IMMUNOREACTIONS INVOLVING PLATELETS

III. Quantitative Aspects of Platelet Agglutination, Inhibition of Clot Retraction, and Other Reactions Caused by the Antibody of Quinidine Purpura

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The antibody present in some instances when thrombocytopenia is due to drug sensitivity not only fixes complement in the presence of platelets and the offending drug, but also agglutinates platelets and acts to inhibit clot retraction (1–4). It has been considered that the antibody also lyses platelets in the presence of complement (4). The antibody has been suspected of acting on endothelium (1, 3), and in one report (5) the antibody present following thrombocytopenia due to quinidine was found to fix complement with red cells and cause their agglutination and lysis.

In the present work, the activity of the antibody as a platelet agglutinin was compared with its activity in complement-fixing reactions, the nature of morphological changes in platelets in the presence of antibody and quinidine was studied, and some aspects of inhibition of clot retraction by the antibody were investigated. Observations were also made concerning specificity and other qualitative properties of the antibody. The studies were directed at determining interrelationships of the different antibody activities, the relative sensitivity of various quantitative and qualitative tests for the presence of antibody, and possible factors of significance in the pathogenesis of thrombocytopenic purpura due to drugs.

Materials and Methods

All reagents, the source of antibody, and methods of measuring complement fixation and determining antibody units were the same as in Paper I.

Measurement of Platelet Agglutination.—Siliconed glassware was used throughout and observations were made at room temperature. Mixtures of platelet suspensions prepared as in Paper I, patient's serum containing antibody, and quinidine were made in 12 × 75 mm. test tubes; and aliquots were transferred immediately to slides and overlaid with coverslips ringed with vaseline to permit prolonged microscopic observation of the wet preparations. Agglutination occurred equally well in slide preparations and in aliquots remaining in the

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tubes. All sera in agglutination mixtures were heated at 56° for 40 minutes before use unless otherwise indicated. Only those platelet suspensions which remained morphologically unaltered and did not show non-specific agglutination for long periods at room temperature were considered satisfactory for agglutination tests. When antibody was titrated, dilutions of patient's serum with serum from normal individuals gave the same results as dilutions with saline. Preliminary mixing of any two reagents 1 hour before adding the third had no effect on the degree of agglutination or the rate at which it occurred.

Measurement of Clot Retraction.—Mixtures of 3 ml. of whole blood with 0.2 ml. of test solution, made in siliconed graduated test tubes, were allowed to clot spontaneously or were clotted with 10 units of thrombin (Parke, Davis & Company) with 0.1 ml. of saline. Spontaneous clotting occurred in approximately 30 minutes in the siliconed tubes; clotting after addition of thrombin took place within 30 seconds. Applicator sticks used for mixing reagents were left in the tubes. After the tubes were incubated 1 hour at 37°, the applicator sticks with adherent clots were removed and the volume of free fluid plus cells not incorporated in the clots was measured. Using normal blood, the volume of free serum and cells following spontaneous coagulation or clotting with thrombin was 54 ± 5 per cent of the original volume of blood used. The technique is similar to one used by Lucia *et al.* (6).

EXPERIMENTS AND RESULTS

1. Agglutination Reactions.—

(*a*) *Effect of antibody concentration on platelet agglutination.* The effect of varying the concentration of antibody on the degree of platelet agglutination is shown in Table I, using the criteria for grading agglutination shown in Fig. 1. The concentration of antibody which produced 1+ agglutination in 1 hour had to be approximately doubled to produce 2+ agglutination, quadrupled to produce 3+ agglutination, and increased eightfold to produce 4+ agglutination. The rate of development of agglutination as well as the final degree of agglutination reflected antibody concentration. Agglutination progressed with time after the 1 hour interval chosen for the final reading; *e.g.*, mixtures showing 1+ agglutination at 1 hour would show as much as 2+ and occasionally 3+ agglutination after 16 hours, and some mixtures showing no agglutination at 1 hour would show ± or 1+ agglutination after 16 hours. Observations were limited to the 1 hour period when quantitative comparisons of results were attempted because beyond that interval visible differences in agglutination with large changes in antibody concentration were less definite, and control mixtures completely free of agglutination were more difficult to obtain.

The concentration of platelets in an agglutination mixture affected the degree of agglutination obtained when antibody concentration was constant. Increasing platelet concentrations resulted in smaller aggregates of platelets until no agglutination occurred, and decreasing platelet concentrations limited the ability to differentiate degree of agglutination based on size of platelet aggregates. In order to compare changes in degree of agglutination, platelet concentration had to be kept constant. The platelet concentration found to be most suitable for reading agglutination and which was used as routine was $2.5 \times 10^5/mm^3$.

The approximate titer of antibody in a serum sample could be measured by the highest final dilution of serum producing 1+ agglutination in 1 hour. The titer was only as precise as the range of dilution giving little detectable difference in degree of agglutination; *e.g.*, $\frac{1}{30}$ to $\frac{1}{40}$ dilution for serum containing a high concentration of antibody, or $\frac{1}{3}$ to $\frac{1}{4}$ dilution for serum with $\frac{1}{10}$ that concentration of antibody. By comparing agglutination titer with the concentration of antibody in units/milliliter determined as in Paper I, it was

TABLE I

Effect of Antibody Concentration on Platelet Agglutination

Tubes contained 0.05 ml. quinidine gluconate (10 mg./ml.), 0.05 ml. platelets (2.5×10^6/mm.^{3}), varying amounts of serum, and saline to bring the final volume to 0.5 ml. Final quinidine concentration, $10^{-2.72}$ M; final platelet concentration, 2.5×10^6/mm.^{3}; reciprocal of final serum dilutions as tabulated. Undiluted serum contained 200 units of antibody/ml. determined as in Paper I. Incubated at room temperature; degree of agglutination graded as shown in Fig. 1. Control mixtures, containing all reagents except for quinidine or all reagents with normal serum substituted for patient's serum, showed no agglutination.

Serum dilution (reciprocal)	Antibody	Degree of agglutination			
		15 min.	30 min.	45 min.	60 min.
	units/ml.				
1.25	160	4+	4+	4+	4+
1.67	120	4+	4+	4+	4+
2.50	80	2+	3+	3-4+	4+
5.0	40	1+	2+	3+	4+
6.0	33.3	1+	2+	3+	3-4+
10.0	20.0	1+	1-2+	2+	3+
13.3	15.0	±	1+	2+	2-3+
20.0	10.0	±	1+	1-2+	2+
30.0	6.7	0	±	1+	1+
40.0	5.0	0	±	±-1+	1+
80.0	2.5	0	0	0	0
∞	0	0	0	0	0

found that 1+ agglutination occurred in the presence of 4 to 6 units of antibody/ml. when quinidine concentration was optimal. If the incubation period was extended to 16 hours, ± or 1+ agglutination occurred when antibody concentration was 2 to 3 units/ml., and this was the order of antibody concentration which could be detected qualitatively by agglutination techniques.

(*b*) *Effect of quinidine concentration on platelet agglutination.* Table II shows changes in degree of agglutination when quinidine concentration was varied, using several different concentrations of antibody. The higher the concentration of antibody, the lower the concentration of quinidine at which agglutination could be measured. If a 16 hour incubation time was used instead of the standard 1 hour period, ± to 1+ agglutination occurred at a quinidine concentra-

tion 0.7 to 1.0 decade on a log scale below the lowest quinidine concentration giving 1+ agglutination with each amount of antibody in Table II. At the lower concentrations of antibody, less agglutination occurred when quinidine concentration was very high ($10^{-1.82}$ M) than at quinidine concentrations giving maximum agglutination.

2. Morphological Changes in Platelets Exposed to Antibody and Quinidine.— Ackroyd considered that the antibody of sedormid purpura acted as an agglutinin in the absence of complement and as a lysin in the presence of

TABLE II

Effect of Quinidine Concentration on Platelet Agglutination

Conditions as in Table I with final quinidine concentration and final antibody concentration as tabulated. Degree of agglutination observed after incubation for 1 hour.

Quinidine gluconate log M concentration	Degree of agglutination				
	Final antibody concentration *units/ml.*				
	150	50	25	12.5	6.25
−1.82*	4+	4+	2-3+	1+	0
−1.94‡	4+	4+	3+	2+	1+
−2.72	4+	4+	3+	2+	1+
−3.72	4+	4+	3+	2+	1+
−4.72	4+	4+	3+	1+	0
−5.34	4+	3+	2+	0	−
−5.72	3+	1+	1+	−	−
−6.04	1+	0	0	−	−
−6.72	±	0	−	−	−
−7.72	0	−	−	−	−

* A profuse precipitate with particles showing Brownian motion occurred in these tubes.
‡ Moderate amount of clumped precipitate present in these tubes.

complement (4). The following observations suggest that the antibody may not necessarily act directly as a platelet lysin in association with complement. In the usual agglutination mixture platelets occasionally became less refractile, swollen, and misshapen, the over-all morphological change perhaps best described as "fading," for they did not completely lose their identity. If fading did occur, it took place either before, during, or after the development of agglutination. With any one platelet preparation, fading either did not occur when platelets were agglutinated (*e.g.* Fig. 1) or occurred regardless of the degree of agglutination. If fading was observed, it was either slight or marked but of the same degree in mixtures containing insufficient antibody or quinidine to cause agglutination as well as enough to cause 4+ agglutination; whereas the same platelets in control mixtures remained morphologically intact. Fading of

platelets was frequently seen when the patient's native serum was used as a source of antibody in agglutination mixtures and was less frequently seen when the patient's native plasma was used. It did occur, however, in mixtures in which serum or plasma as the only source of complement had been heated at 56° for 40 minutes immediately before use.

TABLE III

Inhibition of Clot Retraction by Quinidine in the Presence and Absence of Antibody

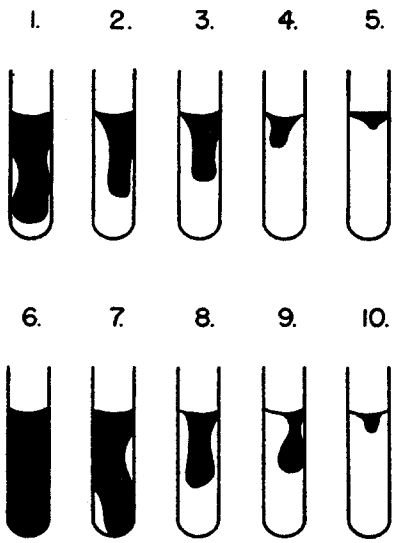
Tests carried out as described in Materials and Methods. Whole blood was used but quinidine concentration is expressed per milliliter of plasma; patient's hematocrit value 42 per cent, that of normal blood sample 44 per cent. Tabulated results of per cent free fluid are the average of two determinations. Patient's blood contained 10.5 units of antibody/ml. of plasma. A final plasma concentration of 0.036 M Na citrate was obtained by adding 0.2 ml. of 0.36 M Na citrate to the clotting mixtures. When thrombin was used for clotting it was the last reagent added. Inhibition of clot retraction could be considered complete when 10 per cent or less free fluid was obtained.

Blood containing antibody 10.5 units/ml. plasma					Normal blood			
Quinidine gluconate concentration		Clot retraction; per cent free fluid			Quinidine gluconate concentration		Clot retraction; per cent free fluid	
		Spontaneous coagulation	Clotted with thrombin	Clotted with thrombin in presence of 0.036 M Na citrate			Spontaneous coagulation	Clotted with thrombin in presence of 0.036 M Na citrate
mg./ml.	*log M*				*mg./ml.*	*log M*		
0	0	51	52	—	0		54	—
0.0015	−5.54	48	49	—	1.1	−2.68	58	—
0.005	−5.02	25	20	—	1.3	−2.61	52	—
0.01	−4.72	24	22	—	1.5	−2.54	45	—
0.05	−4.02	18	15	54	1.9	−2.43	39	—
0.1	−3.72	14	9	49	2.2	−2.38	24	—
0.15	−3.54	5	5	53	2.5	−2.32	23	—
0.2	−3.42	4	6	50	3.0	−2.24	15	17
					3.5	−2.18	11	8
					4.0	−2.12	6	7

3. Inhibition of Clot Retraction.—

The effect of quinidine concentration on degree of inhibition of clot retraction of blood containing antibody and normal blood is shown in Table III. Quinidine inhibited clot retraction of normal blood detectably when the plasma concentration was approximately $10^{-2.54}$ M (1.5 mg. quinidine gluconate/ml.) and completely when the concentration was approximately $10^{-2.18}$ M (3.5 mg. quinidine gluconate/ml.); whereas inhibition of clot retraction of the patient's blood containing 10.5 units of antibody/ml. of plasma was detectable at concentrations of quinidine between $10^{-5.4}$ and 10^{-5} M and complete at approxi-

mately $10^{-3.7}$ M quinidine. Tests done with blood containing different concentrations of antibody showed that an inverse relationship existed between the concentration of antibody and the concentration of quinidine required to produce detectable and complete inhibition of clot retraction. The units of



TEXT-FIG. 1. Inhibition of clot retraction using citrate-plasma. Experimental tubes (1 to 5) contained 0.4 ml. of varying dilutions of patient's heated serum in normal heated serum, 0.02 ml. 10^{-1} M quinidine gluconate, 0.2 ml. platelet-rich citrate-plasma (10^6 platelets/mm.^{3}), 1.35 ml. platelet-free citrate-plasma, and 0.03 ml. of thrombin solution containing 10 units of thrombin. Undiluted patient's serum contained 150 units antibody/ml. Citrate-plasma contained 0.046 molar citrate. Final concentrations: quinidine, 10^{-3} M; platelets, 10^5/mm.^{3}; citrate, 0.036 M; antibody, tube No. 1 = 30 units/ml., No. 2 = 15 units/ml., No. 3 = 7.5 units/ml., No. 4 = 4 units/ml., and No. 5 = 2 units/ml. Control tubes (6 to 10) contained 0.4 ml. of heated normal serum, 0.02 ml. 10^{-1} M quinidine gluconate, 1.55 ml. of varying dilutions of platelet-rich citrate-plasma in platelet-free citrate-plasma, and 0.03 ml. of thrombin. Final concentrations: quinidine, 10^{-3} M; no antibody; platelets, tube No. 6 = 0.625×10^4/mm.^{3}, No. 7 = 1.25×10^4/mm.^{3}, No. 8 = 2.5×10^4/mm.^{3}, No. 9 = 5×10^4/mm.^{3}, and No. 10 = 10×10^4/mm.^{3}.

The method of measuring clot retraction is similar to one described by Hartmann and Conley (8).

antibody required for minimal and complete inhibition of clot retraction were determined using a plasma quinidine concentration of $10^{-2.72}$ M (1 mg. quinidine gluconate/ml. plasma), a concentration which was optimal for complement fixation and agglutination reactions but which did not inhibit clot retraction of normal blood. With this concentration of quinidine complete inhibition of clot retraction occurred in patient's blood when antibody concentration was as low as approximately 5 units/ml. plasma, partial inhibition occurred when anti-

body concentration was from 2.5 to 3.5 units/ml., and no inhibition occurred when antibody concentration was less than approximately 2.0 units/ml. The minimum concentration of antibody which could be detected with certainty using this technique was in the order of 2 to 3 units/ml. of plasma. These data were obtained using whole blood of patients during the rise and decline of antibody concentration following test doses of quinidine (see Paper IV).

Although inhibition of clot retraction could be produced in normal whole blood or in a test system consisting of platelets suspended in normal citrate-plasma by adding patient's serum or plasma containing antibody along with quinidine, the amount of antibody added could not be titrated accurately in this manner. For example, although partial inhibition of clot retraction was produced by adding the patient's serum to a system containing 10^5 platelets/mm.^{3} and 10^{-3} M quinidine in citrate-plasma, complete inhibition was not obtained even when the final antibody concentration in the mixture was as high as 30 units/ml. (Text-figure 1). Similarly, transferring antibody in the form of patient's serum to normal whole blood did not produce the degree of inhibition of clot retraction expected from the final concentration of antibody in the mixture.

Failure of transferred antibody to produce the expected degree of inhibition of clot retraction in the test systems used was related in one case (see Table III, also reference 2) to the presence of citrate[1] which partially interfered with inhibition of clot retraction, and in the other case possibly to some non-specific effect of serum itself when present prior to initiation of coagulation.

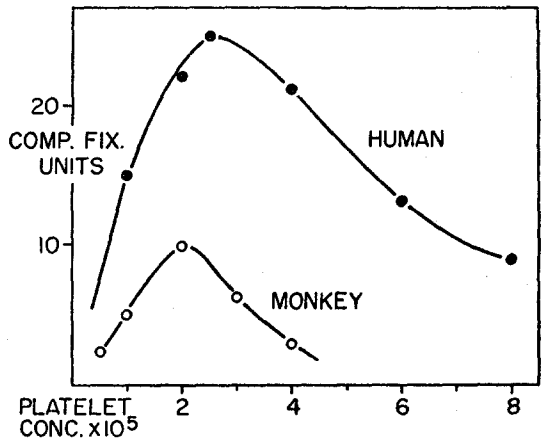
4. Specificity of Antibody Reactions.—

(*a*) *Homologous platelets.* Antibody in the presence of quinidine agglutinated the patient's own platelets and the platelets of 30 other individuals to an equal degree regardless of their blood group or Rh factor, the patient's blood group being Type O, Rh-positive. The patient's platelets and those from individuals of all blood groups were equally effective in complement-fixing reactions. A possibility existed that platelets from individuals taking quinidine might differ from normal platelets, perhaps by containing more firmly bound quinidine. Two normal donors were given 0.2 gm. quinidine sulfate every 8 hours for 4 days; blood was drawn 2 hours after the last dose of quinidine; and sedimented platelets from 25 ml. of platelet-rich plasma from each donor were washed once with 5 ml. of saline. These platelets did not fix complement in the presence of

[1] The anticoagulant, disodium salt of ethylenediaminetetraacetic acid (sequestrene), when substituted for citrate in a system containing plasma or whole blood for evaluating clot retraction, was found to completely inhibit clot retraction of its own accord; and therefore could not be used. Inhibition of clot retraction by sequestrene was not due to a permanent effect on platelets (if due to an effect on platelets at all), for platelets exposed to 0.15 per cent sequestrene solution retained their clot-retracting properties when sequestrene was removed by washing with saline.

antibody unless quinidine was added to the complement-fixing mixture, and then their activity was the same as normal platelets. There was no evidence that quinidine was bound more firmly by platelets *in vivo* than by platelets *in vitro* (see Paper I).

(*b*) *Heterologous platelets.* The platelets of 8 dogs, 7 rabbits, 4 *rhesus* monkeys, and 4 guinea pigs were not agglutinated in the presence of amounts of antibody and quinidine which produced 4+ agglutination with human platelets when the same techniques for preparing platelet suspensions and performing tests were used. Platelets from the rabbits gave 1–2+ agglutination in both control



TEXT-FIG. 2. Comparison of effectiveness of human and monkey platelets in complement-fixing reactions. Reactions carried out as in Paper I, Fig. 1. Antibody concentration 2.3 units/0.5 ml. mixture and quinidine concentration $10^{-2.9}$ M in each case. Similar results were obtained with platelets from two other monkeys.

tubes and tubes containing quinidine. Platelets from all the animals except monkeys did not fix complement or adsorb antibody when tested using a range of platelet concentrations as in Paper I, Fig. 1. Although *rhesus* monkey platelets were not agglutinated, they were effective in complement-fixing reactions and adsorbed antibody. The concentration of monkey platelets required to completely adsorb a given amount of antibody was approximately 3 times that of human platelets. The difference in effectiveness of monkey and human platelets in complement-fixing reactions is shown in Text-fig. 2. The amount of quinidine adsorbed by monkey, dog, and rabbit platelets incubated in quinidine solutions as in Paper I, Table III, was similar in each case to the amount adsorbed by human platelets.

(*c*) *Endothelial cells.* It was considered by Ackroyd that a reaction between antibody and endothelium may account in part for hemorrhagic manifestations of drug purpura. In one report (9) ground choroid plexus did not fix comple-

ment in the presence of the antibody of quinidine purpura and quinidine, and did not adsorb antibody. In the present experiment intact endothelial cells were used in the same manner as platelets in an attempt to fix complement and adsorb antibody.

The endothelial lining of a section of human aorta obtained at autopsy 5 hours after death was scraped off with a knife blade and the membranous material was minced and washed twice with saline. The small sheets of cells obtained had characteristic endothelial morphology (Fig. 1 F). These cells in saline suspension were added to complement-fixing mixtures containing 40 units of antibody and $10^{-2.9}$ M quinidine. The amounts of endothelial cells added were equivalent in dry weight to amounts of platelets covering a suboptimal, optimal, and above optimal range of platelet concentrations for this concentration of antibody (see Paper I, Fig. 1), the dryweight of 10^9 platelets being 3.2 mg.

Under these conditions endothelial cells did not fix complement or adsorb antibody.

(*d*) *Erythrocytes.* An instance in which an antibody associated with thrombocytopenia due to quinidine fixed complement in the presence of the drug and red cells has been reported (5) but corresponding *in vitro* tests with platelets were not presented. Plain washed red cells and red cells treated with trypsin (10), both from the patient and from donors, were used in complement-fixing mixtures, the range of concentration of red cells used being similar to that of platelets in Paper I, Fig. 1. In no case was complement fixed or antibody adsorbed, and the red cells remained morphologically intact and did not agglutinate in the mixtures.

(*e*) *Cinchona alkaloids.* Just as in the case of complement-fixing reactions, cinchonine could be substituted for quinidine at the same molar concentrations for agglutination reactions and for inhibition of clot retraction whereas quinine and cinchonidine were ineffective.

(*f*) *Passive anaphylaxis.*

Four guinea pigs weighing approximately 450 gm. were injected intravenously with 320 units of antibody contained in 2 ml. of heated (56° for 30 minutes) patient's serum. Two days later two of the guinea pigs were injected intravenously with 10^{10} platelets mixed with 8 mg. of quinidine gluconate in a volume of 2.5 ml.; and the other two were injected first with 10^{10} platelets followed 1 hour later by 8 mg. of quinidine gluconate.

None of the guinea pigs developed any symptoms or elevation in temperature suggestive of an anaphylactic reaction.

DISCUSSION

Comparison of Agglutination and Complement-Fixing Reactions.—In Paper I, Fig. 6, it was shown that the amount of antibody adsorbed per platelet when quinidine concentration was $10^{-6.16}$ M was approximately 25 per cent of that adsorbed when quinidine concentration was optimal although no complement fixation could be measured at the suboptimal quinidine concentration. At

$10^{-5.34}$ M quinidine, 60 per cent of the maximum amount of antibody adsorption occurred but complement fixation was only 20 per cent of the maximum. It appeared that decreases in complement fixation at low quinidine concentrations were more marked than decreases in the amount of antibody adsorbed because of changes in the proportion of quinidine in the antibody-quinidine-platelet complex (see Paper I). If degree of agglutination was related only to the amount of antibody adsorbed per platelet, a higher degree of agglutination might have been expected when quinidine concentration was in the range of $10^{-5.34}$ to $10^{-6.04}$ M, particularly with the higher concentrations of antibody (see Table II). It appeared likely, therefore, that agglutination as well as complement fixation was dependent on the proportion of quinidine in the complex. This possibility was also suggested by the decrease in agglutination observed at a high quinidine concentration ($10^{-1.82}$ M) which had been shown in Paper I to produce a decrease in complement fixation with no decrease in the amount of antibody adsorbed per platelet. Results of agglutination experiments at high quinidine concentration may be open to different interpretation, however, because the heavy precipitate which occurred in mixtures containing $10^{-1.82}$ M quinidine may possibly have interfered with agglutination.

Antibody activity could be measured accurately by complement fixation when antibody was present at concentrations in the order of 0.2 units/ml. and could be detected by platelet agglutination and inhibition of clot retraction when antibody was present at concentrations in the order of 2 to 3 units/ml. Complement fixation was at least 10 times more sensitive than platelet agglutination or inhibition of clot retraction for measurement of antibody activity.

Significance of Changes in Platelet Morphology.—The data presented by Ackroyd in favor of the hypothesis that the antibody acts to lyse platelets in the presence of complement is not contradicted by the observations described above. In interpreting the present data, however, it appears that there is equally good evidence that antibody does not lyse platelets directly in the presence of complement but that attachment of antibody to platelets makes them more susceptible to morphologic alteration by non-specific factors *in vitro*.

The same morphological changes which could occur in the presence of antibody and quinidine with platelet preparations which remained unaltered in control mixtures, were occasionally observed without antibody and quinidine in suspensions of platelets in plain saline, heated serum, or plasma. Instability of platelets in such suspensions appeared to be related to conditions which favored production of active coagulation factors during the process of separating platelets from blood, such as unusually slow bleeding of donors, delayed mixing of anticoagulant, or insufficient washes for complete removal of plasma proteins from platelets. The fact that washing platelets in an anticoagulant (1 per cent ammonium oxalate) resulted in unusually stable platelet suspensions and that stable platelets suspended in the presence of even trace amounts of plain

native serum frequently developed morphological changes identical with those produced in the presence of antibody and quinidine suggested that active coagulation factors, which in trace amounts are known to alter platelet morphology (7), may account for the occasional instability of platelets in plain suspension. Platelets exposed to antibody and quinidine may have become more susceptible to morphologic alteration by trace amounts of coagulation factors or other as yet unknown factors which cause instability of platelets in suspending media. If such factors were not present *in vivo*, it is conceivable that morphologic instability (or lysis) of platelets would not occur in association with attachment of antibody. It may be that attachment of antibody to platelets *in vivo* does not result directly in their destruction but alters them in a manner which predisposes to sequestration from the circulation by the usual physiologic processes.

The Nature of Inhibition of Clot Retraction.—Because quinidine alone is capable of inhibiting clot retraction of normal blood, and in view of the possibility that quinidine attaches first to antibody (see Paper I), it might be argued that inhibition of clot retraction which occurs at comparatively low quinidine concentration in the presence of antibody may be due to augmented attachment of quinidine to platelets in association with antibody, quinidine then acting to inhibit clot retraction as it does in normal blood.

Calculations made from data of Table III of this paper concerning the concentration of quinidine required to inhibit clot retraction of normal blood, and from Table III of Paper I concerning the amount of quinidine adsorbed by plain platelets suspended in quinidine solution indicate that the amount of quinidine adsorbed by 1 ml. of platelets at a concentration of 3.0×10^5/mm.^{3} in the presence of a quinidine concentration which inhibits clot retraction in normal blood is in the order of 10^{-8} mole. The amount of antibody required for association with this much quinidine, assuming an average antibody valence of 2 (see Paper I) and an M.W. of 100,000, would be 0.5 mg./ml. Of the 10.5 units of antibody/ml. present in inhibition of clot retraction tests in Table III, approximately 30 per cent would be adsorbed by platelets at a concentration of 3×10^5/mm.^{3} (see Paper I). Therefore 10.5 units of antibody/ml. would be equivalent to approximately 1.5 mg. of antibody/ml. Since antibody concentration in one patient was as high as 300 units/ml., (see Paper IV, Fig. 2), this would imply that the amount of antibody in the patient's serum had been as high as 45 mg./ml. which is too high to be consistent with known antibody levels (11). Furthermore, a serum electrophoretic pattern obtained when antibody concentration was 50 units/ml. showed no increase in gamma globulin although from the above calculations antibody would have accounted for 12% of the total proteins. Any reasonable increase in assumed valence of antibody or decrease in assumed M.W. would not sufficiently reduce the discrepancy between the calculated and usually observed concentrations of antibody. If platelets adsorbed much less quinidine when in plasma than when in saline owing to competition for quinidine by plasma proteins, the possibility that inhibition of clot retraction is due primarily to the amount of quinidine attached to platelets (with or without antibody) could still be considered. However, it was found that 0.036 M Na citrate prevented inhibition of clot retraction caused by $10^{-3.45}$ M quinidine in blood containing antibody but did not interfere with inhibition of clot retraction caused by much higher quinidine concentrations in normal blood (Table III). Interference with inhibition of clot retraction by citrate was not due to interference with attachment of antibody

onto platelets, for platelets were found to adsorb the same amount of antibody at 10^{-3} M quinidine in the presence and absence of 0.056 M Na citrate.

Therefore inhibition of clot retraction in the presence of antibody and quinidine and inhibition of clot retraction of normal blood by quinidine alone appear to be due to different mechanisms.

Inhibition of clot retraction was considered by Ackroyd to be due to lysis of platelets by the action of complement, and the evidence he presented in favor of this hypothesis is substantial (2). However, in the present study, it was found that inhibition of clot retraction did not occur in fresh whole blood when antibody concentration was below 2 units/ml. of plasma in spite of the fact that much complement was fixed *in vitro* at this concentration of antibody (see Paper I); and it could occur in mixtures in which citrate concentration was completely anti-complementary (Text-fig. 1). Morphologic alteration of platelets *per se* does not necessarily imply that they will not cause clot retraction, for disruption of platelets is generally considered to be an integral part of the blood coagulation process (7, 12). It is worthy of note that suspensions of platelets exposed to concentrations of antibody and quinidine sufficient to completely inhibit clot retraction in whole blood retained their ability to promote clot retraction when added to platelet-free plasma after antibody and quinidine had been removed from them by washing with saline. The mechanism of inhibition of clot retraction would appear to be still open to speculation as is the process of clot retraction itself (13, 14). The apparent interaction between platelets and fibrin which leads to clot retraction may be interfered with by the presence of antibody on platelets *per se* and not by subsequent instability of platelets.

Specificity of Antibody Activity.—Of the various cells tested, the only ones which could be substituted for human platelets in antibody reactions were *rhesus* monkey platelets. The finding that monkey platelets did not adsorb as much antibody or fix as much complement as human platelets indicated that monkey platelets contained fewer sites per platelet for attachment of antibody and perhaps, in addition, a different arrangement of sites permitting less complement to be fixed by attached antibodies (see Paper I). Such factors might also account for the inability of monkey platelets with adsorbed antibody to agglutinate. Other animal platelets tested gave no evidence of having any sites for attachment of antibody but did adsorb the same amount of quinidine as human platelets. The ability of platelets to adsorb large amounts of quinidine had no bearing on their ability to enter into reactions with the antibody.

SUMMARY

Quantitative aspects of platelet agglutination and inhibition of clot retraction by the antibody of quinidine purpura were described. The reactions appeared to

depend on formation of types of antibody-quinidine-platelet complexes which could fix complement but complement was not necessary for these reactions. Complement fixation was at least 10 times more sensitive than platelet agglutination or inhibition of clot retraction for measurement and detection of antibody activity.

Although it has been considered that antibodies of drug purpura act as platelet lysins in the presence of complement and that direct lysis of platelets accounts for development of thrombocytopenia in drug purpura, the present study suggests that attachment of antibody produces a change in platelets which is manifested *in vitro* only by increased susceptibility to non-specific factors which can alter the stability of platelets in the absence of antibody. The attachment of antibody to platelets *in vivo* may only indirectly affect platelet survival.

In contrast to human platelets, dog, rabbit, and guinea pig platelets, and normal or trypsin-treated human red cells did not agglutinate, fix complement, or adsorb antibody; and intact human endothelial cells did not fix complement or adsorb antibody.

Rhesus monkey platelets were not agglutinated by the antibody but did adsorb antibody and fix complement although their activity in these reactions differed quantitatively from that of human platelets.

Cinchonine could be substituted for quinidine in agglutination and inhibition of clot retraction reactions but quinine and cinchonidine could not.

Attempts to cause passive anaphylaxis in guinea pigs with the antibody of quinidine purpura were not successful.

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EXPLANATION OF PLATE 58

FIGS. 1 A to 1 E. Criteria for grading agglutination.

FIG. 1 A, homogeneous suspension of discrete platelets with no agglutination; Fig. 1 B, 1+ agglutination, about half the platelets aggregated in clumps of 2 to 6; Fig. 1 C, 2+ agglutination, larger clumps with few free platelets; Fig. 1 D, 3+ agglutination, all platelets agglutinated in large clumps with wide spaces between clumps; Fig. 1 E, 4+ agglutination, very large clumps far apart. Intermediate degrees of agglutination such as ± or 3–4+ could be discerned. The morphology of individual platelets remained the same in agglutinated and control mixtures. Those platelets appearing less refractile in agglutinated clumps are out of focus owing to chamber depth. Magnification 430, bright light.

Fig. 1 F. Smear of endothelial cell preparation used in place of platelets in attempt to fix complement. Papanicolaou stain, 430 magnification.

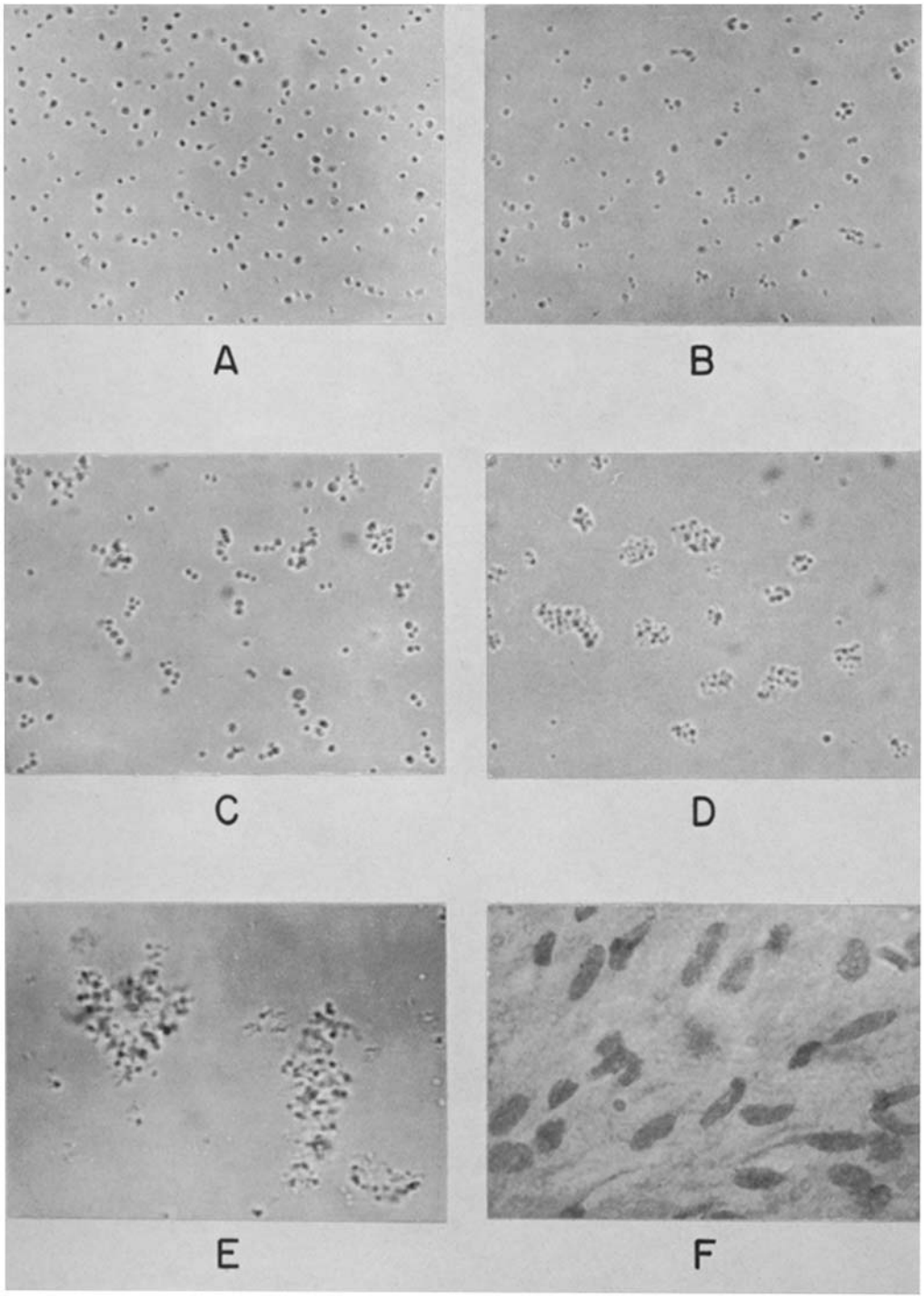

FIG. 1

(Shulman: Immunoreactions involving platelets. III)