IMMUNOLOGICAL INDUCTION OF INCREASED VASCULAR PERMEABILITY

II. Two Mechanisms of Histamine Release from Rabbit Platelets Involving Complement*

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(Received for publication 4 September 1968)

Increased vascular permeability in rabbits may be produced by a variety of immunological mechanisms. One of these has been shown to require complement, platelets, and neutrophils and could be demonstrated by the passive cutaneous anaphylaxis (PCA) reaction (1, 2). Histamine was shown to participate in this reaction, and both it and serotonin have been implicated in other inflammatory conditions of immunological origin (3, 4).

One of the major reservoirs of histamine in the rabbit is the platelet, and since these cells are known to release their histamine in the presence of immunological reactions in vitro (5–9) they may play an important part in the production of increased vascular permeability. In vitro histamine release from platelets by antigen and antibody requires the presence of plasma (5, 10, 11). The precise plasma constituents which are necessary are unknown, and although the complement system has been implicated (5, 11), evidence presented by Barbaro (10) suggested only a partial involvement. Most of these experiments on the release process have involved soluble antigens.

Another way in which antigen-antibody complexes react with platelets is through the production of adherence reactions with the mediation of complement (C) (12, 13). The process has been shown to require the participation of C3 (13), thus resembling the immune adherence reaction with primate erythrocytes. Only platelets from certain species undergo this adherence reaction (13), but both soluble and particulate antigens may be employed.

The studies reported here were undertaken to examine the antibody that induces complement-dependent PCA reactions (1) for its ability to react with platelets in vitro. Since the in vivo lesions were inhibited by depletion of C3 with cobra venom factor, the role of complement in the reactions was of partic-

* Publication 302 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. The work was supported by United States Public Health Service Grant AI-07007 and National Multiple Sclerosis Society Grant 459.

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

§ Established Investigator of the Helen Hay Whitney Foundation.

ular interest. An examination of the adherence reactions of platelets in the light of the histamine-releasing processes was also undertaken and has demonstrated at least two mechanisms of histamine release.

Materials and Methods

Diluent.—Tyrode's solution containing 0.25% gelatin was used for all the release reactions. Platelets were washed, however, in Tyrode's solution with gelatin from which the calcium and magnesium chloride had been omitted.

EDTA and EGTA.—The disodium and tetrasodium salts of ethylenediaminetetraacetic acid were prepared as 0.2 M solutions and mixed to give a pH of 7.4. Ethylene glycol bis(β-aminoethyl ether) N,N_1-tetraacetic acid (EGTA) (K & K Laboratories Inc., Plainview, N. Y.) was prepared as a 0.2 M solution and neutralized with NaOH. These stock solutions were diluted before use.

Antigen.—Bovine serum albumin (BSA) (Armour Pharmaceutical Co., Ill.).

Antisera.—Rabbit anti-BSA antiserum was stimulated by injection of BSA in incomplete Freund's adjuvant. The antiserum produced after 2–4 wk was separated on diethylaminoethyl (DEAE) cellulose, and the fraction not adherent in 0.01 M sodium phosphate buffer, pH 8.1, was concentrated and frozen. This antibody preparation had complement-dependent PCA activity. In addition, antisera prepared and fractionated by methods described in the previous paper (1) were also employed. Quantitative precipitation (14) with the antigen was used to determine the concentration of precipitable antibody nitrogen.

Rabbits.—Adult New Zealand white rabbits were employed as a source of platelets and plasma. C6-deficient rabbits were obtained by breeding a homozygous male (which was kindly supplied by Dr. C. Biro of Mexico City) with New Zealand females and then with the heterozygotes produced. It was possible, therefore, to compare homozygous and heterozygous animals.

Platelets and Plasma.—Siliconized glassware was used throughout. Blood was obtained from the heart or ear artery and immediately mixed with 1:10 vol of 0.2 M EDTA. Platelet-rich plasma was aspirated after centrifugation in the cold (220 *g* for 20 min). The platelets and plasma were separated (1400 *g* for 20 min) and the platelets were resuspended in Tyrode's solution without Ca^{++} or Mg^{++} containing 0.005 M EDTA, and then washed twice and kept at 4°C in Tyrode's solution without Ca^{++} or Mg^{++}. The suspension was standardized to 5×10^8 platelets/ml.

Immediately before use, the plasma calcium and magnesium were restored by the addition of 1:10 vol of a mixture of 2% $CaCl_2$ and 2% $MgCl_2$. To prevent clotting, 10 units/ml heparin was included.

C3 Preparation.—Rabbit C3 was prepared from 1 liter rabbit serum by the method described for human C3 (15). Following euglobulin separation and DEAE cellulose chromatography the fraction rich in C3 (as detected by precipitation with anti-C3 in agar) was fractionated on a column of hydroxylapatite. The fraction eluted with buffer of 14.5 mmho conductivity contained the C3. After concentration the preparation contained 0.5 mg protein/ml. Immune adherence was given by EACl, 4, 2 plus 0.5 μg of this preparation. It was contaminated with some γ-globulin (Ouchterlony analysis), C5 and C6. C5 activity was detected by adding the preparation to EACl, 4, 2 and measuring the hemolysis produced by a guinea pig reagent containing the components C6-C9, which was kindly supplied by Dr. N. Cooper. C6 was assayed by adding the preparation to a mixture of EA and 0.1 ml C6-deficient rabbit serum and measuring the hemolysis produced. In this test system for C6, 50% hemolysis was produced by 10 μg of the preparation.

For the experiment in which the histamine-releasing activity of C6-deficient plasma was

restored, another preparation was used with which 50% hemolysis in the presence of C6-deficient serum was produced by a 1:225 dilution (12 μg).

Preparation of Anaphylatoxins.—Whole rabbit serum was fractionated on triethylaminoethyl (TEAE) cellulose. After applying the serum in 0.03 M sodium phosphate buffer, pH 8.1, and removing the nonadherent proteins, a linear salt and pH gradient using 0.2 M NaH_2PO_4 was applied. The tubes containing C3 were pooled and concentrated. These fractions were also found to contain the β-globulin proinactivator of cobra venom factor (CoF), as well as C5 and other β-globulins. Anaphylatoxin was generated from this preparation with CoF (25 units/mg protein N) by incubation for 30 min at 37°C with 7.5×10^{-5} M $CaCl_2$ and 2.5×10^{-4} M $MgCl_2$. The anaphylatoxin was further purified by gel filtration on Sephadex G-100 in acetate buffered saline at pH 4.0. The active fraction was assayed on the guinea pig ileum and on isolated rat peritoneal mast cells after restoration to neutrality.

Rat anaphylatoxin was produced by CoF treatment of whole rat serum and was separated on Sephadex G-100 as described above.

EAC1, 4, 2 and Immune Adherence.—Rabbit antiserum was used to sensitize the erythrocytes and the procedures used were those described by Linscott and Cochrane (16). EACl, 4, 2 was prepared with guinea pig serum at 0°C.

Zymosan.—After boiling and thorough washing, the zymosan (Nutrional Biochemical Corp., Cleveland, Ohio) was resuspended to a concentration of 2.5×10^9 particles/ml (36 mg/ml). The zymosan was incubated for 15 to 30 min with plasma (1×10^9 particles/ml plasma) and then washed five times.

Plasma Absorption and Depletion.—Plasma was absorbed with antigen-antibody precipitates (100 μg antibody N/ml plasma) prepared at equivalence for 30 min at 37°C.

Plasma C3 was inactivated by treatment with 80 units cobra venom factor (CoF)/ml for 15 min at 37°C. The preparation of CoF and the depletion of C3 in vivo has been described previously (1). 1 unit represents that amount which reduces the hemolysis produced by 1:20 dilution of human serum to 50%.

Platelet Histamine Release.—The total reaction volume was 2.5 ml. Zymosan and alexinated erythrocytes (2.5×10^8 particles or cells) were allowed to react with 2.5×10^8 platelets for 30 min at 37°C with shaking. For tests with soluble antigen, the diluent, antigen, 0.25 ml plasma (10%), platelets, and antibody were added together in that order and also incubated for 30 min at 37°C. Samples were examined microscopically after 10 to 15 min. After the incubation period the tubes were centrifuged and the supernatant fluid was assayed for its content of histamine on the atropinized guinea pig ileum. The sediment was boiled for 2 min in distilled water and also assayed, so that the released histamine could be expressed as a percentage. The background release observed by incubation of platelets alone or platelets with any two of the three reactants varied from 3 to 10% and this background has been subtracted from the reported percentages.

RESULTS

Histamine Release from Platelets Induced by Complement-Dependent PCA Antibody

Clumping and Histamine Release from Platelets in the Presence of Plasma.—Rabbit anti-BSA antibody capable of inducing complement-dependent passive cutaneous anaphylaxis (1) (C-dep PCA antibody) was added to a suspension of washed rabbit platelets in the presence of antigen and normal plasma. Under these circumstances, microscopic examination of the platelet suspension showed platelet clumping (Fig. 4). Clumping was observed after 5 to 10 min

of incubation if all four constituents (antigen, antibody, plasma, and platelets) were included, but was absent if any of the four was omitted. Histamine release was also detected under conditions where the platelets clumped and after 30 min incubation many of the platelets appeared to have lysed. The results of a typical experiment are shown at the top of Table I.

C-dep PCA antibody was fractionated by Pevikon block electrophoresis, column chromatography, and density gradient ultracentrifugation. The frac-

TABLE I

Plasma Factors in Immunological Histamine Release from Platelets

Antigen + antibody +:*		Volume of plasma used				
		0.2	0.1	0.05	0.025	–
Normal plasma	Clumping‡	+	+	+	–	–
	% release§	60	36	20	7	–
Plasma absorbed with Ag + Ab	Clumping	–	–	–	–	–
	% release	–	–	–	–	–
Heated plasma (56°C, 30 min)	Clumping	–	–	–	–	–
	% release	–	–	–	–	–
C3-depleted plasma (cobra factor)	Clumping	–	–	–	–	–
	% release	–	–	–	–	–
C6-deficient plasma	Clumping	+	+	+	–	–
	% release	–	–	–	–	–

* Antibody (12 μg N) was used with 1/20 the quantity of antigen required at equivalence, 2. 5 × 10^8 platelets and decreasing volumes of plasma.

‡ Clumping of platelets was observed microscopically after 15 min.

§ Released histamine was assayed after 30 min at 37°C.

The depletion procedures are described in the Materials and Methods section.

tions were tested for their PCA activity (1) and also for their ability to induce histamine release from platelets in the presence of normal plasma. In all cases the PCA and histamine-releasing activities were found together. Histamine release from platelets by this antigen-antibody reaction had the same properties described previously for immunological release of histamine from rabbit platelets (5–9): the time course of the release process at 37°C was similar, beginning at 10 min and approaching a maximum at 30 min; maximal release occurred in antibody excess (for 10 μg antibody N, $\frac{1}{20}$ of the antigen required for equivalence was optimal) and with the inclusion of 10% plasma; preincubation of antibody, antigen, and plasma before addition of platelets resulted in reduced release (as will be noted below in Fig. 3); and the reaction was inhibited in the presence of 0.01 M EDTA.

Examination of Washed Blood Cells from Rabbits Producing Antibody for Histamine Release Induced by Antigen.—Blood was obtained from four rabbits which were producing homocytotropic antibody and from four producing C-dep PCA antibody as evidenced by skin tests in rabbits with or without C3 depletion (1). The cells from 1 ml of blood were washed four times with Tyrode's solution containing gelatin and resuspended in this medium. After addition of 5 μg BSA nitrogen and incubation for 30 min at 37°C, the released histamine was assayed. Only blood cells from rabbits producing homocytotropic antibody released histamine (up to 70%) under these circumstances. In confirmation of the results of Siraganian and Oliveira (17) and Barbaro and Becker[1] some histamine was released from leukocytes alone, but if platelets were also present, a greatly increased quantity of histamine (coming from the platelets) was found in the supernatant fluid.

C-dep PCA antibody could not be shown to bind to blood cells in this manner in multiple tests.

The Adherence of Platelets and Release of Histamine Using Particulate Antigens: a Requirement for C3.—Platelet clumping was a consistent finding of the in vitro histamine release from platelets described above and, as will be shown later, this clumping was inhibited if C3-depleted plasma was employed. The following experiments were therefore undertaken to examine in detail the role of the platelet adherence reaction, in which C3 has been implicated (13) in the release of histamine. The use of particulate antigens enabled the antigen-antibody complexes to be washed after the addition of complement and allowed the reaction to be carried out in a plasma-free environment. Histamine release was therefore measured after platelets had been allowed to adhere to sensitized sheep erythrocytes or zymosan, to which complement components had been fixed.

Zymosan.—The reaction of zymosan particles with normal serum or plasma causes the fixation of large quantities of C3, as has been shown in many laboratories. Zymosan was therefore incubated with plasma, then washed and added to a suspension of washed platelets. The dose response curve of histamine release is shown in Fig. 1. Control preparations consisted of zymosan incubated with plasma which had not been recalcified (EDTA-plasma), with plasma treated with CoF in vitro, or with plasma from CoF-treated rabbits (CoF-plasma). Such preparations did not produce histamine release. C6-deficient plasma on the other hand, when reacted with zymosan followed by washing, was fully capable of causing histamine release. In all cases where histamine release was detected, adherence of platelets to the zymosan was also observed.

Sheep Erythrocytes.—EAC1,4,2 was prepared with guinea pig complement and was then reacted with a rabbit C3 preparation. Only after the addition of the C3 was platelet adherence observed or histamine release detected (Table

[1] Barbaro, J. F., and E. L. Becker. Personal communication.

II). Moreover, a participation of C6 in the release by particulate antigens can be excluded by the finding that EA treated with C6-def plasma was fully active for histamine release.

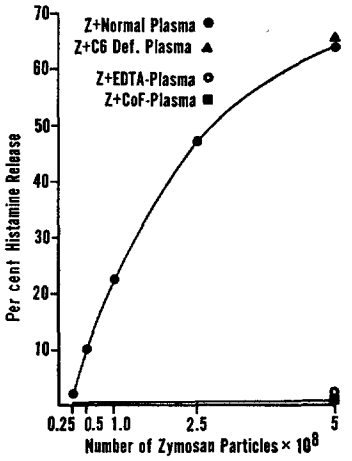

FIG. 1. Dose response curve of histamine release from platelets by zymosan treated with plasma. 5×10^8 zymosan particles were incubated with the different sources of plasma (see text) and washed before addition of washed platelets. Histamine release was assayed after 30 min at 37°C.

TABLE II

Platelet Histamine Release Induced by Washed EAC 1,4,2,3 Preparations

Erythrocyte preparations	Adherence of platelets	Histamine release
		%
EA	−	0
EAC_{gp} § 1,4,2	−	0
EAC_{gp} § 1,4,2 + rabbit C'3 preparation*	+	25
EA + rabbit C6-deficient plasma‡	+	32

* EAC 1,4,2 (2.5×10^8) + C3 preparation (40 μg protein) for 10 min at 30°C and then washed.

‡ EA (2.5×10^8) + 0.1 ml C6-deficient plasma for 20 min at 37°C and then washed. The erythrocytes were reacted with 2.5×10^8 washed rabbit platelets for 30 min at 37°C.

§ gp, guinea pig complement components.

A possible action of C1 on the platelet after it had adhered to the erythrocyte was tested by decaying the same quantity of EAC1,4,2,3 in 0.01 M EDTA at 37°C for 1 hr and then washing before addition to the platelets. Since the release was unaffected by this treatment, such an activity of C1 is unlikely. In an attempt to see if C3 peptidase activity was involved, 1×10^{-2} M glycyl-

tyrosine was included in the reaction mixture (EAC1,4,2,3 or zymosan treated with plasma plus washed platelets), but no inhibition of release was observed.

Another possible releasing mechanism involving complement is by the action of anaphylatoxins. To test this hypothesis, five times as much rabbit or rat anaphylatoxin as was required to contract the guinea pig ileum and release histamine from rat peritoneal mast cells was added to 2.5×10^8 washed rabbit platelets, with or without added plasma. In neither case was any histamine release found.

These results indicate that as far as particulate antigens (zymosan or erythrocytes) are concerned, platelet histamine release requires the reaction of C3, but not of C6 or complement components beyond C6 in the sequence.

Platelet Histamine Release Using a Soluble Antigen

Plasma Requirements for Histamine Release.—Table I shows the clumping and histamine-releasing effect of dilutions of normal rabbit plasma in the presence of antibody in excess, antigen ($\frac{1}{20}$ of the amount required at equivalence), and washed platelets. Neither clumping nor release was observed if the plasma was previously absorbed with BSA–anti-BSA, heated at 56°C, or depleted of C3 with cobra venom factor (CoF). These findings indicated that the complement system was probably involved in this immunological release of histamine from platelets.

The Requirement for C6.—The results shown in Table I indicate that C6-deficient plasma was not able to induce histamine release from platelets in the presence of antigen and excess antibody. Platelet clumping, however, was unaffected. Plasma from seven animals homozygous for C6 deficiency were tested and found to be inactive for histamine release, in contrast to plasma from seven heterozygotes which behaved normally. Addition of 0.025 ml of a preparation rich in rabbit C6 (but also containing C3 and some C5) restored the hemolytic activity of C6-deficient plasma. This restored plasma now behaved like normal plasma, i.e., it supported both platelet clumping and histamine release. Histamine release in this system, therefore, requires the action of C6.

Reduced Histamine Release Following Preincubation of the Immune Complexes.—A notable and unexplained feature of the histamine release produced by soluble antigen, antibody, and plasma is the decay in activity observed if the three components are preincubated together before addition of the platelets (Fig. 2). This was observed with antibody in excess and at equivalence, although it was more marked in the former case. Another property of this in vitro reaction requiring explanation is the reduced release seen if the antigen and antibody are prepared at equivalence, even though the resulting precipitates fix maximal quantities of hemolytic complement.

In an attempt to elucidate the mechanisms responsible for these phenomena, the following experiments were performed.

Antigen and antibody in excess were preincubated together at 37°C before addition of plasma and platelets. This led to a progressive loss of histamine-releasing activity (Fig. 2). The loss in activity was even greater when normal plasma was also present during the preincubation, although this was not observed with EDTA-plasma. If plasma was preincubated by itself, its histamine-releasing properties were not markedly affected. The loss of activity due to preincubation of antigen and antibody in excess was slowed if the reactants were preincubated in larger volumes of diluent or at 0°C.

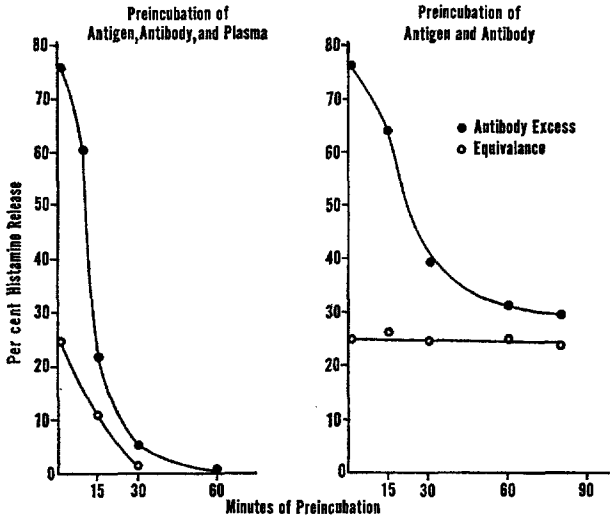

FIG. 2. Histamine release following preincubation of immune reactants. Antigen, antibody, and plasma, or antigen and antibody alone were preincubated at 37°C before addition of platelets. Histamine release was assayed after a further 30 min at 37°C. Antibody (10 μg N); antigen, 0.1 μg N (antibody excess), 2 μg N (equivalence); plasma, 0.25 ml (10%); and platelets (2.5×10^8).

In contrast, when antigen and antibody at equivalence were preincubated, no reduction in releasing activity was observed, although once again some loss occurred if plasma was also present.

These findings suggested that changes in precipitate structure with time might account for some of the loss of activity and since the loss was greater in the presence of plasma, that generation of an inhibitor in the plasma might also contribute.

Generation of an Inhibitor of Histamine Release.—The possibility that an inhibitor of histamine release was being generated was further examined. Zymosan and plasma were incubated in duplicate for different lengths of time at 37°C. At each time interval the zymosan and plasma in one tube were added

to a suspension of washed platelets. The zymosan in the other tube was washed before being added to the platelets. Even after 90 min (and in some experiments 2 hr), the washed zymosan with complement on (ZC) caused the same amount of histamine release (Fig. 3). The zymosan and plasma together, however, showed an increasing inhibition of release.

Table III shows the inhibitory effect of supernatants from zymosan, or BSA and anti-BSA incubated with plasma for 30 min at 37°C. The supernatant from zymosan and plasma was inhibitory to histamine release induced by both zymosan and by BSA–anti-BSA. Inhibitor was also produced by the soluble antigen-antibody system and slightly more was produced by antigen and

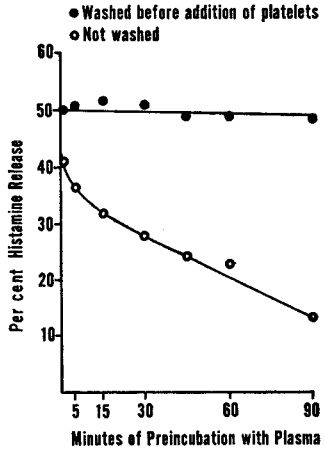

FIG. 3. Generation of an inhibitor of histamine release. Platelets (2.5×10^8) were added to zymosan (2.5×10^8) which had been preincubated with the plasma or to zymosan which had been preincubated and then washed. Histamine release was assayed after 30 min incubation with platelets at 37° C.

antibody at equivalence than in antibody excess. In each case the amount of inhibition generated in the plasma increased with the time of incubation. No inhibitory activity was found in the supernatant from zymosan plus EDTA-plasma.

It had previously been noted that even with antigen and antibody at equivalence, a situation which might be expected to resemble more closely the particulate antigen system, C6-deficient plasma did not support histamine release. Plasma from three homozygous, C6-deficient rabbits was therefore compared with plasma from heterozygous siblings to see if there was any difference in the quantity of inhibitor generated. It was found that more inhibitor was generated in the C6-deficient plasma (Table III).

The Requirement for Calcium.—Chelation of divalent cations prevents the

release of histamine from platelets by the soluble antigen system. In an attempt to characterize the cation required for release by antigen, antibody and plasma. Siraganian, Secchi, and Osler (18) showed that addition of magnesium to EDTA-plasma was sufficient to restore the releasing activity. As complement requires calcium for the activation of C1, the present system was examined for the effect of chelating agents. Ethylene glycol tetraacetic acid (EGTA) was employed because it has a higher binding affinity for calcium than does EDTA.

TABLE III

Generation of an Inhibitor of Platelet Histamine Release from Plasma

Inhibitor-generating system*	Histamine-releasing system	Histamine release	Inhibition
		%	%
—	Ag + Ab + plasma + platelets (antibody excess)‡	76	0
Ag + Ab + plasma (antibody excess)		53	30
Ag + Ab + plasma (equivalence)		49	35
Ag + Ab + C6-def plasma (equivalence)		29	62
Z + plasma		44	42
Plasma alone		80	0
—	Washed ZC + platelets§	60	0
Z + plasma		30	50
Z + C6-def plasma		10	80
Z + EDTA plasma		60	0
Plasma alone		65	0

* The reactants were incubated at 37°C for 30 min, centrifuged, and the supernatant added to the histamine-releasing system. Antibody, 10 μg N; antigen, 0.1 μg N (antibody excess) and 2 μg N (equivalence); zymosan, 2.5×10^8 particles; plasma, 0.25 ml.

‡ Antibody (10 μg N) + antigen (0.1 μg N) + 0.25 ml (10%) plasma. The histamine release was assayed after 30 min at 37°C.

§ Zymosan (2.5×10^8 particles) incubated with 0.25 ml plasma and then washed.

These results are representative of those from three similar experiments.

Calcium chloride, magnesium chloride, or a combination of both was added at different concentrations to a BSA–anti-BSA system which had been prevented from releasing histamine by the presence of EGTA. In addition to examining platelet clumping and histamine release, identical tubes were set up in which sensitized sheep erythrocytes (EA) replaced the platelets, antigen, and antibody. This was done in order to assess the hemolytic activity of the complement under these conditions. The results are presented in Table IV.

If 10% plasma was used, addition of magnesium alone was sufficient to restore some hemolytic, platelet clumping, and histamine-releasing activity, possibly because of insufficient binding of calcium at this high plasma con-

centration. Addition of calcium, on the other hand, permitted more hemolysis and histamine release. If 4% plasma was employed, the different effect of the

TABLE IV

*Cation Requirement for Histamine Release**

Plasma	Molarity × 10^{-4}‡		Hemolysis	Platelet clumping	Histamine release
	Ca^{++}	Mg^{++}			
			%		%
4% plasma + 4 × 10^{-4} M EGTA	—	—	0	0	0
	12	—	100	+	30
	—	12	35	0	0
	8	4	100	+	60
10% plasma + 5 × 10^{-4} M EGTA	—	—	0	0	0
	10	—	100	+	48
	—	10	60	+	28
	6.6	3.3	100	+	60

* Histamine release from 2.5 × 10^8 platelets was produced by addition of 10 μg Ab N, 0.1 μg Ag N in the presence of 4 or 10% rabbit plasma. Platelet clumping was observed microscopically. Duplicate tubes contained 2.5 × 10^7 sensitized sheep erythrocytes instead of Ag and Ab.

‡ Final molar concentration in the reaction mixture.

TABLE V

The Effect of Hirudin on Platelet Histamine Release

Releasing system	Units hirudin	Histamine release	Inhibition
		%	%
Thrombin (0.05 units)	—	74	
	2.5	0	100
	0.25	3	96
Ag + Ab + plasma (antibody excess)*	—	73	
	250	72	0
Washed ZC*	—	50	
	250	50	0

* The histamine-releasing systems are described in Table III.

two cations was clarified. Addition of magnesium was not capable of restoring the platelet clumping or histamine-releasing activity, although a small amount of hemolysis was observed. On the other hand, clumping, histamine release, and hemolysis were found if calcium was added. Due to the different binding

affinities, excess calcium will release bound magnesium so that in this latter circumstance both free calcium and magnesium may be expected to be present. Addition of both cations resulted in increased release. The results indicate a requirement for calcium and possibly magnesium as well, in the histamine release process.

Demonstration That the Release Is Not Due to the Action of Thrombin.—Small quantities of thrombin will cause platelet histamine release. To exclude an action of thrombin in the in vitro release mechanism involving antigen, antibody, and complement, the inhibitory action of heparin and hirudin was examined. Table V shows that hirudin was 10,000 times more effective as an inhibitor of histamine release by thrombin than as an inhibitor of the immunological systems. Heparin also showed a wide difference, but was inhibitory to complement at high concentrations.

DISCUSSION

The release of histamine from platelets induced by homologous antibody and an antigen unreleated to the platelet could be shown to result from two different mechanisms, one involving particulate and the other soluble antigens. Both mechanisms required the participation of complement.

Particulate Antigens.—Zymosan and erythrocytes which had fixed complement (ZC and EAC) could be washed and then reacted with the platelets in a plasma-free environment. Platelets adhered to the ZC or EAC particles and histamine was released into the medium. This reaction required the participation of complement components only through C3 or perhaps C5 in the sequence, since zymosan or EA treated with C6-deficient plasma was fully active. The reaction did not take place when platelets were exposed to EAC1,4,2. Only after the addition of C3 did the platelets adhere and release their histamine.

The immune adherence of platelets from certain species has been described previously (12, 13) and the requirements for adherence herein reported confirm these results. The present studies further these findings by demonstrating that the adherence reaction of platelets with a particulate antigen induces release of histamine. This release may be associated with platelet degranulation similar to that described by Spielvogel (8) for the reaction of platelets with endotoxin.

A number of possible mechanisms were examined that could explain the release of histamine once the platelet was adherent to the particle. It is possible that following adherence, which required fixed C3, other complement components could act on the platelets which have now been brought into contact with the complex. Any effect of C1 or C2 in this way was rendered unlikely by the observation that the releasing action of ZC did not decay after incubation in the presence of EDTA followed by washing. C3 peptidase activity (19) might have been responsible. Again, the lack of decay after 2 hr at 37°C and

the inability of a substrate of the enzyme (glycyl-tyrosine) to inhibit the activity suggest that this enzyme is not responsible. Because it was not possible to separate completely the rabbit C3 from contaminating C5, an action of this latter component cannot be completely ruled out, although rendered unlikely by the decay experiments. Another possibility, that the known histamine-releasing action of anaphylatoxins on mast cells might also affect platelets, is not easily reconcilable with the inability of anaphylatoxins to act on platelets directly. Furthermore, histamine was released from platelets by washed ZC which would have little or no anaphylatoxin activity. The release was in all probability not induced by thrombin because the action of thrombin on platelets was inhibited by small quantities of hirudin, whereas even 10,000 fold larger quantities of this substance were not inhibitory to the action of ZC.

A remaining hypothesis involves the adherence process itself. It is suggested that a reaction of platelets with the C3 (or perhaps γ-globulin or C4) spread out along the surface of a particle itself triggers off a release reaction in the platelet. This release reaction has received some additional study, and it has become apparent that an active participation of the platelet is required, as release of histamine is blocked in the absence of energy metabolism and when certain platelet esterases are inhibited.[2]

This release reaction of the platelet may have some protective function perhaps related to the body's defence against foreign invaders. By adhering to the foreign particles and releasing constituents, greater amounts of antibody and complement would be brought into the site. Only platelets from certain species undergo adherence reactions with complement (13). However, those from at least two of the nonreacting species, man and the pig, do react with antigen-antibody complexes (20). Thus the mechanism of release may be similar although the stimulus (adherence) is caused by different substances.

Soluble Antigens.—The reaction of soluble antigen, antibody, and platelets in the presence of plasma, resulted in platelet clumping. In conditions of antibody excess these clumps were not large and antigen-antibody complexes were not visible. At equivalence, platelets were caught up in the precipitate. Inhibition of this clumping by inactivation or absorption of complement or by depletion of C3 suggested that this too was a manifestation of the immune adherence reaction. The small size of the platelet clumps presumably resulted from the condition of antibody excess. Because of platelet lysis, the clumps were more difficult to see with increasing incubation time. Gocke and Osler (7) did not observe much clumping but looked later in the reaction. Moreover, under their more dilute condition, small complexes may have adhered to the platelets without producing so much cross-linking.

The clumping of platelets with nonparticulate antigens in the complex was

[2] Henson, P. M., and C. G. Cochrane. Manuscript in preparation.

not by itself sufficient to cause release of histamine. This was shown by the use of C6-deficient plasma, which produced clumping but not histamine release. Addition of C6 restored the releasing activity and thus demonstrated that a soluble antigen system, unlike that with a particulate antigen, required later-acting complement components. The evidence for complement participation provided by these studies extends the results of Humphrey and Jacques (5) and of Gocke (11) who presented evidence that hemolytic complement was involved. Siraganian, Secchi, and Osler (18) demonstrated a requirement for magnesium. While a similar requirement was observed in the present study, it has been shown that calcium is needed as well, a finding that agrees with previous reports (5). This is entirely in accord with the known requirements for divalent cations in the complement system.

Since the antibody of the immune complex is not directed against the platelet, the mechanism by which the later-acting components of complement induce the release is perplexing. A possible, but as yet unproven, mechanism is that close contact or even phagocytosis of very small complexes in the presence of the total complement sequence might result in a lytic action of the complement on the cell membrane, even though the antigen is not a part of that membrane. An alternate hypothesis involves an action of the C5-6-7 complex, a substance which is known to activate certain neutrophil enzymes (21).

One of the difficulties with the hypothesis of complement action is that maximal histamine release occurs with antibody excess, whereas maximal complement fixation occurs at equivalence. Such determinations, however, are based on the inactivation of complement for hemolysis. On the other hand, the binding of platelets in immune adherence occurs as a function of the availability of bound C3. This may well be greater in the antibody excess condition where less bound C3 would be internalized in the complex. Evidence for the importance of the size and structure of the complex came from the finding that preincubation of antigen and antibody in antibody excess, before addition of plasma and platelets, resulted in less histamine release. At equivalence there was no such reduction in releasing activity due to preincubation without plasma. This suggests that a configurational change occurs in complexes in antibody excess with preincubation which results in diminished activity. The results differ somewhat from those of Gocke and Osler (7), but may reflect different conditions of preincubation. The reduction of activity following preincubation of antigen and excess antibody may account, in part, for the much greater loss of activity seen if antigen, antibody, and plasma were incubated together before addition of platelets.

Generation of an Inhibitor of Histamine Release.—Prior reaction of the antigen, antibody, and plasma produces an inhibitor of the release reaction when platelets are added to the system. This inhibitor was active on both the re-

lease mechanisms discussed in this paper. Since it was inhibitory to the release induced by ZC, it did not appear to work by preventing complement fixation. Its generation was found to require divalent cations. More inhibitor was generated in C6-deficient plasma than in normal plasma, but this would not account for the inability of C6-deficient plasma to support the release of histamine from platelets when a soluble antigen was employed, since the addition of C6 restored the releasing activity of the plasma. The exact nature of this inhibitor is as yet undetermined, but its production apparently contributes to the decay of activity observed after preincubation of antigen, antibody, and plasma.

Generation of a substance into the surrounding medium from antigen–antibody-complement interaction which causes histamine release from platelets has not, therefore, been demonstrated. The release processes seem to require adherence of the platelet to the complex.

In Vitro Histamine Release by Complement-dependent PCA Antibody.—This antibody, which has been described previously (1), was capable of causing in vitro histamine release from platelets. This process may, therefore, account for the in vivo findings that platelets and histamine participate in the PCA reaction. The reaction of complexes with platelets in these lesions may involve one or both of the release mechanisms described herein as both are inhibited by cobra venom factor. Following the initial reaction of antigen, antibody, complement, and platelets, release of platelet ADP could further produce aggregation of platelets, thus serving to potentiate any effect. In addition, there may also be other as yet undefined processes that are operative. In particular, recent observations have indicated a synergistic action of neutrophils on the release of histamine from platelets in the presence of immune complexes and complement.[2]

SUMMARY

Two mechanisms have been described whereby rabbit platelets in vitro may be induced to release their contained histamine by the reaction of antigen and antibody. Both processes require the participation of the complement system.

In the first, the adherence of platelets to particulate antigens such as zymosan or erythrocytes which have fixed complement through the third component was followed by histamine release. Plasma lacking C6 activity was fully active in this system.

In the second mechanism, the reaction of soluble antigen with antibody in the presence of plasma also caused release of histamine from platelets and platelet clumping was observed. The release process, which appeared to follow the adherence of platelets to the immune complex, required the action of C6 and perhaps the later-acting components. No evidence could be obtained that a soluble factor was produced which caused the release. Instead, an inhibitor

of the release process was detected after incubation of antigen, antibody, and plasma.

Antibody preparations capable of giving complement-dependent PCA reactions in rabbits were also shown to induce the release of histamine from platelets in vitro.

The authors are grateful to Mr. G. M. Standford for preparing the illustrations.

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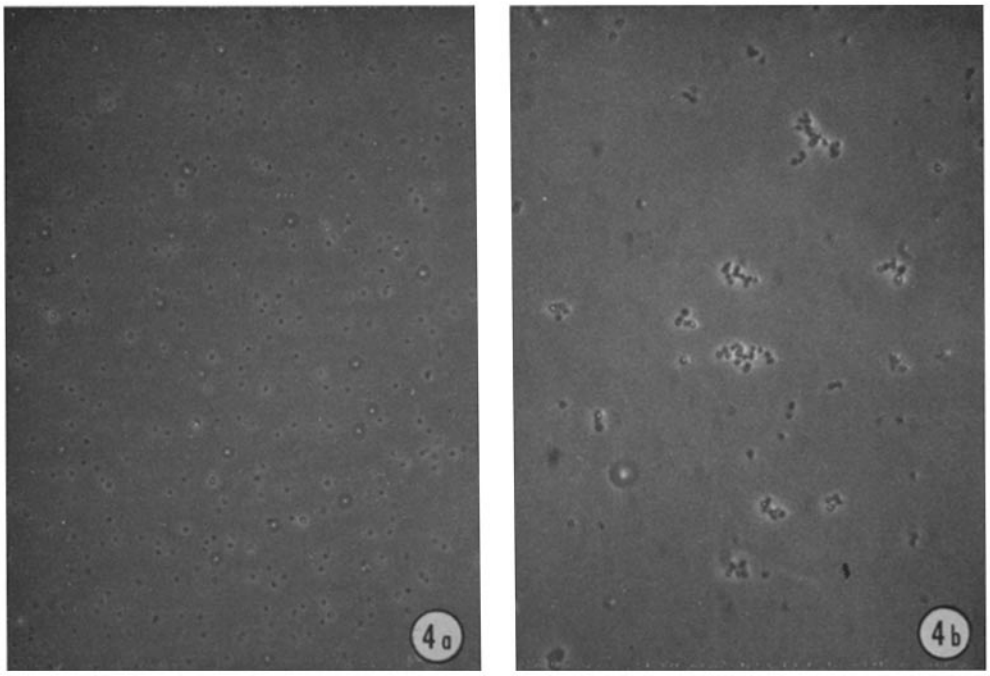

FIG. 4. Clumping of platelets by antigen and antibody in the presence of plasma. *a*. Antigen, plasma, and platelets. *b*. Antigen, antibody, plasma, and platelets (Note clumping of platelets). See Table I for details.