IMMUNOREACTIONS INVOLVING PLATELETS. V. POST-TRANSFUSION PURPURA DUE TO A COMPLEMENT-FIXING ANTIBODY AGAINST A GENETICALLY CONTROLLED PLATELET ANTIGEN. A PROPOSED MECHANISM FOR THROMBOCYTOPENIA AND ITS RELEVANCE IN "AUTOIMMUNITY" *

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A thrombocytopenic state developing in the absence of any apparent underlying disorder and accompanied by an abundance of megakaryocytes in the bone marrow represents the syndrome of idiopathic thrombocytopenic purpura (ITP). Similarities between this syndrome and thrombocytopenic states which can be produced experimentally with heterologous antibody (1, 2), or which occur as a result of the antigen-antibody reactions of drug purpura (1, 3, 4), suggest that an immunologic process may underlie ITP. The observations that plasma from some ITP patients, when transfused into normal individuals, may depress platelets and that plasma containing the "thrombocytopenic factor" may agglutinate platelets *in vitro* (2, 5), support the concept that an antibody may cause the disease. Because no foreign antigen has been implicated as yet in ITP, the disease is generally considered to be an "autoimmune" disorder.

The present work concerns differentiation of a specific immunologic type of purpura which heretofore was indistinguishable from ITP. The pathogenesis of the disease to be described involves mismatch of an inherited platelet antigen during blood transfusion and subsequent development of a complement-fixing antiplatelet isoantibody that is capable of producing severe thrombocytopenia in the sensitized individual.

Circumstances attending development of thrombocytopenia and characteristics of the underlying immunoreaction permitted interpretation of the mechanism responsible for destruction of autologous cells by an antibody formed against a foreign antigen, and provided a rationale for effective therapy of the disorder. This distinctive type of immune reaction is relevant to the problem of ITP and other diseases which have been considered "autoimmune" phenomena. Demonstration of the genetic control and nature of the platelet antigen responsible for post-transfusion purpura helps clarify the problems of inherited platelet types and platelet antigenicity.

CASE REPORTS

The following cases of post-transfusion purpura were studied. *Patient 1,* P.K., a white female, age 40, was admitted to the surgical service of the National Heart Institute, Clinical Center, on May 17, 1959, for correction of cardiac valvular stenosis resulting from rheumatic heart disease. Her last attack of acute rheumatic fever had been at age 17, and symptoms of cardiac failure had been progressively incapacitating for 3 years before admission. There were no other significant diseases or symptoms in her medical history; in particular, no hemorrhagic tendency and no allergic disorder. She had had three normal deliveries and pregnancies. The only drugs taken prior to admission were digitalis and chlorothiazide. Abnormalities on physical examination were limited to signs of valvular heart disease. The week prior to operation she received sulfisoxazole and tetracycline for bronchitis following transbronchial cardiac catheterization. On June 2, 1959, transventricular mitral commissurotomy was performed, during which she received two 500 ml units of whole blood and 220 ml of a third unit which was discontinued because small clots had plugged the tubing filter. The three donors were blood A_1B, Rh positive, the patient's type being A_2B, Rh positive. The operation and first 6 days of the postoperative course were uneventful, except for development of tracheitis which was treated with erythromycin and streptomycin. Early in the morning on the seventh postoperative day (June 9), the patient noticed several small hemorrhagic vesicles on her tongue and later that day developed generalized florid purpura on the trunk and extremities, including numerous 4 to 5 mm hemorrhagic bullae on the skin and mucous membranes. The same day she also had a tarry

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stool, hematuria and vaginal bleeding, and developed large ecchymoses at the site of the thoracotomy incision. Other than the hemorrhagic manifestations, there were no abnormal symptoms or signs. Her platelet count on June 9 was 600 per mm^3 and her bleeding time was longer than 20 minutes; her hemoglobin was 13.4 g per 100 ml, having been 14.7 on June 4, and her white cell count and differential were normal. A unit of fresh whole blood was started the evening of June 9 but had to be stopped after 250 ml had been infused over a 1-hour period, because the patient developed severe shaking chills and slight temperature elevation. No red cell incompatibility was found, and the symptoms promptly subsided. Later that evening 250 ml of fresh platelet-rich plasma was transfused uneventfully over a 3-hour period.

Because the onset of purpura had been acute and the platelet count remarkably low, drug purpura or unusually severe ITP was the suspected diagnosis. Although the drugs being administered (digoxin, erythromycin and streptomycin) are not recognized causes of drug purpura, complement fixation and platelet agglutination tests were performed on June 10 to evaluate that possibility. The patient's serum was found to contain a factor in high titer which fixed complement with platelets from 12 normal individuals and produced marked platelet agglutination whether or not these drugs were added. Nevertheless, medications which had been given postoperatively were discontinued, and prednisone, 50 mg per day, was started. On June 10 the hemoglobin was 10.7 g per 100 ml and the platelet count was 300 per mm^3. Her bone marrow was normal with respect to the myeloid and erythroid series and contained a normal number of megakaryocytes which were almost all in the large mononuclear or megakaryoblastic form. The patient received 1,000 ml of fresh whole blood on June 10, given at a rate of 2 to 3 ml per minute, without reaction; but platelets remained at 700 per mm^3 or below. Prednisone was increased to 100 mg per day.

Because the patient had life-threatening hemorrhage, and the association of a potent antiplatelet factor with thrombocytopenia hardly seemed fortuitous, an exchange transfusion was carried out on the fourth day of purpura in an attempt to decrease the titer of the antiplatelet factor. Over a 16-hour period, a total of 5,500 ml of blood was withdrawn and 6,500 ml of fresh ACD blood replaced in 250- to 500-ml aliquots, alternating withdrawal and infusion. During the first half of the exchange, the infusion rate could not be increased above 2 to 3 ml per minute without producing marked hypotension, and during the course of the exchange the patient frequently developed urticaria which appeared to respond to intramuscular diphenhydramine. By the end of the exchange, the patient's platelet count was 4,900 per mm^3, but the bleeding time had decreased to 6 minutes. There were no further hemorrhagic manifestations, and thereafter the platelet count returned to normal with unusual rapidity, the existing purpura cleared, and the patient remained well without further treatment. The text and Figure 3 contain further details of the effects of exchange transfusion. Prednisone was tapered beginning June 13 and discontinued on June 19. On June 19 megakaryocytes in the bone marrow were normal in number and morphology. Serum protein was 6.5 g per 100 ml with 18 per cent γ-globulin on June 10 and did not change significantly after the exchange transfusion.

Patient 2, G.H., a 43 year old white female, was admitted to the Faulkner Hospital, Jamaica Plain, Mass., on September 20, 1959, with a 2-year history of peptic ulcer symptoms. There were no other illnesses, allergic disorders, or symptoms of significance in her medical history, and she had not received blood transfusions. She had had three normal deliveries and pregnancies. Because her response to medical therapy for peptic ulcer was unsatisfactory, a subtotal gastrectomy was performed on October 13, 1959, at which time she received one 500 ml unit of whole blood and 75 ml from a second unit which was stopped because of clots in the bottle. The patient's and donors' blood type was A_1, Rh positive. The postoperative course was uneventful until the sixth day, when she developed florid purpura over the entire body and hematuria. The platelet count at this time was 28,000 per mm^3, there was poor clot retraction, and the bleeding time was greater than 20 minutes. The bone marrow contained a normal number of megakaryocytes. A diagnosis of ITP was made and steroid therapy was begun. The next day, October 20, the platelet count was 6,000 per mm^3, the hematocrit was 19 per cent, and the white cell count was slightly elevated with a normal differential. Several attempts were made to transfuse fresh whole blood, but each of three units was discontinued after approximately 50 ml was received because of a reaction consisting of chills, fever, urticaria and hypotension. On both October 21 and 22 she was given the red cell content of two units of blood without reaction, the red cells having been washed to remove other cellular elements. Purpura, intermittent hematuria and hematoma formation persisted for 17 days despite high doses of prednisone. Symptoms subsided as the drug was being tapered. The clinical course is shown in Figure 1 and

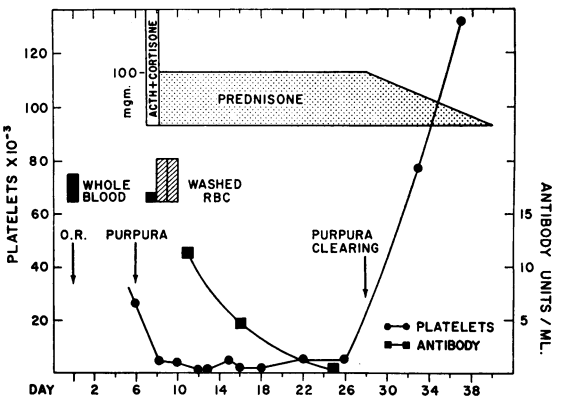

FIG. 1. CLINICAL COURSE, PATIENT 2. Units of antibody are defined in the text.

the results of tests performed with this patient's serum are presented in the text.

METHODS

Methods of *preparing platelet suspensions, counting platelets* (6) and *measuring platelet agglutination, inhibition of clot retraction,* and *complement fixation* (7) were the same as those that have been used in studying the antibody of quinidine purpura (8, 9), with the following exceptions.

The nitrogen content of platelet suspensions was determined by nesslerization. One ml of platelets at a concentration of 2×10^9 per mm^3 was found to contain 800 ± 50 μg N per ml; with this criterion for determining platelet concentration, the error was ± 5 per cent.

The 50 per cent hemolytic unit of complement activity (hereafter denoted by C′), measured by means of a single hemolysin preparation and erythrocytes from one sheep throughout this work, was equivalent to $2.5 \pm 0.2 \times 10^{-3}$ ml of reconstituted lyophilized pooled guinea pig serum. With the same reagents, the C′ content of sera from 15 normal human beings averaged 65 U per ml, with a variation of ± 10. The condition of incubation found to be optimal for maximal C′ fixation in the shortest time with the antigen-antibody system under study was 1 hour at 37° C with frequent agitation of the tubes. The rate of C′ fixation by this system was approximately halved when the incubation temperature was decreased to 27° C.

Fluorescent antibody methods. The patient's antibody-containing serum and antihuman globulin serum (Coombs serum) were conjugated with fluorescein isothiocyanate by the method of Riggs and co-workers (10) as modified by Marshall, Eveland and Smith (11) and adsorbed before use with guinea pig spleen-liver-kidney powder to decrease nonspecific fluorescence. Approximately 35 per cent of the antibody activity in the patient's serum was lost after this treatment. The fluorescein-conjugated Coombs serum used had a titer of 1/64 (see below). Other details of the techniques, such as application of antibody to platelet smears, washing procedures after exposure of smears to antibody, methods of mounting the final preparations, and the optical systems used for microscopy were the same as those reported recently by Silber and co-workers (12), with the exceptions stated in the text.

Preparation of heterologous platelet antisera. Antihuman platelet serum was made in rabbits by injecting suspensions of 3×10^9 washed platelets intravenously on Days 1, 3, 6 and 18 and was harvested on Days 11, 15, 18 and 25. The highest antibody concentration was present in all rabbits on Day 25, and serum harvested on this day was used.

Antihuman platelet serum was made in Sprague-Dawley rats by injecting suspensions of 6.3×10^8 platelets intravenously on Days 1, 3, 6, 14 and 18, and was harvested on Days 12 and 24. Serum harvested on Day 24 contained only slightly more antibody than serum harvested on Day 12.

Antihuman platelet serum was made in NIH-strain guinea pigs by injecting suspensions of 6×10^8 platelets intravenously on Days 1, 3, 6 and 9, and was harvested on Day 13. Guinea pigs were also immunized against rabbit platelets by injecting 10^9 platelets on Days 1, 3, 6, 9 and 16, and harvesting on Day 22.

Although a small amount of erythrocyte material may have contaminated platelets used for immunization, it was insufficient to produce C′-fixing anti-erythrocyte antibodies which could be detected with the maximal amount of erythrocytes contaminating platelet suspensions used experimentally. Antiplatelet serum therefore did not have to be adsorbed with erythrocytes before use. There were also no detectable antileukocyte antibodies formed that would react with the maximal amount of leukocytes contaminating platelet suspensions used in complement fixation tests.

Antihuman globulin (Coombs serum) consumption technique. A commercial preparation of rabbit antihuman serum purchased from Ortho Pharmaceutical Corp., Raritan, N. J., and preparations of horse antihuman globulin and rabbit antihuman globulin were used with identical results. In titering Coombs serum, the agglutination mixture consisted of 0.1 ml of a 1 per cent suspension of group O, Rh positive human erythrocytes sensitized with anti-D serum, plus 0.1 ml of various dilutions of Coombs serum. The titer was the limiting dilution which gave 1 + agglutination (minimal but definite macroscopic clumping) after incubation for 30 minutes at 37° C and centrifugation at 250 G for 3 minutes.

In measuring consumption of Coombs serum (13), platelets were first exposed to patients' sera containing antibody, or to normal control sera, then washed 5 times in large volumes of 0.147 M NaCl. Washing removed at most 10 per cent of antibody attached to platelets (see Section 7 in Results). After the last wash, platelets were resuspended at a concentration of 10^6 per mm^3 in saline and 0.1 ml of suspension (10^8 platelets) was mixed with 5 different progressive twofold dilutions of Coombs serum, the highest final dilution of Coombs serum being one-half the limiting dilution that gave 1 + agglutination with erythrocytes in the preliminary titration. Mixtures were incubated for 10 minutes at 37° C; platelets were then removed by centrifugation, and 0.1 ml of supernatant fluid containing residual Coombs serum was mixed with 0.1 ml of sensitized erythrocytes as described above. The amount of Coombs serum consumed by platelets was evident from the limiting dilutions which still produced erythrocyte agglutination (see Table III).

Coombs consumption tests done in an attempt to obtain evidence for a possible "blocking" antibody on platelets that were not preincubated with known antibody are referred to in the text as "direct" Coombs consumption tests. Controls for these tests were simply suspensions of platelets from normal individuals treated in the same manner as patients' platelets.

Trypsin treatment of platelets and platelet fractions. Washed platelets, prepared as described above, were suspended in 0.145 M NaCl containing 0.04 M phosphate buffer, pH 7.5, to which crystalline trypsin was added to

give a final concentration of 0.5 mg per ml. After incubation, platelets were washed three times in 0.147 M NaCl. Platelets incubated in this way for as little as 10 minutes at 37° C did not sediment as readily as untreated platelets and could not be recovered quantitatively if the usual centrifugal force was used for washing. Platelet fractions which were treated with trypsin (Section 6) were not washed; instead, the trypsin was neutralized with an equimolar amount of soybean trypsin inhibitor. The trypsin-inhibitor complex, at the concentrations used experimentally, was found to be inert in C'-fixing mixtures. Trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp., and the specific activity of each preparation was assayed before use.

Measurement of sugars. Assays for total hexose by the indole and cysteine-H_2SO_4 reactions (14), for deoxy sugars by the thiobarbituric acid reaction (15), for glucuronic acid by the orcinol reaction (14), and for uronic acid by the carbazole reaction (14) were done on platelets and platelet fractions with techniques which detected with certainty 0.1 μmole of each substance assayed.

EXPERIMENTAL RESULTS

1. C' fixation reactions of the serum factor in Patient 1 and definition of an antibody unit. Serum from Patient 1, obtained during the period of purpura, when mixed with suspensions of platelets from normal individuals, fixed C' in the manner shown in Figure 2. No C' was fixed when the same serum was mixed with suspensions of human erythrocytes or leukocytes.

There was no loss of activity when serum containing the factor responsible for C' fixation was dialyzed for 72 hours at 5° C against 10^3 vol of 0.147 M sodium chloride. The factor was recovered in serum precipitates produced at one-third saturation with ammonium sulfate, solutions of which gave a visible band only in the γ-globulin region on paper electrophoresis. When serum was subjected to 76,000 G for 10 hours, by a sucrose density gradient, C'-fixing material had sedimentation characteristics of a 7S γ-globulin (16). The factor also had stability characteristics of γ-globulin in that it did not lose activity when whole or diluted serum was kept for at least 60 minutes at 56° C, 2 weeks at 5°, or for 18 months at − 20°, but did lose activity at an increasingly rapid rate when serum was incubated at temperatures above 65° or when the pH of the serum was adjusted to values above 11.2 or below 3.0 at 20°. In view of these various characteristics, the C'-fixing serum factor will hereafter be referred to as an antibody.

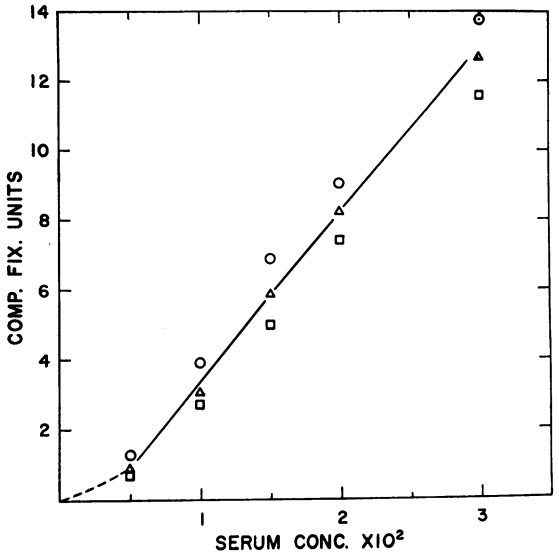

FIG. 2. COMPLEMENT FIXATION WITH VARIOUS ANTIBODY CONCENTRATIONS AND FIXED PLATELET CONCENTRATION. Fixation of C' was carried out in a 0.3 ml volume, the mixture consisting of serum from Patient 1 obtained on June 12 before transfusion, a saline suspension of platelets to give a final concentration of 200,000/mm³, guinea pig serum (either 0.02 or 0.04 ml, containing 8 or 16 U of C', respectively), and 0.147 M NaCl to maintain a fixed volume. Concentration of serum is expressed as ml of patient's serum per 0.3 ml reaction mixture. Each symbol represents results obtained on a different day with a platelet suspension made from a different donor, and individual points are the average of two determinations. The amount of C' fixed when antibody and platelets were preincubated for 1 hour at 37° C, before C' was added, was the same as the amount fixed when all three reagents were mixed together initially.

Standard curves relating the amount of C' fixed to the concentration of antibody used (Figure 2) showed little variation in different experiments. There was no greater variation with platelets from different donors than with different platelet preparations from a single donor. In comparing antibody content of different sera, several dilutions of each sample were used, as in Figure 2, to fix amounts of C' in the range of 2 to 12 U with platelets at 200,000 per mm³ final concentration in the reaction mixture. Although the curves obtained were not strictly linear, relative antibody content of different sera could be determined by comparing amounts of each required to fix an equivalent amount of C', there being a simple inverse rela-

tionship between antibody content and the amount of serum necessary to fix a given amount of C′. Relative antibody concentration in different sera could be determined in this way with an error of approximately ± 10 per cent. Serum from Patient 1, obtained before the exchange transfusion, was considered the reference standard and arbitrarily assigned the value of 100 U per ml. In the linear portion of the standard curve (Figure 2), 1 U of antibody fixed 4 U of C′.

The concentration of platelets used in Figure 2 is in excess of the amount necessary to produce maximal C′ fixation by the highest concentration of antibody used. In titering antibody it was necessary to use an excess of platelets in order to obtain uniform results with platelets from random donors; for as will be seen in Figure 8, platelets from different donors in suboptimal concentrations did not give uniform results.

2. Effect of exchange transfusion in Patient 1. Figure 3 shows the effects of the exchange transfusion (see case report) on the level of circulating antibody, platelets, and serum C′. The "exchange" curve represents the dilution produced as calculated from the initial blood volume (4,900 ml) and the amount of blood removed (5,500 ml) and replaced (6,500 ml), a fixed blood volume being assumed. The increments of dilution for each 500 ml exchanged are plotted, the total exchange being approximately 70 per cent effective.

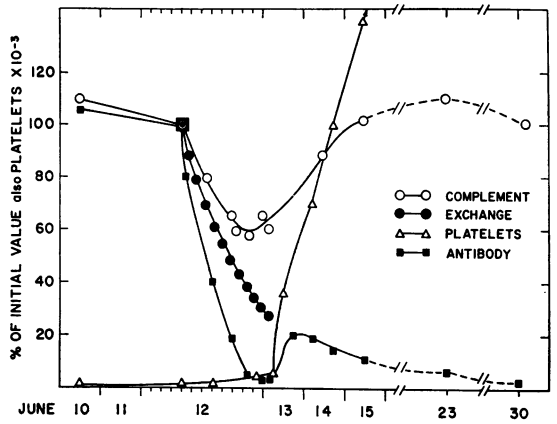

FIG. 3. EFFECTS OF EXCHANGE TRANSFUSION IN PATIENT 1. Immediately preceding the exchange, the patient's red cell volume was found to be 1,700 ml, by a Cr^{51}-labeled red cell technique. The initial hematocrit was 35 per cent and the hematocrit at the end of the exchange was 41 per cent. Antibody was assayed as described in the legend of Figure 2. Initial values for antibody and C′ were those present in the June 12 pretransfusion sample, i.e., 100 U of antibody per ml and 55 U of C′ per ml. On June 14, 250 ml of fresh whole blood was given between the two antibody determinations. On June 12 the abscissa is enlarged and divided into 2-hour intervals; the scale is interrupted between June 15 and 23, and between June 23 and 30.

Antibody level fell more rapidly than could be accounted for by simple dilution alone, for only 3 per cent of the initial concentration of antibody remained at the end of the exchange. Although replaced blood was drawn in plastic bags and stored at 5° C no longer than 12 to 24 hours, the platelet content of the transfused blood produced only a slight rise in the patient's platelet count—from 500 per mm^3 at the beginning to 4,900 per mm^3 at the end of the exchange. The C′ content of the patient's serum fell during the exchange in spite of the large amount of C′ replaced in the transfused blood. These various observations taken together suggested that antibody, combined with transfused platelets, fixed C′ *in vivo* and was sequestered with destroyed platelets.

Immediately after the exchange transfusion, the patient's platelet level rose at a rate of approximately 60,000 per mm^3 per day to reach 140,000 per mm^3 on June 15 and 350,000 per mm^3 on June 19, after which it remained at a normal level. Note that the platelet rise occurred despite reappearance of significant amounts of antibody in the patient's plasma, for although antibody level was 3 U per ml at the end of the exchange, it was 20 U per ml 13 hours later.

3. Platelet agglutination and inhibition of clot retraction by the antibody from Patient 1. In Table I the titer of the patient's C′-fixing antibody in different serum samples is compared with the titer of the platelet agglutinin and the inhibitor of clot retraction in the same samples. Since C′ fixation, agglutination, and clot retraction inhibition activities varied quantitatively in the same way in different serum samples, it appeared that the several reactions were produced by a single antibody. In support of this were the findings that amounts of normal platelets which partially or completely adsorbed the C′-fixing activity out of the patient's serum (see Figure 7) also adsorbed the agglutinin and clot retraction inhibitor to an equal degree, and that the three activities

TABLE I

*Comparison of C′ * fixation, agglutination and inhibition of clot retraction reactions †*

Sample	Agglutination		Inhibition of clot retraction	
	C′-fixing antibody	Dilution titer	C′-fixing antibody	Dilution titer
	U/ml		*U/ml*	
6–12, 1	100	1/20	5.0	1/28
6–12, 10	19	1/4	4.8	1/5
6–12, 13	5			(1/2.5)
6–13, 3 a.m.	3	(1/1.25)	(2.4)	(1/?)
6–13, 4 p.m.	20	1/3	6.7	1/5
6–15	10			1/2.5
6–23	6	1/1.25	4.8	(1/2.5)
6–30	2	(1/1.25)	(1.6)	(1/2.5)

Sample	C′-fixing antibody
	U/ml
6–12, 1	3.6
6–12, 10	3.8
6–12, 13	(2.0)
6–13, 3 a.m.	(1.5)
6–13, 4 p.m.	4
6–15	4
6–23	(2.4)
6–30	(0.8)

* C′ = the 50% hemolytic unit of complement activity.

† Titer of antibody by C′ fixation was measured as described in text. Titer of agglutinin expressed as the highest final dilution of patient's serum which produced 2+ agglutination (9) in reaction mixtures. Titer of inhibitor of clot retraction expressed as the highest final dilution of patient's serum which produced less than 15 per cent free fluid in mixtures with normal whole blood, dilutions being based on the plasma volume rather than the total volume of whole blood used. With this antibody, inhibition of clot retraction occurred uniformly at 37° C, but inconstantly at room temperature. The various dilutions correspond to *final* concentrations of C′-fixing antibody in units/ml as listed. Dilutions in parentheses did not produce agglutination or inhibition of clot retraction. Sample 6–12, 1 was obtained before transfusion; 6–12, 10 after 3,400 ml blood transfused; 6–12, 13 after 4,500 ml blood transfused, and 6–13, 3 a.m. at the end of the exchange after 6,500 ml blood transfused.

were recovered quantitatively in the same ammonium sulfate fraction of serum and had the same stability characteristics (see Section 1).

Platelet agglutination and inhibition of clot retraction were about equally sensitive techniques for detecting antibody. In performing platelet agglutination tests, a small volume of concentrated platelets could be added to antibody-containing serum without significantly diluting the serum, so that 0.1 ml of serum containing 0.5 U of antibody (i.e., 5 U per ml) would produce agglutination (Table I). Since a minimal final concentration of 4 U of antibody per ml (Table I) was required for inhibition of clot retraction and performance of the test necessitated diluting serum at least one-half (0.5 part antibody-containing serum to 1 part whole blood), a serum sample had to contain at least 8 U per ml to inhibit clot retraction. Although platelet agglutination was slightly more sensitive than inhibition of clot retraction, the simplicity of the latter test and the certainty of the result made it the preferable test for screening a large number of individuals for the ability of their platelets to react with the antibody (see Section 5).

C′ fixation was the most sensitive and accurate procedure for detecting and measuring this antibody, for antibody at a concentration as low as 1.0 U per ml could be measured with an error of ± 30 per cent. In measuring antibody in serum containing less than 5 U per ml, greater sensitivity was obtained by using platelets at 20,000 to 50,000 per mm^3 final concentration, rather than at 200,000 per mm^3, because relatively high concentrations of platelets produced decreases in C′ fixation (see Figure 8).

4. Reactions of the antibody from Patient 2. The initial serum sample obtained from the second patient 5 days after onset of purpura (Day 11, Figure 1) contained 11.2 U of antibody per ml, as assayed by the C′ fixation technique described above. This serum did not inhibit clot retraction and produced only equivocal platelet agglutination when used at one-third final concentration in reaction mixtures. These findings were in keeping with the relative sensitivity of C′ fixation compared with other reactions of the antibody of Patient 1. Only C′-fixation tests could be used for further evaluation of the second patient's antibody. Serum obtained 10 days after onset of purpura contained 4.2 U of antibody per ml, and antibody could not be measured in the serum obtained just before purpura cleared. It is significant that, although the first patient recovered after exchange transfusion in the face of a subsequent rise in serum antibody to 20 U per ml, the second patient had continued severe purpura when serum antibody concentration was 4.2 U per ml and less.

5. Identification of a specific inherited antigen involved in the platelet-antibody reaction. Clotted whole blood obtained from the first patient 1 day after the exchange transfusion showed partial clot retraction, the platelet count being 90,000 per mm^3; blood obtained 2 days after the exchange, when the platelet count was 140,000 per mm^3, showed normal clot retraction. This finding was unexpected, for the concentration of antibody in the patient's plasma at the time was 15 U per ml, or three times the concentration necessary to completely inhibit clot retraction of normal blood. The discrepancy proved to be due to complete refractoriness of the patient's platelets to her own antibody, a circumstance that has been reported in two other cases (17, 18).

Platelet suspensions made from Patient P.K. on June 15 (Figure 3) and thereafter did not fix C′ or agglutinate with any amount of the antibody present in her serum before or after the exchange transfusion. In addition, platelets from this pa-

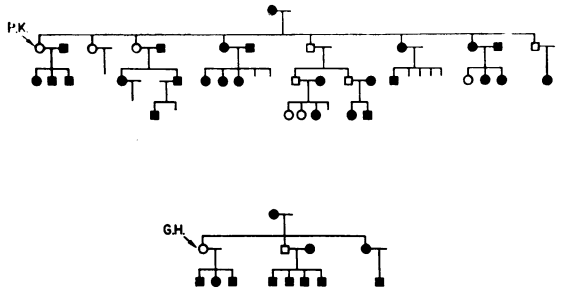

FIG. 4. INHERITANCE OF REACTIVE AND NONREACTIVE TRAITS. Circles are females, squares males. Open symbols represent individuals whose platelets did not react with the antibody in C′ fixation, agglutination and inhibition of clot retraction tests. Solid symbols represent those whose platelets reacted in all of these tests.

tient did not fix C′ with the antibody from Patient 2. Just as in the first case, platelets obtained from the second patient on Day 32 (Figure 1), shortly after recovery, did not fix complement with the antibody which was in the initial sample of her own serum and did not react with the antibody from Patient 1 in C′ fixation, platelet agglutination, and inhibition of clot retraction tests. When platelets were obtained from the patients' relatives, it was apparent that failure of the patients' platelets to react was not owing to an interfering substance in the patients' recovery sera or on their platelets,[1] but to lack of an inherited antigen against which the antibody had developed.

Figure 4 demonstrates the manner in which reactive platelets (solid symbols) and nonreactive platelets (open symbols) are inherited. The antigen appears to be a dominant mendelian character; but as will be seen, the reactive trait does not fit the strict definition of dominance which demands that the character be as fully expressed when the gene is in the heterozygous state as it is when the gene is in the homozygous state. The reactive trait—i.e., the gene responsible for the antigen that reacted with the antibody—will be labeled Pl^{A1}, and the nonreactive trait, Pl; reasons for this notation will be discussed.

Of the 206 randomly selected normal individuals subsequently tested, 3, or 1.5 per cent, had nonreactive platelets. Moreover, it was found that reactive platelets could be separated into two groups based on differences in the amount of C′ fixed per platelet (Figure 5).

The over-all variation in C′ fixation observed when suboptimal concentrations of platelets from different normal donors were used was much more than could be accounted for on the basis of errors in measuring C′ fixation (cf. Figure 2) or in counting platelets. Platelet activities fell into two groups without overlap, even though there was considerable spread of values in each group. The

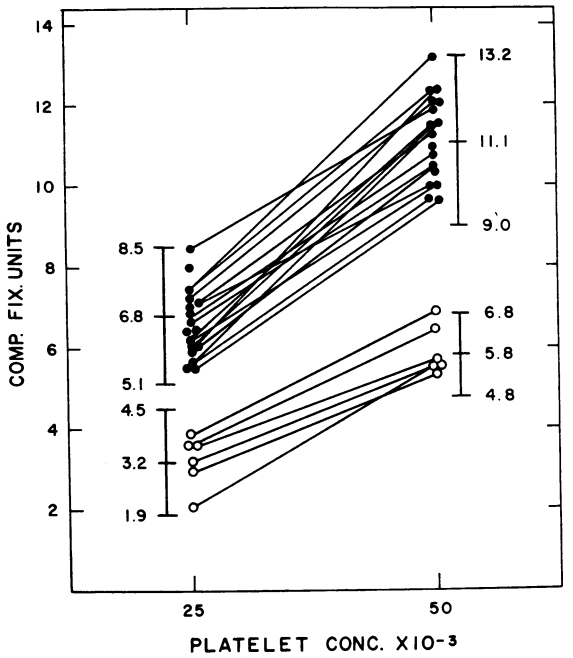

FIG. 5. QUANTITATIVE ACTIVITY OF PLATELETS FROM RANDOM DONORS. Platelets were prepared in the usual way and counted with 3 pipets and 6 chambers. The two final platelet concentrations used were 25,000/mm³ and 50,000/mm³ in reaction mixtures containing 4 U of antibody. This amount of antibody fixed 15 to 17 U of C′ in the presence of an excess (200,000/mm³) of any donor's platelets (see Figure 2). The brackets delimit 2 standard deviations from the mean for each group. The graph is a composite of results obtained with different donors on different days.

[1] Because platelets from all normal individuals tested up to this point had reacted with the antibody from both patients, numerous attempts were made to detect a possible "blocking" antibody on the patients' platelets. The general techniques used were the direct Coombs test, "direct" Coombs consumption, and various elution procedures, as well as incubation of antibody or normal platelets with patients' recovery sera before completing usual C′ fixation or agglutination reaction mixtures. None of the techniques gave presumptive evidence for a blocking antibody on platelets or a factor in serum which would inhibit the activity of the original antibody; but with all of these techniques, it is impossible to draw definite conclusions from negative results.

intragroup spread could be decreased by preparing suspensions of platelets from different donors simultaneously, by performing C′ fixation tests with all platelets on the same day, and by using nitrogen content of platelet suspensions rather than counts as the criterion of platelet concentration. With these refinements, the values for standard deviations shown in Figure 5 were approximately halved, but the mean values for the two groups remained essentially the same. Thus, of 23 randomly selected individuals tested, 74 per cent had platelets which fixed 1.7 to 2.4 times more complement than did platelets from the remaining 26 per cent.

If inheritance of the antigen were controlled by a pair of alleles, all children of a parent who had nonreactive (Pl Pl) platelets would have at least one *Pl* gene; and if the children's platelets were reactive, their genotype would have to be Pl^{A1} *Pl*. Quantitative C′-fixing activity of platelets from five family groups containing a nonreactive parent and reactive children is shown in Figure 6. In all 11 instances (children in Families 1 to 5) there was 100 per cent correlation between low-activity platelets and the postulated heterozygous state. Three mates of individuals with nonreactive platelets had high-activity, presumably Pl^{A1} Pl^{A1}, platelets; all seven children (in Families 1, 3, and 4) were apparent heterozygotes. In Family 2, the mate of a nonreactor had low-activity, presumably Pl^{A1} Pl, platelets, and two of the three children tested were nonreactors, the third being an apparent heterozygote. Because, in all families tested, there was strict correlation between platelet activity and the postulated mode of inheritance, random donors whose platelets gave low activity were most likely Pl^{A1} *Pl* individuals, and those whose platelets gave a high activity were most likely Pl^{A1} Pl^{A1}. In Family 6, the mother and eight children had high-activity platelets; this would be the circumstance if both parents were genotype Pl^{A1} Pl^{A1}.

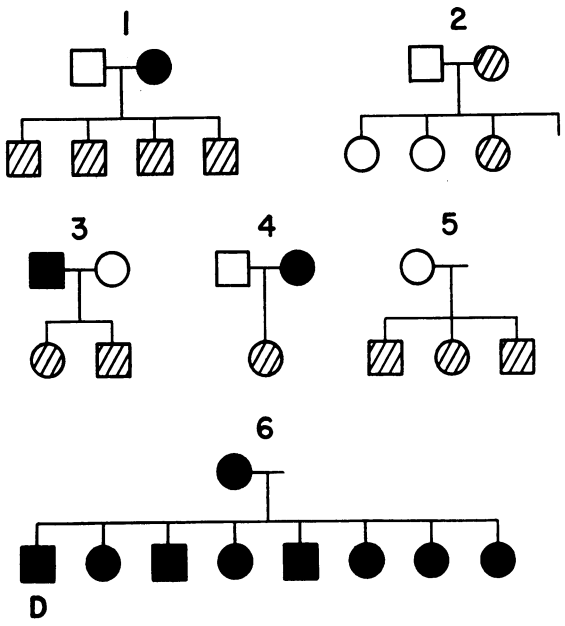

FIG. 6. QUANTITATIVE ACTIVITY OF PLATELETS FROM FAMILY GROUPS. Individuals whose platelets, at a final concentration of 50,000/mm³, fixed less than 5.8 U of C′ in the presence of excess antibody are represented by hatched symbols and those whose platelets fixed greater than 9.2 U of C′ under the same conditions are represented by solid symbols (see Figure 5). No values for C′ fixation were obtained in the range of 5.8 to 9.2 U. Open symbols represent individuals whose platelets did not react. Family 6 was the family of the individual (D) who donated blood to Patient 2.

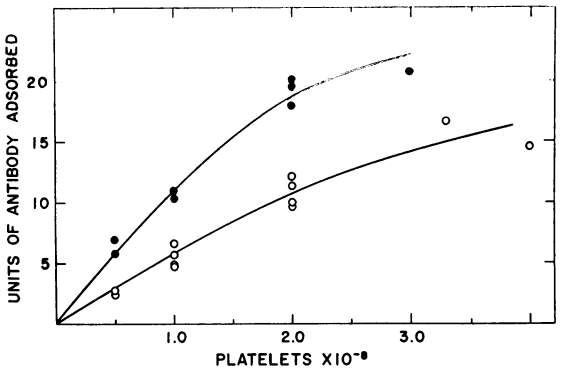

FIG. 7. ADSORPTION OF ANTIBODY BY HIGH-ACTIVITY AND LOW-ACTIVITY PLATELETS. Buttons prepared from suspensions of high-activity and low-activity platelets were resuspended in 0.25 ml serum containing 25 U of antibody. The mixtures were incubated for 90 minutes at 37° C, centrifuged at 15,000 G for 15 minutes, and antibody remaining in the supernatant serum was titered as described in Figure 2, using the average results of 3 different dilutions. Individual points for each amount of platelets used were obtained with platelets from a different donor. The C′-fixing activity of the platelets (● high-activity, ○ low-activity) was determined as described in Figure 5.

The amounts of antibody adsorbed by the different types of platelets are compared in Figure 7. Nonreactive platelets at any concentration did not adsorb antibody. High-activity, presumably Pl^{A1}

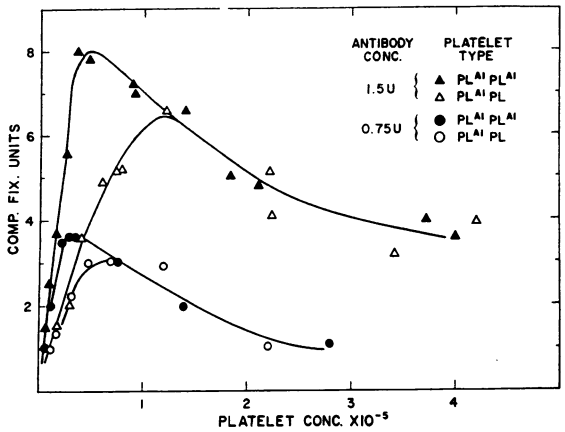

FIG. 8. EFFECTS OF VARYING THE CONCENTRATION OF THE TWO TYPES OF PLATELETS WITH ANTIBODY CONCENTRATION FIXED. Amount of C′ fixed was determined as in Figure 2. Reaction mixtures contained either 0.015 or 0.0075 ml of patient's serum in a final volume of 0.3 ml. The various final concentrations of Pl^{A1} Pl^{A1} or Pl^{A1} Pl platelets used with each amount of serum are indicated on the abscissa.

Pl^{A1}, platelets adsorbed approximately twice as much antibody per platelet as did low-activity, presumably Pl^{A1} Pl, platelets. In partially adsorbed serum samples, decreases in titer of agglutinin and inhibitor of clot retraction corresponded to decreases in titer of C′-fixing antibody.

Because of the variation in platelet activity, it was not possible to establish an alternate antibody unit by determining the minimal concentration of platelets required for maximal C′ fixation, as has been done in the case of the quinidine purpura antibody (8). Titrations of this type (Figure 8) with platelets of different activity gave approximately a twofold difference in the platelet concentrations necessary to produce maximal C′ fixation with a single concentration of antibody. However, in the presence of an excess of either type of platelet, a single concentration of antibody fixed the same amount of C′. Either type of reactive platelet could be used with uniform results when antibody was assayed as in Figure 2.

From the data shown in Figures 5, 6, 7 and 8, it was evident that three platelet phenotypes could be differentiated which corresponded most likely to the genotypes *Pl^{A1} Pl^{A1}*, *Pl^{A1} Pl* and *Pl Pl*.

Differences evident in the C′-fixation reaction were not detectable by agglutination or inhibition of clot retraction techniques, for within the limits of error, both types of reactive platelets were agglutinated and their clot retraction activity inhibited to an equal degree by the same final effective concentrations of antibody. There were no microscopic morphologic differences between platelets that could be identified as phenotypes Pl^{A1} Pl^{A1}, Pl^{A1} Pl, or Pl Pl, no differences in the mean volume determined by a microhematocrit method, and no differences in the nitrogen or total hexose content (see Section 6) per platelet. Sera of all normal individuals with Pl Pl platelets showed no evidence of a naturally occurring antibody against Pl^{A1} antigen by agglutination and C′ fixation tests; similarly, sera of individuals with Pl^{A1} Pl^{A1} platelets had no detectable antibody against Pl Pl platelets. There was no correlation between the different platelet types and the presence or absence of erythrocyte antigens, A_1, A_2, A_3, B, O, M, N, S, P, C, D, E, c, e, D^u, K and Fy^a, based on typing erythrocytes from a total of 52 individuals (22 with Pl^{A1} Pl and 12 with Pl Pl platelets). All individuals tested had erythrocyte antigens s and k and lacked antigens C^w and Di^a. The secretor trait was found to be either present or absent in individuals with Pl^{A1} Pl^{A1}, Pl^{A1} Pl, or Pl Pl platelets.

6. The nature of the Pl^{A1} antigen. The Pl^{A1} antigen on platelets suspended in 0.147 M NaCl retained full ability to fix C′ with antibody after at least a month in the frozen state at − 20° C, after repeated freezing and thawing, and after incubation for at least 5 hours at 37° C, but lost 3 to 4 per cent of remaining activity per day at 5° C. Neither exposure to hypotonic and hypertonic solutions (i.e., distilled water to 2.56 M NaCl) for at least several hours at 20° C, nor prolonged incubation at pH values from 4.8 to 9.4 affected the C′-fixing activity of the antigen.

Although platelets in ACD whole blood stored under blood bank conditions for 20 days were not morphologically intact, approximately 50 per cent of the initial Pl^{A1} antigen could be recovered in suspensions of particulate material made in the same way as platelet suspensions from fresh blood. In an occasional blood sample, some of the particulate antigen-containing material which was present in plasma after storage for 10 to 30 days at 5° C would not sediment at 80,000 G, but this material could be removed by filtering plasma through a 300 mμ pore-size Millipore filter.

Suspensions of reactive platelets heated at 56° C

TABLE II

Preparation and properties of platelet fractions *

Platelet preparations	Characteristics	C′-fixing activity		N content		Sugar content	
		U/ml	%	*μg/ml*	%	*μg/ml*	%
No. 1: Suspension of Pl^{A1} Pl^{A1} platelets in saline, 1.5 × 10^6/mm³	Turbid, not transparent	903	100	577	100	219	100
No. 2: Prep. 1 treated in Raytheon 9 KC oscillator at max. voltage and frequency for 7 min at 3° C	Transparent opalescence, no visible platelets at 800 ×	866	96	577	100	219	100
No. 3: Prep. 2 centrifuged 8,000 G for 15 min; supernatant fluid assayed	Crystal clear	650	72	462	80		
No. 3A: Alternately, Prep. 2 filtered through 1 μ sintered glass filter; filtrate assayed	Crystal clear	497	55	363	63		
No. 4: Prep. 3 (i.e., clear superantant fluid) centrifuged 80,000 G for 45 min; precipitate resuspended in original volume 0.147 M NaCl and assayed	Yellow button; suspension in original vol clear; in 1/10 original vol, opalescent	268	30	47	8	21	9
Supernatant fluid of no. 4	Clear	9	1	335	58		

* C′-fixing activity of each preparation determined as shown in Figure 8 by using several dilutions of antigen with excess antibody. Nitrogen determined by nesslerization. Sugar expressed as μg glucose based on total hexose assay by indole reaction after hydrolysis with conc. H_2SO_4 for 20 minutes at 100° C. Particulate material obtained in Prep. 2 when oscillation time was less than 3 minutes did not pass a 1 μ pore-size filter, and when greater than 9 minutes, the C′-fixing activity of the sonicate decreased progressively. Concentration of platelets had to be less than 2 × 10^6/mm³ for most effective disintegration to occur in the oscillator.

for 10 minutes, incubated at pH 3.2 or 10.0 for 30 minutes at 20° C, and incubated in 50 per cent ethanol-water for 10 minutes at 20° C lost completely and irreversibly their ability to fix C′ in the presence of antibody. However, these forms of treatment had relatively little effect on the ability of platelets to combine with antibody, for the same platelets which had lost their C′-fixing property were at least 80 per cent as effective as untreated platelets in adsorbing antibody (see Figure 7). True inactivation of the antigen (i.e., loss of both C′-fixing and antibody-binding properties) was not complete after heating at 56° C for 2 hours, but was almost complete after heating at 100° C for 10 minutes or after incubation in 50 per cent acetone-water for approximately 15 minutes.

The antigen on platelets or in a particulate fraction of platelets (preparation 4, Table II) retained full activity when incubated in trypsin at a concentration of 0.5 mg per ml for up to 4 hours at 37°, in addition to 16 hours at 25° C. The antigen appeared to be associated with the stromal fractions of platelets. To prepare platelet stroma, platelet suspensions were frozen and thawed three times, and material that sedimented in 15 minutes at 14,000 G was then incubated for 1 hour in distilled water and washed three times in 0.147 M NaCl (8). Several stroma preparations of this type contained approximately 30 per cent of the nitrogen and 70 per cent of the complement-fixing activity present in the initial suspension.

Some further purification was obtained by exposing suspensions of whole platelets or platelet stroma to ultrasonic vibration and separating antigen from residual platelet material by differential high speed centrifugation or fine filtration. Table II lists the conditions of preparation and properties of the sonicate.

The ratio of C′-fixing activity to N content, as well as to sugar content, could be increased four- to fivefold by these procedures. Suspensions of preparation 4, which contained 12 μmoles of hexose (as glucose) per ml, had no detectable uronic acid, glucuronide, or deoxy sugars. C′-fixing material of preparation 4 was in particulate form which passed a 300 mμ pore-size filter but was 95 per cent retained by 50 mμ pore-size. Pl^{A1} Pl platelets treated in the same way as Pl^{A1} Pl^{A1} platelets gave approximately 50 per cent the yield of C′-fixing material at each step of fractionation, and Pl Pl platelets gave none.

Antigen in platelet fractions had stability properties similar in all respects to those described above for the antigen on platelets. Moreover, the

relationship between the amount of C′ fixed and the amount of antibody bound by platelet fractions was quantitatively the same as in the case of whole platelets—i.e., curves in Figures 7 and 8 could be reproduced with the fractions. Preparation 4, Table II, did not produce a precipitin reaction when mixed in suboptimal, optimal or above optimal amounts with serum containing 100 U of antibody per ml, or when layered on serum containing this concentration of antibody. Nonreactive Pl Pl platelets, incubated in preparation 4 and washed once, did not acquire the ability to fix C′.

7. The nature of the antigen-antibody complex. The following experiments indicated that the antigen-antibody complex was very stable. Platelets in complexes formed in the presence of excess antibody could be washed four times in distilled water or various concentrations of NaCl up to 2.56 M at temperatures from 0° to 40° C and remain at least 90 per cent saturated with antibody. There was no detectable antibody in supernatant fluid of complexes heated for as long as 120 minutes at 56° C in 0.147 M or 2.5 M NaCl, although any free antibody which might have been released would have been stable under these conditions (Section 1). The supernatant fluid of complexes incubated for 30 minutes at 20° C in 0.147 M NaCl adjusted to pH 10.1 with NaOH, then neutralized with HCl after sedimenting platelets did not contain detectable free antibody, although antibody was stable at this pH (Section 1). Thus, heat and high pH, which completely abolished the ability of complexes to fix C′, did not effect release of antibody.

Of the various techniques tried, only incubation at low pH produced significant dissociation of platelet-antibody complexes. The optimal pH for maximal dissociation was found to be 3.1 in 0.147 M NaCl adjusted with HCl; the maximal yield of free antibody was 18 to 23 per cent of the amount attached to platelets. Platelet material was separated from the acid medium by centrifugation, and the supernatant fluid was neutralized before being assayed. Most of the antibody that would elute at pH 3.1 did so within 15 minutes at 20° C. Prolonged incubation at pH 3.1 at other temperatures from 0° to 56° C and in 2.56 M NaCl did not increase the yield of free antibody. At pH values below 3.0, antibody inactivation (see Section 1) apparently proceeded along with elution, for the yield of free antibody was progressively less; at pH values above 3.4, no free antibody was obtained.

8. Antiglobulin consumption and fluorescent antibody studies. Typical results of experiments with Coombs serum are shown in Table III. Nonreactive platelets incubated in normal serum (column 3) or in patient's serum (column 4) and reactive platelets incubated in normal serum (column 5) contained sufficient nonspecifically adsorbed material after five washes so that 10^8 platelets consumed approximately that amount of antihuman globulin contained in 0.1 ml Coombs serum with a titer of 1/2 (see Methods). Pl^{A1} Pl^{A1} platelets saturated with antibody consumed more Coombs serum (column 6) than did control platelets. Approximately 10 U of antibody attached to 10^8 platelets (see Figure 7) appeared to account for the specific binding of that amount of antihuman globulin contained in 0.1 ml of Coombs serum with a titer of 1/4. Approximately the same amount of Coombs serum was adsorbed by 10^8 erythrocytes saturated with anti-D.

Fluorescent antibody methods which have been used to study reactions between platelets and heterologous antiplatelet antibodies (12, 19) were applied to the present antigen-antibody system with both the direct and indirect "staining" techniques. In the direct method, fluorescein-conjugated patient's serum was applied to platelet

TABLE III

Fixation of antihuman globulin by platelet-antibody complexes *

1 Coombs serum, ▶ final dilution	2 Preliminary titration	3 Pl Pl platelets + normal serum	4 Pl Pl platelets + patient's serum	5 Pl^{A1} Pl^{A1} platelets + normal serum	6 Pl^{A1} Pl^{A1} platelets + patient's serum
1/40	4+	4+	4+	4+	2+
1/80	4+	3+	4+	3+	2+
1/160	3+	3+	3+	2+	1+
1/320	3+	3+	2+	2+	0
1/640	2+	1+	1+	1+	0
1/1,280	1+	0	0	0	0

* General technique described in Methods. The initial adsorption mixtures consisted of 10^9 platelets suspended either in 1 ml of patient's serum containing 100 U of antibody/ml, or in 1 ml of normal serum from an individual with type AB erythrocytes. After incubation for 30 minutes at 37° C and 5 washes, 10^8 platelets from each mixture were suspended in 0.2 ml of Coombs serum at 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640 dilutions. Supernatant Coombs serum from these mixtures was then added to an equal volume of a suspension of sensitized erythrocytes to give the final dilutions of Coombs serum listed in column 1. The preliminary titration of plain Coombs serum is shown in column 2. In columns 3, 4, 5 and 6 the initial adsorption mixtures used and the degree of agglutination obtained are listed. Similar results were obtained in 4 separate experiments with platelets from a different donor each time.

smears and incubated for 60 minutes at 37° C; excess serum was then washed off and the slide dried and mounted. In all instances the content of antibody in the conjugated serum used was at least 20-fold that necessary to saturate platelet smears; smears were used immediately after being dried in air, not fixed by organic solvents. The washing procedures necessary to remove excess serum were known not to dissociate antigen-antibody complexes (see Section 7), but the degree of fluorescence shown by preparations made with Pl^{A1} Pl^{A1} platelets could not be distinguished from background or from that shown by similar preparations made with Pl Pl platelets. In the indirect technique, unconjugated serum containing antibody in great excess of that necessary to saturate the antigen was applied to platelet smears or mixed with platelet buttons. Platelet smears coated with anti-Pl^{A1} serum or platelets suspended in the serum were incubated for 60 minutes at 37° C. Smears were washed after incubation and coated with fluorescein-labeled Coombs serum. Suspensions were washed after incubation, then smeared on slides and coated with Coombs serum. In each case Coombs serum was kept on the slides for 60 minutes at 20° C, then washed off. Again, none of the preparations with Pl^{A1} Pl^{A1} platelets showed fluorescence that differed from that of control preparations.

9. Reactions of patient's antibody with heterologous platelets. Quantitative C′-fixation activity of platelets obtained from various animals was determined, the same final concentrations of platelets and the same concentration of antibody as in Figure 5 being used. Platelets from 11 mongrel dogs, when used at a concentration of 25,000 per mm^3, fixed 4 to 7 U of C′, and at 50,000 per mm^3, fixed 7.5 to 11 U of C′. The dogs were therefore a homogeneous group with respect to platelet activity, and the activity was comparable with that of Pl^{A1} Pl^{A1} human platelets. Platelets from 25 rabbits, all from different litters, fixed C′ with the patient's antibody. Rabbits also appeared to be a homogeneous group with respect to platelet activity; for at a concentration of 25,000 per mm^3, rabbit platelets fixed 2 to 4 U of C′, and at 50,000 per mm^3, fixed 5 to 8 U. Relatively low activity (cf. Figure 5) of the apparently homozygous rabbit platelets was probably due to their very small size, for the hematocrit of rabbit platelets in a suspension containing 2×10^6 per mm^3 was 6 vol per cent, whereas the hematocrit of the same concentration of human platelets was 15 vol per cent. Platelets from 3 Cynamacus and 3 Rhesus monkeys reacted with the antibody. Quantitative activity of monkey platelets was relatively low on the basis of cell count, but again, monkey platelets are smaller than human platelets. Animal platelets which fixed C′ with the patient's serum adsorbed all of the antibody that reacted with human platelets and the reverse was also true. None of the animal platelets tested reacted with normal human serum to produce C′ fixation.

Although complexes of dog platelets with patient's antibody fixed guinea pig C′ in amounts similar to that fixed by human platelet-antibody complexes, the patient's antibody, in concentrations up to 20 U per ml, would not inhibit clot retraction of dog blood (cf. Table I). The discrepancy between the ability of dog platelets to react with antibody and the inability of antibody to inhibit clot retraction of dog blood may be explained by failure of dog C′ to be fixed as effectively as human C′ by platelet-antibody complexes; for platelet-antibody complexes fixed very little, if any, dog C′ when dog serum was substituted for guinea pig serum as a source of equivalent C′ activity in reaction mixtures. The same discrepancy was observed in the case of rabbit blood.

Although all dog, rabbit and monkey platelets tested reacted with the antibody, platelets from 16 Sprague-Dawley rats and 12 NIH-strain guinea pigs, and thrombocytes from 6 New Hampshire chickens did not react with the patient's antibody in C′ fixation tests and did not adsorb antibody from the patient's serum.

10. Reactions of heterologous antisera with human platelets. Antisera against human Pl^{A1} Pl^{A1}, Pl^{A1} Pl, and Pl Pl platelets were made both in animals whose platelets contained the Pl^{A1} antigen (10 rabbits) and in animals whose platelets lacked the Pl^{A1} antigen (11 rats and 9 guinea pigs). Regardless of the animal used or the type of human platelet against which it was sensitized, the antibody that formed reacted with the three types of human platelets to give the same quantitative relationships between the amount of C′ fixed per platelet in the presence of a fixed concentration of antibody (e.g., Figure 9, curve 1), and between the amount of C′ fixed by increments of antibody

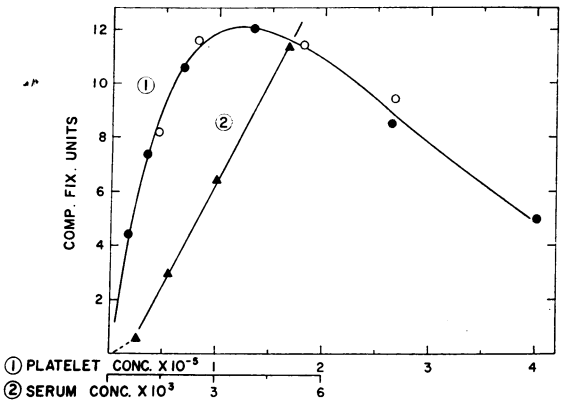

FIG. 9. COMPLEMENT FIXATION WITH HETEROLOGOUS ANTIBODY. Curve 1 is comparable with curves obtained in Figure 8. Reaction mixtures contained a fixed amount (0.005 ml) of serum from a rabbit sensitized with Pl^{A1} Pl^{A1} platelets. The solid circles are results obtained with the same platelets used for sensitization, and the open circles obtained with Pl Pl platelets. Final concentration of platelets in the mixtures are on the abscissa. Curve 2 is comparable with the curve in Figure 2. The reaction mixture consisted of a fixed final concentration (100,000/mm³) of the same platelets that had been used for immunization and various concentrations of the same immune rabbit serum used to obtain curve 1. Concentration of serum is expressed as ml of rabbit serum per 0.3 ml reaction mixture. Similar results were obtained with all antihuman platelet sera, but antibody titers in different animals varied from 10 to 625 U/ml, a unit being defined in the same way as a unit of patient's antibody.

in the presence of a fixed concentration of platelets (e.g., Figure 9, curve 2). Moreover, antibody made in rabbits, rats, or guinea pigs against any one of the three types of human platelets was adsorbed completely by each type of human platelet, leaving no residual antibody that would react with the other two types. Thus, neither the Pl^{A1} antigen on human platelets injected into animals which lacked the antigen on their platelets, nor the possible antigen "allelic" to Pl^{A1} (see Discussion), injected into animals which were apparently homozygous for the Pl^{A1} antigen, provoked a type-specific antibody. Since these experiments involved a total of 28 antisera made against and cross-adsorbed with platelets from 18 different human beings, it appears either that those human platelet antigens which provoke C'-fixing antibodies in animals are shared by many individuals, or that the animals used do not discriminate (20) between type-specific antigen and other human platelet antigens.

11. In vivo effects of the patient's antibody in animals and human beings. In view of the finding in Patient 2 (Figure 1) that thrombocytopenia persisted when antibody concentration was too low to be detected by *in vitro* techniques, it appeared that very little of the antibody from Patient 1 would be necessary to produce thrombocytopenia experimentally *in vivo*. The plasma used for all *in vivo* tests was obtained from Patient 1 and contained 72 U of antibody per ml. One ml of plasma injected intravenously in an individual who had Pl^{A1} Pl^{A1} platelets did not change the platelet count. As shown in Figure 10, 5 ml of plasma containing 360 U of antibody, given to the same individual, depressed the platelet count 60,000 per mm³ below the baseline value within 90 minutes, and a sustained depression of 40 to 50,000 per mm³ lasted for approximately 24 hours. A second individual who had Pl^{A1} Pl^{A1} platelets was given 10 ml of plasma intravenously; platelets gradually fell from 170,000 per mm³ to 50,000 within 2 hours and remained below the baseline value for more than 2 days. There were no ill effects whatsoever

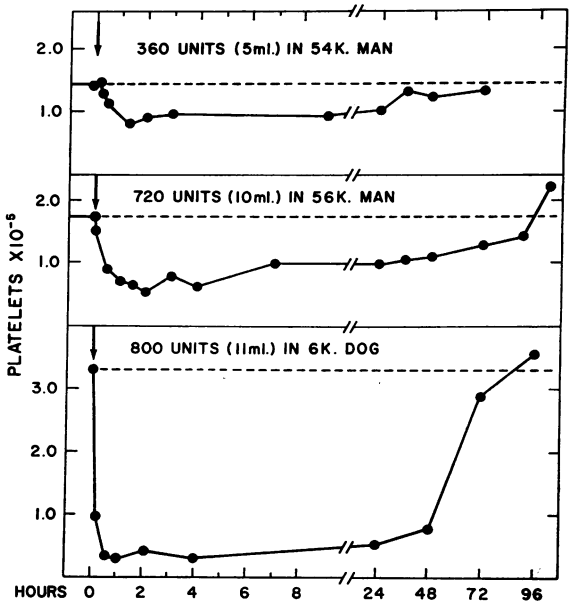

FIG. 10. *In vivo* EFFECTS OF PATIENT'S ANTIBODY. All platelet counts were performed with a minimum of 4 pipets and 8 chambers, with venous blood. The same plasma sample was used in all experiments and was titered for antibody just before use. The dog was not anesthetized and plasma given to the dog had been heated at 56° C for 30 minutes. Horizontal dashed line is the baseline platelet value.

after administration of the antibody and no change in the leukocyte count or serum C′ content.

Antibody which produced the decrease of 120,000 platelets per mm³ *in vivo* could not be detected in the recipient's blood by *in vitro* measurements. The 720 U would have resulted in a final plasma concentration of approximately 0.35 U per ml on the basis of dilution alone, and several minutes after injection free antibody could not be detected in the recipient's serum by C′ fixation techniques. Moreover, any antibody adsorbed on circulating platelets was too small an amount to detect by agglutination, inhibition of clot retraction or Coombs consumption tests, with the recipient's own platelets or whole blood being used.

One year after Patient P.K. had recovered, she was given 68 ml of her own plasma containing a total of 4,900 U of antibody assayed before injection. Although the amount of intravenous antibody given was 13 times greater than had been used to depress platelets in an individual with Pl^{A1} Pl^{A1} platelets, her platelet count remained unchanged. The antibody did not produce ill effects or change her white cell count.

The antibody was not so effective in dogs as in man. As much as 3.5 ml of plasma (250 U of antibody) given to a dog weighing 6 kg did not produce a sustained thrombocytopenia, but 11 ml of plasma (800 U of antibody) produced marked and lasting thrombocytopenia (Figure 10). A second dog given the same dose, 133 U (1.8 ml) of antibody per kg, had an identical response. The dogs did not have hemorrhagic symptoms, discernible ill effects, change in leukocyte count, or a decrease in serum C′. Because several normal human sera, as well as the patient's serum, contained an agglutinin for dog platelets which was present in dilutions up to 1/10 after adsorption with human platelets, the same dogs were also injected with the patient's plasma from which anti-Pl^{A1} had been adsorbed completely with human Pl^{A1} Pl^{A1} platelets, as well as with plasma obtained from the patient 6 months after recovery. These control injections (2 ml of plasma per kg) produced approximately a 30,000 per mm³ decrease in platelet count which lasted less than 1 hour. Control injections were given in one dog 3 days before antibody, and in the other dog, 4 days after antibody.

12. Other observations in human beings on mismatch of Pl^{A1} and the possible antigen "allelic" to Pl^{A1}. During the period of purpura, the first patient received a total of 17 U of fresh whole blood and the second patient received small amounts of fresh blood from three different donors, without evidence of further provocation of antibody in each case. It was considered that neutralization of transfused antigen by remaining antibody may have prevented stimulation of antibody production in these instances, and that antigen might be effective if given after antibody had disappeared. However, after administration of various forms and amounts of antigen (listed in Table IV) to Patient 1 long after disappearance of detectable antibody, all tests for C′ fixing and agglutinating antibodies remained negative, as did tests for possible "blocking" antibody with the direct and indirect Coombs consumption techniques. There was also no evidence of a "block-

TABLE IV

Attempt to provoke anti-Pl^{A1} antibody in a previously sensitized individual *

Infusion no.	Interval after recovery	Material infused
1	7 mos.	Pl^{A1} Pl^{A1} platelets from 500 ml fresh ACD blood, washed once in saline
2	8 mos.	500 ml Pl^{A1} Pl^{A1} whole blood stored 1 week
3	10 mos.	25 ml Pl^{A1} Pl^{A1} platelet-rich plasma, stored 5 days, from the donor to Pat. 2
4	10 mos. + 11 days	200 ml fresh whole blood from a donor to Pat. 1
5	10 mos. + 17 days	125 ml platelet-rich plasma, stored 22 days, from the donor to Pat. 2
6	14 mos.	Fractionated Pl^{A1} Pl^{A1} platelets, Prep. 4 in Table II, 32% yield from total of 5×10^{10} platelets
7	18 mos.	225 ml whole blood, stored 3 days, from the donor to Pat. 2

* The recipient in each instance was Patient 1. The donor to Patient 2 was the individual who supplied the one full unit of blood for the transfusion at operation, and the donor to Patient 1 was one of three whose blood the patient had received during operation (see Case Reports). None of the infusions produced symptoms and the recipient's platelet count did not change during postinfusion periods. Tests for antibody, by a C′-fixation technique that could detect 1.0 U of antibody/ml performed on sera obtained daily for 8 to 12 days after each infusion, were negative. The same sera were also used in agglutination tests and in various tests for possible "blocking" antibody (see text). After infusions 1 and 7, a "direct" Coombs consumption test was done, using the recipient's own platelets. All of these tests were negative.

TABLE V

Comparison of patients who developed purpura after transfusion

Sex	Age	Initial transfusion	Post-transfusion thrombocytopenic state				
			Onset	Duration	Megakaryocytes	Transfusion reaction	Treatment
			days	*days*			
F	40	3 U*	7	3	plentiful	+	Exchange transfus.
F	42	1 U† (75 ml of 2nd)	6	20	plentiful	+	Steroids
F	56(17)	1 U‡	5	17	plentiful	+	Steroids
F	51(18)	1 U	7	8	increased	+	None

* Each unit stored 20 days in blood bank; small clots in one unit.
† Each unit stored 16 days in blood bank; large clots in second unit.
‡ Blood stored 10 days in blood bank (personal communication from M. Zucker).

ing" antibody which might have been detected by its inhibitory effects in C′-fixation reactions; for 10^8 Pl^{A1} Pl^{A1} platelets, incubated in 1.0 ml of the various post-transfusion sera (see legend, Table V) for 1 hour at 37° C, retained full ability to react with C′-fixing antibody, and the activity of small amounts of C′-fixing antibody (0.5 to 1.0 U) was not diminished in the presence of 0.25 ml of the various post-transfusion sera. Moreover, there was no indication that hypersensitivity of the delayed type had developed, for the infusions of antigen caused no systemic symptoms and intradermal injection of antigen produced no local reaction.

Of the large group of normal individuals whose platelets were tested with anti-Pl^{A1}, two whose platelets were nonreactive (Pl Pl) had received several transfusions during operative procedures 2 and 3 years before, respectively. They had not developed hemorrhagic complications after transfusion, although the chances were at least 50 to 1 that each unit of blood they received contained Pl^{A1} antigen.

A normal individual with Pl Pl platelets, who had never been transfused, was observed daily for 11 days after receiving 50 ml of 20-day old blood (containing Pl^{A1} Pl^{A1} platelets) from the initial donor of the full unit of blood to Patient 2. There was no evidence of antibody formation by C′ fixation, agglutination, and direct and indirect Coombs consumption techniques, and the above tests for "blocking" antibody were negative. A second injection of a suspension of 1.6×10^{11} fresh Pl^{A1} Pl^{A1} platelets, given to the same individual 11 days after the first injection, also did not produce a detectable form of anti-Pl^{A1} antibody based on determinations done on serum samples obtained daily for 2 weeks thereafter.

It was conceivable that individuals with Pl^{A1} Pl^{A1} platelets might lack a possible antigen "allelic" to Pl^{A1} and develop antibodies against it. Platelet-rich plasma from a normal *Pl Pl* individual was given intentionally to an individual with Pl^{A1} Pl^{A1} platelets. After administration of 125 ml on two separate occasions, the recipient did not develop antibody against Pl Pl platelets that could be detected with the same C′ fixation, agglutinin, and Coombs consumption techniques employed to measure the anti-Pl^{A1} antibody, and the recipient's platelet count was unaffected.

13. Evaluation of other relevant factors. In accounting for the infrequency of post-transfusion purpura, the possibility that a rare donor's blood might contain an unusual amount or form of antigen had to be considered. Attempts were made to detect a free or coating form of antigen in fresh samples of ACD blood obtained from the five donors whose blood had been given to both patients at operation; platelets from each donor reacted with the antibody, the donor of the full unit of blood given to Patient 2, and two of the three donors to Patient 1 having platelet type Pl^{A1} Pl^{A1}. Serum and plasma from each donor did not produce a visible band against antibody in agar-gel diffusion plates, and did not form a precipitin when layered on or mixed with serum containing 100 U of antibody per ml. Unheated, platelet-free sera from three of the five donors, used as

a source of possible antigen as well as C′ in incubation mixtures with antibody, did not fix C′; and prior incubation of small amounts of antibody (1.5 U) in 0.3 ml of the various donors' fresh platelet-free sera did not decrease the amount of C′ fixed by the antibody on subsequent addition of reactive platelets.[2] Thus, no evidence could be obtained for free Pl^{A1} antigen that would react directly with anti-Pl^{A1}. With the same series of tests for free antigen, none was detectable in Pl^{A1} Pl^{A1} blood after storage for up to 21 days at 5° C or after partial clotting was permitted before citrate anticoagulant was added. If age of blood or inadequate anticoagulant were involved in producing a free form of Pl^{A1}, the antigen was not detectable by the *in vitro* tests used.

Attempts to detect a soluble form of Pl^{A1} antigen in donor blood, which might be demonstrable by C′ fixation after adsorption on Pl Pl platelets, were unsuccessful, but two samples of plasma from the donor to Patient 2 contained large amounts of finely particulate antigen which did not sediment (see Section 6) unless Pl Pl platelets were mixed with the plasma. Pl Pl platelets, incubated in plasma of the donor to Patient 2 and washed, acquired the ability to fix C′ with antibody. However, filtration of the donor's plasma (300 mμ pore-size; see Section 6) removed the particulate antigen which apparently could be adsorbed by Pl Pl platelets.

Excretion of Pl^{A1} antigen in saliva could not be evaluated conclusively, for relatively undiluted saliva had marked anticomplementary activity. However, saliva from four individuals with Pl^{A1} Pl^{A1} platelets, including the donor to Patient 2, used at 1/10 dilution, did not fix C′ with antibody or interfere with complement fixation by Pl^{A1} Pl^{A1} platelets; and Pl Pl platelets, incubated in these undiluted salivas, did not acquire the ability to fix complement with antibody.

It was of interest to determine whether the Pl^{A1} antigen was present on platelets at birth. Platelets in cord blood of two infants were found to have the same antigen content as the Pl^{A1} Pl^{A1} platelets of the mothers.

14. Observations on typical cases of idiopathic thrombocytopenic purpura. None of 16 cases of classical ITP was found to have a platelet agglutinin, with a test system using sera at 8/10 or greater final dilution. Of the 16 cases, 5 were less than 15 years old; 4 of the 5 were males; 2 of the 5 had acute and 3 had chronic ITP. Eight of 11 adults were females; 3 of the 11 had acute and 8 had chronic ITP. Sera from 12 patients with ITP were used in C′-fixation tests with platelets from 6 different normal individuals, and in no instance was C′ fixed. Final platelet concentrations used in C′-fixation tests with ITP sera varied from 25,000 to 100,000 per mm^3, the final serum dilution was 1/2 to 1/4, and the amount of guinea pig C′ used permitted detecting with certainty fixation of 0.5 U. All C′-fixation mixtures were incubated for 90 minutes at 37° C. In addition, in three instances in which the ITP patient's own serum was used as a source of possible antibody as well as C′, C′ was not fixed. Thus, the various *in vitro* techniques, used at the limit of their sensitivity for detecting the anti-Pl^{A1} antibody and other definite platelet antibodies (8, 9), gave no evidence for the presence of a similar antibody in the sera of typical ITP cases.

Platelets from eight consecutive cases of ITP reacted with the antibody from Patient 1 in C′ fixation and agglutination tests in the same way as did platelets from normal individuals. Antigen content of these platelets was not determined quantitatively.

DISCUSSION

Development of the disease. The correlation in the two patients studied of transfusion of mismatched platelets, thrombocytopenia 1 week later, and the presence of an antibody which reacted with the donors' platelets, but not with the patients' own platelets obtained after recovery, indicates that an isoantibody was provoked which paradoxically was capable of destroying platelets of the sensitized individuals.

There are two published reports of patients with thrombocytopenic purpura associated with a platelet agglutinin (17, 18) and inhibtor of clot retraction (17) which did not react with the patients' own platelets obtained after recovery. Rea-

[2] When human serum instead of guinea pig serum was used as a source of C′, antigen-antibody mixtures fixed half as many units of human C′ as guinea pig C′. The decrease in C′ fixation was not due to specific inhibition produced by free Pl^{A1} antigen; for all human sera used as a source of C′, whether from individuals with Pl^{A1} Pl^{A1} or Pl Pl platelets, gave identical results in C′-fixation tests.

sons for development of the antibody and the thrombocytopenic state in these cases were not clearly defined. Actually, the two reported patients had received a blood transfusion shortly before onset of purpura and the clinical course of all four patients was strikingly similar (Table V). Serum from the patient of van Loghem, Dorfmeijer, van der Hart and Schreuder (18), had agglutinated platelets from 97.6 per cent of the normal individuals tested, and the antigen which reacted with the antibody was labeled "Zw." We sent antibody which reacted with antigen, that we have labeled Pl^{A1}, to Dr. van Loghem who found that it reacted with Zw-positive platelets, but not with Zw-negative platelets. The antigen Pl^{A1} is, therefore, the same as Zw, and the term, Zw, has precedence; but the designations Pl^{A1} and *Pl,* for the genetic determinants of platelet antigenicity, facilitate presentation of the concepts derived from the present study and are in accord with systematic notation suggested by Ford (21) (see below). No doubt three of the cases represent immunization of *Pl Pl* individuals against Pl^{A1} antigen, and there is little doubt that the patient of Zucker and co-workers (17) represents immunization against a platelet antigen but, unfortunately, the specificity could not be determined.

During the period of purpura, the patients' platelets, nonreactive on a genetic basis, evidently were susceptible to the antibody. This susceptibility was not due to some obscure reaction between antibody and normal Pl Pl platelets which might occur only *in vivo,* for 13 times the amount of antibody that produced sustained depression of platelets in a Pl^{A1} Pl^{A1} individual had no effect on platelets of a *Pl Pl* individual (Section 11). Since recovery took place in Patient 1 (Figure 3) in the presence of an antibody level much higher than the highest level measured in Patient 2 during purpura (Figure 1), it was apparent that beneficial effects of exchange transfusion were not due simply to decrease in antibody. Rather, the abrupt cure appeared to be caused by suppression of an additional factor which must have been present to make Pl Pl platelets susceptible to antibody during the period of thrombocytopenia. The observed phenomena could be explained if Pl^{A1} antigen in transfused blood not only provoked antibody, but also persisted *in vivo* in a form capable of effecting attachment of antibody to Pl Pl platelets. The following considerations lend weight to this hypothesis.

There are at least two ways in which antibody against Pl^{A1} antigen could become attached to Pl Pl platelets; these platelets could be coated *in vivo* with some form of antigen that would react with antibody, or antigen-antibody complexes formed *in vivo* could be adsorbed onto platelets. The first possibility is supported by well documented examples that free antigens are adsorbed on erythrocytes both *in vitro* and *in vivo*—e.g., Lewis Groups (22a) and the J antigen in cattle (23); but, as yet, there are no examples that transfused erythrocyte coating-antigens provoke antibodies which cause destruction of the recipient's erythrocytes. The second possibility is supported by observations which indicate that adsorption of antigen-antibody complexes onto platelets is the most likely mechanism of drug purpura (4, 8, 24), and that nonspecific adsorption of antigen-antibody complexes by platelets is a general immunologic phenomenon (25) which can produce thrombocytopenia *in vivo* (26). Either possibility would depend on survival of Pl^{A1} antigen *in vivo* for a longer time than the period of antibody induction.

Results of infusions of antibody in normal Pl^{A1} Pl^{A1} individuals (Figure 10) indicate the amount of Pl^{A1} antigen that would have to survive to cause thrombocytopenia by the above mechanisms. As little as 360 U of injected antibody, an amount which could saturate at most the 3.6×10^9 Pl^{A1} Pl^{A1} platelets (Figure 7) contained in approximately 20 ml of the recipient's blood, produced sustained depression of platelets in a 54 kg individual. By analogy, it appears that if Pl^{A1} antigen from as little as 20 ml of blood were distributed over the platelets of a *Pl Pl* recipient in association with antibody, thrombocytopenia would no doubt occur. A form of antigen that might coat Pl Pl platelets or be adsorbed onto platelets in complex with antibody would have to be detached from antigenic platelets. The *in vivo* survival of antigen which became detached from a donor's platelets (either before blood was transfused or by some *in vivo* process in the recipient) would not necessarily be comparable with survival of intact transfused platelets. Because the antigen is so stable (Section 6), and such small amounts apparently would suffice to make Pl Pl platelets susceptible to anti-

body *in vivo,* it is conceivable that, after transfusion of 500 ml of Pl^{A1} Pl^{A1} blood, an effective antigen concentration could persist for weeks *in vivo.*

Numerous attempts were made to demonstrate a form of antigen that might effect attachment of antibody to Pl Pl platelets (Sections 6 and 13), but none could be detected in blood from normal individuals, including three of the patients' initial donors, or in various preparations of disrupted platelets. It is possible that conditions for producing the appropriate form of antigen may exist only *in vivo.* The finding that Pl^{A1} antigen could be altered so that its complexes with antibody would not give the usual *in vitro* reactions (Section 6) suggests that a form of antigen, not necessarily detectable by techniques used so far, could be involved in development of the disease.

It is apparent from the various conditions which failed to dissociate antibody-Pl^{A1} antigen complexes (Section 7), and from the completeness of antibody adsorption in mixtures containing an approximately equal ratio of antigen and antibody (Figure 7), that the association constant of the complex must be very high. This favors the possibility that circulating antigen remaining at the time antibody appears exists thereafter in complex with antibody, and that the complex is adsorbed by Pl Pl platelets. The high affinity between antibody and antigen would assure complex formation even at very low concentration of both reactants and could account for persistence of thrombocytopenia under the conditions present in Patient G.H. Smaller amounts of antigen-antibody complex may be required to perpetuate the thrombocytopenic state than to establish it, for megakaryocytes apparently have a limited capacity to replenish platelets after induction of severe thrombocytopenia (4).

The above considerations, therefore, make it possible to explain the occurrence of post-transfusion purpura in terms of *in vivo* survival of minute amounts of foreign antigen which, in complex with the antibody it provokes, can attach to platelets of the recipient. Other conceivable explanations for the paradoxical thrombocytopenia would require such hypotheses as the presence of a different population of platelets before immunization in individuals not showing evidence of chimerism (27), the presence of a different population of platelets after the thrombocytopenic state as a result of somatic mutation, or the temporary presence of acquired Pl^{A1}-like substance on Pl Pl platelets analogous to acquired B-like substance on Group A erythrocytes (28). The likelihood that these hypothetical circumstances would associate by chance with immunization against Pl^{A1} antigen, and account for thrombocytopenia, seems more remote than the explanation discussed above.

The infrequency of post-transfusion purpura. The problem of explaining the infrequency of post-transfusion purpura, in spite of frequent antigenic mismatch (approximately 1.5 per cent of general transfusions), has two aspects. One aspect involves factors affecting development of thrombocytopenia if antibody does form; the other, factors affecting formation of antibodies against so-called "weak" antigens. Development of antibody alone without accompanying thrombocytopenia conceivably could occur, for antibody might be provoked in susceptible *Pl Pl* individuals by any form of Pl^{A1} antigen, but the form of antigen necessary to effect attachment of antibody to platelets may not develop or survive the period of antibody induction so frequently. The frequency with which antibody alone may develop is not known, because purpura is the only symptom that draws attention to immunization. Failure of attempts to provoke antibody in a normal *Pl Pl* individual (Section 12) established the fact that *Pl Pl* individuals do not always form detectable antibodies or develop thrombocytopenia after initial exposure to Pl^{A1} antigen. Alternatively, the form of antigen that may survive *in vivo* and effect attachment of antibody to Pl Pl platelets may be the only type that gains access to antibody-forming tissue. Failure of Patient 1 to respond to attempts at re-immunization (Section 12) suggests that some unique characteristic of antigen in donor blood may be a prerequisite for immunization. Otherwise, it would appear that Pl^{A1} antigen either could produce "immunologic paralysis" (29), a phenomenon which has not been observed in human beings as yet, or provoke another form of antibody undetectable by techniques used.

There are other possible factors which could affect initial "susceptibility" to transfused Pl^{A1} antigen. Some *Pl Pl* individuals may acquire immunologic tolerance (30) as a result of placental transfer of maternal Pl^{A1} antigen, since most

Pl Pl individuals would have Pl^{A1} *Pl* parents (see below). If placental transfer occurred invariably, only children of *Pl Pl* mothers might be expected to be sensitive to Pl^{A1} antigen; but the two patients observed had Pl^{A1} *Pl* mothers (Figure 4), indicating that, if immunologic tolerance does occur, it is not predictable. The two observed patients each had three Pl^{A1} *Pl* children, and the two other patients (Table V) also had had children. If the disease proves to be limited to parous females, conditioning to placental transfer of Pl^{A1} antigen present in fetal blood may have to be considered as a susceptibility factor. The meaning of the equivocal terms "weak antigen" and "susceptibility," applied in the case of platelet mismatch, may become clearer when these possibilities are further evaluated.

Therapy of the disease. Antibody production in this disease is typical of that following an initial single exposure to an antigen; for after the peak response, by the sixth to eighth post-transfusion day, there was a gradual decline in titer (Figure 1) (31). Thrombocytopenia in Patient 2 cleared concomitantly with decay of antibody, and in Patient 1, possibly after removal of the postulated antigen-antibody complex. The disease is therefore self-limited, and purpura apparently clears spontaneously when antibody or the postulated antigen-antibody complex, or both, reach a level low enough to permit regenerating platelets to circulate. However, since purpura is characteristically severe and lasts as long as 3 weeks, treatment may become necessary.

Exchange transfusion may prove to be the treatment of choice, for all evidence indicates that steroids are not beneficial (see Table V) and splenectomy does not seem to be indicated; but there is some hazard attending exchange transfusion. During the period of purpura, all patients had reactions to whole blood or platelet-rich plasma, such as chills, fever, urticaria and shock, all of which might be attributable to reaction between Pl^{A1} antigen on transfused platelets and antibody. The long time required to effect the exchange in Patient 1 was due to the inability to infuse whole blood at a rate faster than 2 to 3 ml per minute without causing severe hypotension. The severity or type of symptomatology other patients may develop when similarly treated cannot be predicted. Although Pl^{A1} antigen given during the exchange did not provoke further antibody or perpetuate thrombocytopenia, until further information is obtained, it must be considered that additional Pl^{A1} antigen potentially may prolong, rather than cure, the disease. Perhaps exchange transfusion with Pl Pl blood, or administration of large amounts of Pl Pl platelets alone, if available, would provide equally effective treatment and entail less risk.

The exchange transfusion involved alternate removal and replacement of 100- to 300-ml aliquots of blood until a total of 5,500 was withdrawn and 6,500 replaced. Assuming a fixed blood volume of 4,900 ml (see Section 2 and Figure 3) and that excess transfused blood elevated the hematocrit but did not dilute plasma components, the final dilution produced, calculated simply as a series of sequential dilutions, was 31 per cent (or 69 per cent effective). Since this value would vary no more than $\pm$ 1 per cent whether the alternate aliquots withdrawn and replaced were 10 per cent or 1 per cent of the initial blood volume, not less than approximately 31 per cent of the initial antibody should have been left as a result of dilution alone; but only 3 per cent could be measured (Figure 3). However, the 6,500 ml of transfused blood, containing approximately 200,000 platelets per mm^3 (assumed 74 per cent Pl^{A1} Pl^{A1}, 24 per cent Pl^{A1} Pl), was sufficient to bind approximately 1.14×10^5 U of antibody based on the data of Figure 5 (i.e., 10^8 Pl^{A1} Pl^{A1} and 2×10^8 Pl^{A1} Pl platelets bind 10 U of antibody). Of the 3.2×10^5 U of circulating antibody (3,200 ml plasma $\times$ 100 units per ml) present initially, apparently 69 per cent, or 2.2×10^5 U, was removed by exchange and 1.14×10^5 U could have been sequestered with the transfused platelets which did not circulate. Thus, combined removal and sequestration could account for loss of approximately as much antibody as circulated initially, and hence for the finding of 3 per cent antibody left. Within 13 hours after the exchange, circulating antibody had increased to 6.4×10^4 U (3,200 ml plasma $\times$ 20 U per ml). The elevation was too prompt and too moderate to be considered an anamnestic response and suggested that re-equilibration with extravascular γ-globulin occurred, which in part could have reflected some release of the 1.14×10^5 U of sequestered antibody.

Since approximately 2 U of human C′ (one-half

as much as guinea pig C′, Section 13) was fixed by 1 U of antibody, administration of enough antigen to complex with 1.14×10^5 U of antibody (see above) could have resulted in fixation of as much as 2.28×10^5 U of C′ *in vivo*. The patient's total circulating complement was initially 1.76×10^5 U (55 U C′ per ml × 3,200 ml plasma), and the difference between the 2.34×10^5 U of C′ in transfused plasma (an average of 60 U C′ per ml × 3,900 ml) and the 1.54×10^5 U of C′ in removed plasma (an average of 44 U C′ per ml × 3,500 ml) would have provided an additional 0.8×10^5 U of circulating C′. Thus, 2.56×10^5 total units of C′ were available and 2.28×10^5 (or approximately 90 per cent) could have been fixed. The measured drop of C′ *in vivo* was the result of fixation, for it was subsequently observed that exchange transfusion with even larger amounts of ACD blood in a nonimmunologic disease did not produce a drop in C′. The patient's C′ level fell as little as it did (Figure 3) possibly because additional amounts were produced rapidly or were available from extravascular spaces, or both. Because such large amounts of C′ may be fixed with relatively little effect on the circulating C′ level, it is not surprising that both patients had normal levels of C′ during the height of purpura, for the amounts of antigen conceivably involved in development of the disease were quite small.

Characteristics of the platelet system containing Pl^{A1} antigen. The ability to differentiate clearly three cell phenotypes based on quantitative reactions with an antibody against one antigen is unique. Since platelets of individuals who were most likely Pl^{A1} *Pl* uniformly contained half as much antigen as platelets of individuals who were most likely Pl^{A1} Pl^{A1}, it appears that the known antigen, Pl^{A1}, is inherited as a character, dependent on a gene capable of expressing itself in a single or double dose. In view of the dominance of blood group antigens in general (22b), there is a good possibility that the allele of Pl^{A1} controls another antigen which is inherited as a "co-dominant" character. Attempts to find or provoke an antibody against this hypothetical antigen (or antigens, for conceivably there could be more than one allele) have been unsuccessful so far (Sections 10 and 12). Since similar attempts to provoke antibody against the known Pl^{A1} antigen also failed, the possibility of finding the "missing" antigen(s) still seems more likely than the possibility that the *Pl* gene has no activity or only modifies (22c) the expression of Pl^{A1}.

The gene frequency of Pl^{A1} and *Pl*, estimated by translating low and high activity phenotypes into genotypes Pl^{A1} *Pl* and Pl^{A1} Pl^{A1}, respectively, was in close agreement with the frequency that would be required to give the observed 1.5 per cent nonreactors in the group of 206 individuals tested. Since there were 26 per cent apparently heterozygous and 74 per cent apparently homozygous Pl^{A1} individuals among the 23 individuals screened by quantitative C′ fixation, the gene frequency (22d) of Pl^{A1} would be $0.74 + 0.26/2 = 0.87$, and of *Pl*, $0.26/2 = 0.13$. The expected frequency of *Pl Pl* individuals on this basis would be $(0.13)^2 = 0.017$ or 1.7 per cent, which compares well with the observed frequency of 1.5 per cent in the large group.

Effects of various physical and chemical forms of treatment (Section 6) indicated that Pl^{A1} antigen could be either a relatively stable protein or polysaccharide, and the ratios of C′-fixation activity to nitrogen or sugar content of platelet fractions (Table II) did not distinguish the possibilities. However, since prolonged exposure to high concentration of trypsin had no effect on the antigen, there is a good possibility that Pl^{A1} is a polysaccharide, perhaps similar to the mucopolysaccharide erythrocyte group substances which have been characterized (32, 33). The Pl^{A1} antigen could not be detected by fluorescein-labeled antibody techniques (Section 8). In this respect Pl^{A1} antigen resembles A substance on erythrocytes (34) and differs from the human species-specific platelet antigens which provoke heterologous antibodies (12, 19) which do not distinguish type-specific human platelet antigens (Section 10).

By determining platelet genotypes and erythrocyte phenotypes in the same individuals, it was possible to conclude that Pl^{A1} antigen was not identical with a number of different erythrocyte antigens (Section 5), but more observations on appropriate family groups will be necessary to evaluate possible linkage between platelet and erythrocyte group systems. Since the platelet antigen system is another example of a genetic polymorphism (35), some correlation between this system and a factor influencing natural selection may exist. The selection factor conceivably could be

neonatal purpura (36) due to mother-fetal Pl^{A1} incompatibility; for we have recently seen a family in which the mother has type Pl Pl platelets, the father has type Pl^{A1} Pl^{A1}, and a child born a year ago with neonatal purpura has type Pl^{A1} Pl.

Definite demonstration of a direct proportionality between gene dose and quantity of antigen on platelets was made possible by use of a quantitative C′-fixation technique. Other serologic procedures used did not lend themselves to sufficient refinement for differentiating cells containing a twofold difference in antigen (Section 5). Over the years many attempts have been made to distinguish heterozygous erythrocyte phenotypes by agglutination techniques (22) and, more recently, by employing radioactively labeled antibodies (37, 38), but the results have not been clear-cut, possibly due to limitations of techniques used. It appears likely that application of a C′-fixation technique, similar to the one used herein, will facilitate more precise evaluation of erythrocyte antigen systems (and gene-dose effects) in the instances in which this technique is applicable.

Notation of platelet antigen systems. Because it is likely that at least one allele and possibly other alleles of Pl^{A1} will be identified, and because we have recently identified several other inherited platelet antigens [3] which are not produced by an allele of Pl^{A1}, some uniform notation of platelet antigen systems should be adopted. Many of the suggestions of Ford (21) have been followed in this report. Genes are italicized, antigen not italicized; the gene whose antigen has been recognized by an antibody is indicated by the suffix Al, and no suffix has been given to the gene whose antigen has not been recognized as yet. It is suggested that if an allele of Pl^{A1} is recognized by an antibody, it be labeled Pl^{A2}; and if multiple alleles are recognized, they be labeled sequentially, Pl^{A3}, and so on.

The matter of labeling loci of platelet antigen systems to distinguish them from erythrocyte systems can be overcome if new loci controlling platelet antigens are designated by a new suffix letter after *Pl*—i.e., Pl^{B}, Pl^{C}, and so on,[4] and if the alleles at each locus are identified by suffix numbers. A gene whose antigen has not been recognized by an antibody could be labeled with the appropriate suffix letter, but not a number. For example, the system reported herein would be called the Pl^{A} system; the recognized allele would be designated as it has been, Pl^{A1}, and the unrecognized allele would be Pl^{A} instead of *Pl*. This is a departure from Ford's suggestion but would provide an open end for any number of alleles in any one system; each system would have its distinguishing suffix letter, and there would be no confusion with erythrocyte or other systems. Because phenotypes can be translated directly into genotypes in the Pl^{A} system, antigen-dose and gene-dose could be indicated by numbers in brackets after the locus symbol, and absence of a "dose" could be indicated simply by a minus, i.e., Pl^{A} (1,1) for Pl^{A1} Pl^{A1} platelets, and Pl^{A} (1,1) for the corresponding genotype; Pl^{A} (1,-) for Pl^{A1} Pl^{A} platelets, and Pl^{A} (-,-) for Pl^{A} Pl^{A} platelets. The number, 2, would be substituted for a minus when the antigen is recognized. If only the presence or absence of an antigen were detectable in another platelet antigen system, only one number or minus would be placed in a bracket. The suggestions by Ford concerning recessive antigens could be followed by using a small suffix number.

Implications in ITP and suspected "autoimmune" disorders. Post-transfusion purpura is the second definite immunologic entity to be differentiated from the ITP syndrome. The first was drug purpura. Although the underlying causes of sensitization are widely different in post-transfusion and drug purpura, the antibodies in both diseases have similar qualitative and quantitative activities *in vitro* and *in vivo*. The similar relationships in the two antibody systems, between the amount of C′ fixed and the relative concentrations of antibody and platelets (see Figure 8 and Reference 8) indicate that the theoretical considerations concerning antibody adsorption and C′ fixation which apply to drug antibodies (8, 39) also are applicable to anti-Pl^{A1} (as well as to heterologous antibodies; e.g., Figure 9) if variables de-

[3] During preparation of this report, the mother of an infant with neonatal purpura was found to have an antibody in her serum which reacts with an antigen inherited independently of Pl^{A1}. An abstract concerning this finding appeared in the April, 1961, issue of Clinical Research. Moreover, antibodies against two additional platelet antigens, each apparently genetically unrelated to the other three, have been found in individuals who had received multiple transfusions.

[4] The additional loci, Pl^{B}, Pl^{C}, and Pl^{D} are already accounted for in this laboratory (Footnote 3).

pendent on drug concentration are excluded. Thus, the mechanism proposed for C′ fixation by drug antibodies (8) may operate generally with many antibodies reacting with antigens on cellular surfaces.

The concentration of anti-Pl^{A1} required to depress Pl^{A1} Pl^{A1} platelets experimentally *in vivo* was much lower than the concentration that could be detected *in vitro* with the most sensitive assay techniques developed so far (Figure 10). Platelets which were destroyed *in vivo* were quite unsaturated with antibody. The degree of unsaturation was such that platelets probably were not "lysed" directly by the small amount of C′ which could have been fixed and probably were not agglutinated in the circulation. Rather, platelets with minimal amounts of antibody attached apparently were rendered more susceptible to processes of *in vivo* sequestration, just as in the case of thrombocytopenia due to the antibody of quinidine purpura (4). The observations that dogs are resistant to *in vivo* effects of anti-Pl^{A1}, and that dog C′ is not fixed as effectively as human C′ by this antibody system (Sections 9 and 11), indicate that C′ fixation may play a role in sequestration of antibody-coated cells.

The fact that a C′-fixing antibody is not demonstrable in the usual case of idiopathic thrombocytopenic purpura, by techniques used so far, does not conflict with the possibility that ITP is an immune disease. Analogies drawn from a study of quinidine purpura (4, 8, 9) made it possible to explain the characteristics of ITP in terms of immunoreactions occurring at low levels of activity which could not be detected by usual *in vitro* techniques, and similar analogies can be drawn from the present study of post-transfusion purpura. Since drug purpura and post-transfusion purpura are due to immunization against foreign antigens and the mechanism of thrombocytopenia in both diseases appears to be similar, there is reason to believe that a variety of other foreign antigens may be identified as the underlying cause of a group of specific disorders falling within the syndrome of ITP.

The ITP syndrome resembles a variety of other diseases in which an individual's own cells are injured or destroyed by apparent immune processes (2, 40). Demonstration that the manifestations of ITP can be duplicated by antibodies provoked by foreign antigens suggests that sensitization by foreign antigens may underlie other so-called "autoimmune" disorders.

SUMMARY

1. The clinical picture of post-transfusion purpura and a successful method of treatment was presented.

2. An isoantibody provoked by a mismatched platelet antigen destroyed platelets in the sensitized individuals. It is proposed, as a mechanism of thrombocytopenia, that foreign antigen survives *in vivo* longer than the period of antibody induction and that antibody, complexed with foreign antigen, is adsorbed by autologous platelets.

3. Infrequency of purpura, despite frequent mismatch of platelet antigens in general transfusions, may be accounted for by various factors that affect development of antibody as well as survival of antigen *in vivo*.

4. Experimental infusions of antibody indicate that thrombocytopenia can occur when *in vivo* concentrations of antibody are below levels detectable by currently available *in vitro* tests. Minimal amounts of antibody and complement attached to platelets appear to increase their susceptibility to sequestration.

5. Similarities which were found between the immunoreactions of post-transfusion and drug purpura permit interpreting the pathogenesis of idiopathic thrombocytopenic purpura (and possibly other disorders thought to be "autoimmune") in terms of sensitization by foreign antigens.

6. The antigen responsible for the disorder is inherited as a character dependent on a gene capable of expressing itself in a single or double dose. A clear-cut proportionality between gene dose and quantity of antigen per cell was demonstrated.

7. A uniform notation for platelet antigen systems is proposed.

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REFERENCES

1. Ackroyd, J. F. Platelet agglutinins and lysins in the pathogenesis of thrombocytopenic purpura, with a note on platelet groups. Brit. med. Bull. 1955, **11**, 28.
2. Harrington, J. H., Minnich, U., and Arimura, G. The autoimmune thrombocytopenias *in* Progress in Hematology, L. M. Tocantins, Ed. New York, Grune & Stratton, 1956, vol. I, p. 166.
3. Ackroyd, J. F. The cause of thrombocytopenia in sedormid purpura. Clin. Sci. 1949, **8**, 269.
4. Shulman, N. R. Immunoreactions involving platelets. IV. Studies on the pathogenesis of thrombocytopenia in drug purpura using test doses of quinidine in sensitized individuals; their implications in idiopathic thrombocytopenic purpura. J. exp. Med. 1958, **107**, 711.
5. Stefanini, M., and Dameshek, W. The Hemorrhagic Disorders. A Clinical and Therapeutic Approach. New York, Grune & Stratton, 1955, p. 88.
6. Brecher, G., and Cronkite, E. P. Morphology and enumeration of human blood platelets. J. appl. Physiol. 1950, **3**, 365.
7. Kabat, E. A., and Mayer, M. M. Experimental Immunochemistry. Springfield, Ill., C. C Thomas, 1948.
8. Shulman, N. R. Immunoreactions involving platelets. I. A steric and kinetic model for formation of a complex from a human antibody, quinidine as a haptene, and platelets; and for fixation of complement by the complex. J. exp. Med. 1958, **107**, 665.
9. Shulman, N. R. Immunoreactions involving platelets. III. Quantitative aspects of platelet agglutination, inhibition of clot retraction, and other reactions caused by the antibody of quinidine purpura. J. exp. Med. 1958, **107**, 697.
10. Riggs, J. L., Siewald, R. J., Burckhalter, J. H., Downs, C. M., and Metcalf, T. J. Isothiocyanate compounds as fluorescent labeling agents for immune serum. Amer. J. Path. 1958, **34**, 1081.
11. Marshall, J. D., Eveland, W. C., and Smith, C. W. Superiority of fluorescein isothiocyanate (Riggs) for fluorescent-antibody technic with a modification of its application. Proc. Soc. exp. Biol. (N. Y.) 1958, **98**, 898.
12. Silber, R., Benitez, R., Eveland, W. C., Akeroyd, J. H., and Dunne, C. J. The application of fluorescent antibody methods to the study of platelets. Blood 1960, **16**, 958.
13. Steffen, C. Results obtained with the antiglobulin consumption test and investigations of autoantibody eluates in immunohematology. J. Lab. clin. Med. 1960, **55**, 9.
14. Ashwell, G. Colorimetric analysis of sugars *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1957, vol. III, p. 73.
15. Waravdekar, V. S., and Saslaw, L. D. A method of estimation of 2-deoxyribose. Biochim. biophys. Acta 1957, **24**, 439.
16. Fudenberg, H. H., and Kunkel, H. G. Physical properties of the red cell agglutinins in acquired hemolytic anemia. J. exp. Med. 1957, **106**, 689.
17. Zucker, M. B., Ley, A. B., Borrelli, J., Mayer, K., and Firmat, J. Thrombocytopenia with a circulating platelet agglutinin, platelet lysin and clot retraction inhibitor. Blood 1959, **14**, 148.
18. van Loghem, J. J., Jr., Dorfmeijer, H., van der Hart, M., and Schreuder, F. Serological and genetical studies on a platelet antigen (Zw). Vox Sang. (Basel) 1959, **4**, 162.
19. Vazquez, J. J., and Lewis, J. H. Immunocytochemical studies on platelets. The demonstration of a common antigen in human platelets and megakaryocytes. Blood 1960, **16**, 968.
20. Dray, S. Three γ-globulins in normal human serum revealed by monkey precipitins. Science 1960, **132**, 1313.
21. Ford, E. B. A uniform notation for the human blood groups. Heredity 1955, **9**, 135.
22. Race, R. R., and Sanger, R. Blood Groups in Man, 3rd ed. Oxford, Blackwell, 1958, (a) 200, (b) 7, (c) 28, (d) 22.
23. Stormont, C. Acquisition of the J substance by the bovine erythrocyte. Proc. nat. Acad. Sci. (Wash.) 1949, **35**, 232.
24. Miescher, P., and Miescher, A. Die Sedormid-Anaphylaxie. Schweiz. med. Wschr. 1952, **82**, 1279.
25. Miescher, P., and Cooper, N. The fixation of soluble antigen-antibody-complexes upon thrombocytes. Vox Sang. (Basel) 1960, **5**, 138.
26. Miescher, P., and Straessle, R. Experimentelle Studien über den Mechanismus der Thrombocyten-Schädigung durch Antigen-Antikörper-Reaktionen. Vox Sang. (Basel) 1956, **1**, 83.
27. Dunsford, I., Bowley, C. C., Hutchison, A. M., Thompson, J. S., Sanger, R., and Race, R. R. A human blood-group chimera. Brit. med. J. 1953, **ii**, 80.
28. Giles, C. M., Mourant, A. E., Parkin, D. M., Horley, J. F., and Tapson, K. J. A weak B antigen, probably acquired. Brit. med. J. 1959, **ii**, 32.
29. Sercarz, E., and Coons, A. H. Specific inhibition of antibody formation during immunological paralysis and unresponsiveness. Nature (Lond.) 1959, **184**, 1080.
30. Billingham, R. E., Brent, L., and Medawar, P. B. Actively acquired tolerance of foreign cells. Nature (Lond.) 1953, **172**, 603.
31. Boyd, W. C. Fundamentals of Immunology, 3rd ed. New York, Interscience, 1956, p. 77.
32. Kabat, E. A. Blood Group Substances; Their Chemistry and Immunochemistry. New York, Academic Press, 1956.

33. Morgan, W. T. J., and Watkins, W. M. Some aspects of the biochemistry of the human blood-group substances. Brit. med. Bull. 1959, **15**, 109.
34. Alexander, W. R. M. The application of the fluorescent-antibody technique to haemagglutinating systems. Immunology 1958, **1**, 217.
35. Sheppard, P. M. Blood groups and natural selection. Brit. med. Bull. 1959, **15**, 134.
36. Hugh-Jones, K., Manfield, P. A., and Brewer, H. F. Congenital thrombocytopenic purpura. Arch. Dis. Childh. 1960, **35**, 146.
37. Masouredis, S.. P. Relationship between Rh_0 (D) genotype and quantity of I^{131} anti-Rh_0 (D) bound to red cells. J. clin. Invest. 1960, **39**, 1450.
38. Barnes, A. E. Failure of the Rh_0 (D) genotype to influence the quantity of erythrocyte Rh_0 (D) antigen (abstract). J. Lab. clin. Med. 1960, **56**, 789.
39. Hill, T. L. Immunoreactions involving platelets. II. Theoretical analysis of the model. J. exp. Med. 1958, **107**, 691.
40. Dameshek, W., and Schwartz, R. S. Leukemia and auto-immunization—Some possible relationships. Blood 1959, **14**, 1151.

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