RELEASE OF VASOACTIVE AMINES FROM RABBIT PLATELETS INDUCED BY SENSITIZED MONONUCLEAR LEUKOCYTES AND ANTIGEN*

P. M. HENSON,‡ PH.D.

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

(Received for publication 8 September 1969)

Release of vasoactive amines from rabbit platelets has been implicated in a number of immunologic reactions in vivo (1). Studies in vitro have revealed immunologic release of histamine and serotonin from platelets by processes involving immune complexes and complement (1–3) and immune complexes, complement, and neutrophils (4). A reaction which occurs in the absence of complement or plasma has also been described, initially in rabbits infected with *Shistosoma mansoni* (5–7), but also in rabbits after immunization with protein antigens (1, 8–10).

This complement-independent reaction has been shown to require blood leukocytes acting in the presence of antigen to induce release of histamine from platelets (1, 6, 8). In studies attempting to relate the mechanisms of release of vasoactive amines to the deposition of immune complexes in acute immune complex disease (1, and manuscript in preparation), evidence was obtained that this latter reaction, requiring antigen, leukocytes, and platelets, was involved. Studies on the mechanism of the leukocyte-dependent release of vasoactive amines from platelets are reported herein.

Materials and Methods

Platelets.—The methods employed for harvesting and washing rabbit platelets have been described elsewhere (3) and essentially follow the method of Ardlie (personal communication from Dr. N. Ardlie, McMaster University, Hamilton, Canada). Adult New Zealand white rabbits were bled into acid citrate dextrose (ACD)[1] and platelets were obtained by differential

* This is publication No. 368 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation La Jolla, California 92037. The work was supported by United States Public Health Service Grant AI-07007.

‡ Supported by United States Public Health Service Training Grant 5 TI GM 683.

[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol tetraacetic acid; LDHR, leukocyte-dependent histamine release; Mg EGTA, magnesium EGTA; MIF, macrophage migration inhibition factor; P, protein.

centrifugation. They were washed with Tyrode's solution containing 0.25% gelatin. Calcium was omitted from the first and second wash solution and 10^{-4} M ethylene glycol tetraacetic acid (EGTA) was included in the first wash. In some experiments, ethylenediaminetetraacetate (EDTA) was used as the original anticoagulant. For work with platelets, siliconized glassware or plastic containers and pipettes were used throughout.

Leukocytes.—Rabbit blood was taken from the ear artery into 0.005 M EDTA. After light centrifugation (400 *g* for 20 min) the platelet-rich plasma was removed. The layer of cells (platelets and leukocytes) on top of the erythrocytes was removed with a siliconized Pasteur pipette with the tip bent at right agles. These cells were washed three times (300 *g* for 10 min) with the same Tyrode's solutions as used for the platelets and finally suspended in whole Tyrode's solution (containing Ca^{++}) with gelatin. These preparations contained erythrocytes, but usually only a small percentage of granulocytes (5–10%). The majority of the leukocytes were mononuclear cells, both large and small.

For some experiments, such buffy coat cells were passed through columns of acid-washed, nonsiliconized glass beads (No. 12 Ballotini, Atlas Chemical & Mfg. Co., San Diego, Calif.). The columns were prepared in 10 ml plastic pipettes and 1–2 ml cells were allowed to run into the column in the Tyrode's solution with gelatin. After 30 min at 37°C the nonadherent cells, including the erythrocytes, were eluted with the same diluent and then the adherent cells were removed with Tyrode's solution containing 0.01 M EDTA. The cells were finally washed to remove the EDTA.

Spleen and lymph node cells were obtained by passing the organs through sieves in Tyrode's solution with 0.1 M EDTA sand washing the cell suspensions obtained. Bone marrow cells came from the femur. Macrophages were washed from normal, unstimulated peritoneal cavities. The cells remaining after removal of platelet-rich plasma and the buffy coat layer contained most of the blood neutrophils and were either used as such, or the erythrocytes were sedimented with an equal volume of 2% gelatin. Thoracic duct lymphocytes were obtained by cannulation of rabbit thoracic ducts, kindly performed by Dr. Sun Lee. All cells were washed and counted before testing.

Immunization of Rabbits.—Two antigens were employed, bovine serum albumin (BSA) (Armour Pharmaceutical Co., Chicago, Ill.) and horse spleen ferritin (cadmium-free) (Nutritional Biochemicals Corp., Cleveland, Ohio). Rabbits with cells reactive to BSA were generally produced by an injection regimen identical to that used for acute immune complex disease (serum sickness). It consisted of an intravenous injection of rabbit anti-BSA antiserum (5 μg antibody N/kg) 1 day before a large intravenous injection of BSA (250 mg P (protein)/kg). Other injection schedules consisted of 5 mg BSA/kg into the footpads with complete Freund's adjuvant or single injections (250, 100, 20, and 2 mg P BSA) intravenously without the previous antiserum. Subsequent injection courses comprised 250 mg or 20 mg BSA intravenously as a single injection. Ferritin was also injected intravenously as one dose (20 mg P/kg)

Leukocyte-Dependent Release of Histamine (LDHR) Test for Screening of Rabbits.—This leukocyte-dependent histamine release test was used to examine rabbits for the presence of the reaction. The total formed elements from 2 ml blood taken into 0.01 M EDTA were washed three times with the Tyrode's solutions used for platelets. Two equal portions of the cells were taken and made to 2.5 ml with Tyrode's solution containing gelatin. Antigen (62 μg P BSA or 190 μg P ferritin) was added to one of the tubes and both were incubated for 30 min at 37°C. After centrifugation the supernatant was assayed for histamine. The percentage released was calculated after assay of the boiled sediments for their content of histamine.

Reaction Conditions.—The reactions were performed in whole Tyrode's solution containing 0.25% gelatin. Platelets (2.5×10^8) from normal rabbits, varying numbers of leukocytes and antigen were incubated in a total volume of 2.5 ml for 30 min at 37°C. After centrifugation the supernate and sediments (boiled for 2 min) were assayed for released materials.

For examination of enzymes and nucleotides or electron microscopy, 5×10^9 platelets in

a final volume of 5 ml and correspondingly increased quantities of leukocytes (2×10^7) and antigen (1.5 mg P ferritin) were used. The soluble factor was produced by incubation of buffy coat leukocytes (5×10^7 or more) with antigen (1.5 mg ferritin) for 20 min at 37°C. After centrifugation, the supernatant fluid containing the soluble factor was incubated with platelets. After the reaction was completed, sedimented cells were lysed with two drops of 1 mg/ml Triton ×100 for enzymes, or boiled for histamine and serotonin.

Assays.—Histamine and serotonin were assayed on the guinea pig ileum and estrous rat uterus, respectively, using appropriate standards.

Acid phosphatase, lactic dehydrogenase, β glucuronidase, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) were assayed by standard procedures as described elsewhere (3). The samples were extracted with 4% perchloric acid for the nucleotide assays. ^{86}Rubidium was incorporated into the platelets before the reaction (11) and both sediments and supernates were counted in a well-type counter to determine the per cent released.

Inhibitors.—Diisopropyl fluorophosphate (DFP) (Boots Pure Drug Co., Ltd., Nottingham, U.K.), adenosine, and acetyl salicylate were prepared at 10 times the concentration used in saline and one-tenth volume was added to the tubes before the reactions. Mg EGTA (magnesium EGTA) consisted of 0.005 M Na EGTA with 0.01 M $MgCl_2$. 2-deoxyglucose was used at 10^{-3} M in a glucose-free environment, i.e., the cells were washed in, and the test performed in Tyrode's solution from which the glucose had been omitted.

Electron Microscopy.—Platelets to be used for electron microscopic examination were prepared with ACD and at room temperature. After 15 min of reaction a portion was added to 3 volumes of 3% glutaraldehyde, pH 7.3, previously warmed to 37°C. After 20 min, the cells were centrifuged, fixed in osmic acid in the cold and embedded in Vestopal. Sections were cut, stained with uranyl acetate and lead citrate and examined in the Hitachi HU11 microscope.

RESULTS

A Test for the Release of Histamine from Platelets by Leukocytes from Sensitized Rabbits Together with Antigen.—A simple test was devised for screening of rabbits for the presence of the requisite sensitized leukocytes. The formed elements from 2 ml (or less) of whole blood were washed to remove the plasma, since antibody and complement could induce platelet histamine release by other mechanisms (3, 4). Incubation of antigen with the washed cells resulted in release of histamine, the specificity of which could be determined by comparison with an identical tube lacking the antigen. Since most of the blood histamine in the rabbit is present in the platelets, the amount of histamine released was a rough guide to the strength of the reaction, i.e., the number and/or the reactivity of the leukocytes inducing release of histamine from the platelets.

Presence of LDHR after Immunization of Rabbits.—Fig. 1 shows the LDHR reaction in groups of rabbits immunized with BSA. The reaction is expressed as the percentage of histamine released in the test described above. The primary injection course consisted of first an intravenous injection of anti-BSA antiserum (5 mg N/kg) and then 250 mg BSA/kg 1 day later. This regimen is also used to induce acute serum sickness (12), the lesions of which occur during elimination of antigen when antibody-antigen complexes are circulating. Immediately after this immune elimination, but not before, the LDHR was detected in most

of the rabbits. The reaction was still present, though weaker, in many rabbits after 22 days and some rabbits have shown it after 35 days.

The second injection course comprised an intravenous injection of 250 mg/kg BSA given 2 months after the first course. The immune elimination of antigen was faster in such secondary reactions and the onset of the LDHR was earlier and had disappeared faster than in the primary response. Some rabbits did not react to such high doses of BSA, either by immune elimination or by development of the LDHR. Ferritin has also been used for the production of the LDHR reaction. Intravenous injection of 20 mg/kg resulted in a more consistent reaction which appeared earlier than with BSA (about the 7th day in a primary reaction). Lower doses of BSA, or BSA in adjuvant into the footpads

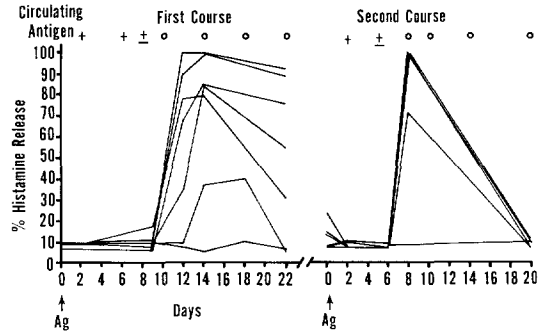

FIG. 1. Leukocyte dependent histamine release (LDHR) reaction in rabbits after immunization with BSA. The immunization procedure is described in the text. The use of ^{131}I-BSA permitted determination of the circulating antigen (12). The formed elements from 2 ml of blood taken at intervals after immunization were incubated for 30 min at 37°C with and without antigen (62 μg P) and the percentage of histamine released from the platelets was determined.

did induce the LDHR, but more variably, to a lesser degree, and for a shorter duration.

The Reaction of Buffy Coat Leukocytes, Antigen, and Platelets.—Fig. 2 depicts the time course of the histamine release from 2.5×10^8 washed platelets induced by 2×10^6 mononuclear leukocytes in a preparation of washed buffy coat cells from an immunized rabbit and 62 μg P BSA. The histamine release commenced after 2.5 min and was complete after 10–15 min of incubation at 37°C. Buffy coat leukocytes from a nonimmunized rabbit were included as a control and did not induce histamine release.

The number of leukocytes required to produce the histamine release reaction varies from rabbit to rabbit and with the time of the immunization course. A representative titration of mononuclear cells in the buffy coat from a single rabbit with constant antigen and platelets is shown in Fig. 3. 50% release could have been produced with about 5×10^5 leukocytes.

The amount of antigen was varied in experiments shown in Table I. 5–10 μg BSA or ferritin induced only slight release of histamine from platelets in the presence of leukocytes. 30 μg or more induced maximal release with the leukocytes employed. Use of ferritin with leukocytes from rabbits immunized with

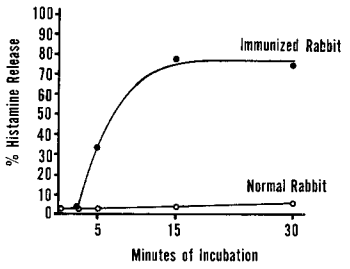

FIG. 2. Time course of leukocyte-dependent release of histamine from platelets. Buffy coat leukocytes (2×10^6) from a rabbit sensitized with BSA and from a normal animal were incubated at 37°C with 62 μg P BSA and 2.5×10^8 platelets in a total volume of 2.5 ml. At intervals, tubes were removed and the reaction was stopped by cooling and the addition of EDTA (0.01 M). After centrifugation the percentage of histamine released from the platelets was determined.

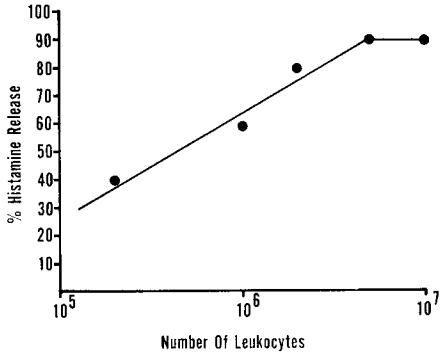

FIG. 3. Titration of leukocytes. Increasing numbers of buffy coat cells (94% mononuclear cells) were incubated with antigen (62 μg BSA) and 2.5×10^8 platelets for 30 min at 37°C.

BSA or BSA with cells sensitized to ferritin did not lead to release of histamine from the platelets.

The Ability of Cells from Different Sources to Participate in the Leukocyte-Dependent Release of Histamine from Platelets.—Cells from various lymphoid organs of rabbits which had an active LDHR reaction in the blood were examined in the presence and absence of antigen for their effect on normal platelets. The results from two representative animals so studied are depicted in Table II. The number of cells which induced 50% histamine release from 2.5×10^8 platelets or the maximal number tested is shown.

Cells from no other source approached the activity of leukocytes from the blood. Release of 50% of histamine from the platelets could be obtained with spleen and bone marrow cells in the presence of antigen, but only if 1 or 2 $\times$ 10^8

TABLE I

*Leukocyte-Dependent Release of Histamine by Different Quantities of Antigen**

Ferritin		BSA	
P	Histamine released	P	Histamine released
μg	%	μg	%
750	75	125	43
30	75	31	43
7.5	15	6	15
3	11	1.6	8
—	10	—	4

* Buffy coat mononuclear leukocytes (2 $\times$ 10^6), platelets (2.5 $\times$ 10^8) and antigen were incubated for 30 min at 37°C.

TABLE II

The Number of Cells from Different Organs of a Sensitized Rabbit Required to Initiate Histamine Release from Platelets in the Presence of Antigen

Source of cells	Rabbit immunized with ferritin		Rabbit immunized with BSA	
	Number of cells $\times$ 10^6*	Histamine released	Number of cells $\times$ 10^6	Histamine release
		%		%
Buffy coat	0.75	50	0.5	50
Spleen	100	50	20	16
Bone marrow	200	50	ND‡	ND
Mesenteric lymph node	200	25	20	0
Popliteal lymph node	10	9	20	0
Thoracic duct cells	ND	ND	100	0
Peritoneal cells (macrophages)	2	4	20	0
Peripheral neutrophils	2	0	ND	ND

* The maximal number of cells tested, or the number required for 50% release of histamine from 2.5 $\times$ 10^8 platelets in the presence of antigen (350 μg P ferritin or 62 μg P BSA). The results are representative of six similar experiments.

‡ ND, not done.

cells were employed. Cells from mesenteric and popliteal lymph nodes occasionally showed some reactivity, but always required many more cells than from the blood. No activity could be found with thoracic duct lymphocytes obtained from four rabbits. Peritoneal macrophages, obtained without stimulation

of the peritoneal cavity, did not show antigen-specific activity, although some histamine release (10–20%) did result from incubation of the maximal number tested (2×10^6) with platelets in the absence of antigen. To examine the possibility that lymphocytes were interacting with leukocytes in the blood to produce the effect on platelets, mesenteric lymph node cells (5×10^7) from a sensitized rabbit were incubated with platelets and buffy coat cells (2×10^7) from a normal animal. No antigen-specific release of histamine occurred, although 30% of the platelet histamine was released even in the absence of antigen.

The preparation of buffy coat cells in the manner described usually contained 10% or less granulocytes (8% in the example shown), the great majority of which were neutrophils. The remaining neutrophils were found below the buffy coat layer and when tested did not induce histamine release from platelets.

TABLE III

*The Adherence to Glass Bead Columns of Leukocytes Reacting with Antigen and Platelets**

Number of leuocytes required for 50% histamine release from 2.5×10^8 platelets		
Whole buffy coat leukocytes	Nonadherent leukocytes	Adherent leukocytes
10^5	5×10^6	10^5
10^5	1.6×10^6	5×10^4
3×10^5	$>2 \times 10^6$	3×10^5
10^6	$>2 \times 10^6$	5×10^5
$<5 \times 10^5$	$>5 \times 10^6$	—
5×10^5	$>5 \times 10^6$	—
$<5 \times 10^5$	5×10^7	8×10^5
$<3 \times 10^5$	$>10^7$	3×10^5

* The reaction conditions were those shown in Table II.

Erythrocytes did not participate in the reaction, since they could be removed by hypotonic lysis and the remaining blood leukocytes were still active.

Adherence of Reactive Cells to Glass Bead Columns.—Preparations of reactive, washed buffy coat cells were passed through columns of glass beads. The reactivity of the starting preparation, the nonadherent cells, and the cells which were eluted from the column with EDTA were compared (Table III). It was found that the leukocytes which did not adhere to the column in the Tyrode's solution containing gelatin, which was used as a diluent, were much less active in inducing histamine release from platelets in the presence of antigen than either the starting preparation of cells or those eluted with EDTA. In general, 10–100 times as many nonadherent cells as whole buffy coat leukocytes were required for 50% histamine release. Adherent cells and starting buffy coat cells were about equally active with occasional increased (two-fold) activity of the adherent cells. The numbers of cells shown are of mononuclear leukocytes. Some of the low percentage of neutrophils which were present in the whole

buffy coat were eluted with the EDTA (0–5%). The nonadherent cells consisted largely of small lymphocytes when examined by light microscopy. Larger mononuclear cells were present in appreciable numbers among the adherent cells, although the exact percentage varied and could not be accurately determined due to even gradations in size, amount of cytoplasm, etc., from that characteristic of small lymphocytes to that of large lymphoid cells.

In the electron microscope, the same variation was seen. Some cells resembled small lymphocytes with minimal cytoplasm, no vacuoles, granules, or endoplasmic reticulum and dense chromatin in the nucleus. Others had the

TABLE IV

*Decreasing Activity of Sensitized Leukocytes Incubated with Antigen**

First incubation (leukocytes)		Second incubation (leukocytes and platelets)		Histamine release
Time	Antigen	Time	Antigen	
min		*min*		%
15	+	30	−	77
30	+	30	−	62
60	+	30	−	38
120	+	30	−	26
180	+	30	−	14
180	+	30	+	19
15	−	30	−	22
180	−	30	−	18
180	−	30	+	66

* Leukocytes adherent to a glass bead column (See Materials and Methods section.) were incubated at 37°C with or without antigen (6×10^6 leukocytes +2.1 mg P ferritin). Portions containing 10^6 cells were removed at intervals, washed, and incubated for a further 30 min with or without antigen (350 μg P) in the presence of 5×10^8 platelets. The percentage of histamine released from the platelets was then determined.

appearance of monocytes, a larger cytoplasm containing endoplasmic reticulum, golgi apparatus, vacuoles, and sometimes granules, as well. Other cells were intermediate between these extremes.

The Effect of Pretreatment of Leukocytes with Antigen on Their Reaction with Platelets.—In order to examine the ability of the leukocytes to react with platelets following an initial exposure to antigen (7) the following experiments were performed.

Leukocytes from a rabbit immunized with ferritin were incubated with ferritin for increasing lengths of time. They were centrifuged, resuspended, and added to platelets without additional antigen. Such cells did induce release of histamine from platelets, but the release diminished with the time of preincubation of the leukocytes with antigen (Table IV). After 3 hr, the cells induced

only 14% release, i.e., showed loss of activity. Addition of antigen during incubation of these "exhausted" cells with platelets did not restore the activity. However, leukocytes incubated without antigen for 3 hr did induce release of histamine when added to platelets and antigen, showing that activity could be retained over this time period at 37°C in the absence of antigen.

This "exhaustive" effect of antigen on the leukocytes was shown in another way (Table V). Leukocytes were preincubated with antigen for 20 min, washed, and then incubated with platelets either with or without additional antigen. Leukocytes preincubated with antigen did induce release of histamine from platelets after washing without additional antigen (60%), although the amount

TABLE V

*Inhibitory Effect of Antigen on Sensitized Leukocytes Which Have Been Preexposed to Antigen**

First incubation (leukocytes)			Second incubation (leukocytes and platelets)		Histamine release
Time	Antigen	EDTA (10^{-2} M)	Time	Antigen	
min			*min*		%
−	−	−	30	+	70
20	+	−	30	−	60
20	+	−	30	+	40
20	+	+	30	−	32
20	+	+	30	+	24
20	−	−	30	−	22
20	−	−	30	+	65
20	−	+	30	−	22
20	−	+	30	+	66

* Buffy coat leukocytes (10^6) were incubated for 20 min at 37°C with or without antigen (62 μg P BSA) and with or without EDTA (0.01 M). After washing, they were incubated for a further 30 min with or without antigen (31 μg P) in the presence of 2.5×10^8 platelets. The percentage of histamine released from the platelets was then determined.

was less than if platelets, leukocytes, and antigen were all reacted together (70%). If, however, antigen was included in the second incubation, i.e., during the reaction with platelets, less histamine release occurred (40%). These effects could be accentuated by performing the preincubation of antigen and leukocytes in the presence of 0.01 M EDTA. Such leukocytes now reacted poorly with platelets after washing (32% release), and if antigen was present during the second incubation, the histamine release was even lower and approached the background level. Controls consisted of leukocytes preincubated in the absence of antigen and these were shown to be normally reactive with antigen and platelets after washing.

The results indicated that preincubation of leukocytes with antigen reduced their ability to react with antigen and platelets and that the presence of EDTA

during the preincubation increased the inhibition. Omission of glucose from the preincubation reaction or, to a lesser extent, performing it in the cold, had the same effect as EDTA, i.e., increased the inhibitory effect of the preincubation. The results also confirm that leukocytes reacted with antigen and washed can still induce release of histamine from platelets, though they are not as active as if all three reactants were incubated together.

The Production of a Soluble Factor from the Leukocytes.—A possible mechanism for the LDHR reaction could involve the release of a material from the leuko-

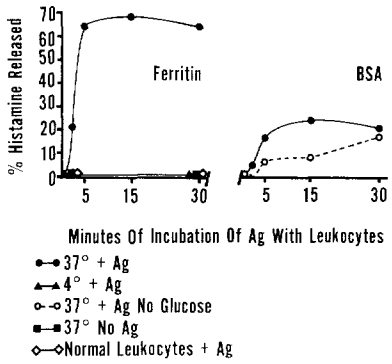

FIG. 4. Release of a soluble factor from leukocytes measured by its effect (release of histamine) on platelets. Buffy coat leukocytes (5×10^6) from normal rabbits or those sensitized with ferritin or BSA were incubated with antigen (700 μg P ferritin or 125 μg P BSA). At intervals the cells were sedimented and the supernatant fluid (containing releasing factors) was incubated with 2.5×10^8 platelets for 30 min. at 37°C. The percentage of histamine released from the platelets was then determined.

cytes which would cause platelets to release their histamine. Experiments described in Fig. 4 demonstrated such a substance.

Antigen (BSA or ferritin) was incubated with buffy coat leukocytes from appropriately immunized rabbits. After varying intervals, the cells were centrifuged and the supernatant was incubated with platelets for 30 min at 37°C. The per cent release of histamine from the platelets was assayed by determination of the levels in the fluid and in the sedimented platelets after the incubation period. Since the buffy coat usually contained a few platelets, some histamine was already present in the supernatants used, and these values were subtracted from the amount found after the final incubation with platelets so that the quantity released by the platelets could be determined. The finding of less histamine remaining in the platelets after the incubation served to confirm the releasing effect.

Fig. 4 shows that with both ferritin and BSA as antigen a soluble releasing factor was liberated from leukocytes within 2–5 min of incubation. It was not produced in the absence of antigen or with a different antigen. Moreover, incubation of antigen and buffy coat cells from a normal rabbit did not yield such a

factor. Omission of glucose from the incubation step of antigen with leukocytes resulted in reduced and delayed production of the releasing factor.

The soluble factor from the leukocytes was found to be considerably labile. Storage at different temperatures, freezing, or lyophilization have resulted in varying, but usually low, recoveries. Attempts to characterize the factor by dialysis, passage through filters or through gel columns have likewise yielded variable recoveries. Consequently, the experiments on the soluble factor that are reported herein were performed soon after sedimentation of the leukocytes so that loss of activity was minimized.

Inhibition of the Production of the Soluble Factor and of its Effect on Platelets.—A number of inhibitors or inhibiting conditions which have been used for study of reactions resulting in release of materials from platelets were examined for their effect on the leukocyte-dependent reaction. Fig. 5 shows the effect of the inhibitors on the production of soluble factor by the leukocytes. The leukocytes were incubated with antigen and inhibitor in a small volume, so that when 0.25 ml of the supernatant was transferred to 2.25 ml of diluent containing platelets, the inhibitors were diluted 1:10. As controls, inhibitors diluted 1:10 were checked in the reaction of soluble factor with platelets, so that the inhibitory effect on the production of the soluble factor could be determined.

If the glucose in the Tyrode's solution was replaced with 10^{-3} M 2-deoxyglucose, an inhibitor of glucose metabolism, less soluble factor (as measured by histamine release from platelets) was produced. Mg EGTA was completely inhibitory and acetyl salicylate partially so. Adenosine, however, did not diminish the amount of soluble factor produced. DFP produced an anomalous effect, since treatment of the leukocytes with DFP even in the absence of antigen caused the supernatant material to release 79% histamine from platelets. In a similar way, pretreatment of the leukocytes with DFP has also yielded antigen-independent, histamine-releasing activity, although the presence of DFP *during* the reaction of platelets, leukocytes, and antigen is inhibitory.

Fig. 5 also depicts the effect of the inhibitors on the whole reaction of antigen, leukocytes, and platelets incubated together. 2-deoxyglucose replacing glucose was markedly inhibitory and Mg EGTA abolished the reaction completely. DFP, acetyl salicylate, and adenosine also inhibited the reaction, but to a lesser extent.

The effect of the soluble factor on platelets was also examined with similar effects. All five materials were inhibitory, acetyl salicylate being the least effective.

The Effect of the Reaction on Platelet Morphology.—Platelets undergoing the reaction with leukocytes and antigen or with the soluble factor were examined after 15 min of incubation by electron microscopy (Figs. 7 and 8). In both situations loose clumps of platelets were seen, often consisting of only a few cells. The platelets involved in these clumps had irregular outlines with loosely

interdigitating processes, and in many, the granules and canalicular system had disappeared. In other platelets near the outside of the clump, these organelles were concentrated in the center of the cell in a basket-like network of microtubules. Platelet lysis was not seen. Platelets treated with supernatant from leukocytes incubated without antigen showed few clumps and only slight changes, consisting of some grouping of intracellular granules and canaliculi in

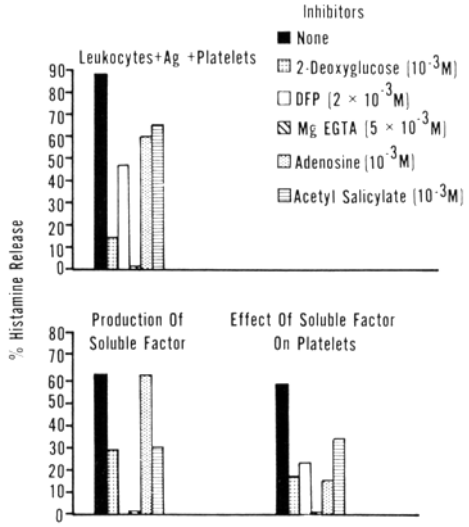

FIG. 5. The effect of inhibitors on the leukocyte-dependent release of histamine from platelets. Inhibition of the whole reaction was achieved by including the inhibitors in the reaction with sensitized buffy coat leukocytes (3×10^6), antigen (62 μg P BSA) and platelets (2.5×10^8). Incubation of the inhibitors, antigen, and leukocytes for 20 min at 37°C in a volume of 0.25 ml with subsequent addition of the supernatant fluid to platelets was used to show inhibition of production of the soluble factor. Soluble factor was also produced from the buffy coat leukocytes in bulk (in the proportions of cells and antigen noted above) and then incubated with platelets in the presence of the inhibitors.

the center of the cells. In the reaction with buffy coat leukocytes, only occasionally were leukocytes seen in the platelet clumps, and then, generally at the edge. In nearly every case, the leukocytes thus involved contained extensive cytoplasm with some vacuoles and granules.

Release of Platelet Constituents.—Since, morphologically, the reaction did not appear to result in platelet cytolysis and since a requirement for platelet energy (glucose) was shown, the process may require an active participation of the platelet. Accordingly, a variety of platelet constituents was examined to determine what types of substances were released. Since the reaction is complex, the figures shown represent the release of materials from the platelet, and amounts

released from the leukocytes or contained in the source of soluble factor have been subtracted. Representative control values (release from platelets alone) and total levels within 5×10^9 platelets have been described elsewhere (3). Fig. 6 shows some representative results. Histamine and serotonin behaved alike and were released to the greatest degree. Nucleotides (ATP, ADP, and AMP) were not released in great quantity over and above that found in the controls, although the degree of metabolism of any released nucleotides during the reaction was not determined. ^{86}Rubidium was incorporated into the platelets and its release is an indication of potassium loss. Levels only slightly greater than the control values were found. The intracytoplasmic enzyme lactic dehydrogenase was not released. Of the two lysosomal enzymes, acid phosphatase β-glucuronidase, only a very low level of the latter was liberated.

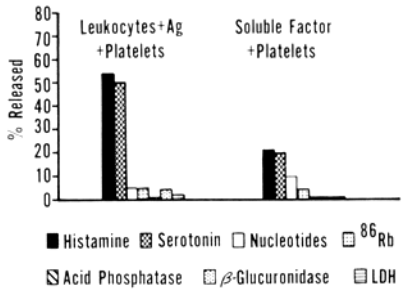

FIG. 6. Release of platelet constitutents by the leukocyte-dependent reaction. The reactions were performed as described in the Materials and Methods section. The results are representative of four similar experiments.

DISCUSSION

Incubation of antigen, blood leukocytes from an immunized rabbit and rabbit platelets results in release of vasoactive amines from the platelets. This process, unlike other immunologic reactions of rabbit platelets leading to vasoactive amine release (3, 4), did not apparently require complement, since thoroughly washed leukocytes and platelets could be employed. Moreover, rabbits depleted of C3 with cobra venom factor were still capable of exhibiting the leukocyte-dependent histamine release reaction in the blood after immunization (1, and manuscript in preparation).

Presence of the Reaction in the Blood after Immunization.—In the present studies, BSA or ferritin was used as antigen and the animals were immunized by the intravenous route. A large injection of BSA was found most efficacious, but smaller quantities sufficed if ferritin was employed. The reaction was detected by incubating antigen with whole washed blood cells and determining the quantity of histamine released. The possible involvement of blood basophil reactions (13, 14) in this preliminary test

cannot be excluded, but since most of the blood histamine in the rabbit is contained within the platelets (13), the procedure served as a useful screening test for the presence of the LDHR reaction in rabbit blood. After injection of BSA, the reaction could be elicited (i.e., reactive leukocytes were present in the blood) after the circulating antigen had been eliminated. Before that time, even if the leukocytes were present, they would presumably have already reacted with antigen and thus be undetectable. After a primary injection the reaction persisted for from 3–5 wk. After a second or subsequent injection of BSA, however, the reaction was detectable earlier (reflecting earlier antigen elimination) and disappeared more quickly. Rabbits which did not give immune elimination of the antigen (tolerant animals) did not exhibit the reaction in the blood. It appeared, therefore, that the time when the reactive leukocytes could be detected in the blood corresponded with the time of most active antibody synthesis.

The Nature of the Participating Leukocytes.—The type of leukocyte which reacts with antigen and platelets, resulting in histamine release, was partially purified from the blood in an attempt to identity it. Since the most reactive preparation contained less than 10% granulocytes and, moreover, blood granulocytes by themselves were inactive, mononuclear cells were implicated. Schoenbechler and Barbaro (6) have suggested that the cell is a small lymphocyte. However, in our hands the reactive leukocytes did not pass through columns of glass beads but remained adherent and could then be eluted off with EDTA. The cells were examined by electron microscopy, but in the absence of any evidence to date concerning the reactivity of individual cells, it is impossible to determine the cell type involved. The morphology of the adherent leukocytes varied from that corresponding to the small lymphocyte to that more closely resembling the monocyte. Many of the cells had somewhat scattered chromatin and relatively extensive cytoplasm with occasional vacuoles. In an examination of reactions with platelets, the only cell types seen regularly associated with platelet clumps were cells with granules and vacuoles. However, this association was not common, and it was felt unlikely to lead directly to such extensive release of histamine.

The blood was the best source of leukocytes which could participate in this reaction. The inactivity of thoracic duct cells and lymph node cells again suggested that the general circulating pool of small lymphocytes was not involved. At the time of greatest activity in the blood, other parts of the body appeared to be relatively deficient in reactive cells. However, examination of lymphoid organs at times later in the immunization might reveal a somewhat different pattern. The possibility that "sensitized" lymphocytes were reacting with cells such as monocytes in the blood and then inducing histamine release from platelets was felt to be unlikely since lymph node cells from an immunized rabbit did not confer reactivity upon buffy coat leukocytes from a normal rabbit. It has been suggested that homocytotropic antibody may be involved in this release of vasoactive amines from platelets (15) and indeed some correlation of homocytotropic antibody production and the presence of the leukocyte-dependent release reaction in the blood has been found (10). However, attempts to passively sensitize blood leukocytes from a normal rabbit with plasma or serum from sensitized rabbits with or without homocytotropic antibody have consistently met with failure. Despite this, the possibility that the blood leukocytes have passively absorbed antibody on their surfaces cannot yet be excluded.

The Production of a Soluble Factor from the Leukocytes.—The release of a soluble

factor from the leukocytes, capable of acting on platelets, was suggested by the small numbers of leukocytes required. A ratio of 1 leukocyte to 1000 or more platelets has been frequently observed and the actual ratio may be very much higher, since the proportion of active cells in the leukocyte preparation was not known. The possibility also exists that the leukocytes may contact many platelets for a brief period, thereby leading to release of histamine. However, this was felt to be unlikely as a complete explanation, since platelet clumping and release occur very quickly. In addition, association of leukocytes with the platelets was not regularly seen, and occurred just as frequently when antigen was omitted as when it was present.

A search for a soluble agent was therefore pursued and resulted in demonstration of the release of such a substance from the leukocytes. Addition of the supernatant medium from the reaction of antigen with reactive leukocytes to normal rabbit platelets resulted in release of vasoactive amines. The soluble factor was easily lost, however, and its lability may account for earlier reports of its absence, (7) and have prevented, as yet, its characterization. It appears to be released from leukocytes by an active process which may be inhibited in the cold or by chelation of calcium. Its liberation depends upon the presence of specific antigen and proceeds within 2 min of addition of the antigen.

The Effect of the Reaction on the Platelets.—The effect of the soluble factor, or of the leukocytes and antigen upon the platelets was similar. The release of histamine and serotonin was not accompanied by release of intracellular enzymes or by large amounts of rubidium. A nonlytic process was thus suggested and was confirmed morphologically. The platelets formed loose clumps and exhibited some of the changes (disappearance of granules and canaliculi) described for other active, immunologic release mechanisms (3, 4), but no lysis was observed. Active participation of the platelet in the release of the vasoactive amines was also indicated by the requirement for glucose, esterases, and environmental calcium and by the inhibitory action of aspirin and adenosine. It was of interest that while aspirin was inhibitory to both the release of soluble factor from the leukocytes and release of vasoactive amines from the platelets, adenosine had an effect only on the latter process.

The Possible Relationship of the Reaction to Those Involving Release of Factors from Lymphocytes.—Cytotoxic factors (16–18), chemotactic agents (19, 20) and substances inhibitory to macrophage migration (MIF) (21, 22) are liberated from lymphocytes and the platelet-releasing factor may be related to them. However, in contrast to these materials, the platelet-releasing factor was produced by blood leukocytes and was released immediately after contact with antigen. Moreover, the releasing factor was not cytotoxic to the platelets, but this may be an effect of the dose level. Preliminary studies with gel filtration have suggested a size for the releasing factor similar to lymphotoxin and MIF. One possibility that is at present under investigation is that the leukocytes in the blood are lymphocytes already "transformed" by the antigen (injected intravenously) and that subsequent addition of antigen serves to cause release and/or synthesis and release of the factor which acts on platelets and which may or may not be related to the other lymphocyte factors. Another possible explanation for the activity of supernatants from leukocytes incubated with antigen could be the presence of ADP, which is known to induce platelet clumping and some release of constituents. Little increased ADP was detected (4-8 nanomoles) in supernatants from

leukocytes incubated with antigen when compared to those without antigen, and equivalent quantities of ADP added to platelets did not induce a comparable amount of vasoactive amine release. Consequently, although ADP may contribute to the reaction, it did not appear to be the only factor involved.

The reaction of antigen with leukocytes in the *presence* of platelets resulted in the greatest release of vasoactive amines. Moreover, as reported by Barbaro and Schoenbechler (7), incubation of leukocytes which were previously treated with antigen and then washed still induced histamine release. In contrast to their findings, however, the amount of release decreased with increasing time of pretreatment of the leukocytes with antigen. This suggested either an exhaustion of the cells, perhaps due to release of all their soluble factor, or inactivation by prolonged contact with antigen. Contact of the leukocytes with antigen under conditions where soluble factor release did not occur (4°C or with EDTA), followed by washing, yielded leukocytes which were much less active when incubated with platelets and additional antigen. This may represent decay of activities in the membrane, responsible for release of the soluble factor, and if so it may open up an approach to the study ot this release process. This effect could account for the inability to detect the LDHR during the presence of antigen in the circulation (Fig. 1) although clearance of cells which have already reacted with antigen may also occur.

Involvement of the Reaction in Acute Immune Complex Disease.—The release of vasoactive amines from platelets by this reaction with antigen and leukocytes has been implicated in the pathogenesis of acute immune complex disease in rabbits (1, and manuscript in preparation). The deposition of immune complexes in blood vessel walls and in the glomerulus involves an increase in vascular permeability apparently induced by vasoactive amine release from platelets (23). Since complement depletion was not inhibitory to this process (1), the complement-dependent histamine release mechanisms (3, 4) did not appear to be involved, suggesting that the leukocyte-dependent process might be required. Correlation of the presence of the leukocyte-dependent reaction in the blood and the occurrence of lesions of acute immune complex disease (1, 10) provided additional evidence of its involvement.

SUMMARY

The immunological release of vasoactive amines from rabbit platelets by a mechanism requiring blood leukocytes has been described. The reaction involved leukocytes from an immunized rabbit, antigen, and platelets and did not require the complement system. The leukocytes appeared to be mononuclear, had the ability to adhere to glass, and were found in greater numbers in the blood than in spleen, lymph nodes, thoracic duct lymph, bone marrow, and peritoneal cavity washings.

Part or all of the effect on the platelets appeared to result from active release of a soluble factor from the leukocytes, which induced vasoactive amine release from the platelets. The platelets were not lysed during this reaction and the release of vasoactive amines required the active participation of metabolic pathways in the platelet.

The author thanks Dr. C. Cochrane for his help and advice, Mrs. D. Durham for preparing the sections for electron microscopy, Miss C. Brown for technical assistance, and Mrs. K. Prescott and Mr. G. Sandford for the illustrations.

BIBLIOGRAPHY

1. Henson, P. M. 1969. The role of complement and leukocytes in the immunologic release of vasoactive amines from platelets. *Fed. Proc.* **28:**1721.
2. Henson, P. M., and C. G. Cochrane. 1969. Immunological induction of increased vascular permeability. II. Two mechanisms of histamine release from rabbit platelets involving complement. *J. Exp. Med.* **129:**167.
3. Henson, P. M. 1970. Mechanisms of release of constituents from rabbit platelets by antigen-antibody complexes and complement. I. Lytic and nonlytic reaction. *J. Immunol.* In press.
4. Henson, P. M. 1970. Mechanisms of release of constituents from rabbit platelets by antigen-antibody complexes and complement. II. Interactions with neutrophils. *J. Immunol.* In press.
5. Schoenbechler, M. J., and E. H. Sadun. 1968. In vitro histamine release from blood cellular elements of rabbits infected with *Schistosoma mansoni*. *Proc. Soc. Exp. Biol. Med.* **127:**601.
6. Schoenbechler, M. J., and J. F. Barbaro. 1968. The requirement for sensitized lymphocytes in one form of antigen-induced histamine release from rabbit platelets. *Proc. Nat. Acad. Sci. U.S.A.* **60:**1247.
7. Barbaro, J. F., and M. J. Schoenbechler. 1969. The nature of the reaction of antigen with sensitized lymphocytes in the lymphocyte-dependent release of histamine from rabbit platelets. *Fed. Proc.* **28:**378.
8. Siraganian, R. P., and B. Oliveira. 1968. The allergic response of rabbit platelets and leukocytes. *Fed. Proc.* **27:**315.
9. Siraganian, R. P., and A. G. Osler. 1969. Histamine release from sensitized rabbit leukocytes and associated platelet involvement. *J. Allergy.* **43:**167.
10. Henson, P. M., and C. G. Cochrane. 1969. Antigen antibody complexes, platelets and increased vascular permeability. *In* Cellular and Humoral Mechanisms of Anaphylaxis and Allergy. H. Z. Movat, editor. S. Karger, Basel. 129.
11. Siraganian, R. P., A. G. Secchi, and A. G. Osler. 1968. The allergic response of rabbit platelets. I. Membrane permeability changes. *J. Immunol.* **101:**1130.
12. Kniker, W. T., and C. G. Cochrane. 1965. Pathogenic factors in vascular lesions of experimental serum sickness. *J. Exp. Med.* **122:**83.
13. Greaves, M. W., and J. L. Mongar. 1968. The histamine content of rabbit leucocytes and its release during *in vitro* anaphylaxis. *Immunology.* **15:**733.
14. Greaves, M. W., and J. L. Mongar. 1968. The mechanism of anaphylactic histamine release from rabbit leucocytes. *Immunology.* **15:**743.
15. Barbaro, J. F., and N. J. Zvaifler. 1966. Antigen induced histamine release from platelets of rabbits producing homologous PCA antibody. *Proc. Soc. Exp. Biol. Med.* **122:**1245.
16. Kolb, W. P., and G. A. Granger. 1968. Lymphocyte in vitro cytotoxicity: Characterization of human lymphotoxin. *Proc. Nat. Acad. Sci. U.S.A.* **61:**1250.
17. Williams, T. W., and G. A. Granger. 1969. Lymphocyte in vitro cytotoxicity:

Mechanism of lymphotoxin-induced target cell destruction. *J. Immunol.* **102:** 911.

18. Kikuchi, K., J. Reiner, and C. M. Southam. 1969. Diffusible cytotoxic substances and cell-mediated resistance to syngeneic tumours: In vitro demonstration. *Science (Washington).* **165:**77.
19. Ward, P. A., and J. R. David. 1969. A leukotactic factor produced by sensitized lymphocytes. *Fed. Proc.* **28:**630.
20. Ward, P. A., H. Gremold, and J. R. David. 1969. Leukotactic factor produced by sensitized lymphocytes. *Science (Washington).* **163:**1079.
21. David, J. R. 1968. Macrophage migration. *Fed. Proc.* **27:**6.
22. Bennett, B., and B. R. Bloom. 1968. Reactions in vivo and in vitro produced by a soluble substance associated with delayed type hypersensitivity. *Proc. Nat. Acad Sci. U.S.A.* **59:**756.
23. Kniker, W. T., and C. G. Cochrane. 1968. The localization of circulating immune complexes in experimental serum sickness. The role of vasoactive amines and hydrodynamic forces. *J. Exp. Med.* **127:**119.

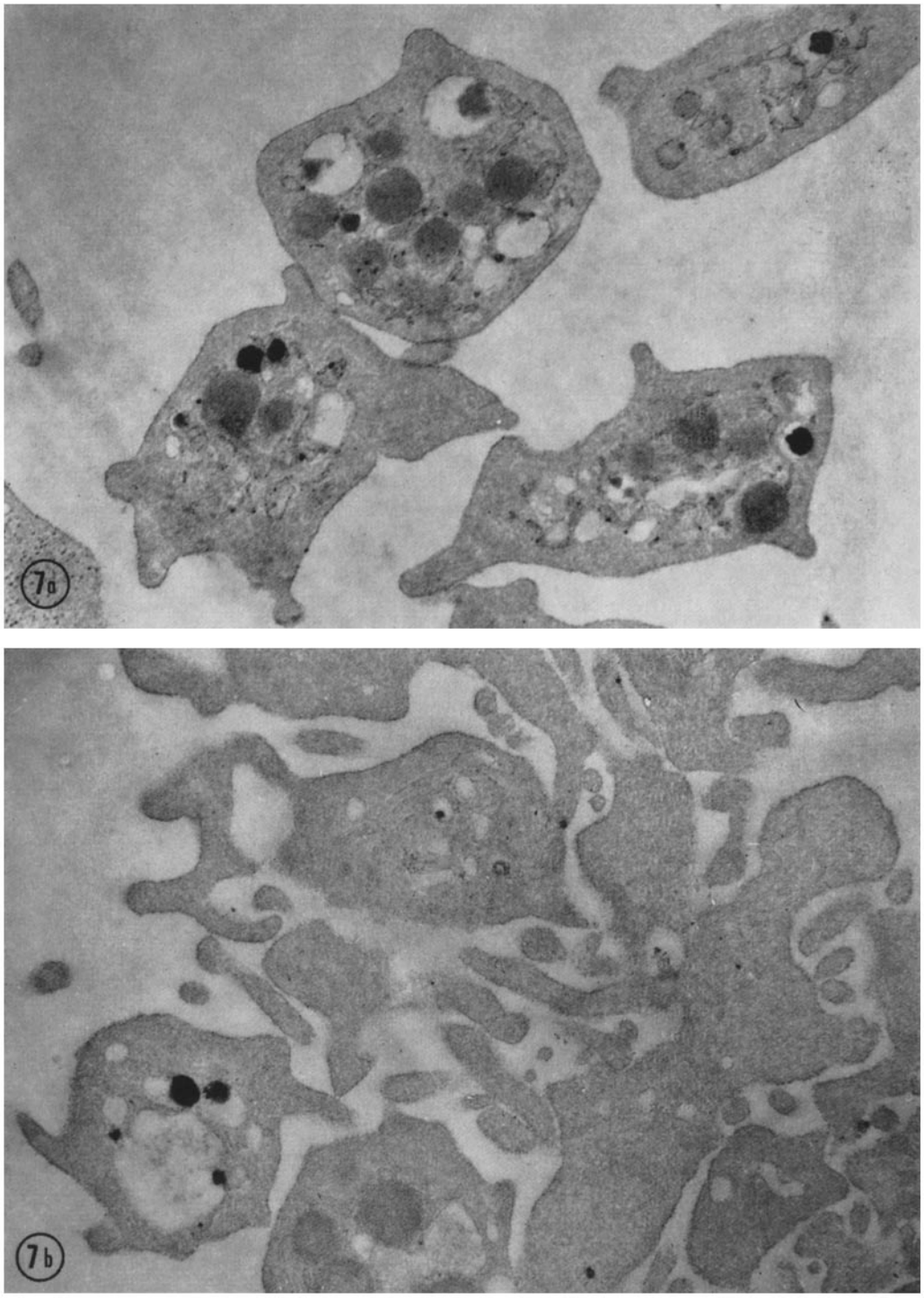

FIG. 7. Morphology of platelets incubated with soluble factor. (a) factor from normal leukocytes; (b) factor from sensitized leukocytes. The supernatant fluid from the reactions of buffy coat leukocytes and antigen (See Materials and Methods section.) was incubated with 2.5×10^9 platelets for 15 min before fixation in glutaraldehyde at 37°C. The soluble factor from sensitized leukocytes induced loose clumps of platelets and disappearance of granules and canaliculi. The supernatant fluid from normal leukocytes caused less change in the platelets (loss of regular discoid shape and some clustering of organelles in the center of the cells). × 28,000.

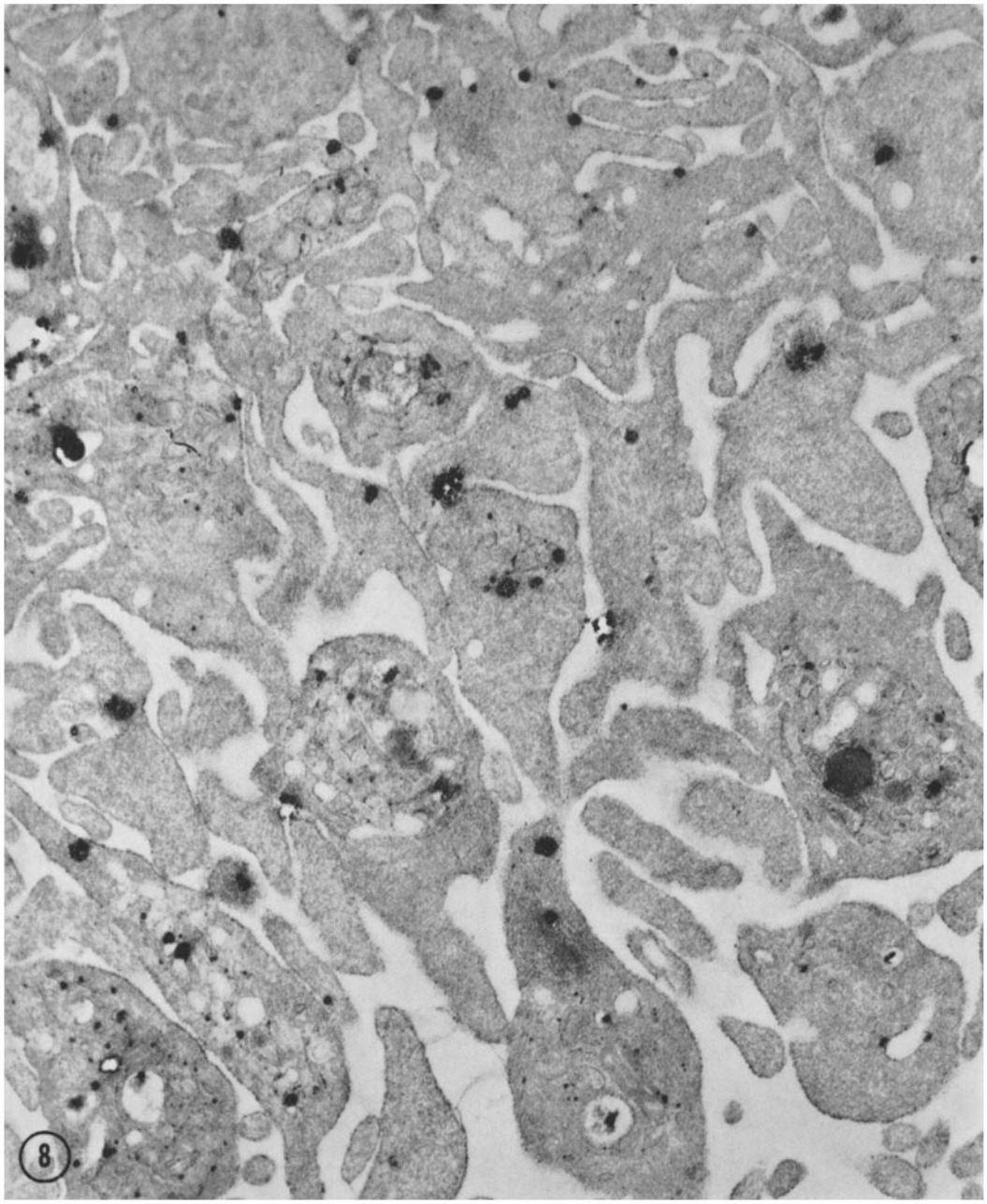

FIG. 8. Morphology of platelets undergoing leukocyte dependent release of histamine. Buffy coat leukocytes (2×10^7), antigen (620 μg P BSA) and platelets (2.5×10^9) were incubated together for 15 min at 37°C before glutaraldehyde fixation. Loose clumps of platelets are apparent with some disappearance of granules and canaliculi. × 30,000.