability-enhancing activity (Fig. 4). Cationic gel electrophoresis of this protein peak revealed one major and 1–2 minor bands.

Partial purification of permeability-enhancing activity from human platelet granule extract. Previous studies have demonstrated that the human platelet permeability factor(s) was present in the isolated subcellular granule fraction. The separation of acid-extracted protein obtained from the isolated granules of human platelets on

TABLE IV
Effect of Human Platelet Acid Extract on Rabbit Platelet Histamine

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Test substance</th>
<th>Histamine released †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed platelets in buffer‡</td>
<td>Saline</td>
<td>13</td>
</tr>
<tr>
<td>Washed platelets in buffer</td>
<td>HPAE§</td>
<td>6</td>
</tr>
<tr>
<td>Washed platelets in buffer</td>
<td>Thrombin‖</td>
<td>100</td>
</tr>
<tr>
<td>Platelet rich plasma¶</td>
<td>Saline</td>
<td>12.4</td>
</tr>
<tr>
<td>Platelet rich plasma</td>
<td>HPAE§</td>
<td>9</td>
</tr>
<tr>
<td>Platelet rich plasma</td>
<td>Collagen*</td>
<td>83</td>
</tr>
</tbody>
</table>

* The amount of histamine extracted from an untreated portion of platelets was considered 100%.
† Platelet count, 855,000/mm² in Tyrode's solution containing Ca²⁺ and Mg²⁺.
§ HPAE, human platelet acid extract, 100 μg protein.
‖ 5 NIH U/ml.
¶ Platelet count, 800,000.
** 50 μl of acid-soluble collagen, 4 mg/ml.

Human Platelet Vascular Permeability-Enhancing Activity 553
activity also counteracted the prolongation of the thrombin time produced by heparin.

DISCUSSION

We have demonstrated that a nondialyzable relatively heat-stable human platelet cationic protein fraction of approximate mol wt 30,000 increases vascular permeability in rabbit skin. Our previous studies on platelet granule extract (3) as well as the present purification studies on the granule acid extract strongly suggest that the platelet permeability activity resides in the intracellular granule (presumably lysosomal) fraction.

The most striking feature of the increased permeability effect emphasized by the present studies is the biphasic (acute and delayed) permeability-enhancing response in the rabbit skin. This phenomenon was not demonstrated in the previous permeability studies using mice (3). Our prior permeability studies on rabbits were designed to clarify the nature of the activity and no attempt was made to define an acute versus delayed response (3). Repeated studies using numerous mice which have been pretreated with intraperitoneal antihistamine have revealed complete inhibition of permeability-enhancing effect for as long as 2-3 hr after the intradermal challenge of the platelet acid extract. Thus it appears that there may be significant species differences in the response to the delayed permeability-enhancing effect of the human platelet permeability factor.

The acute permeability-enhancing effect of the HPAE and of the purified fractions was completely inhibited by prior treatment of the challenged animal by local or parenteral antihistamine. Our previous studies demonstrated that the platelet granule permeability-inducing extract produced degranulation of mouse and rat peritoneal mast cells leading to histamine release (3). There is no evidence from our present studies to suggest that the HPAE caused release of rabbit platelet or rabbit leukocyte intracellular histamine. It thus seems logical to assume that the acute permeability effect in rabbit skin is related to the local accumulation of histamine secondary to tissue mast cell degranulation by the permeability factor. In contrast, the delayed permeability effect in rabbit skin was not inhibited by local or systemic antihistamine administration. The possibility that the delayed response in the antihistamine-treated animal might represent the slow inactivation of antihistamine seems unlikely in view of the findings that the antihistamine effect was demonstrable for as long as 2 hr after intradermal injection (Table III). The histologic changes in the skin associated with the delayed permeability response were characterized primarily by tissue leukocytosis. These observations suggest that the delayed response was due to direct or indirect in vivo

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chemotaxis, initiated by the platelet permeability factor. It is of interest that Packham, Nishizawa, and Mustard have also previously noted a chemotactic effect of pig platelet permeability factor after injection into rabbit skin (1). The direct cause of the intradermal delayed response is not evident from our studies; however, it is possible that the cellular exudation with subsequent leukocytic release of lysosomal cationic proteins led to the eventual increase in vascular permeability. Ranadive and Cochrane have demonstrated that three of the isolated cationic rabbit permeability factors can lead to increased vascular permeability independent of histamine release (11).

The question arises whether the biphasic rabbit response is due to one or more individual human platelet permeability factors. We have been unable to separate the acute and delayed permeability effects using the crude platelet acid extract as well as purified fractions. In view of the recently described differentiation of the histamine-dependent and nonhistamine-dependent cationic permeability factors in rabbit exudative neutrophils, it seems logical to assume that the human platelet histamine-dependent permeability factor may be different from the delayed permeability-enhancing chemotactic factor. Such a differentiation must await further biochemical purification.

It is of interest that the permeability-enhancing fractions also possessed antiheparin activity. Recent studies by Pepper, Moore, and Cash suggest that human platelet antiheparin activity is not homogeneous and may exist in two separate platelet protein fractions (12). One of these fractions has a high mol wt of approximately 500,000 while the second is apparently a much smaller protein. Pretreatment of rabbits with intradermal heparin did not block the acute or delayed enhanced vascular permeability effects of HPAE. It is not clear from our studies whether the antiheparin effect of the platelet permeability-enhancing fraction is due to contamination with one of the forms of platelet factor 4 or to the strongly positive charge of the permeability protein.

Our studies in addition to the earlier findings further substantiate the important role that the platelet may play in inflammatory responses. Recent studies have shown that platelets can synthesize prostaglandin E2 (13), a dialyzable small molecular weight compound (mol wt 350) which increases vascular permeability (14). Thus the platelet may possess multiple potential mechanisms for influencing inflammatory reactions. It has been well demonstrated over a number of years that early inflammatory changes in injured tissues involve the release of various biologically active materials from leukocyte lysosomes (15-17). Included among the materials are a family of cationic proteins which possess antibacterial activity (18), enhance vascular permeability (17), and are pyrogenic (19). Our studies demonstrate that the circulating platelet may also contribute to the cationic protein mediators of inflammatory responses. In separate experiments it has been demonstrated that bactericidal activity may be induced in human platelets during the active stage of an inflammatory process (20). In addition, we have recently isolated and characterized a cationic rabbit platelet protein with bactericidal activity (21). Thus in these respects, the circulating platelet resembles a significant extent the exudative polymorphonuclear leukocyte.

ACKNOWLEDGMENTS

This study was supported by grants from the American Heart Association, U. S. Public Health Service, and the Krakower Foundation.

<table>
<thead>
<tr>
<th>TABLE V Antiheparin Activity</th>
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<tbody>
<tr>
<td>Material tested</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Saline</td>
</tr>
<tr>
<td>HPAE*</td>
</tr>
<tr>
<td>DEAE− peak 1 of HPAE</td>
</tr>
<tr>
<td>Sephadex− peak of DEAE fraction</td>
</tr>
</tbody>
</table>

Permeability Activity: The permeability reaction (15 min) was interpreted as present (+) or absent (−). Activity was defined as an intradermal bluing reaction of greater than 8 mm.

The platelet factor 4 assay (antiheparin) was performed as follows: the clotting time was determined in a mixture consisting of 0.3 ml platelet poor plasma, 0.1 ml saline or test reagent, 0.1 ml heparin (0.3 μ/ml) and 0.1 ml thrombin (10 μ/ml). All materials were incubated at 37°C and all tests performed in duplicate.

* HPAE, human platelet acid extract, protein concentration, 300 μg/ml.
† Protein concentration, 340 μg/ml.
‡ Peak B of a Sephadex G-75 separation of the active DEAE peak; protein concentration, 40 μg/ml.
REFERENCES