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

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(Received for publication, December 23, 1957)

In Papers I and III of this series, quantitative relationships between antibody, quinidine, and platelets in various *in vitro* reactions were described. This provided a basis for evaluating *in vivo* relationships between these reactants when test doses of quinidine were given to patients with the antibody of quinidine purpura. Regulated test doses of quinidine were given to one patient at intervals during the period of decline of a high serum antibody concentration present after quinidine purpura, and to another patient after antibody was present following an anamnestic response to quinidine given as a diagnostic test for sensitivity.

By comparing the *in vivo* conditions which resulted in thrombocytopenia with the *in vitro* conditions necessary for various antibody reactions, the *in vivo* reactions which led to a decrease in circulating platelets could be deduced. Results of the present work appeared to be pertinent to other diseases of sensitivity. In light of these results, a detailed interpretation of the pathogenesis of idiopathic thrombocytopenic purpura (ITP),[1] which had been considered to be a disease of sensitivity, is presented.

Materials and Methods

All reagents and techniques used are described in Papers I and III of this series.

Case Reports

Case 1, E. T. S., a white female, age 74, was admitted to the United States Naval Hospital, Bethesda, Maryland, on March 8, 1955, for weakness associated with a rapid and irregular pulse. She had had similar episodes previously, the first about 6 years prior to admission, with recurrences at 3 to 6 month intervals. About 3 years prior to admission, she was advised to take quinidine at the onset of such attacks and had taken a single tablet of 0.2 gm. quini-

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[1] ITP, idiopathic thrombocytopenic purpura.

dine sulfate on about five occasions with prompt relief of symptoms. Between 3 and 7 p.m. on the day of admission she had taken two quinidine tablets, each containing 0.2 gm. quinidine sulfate, and was admitted at 10 p.m. because she had not obtained the customary relief with this medication. She had taken no quinidine for 4 to 6 months prior to the day of admission, and had never taken any other drug except for an occasional aspirin. She had never had any allergic disorder.

The chief physical findings on the evening of admission were moderate cardiac enlargement, irregularity of the pulse typical of auricular fibrillation, and a moderate tachycardia. The

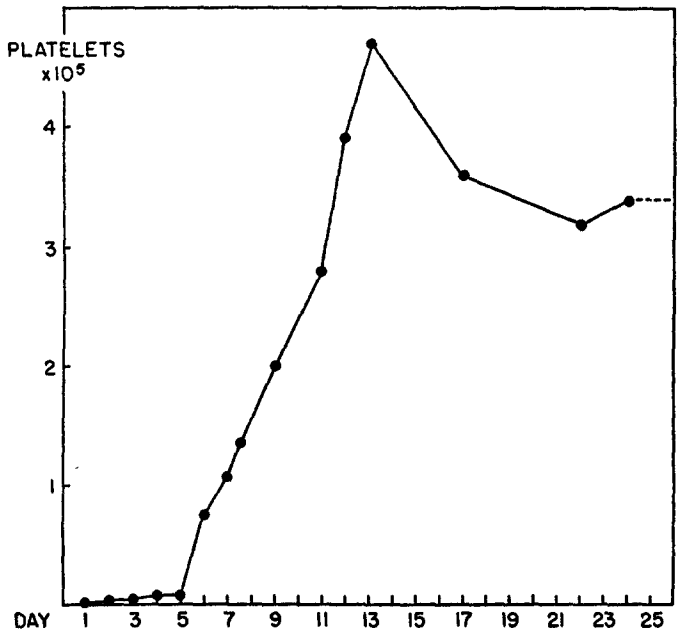

FIG. 1. Case 1, platelet counts following onset of quinidine purpura. Day 1 was the morning after admission when purpura was first noted, approximately 18 hours after therapeutic doses of quinidine. The platelet count in subsequent months of observation remained stable at approximately 3×10^5/mm.^{3}.

physical examination was otherwise normal. No abnormalities of the skin or mucous membranes were noted. An electrocardiogram showed auricular fibrillation. Digitalization with digitoxin was begun and nembutal was given as a sedative. No other drugs were given.

The following morning, March 9, the patient felt well, her pulse was regular at 78 per minute, and an electrocardiogram was normal except for digitalis effect. However, at this time, the patient had numerous petechial hemorrhages in the skin over the lower abdomen and on the extremities, increasing in number peripherally. There were also numerous hemorrhagic bullae on the mucous membranes of the mouth and pharynx and there was bleeding from the gums. The peripheral blood on the morning of March 9 contained 500 platelets/mm.^{3}, 8,350 white cells/mm.^{3} with a normal differential, and 12.7 gm. per cent of hemoglobin. The bleeding time (forearm) was greater than 20 minutes, the clotting time was normal, and there was no clot retraction in 1 hour. A diagnosis of quinidine purpura was established by the presence of marked platelet agglutination in mixtures of the patient's plasma, platelet-

rich plasma from normal individuals, and quinidine sulfate solution; and the absence of agglutination in mixtures in which saline was substituted for quinidine solution.

Purpura extended during the first 2 hospital days, but on the 3rd day there were no new petechiae or ecchymoses, and by the 4th day the hemorrhagic bullae in the mouth had disappeared and ecchymoses were fading in spite of the fact that the platelet count had not yet risen above 8,000/mm.^{3}. A bone marrow aspiration taken on the 4th day was normal with respect to the myeloid and erythroid series and megakaryocytes were normal in number and morphology.

The patient's white count and differential remained normal throughout. The total protein and albumin:globulin ratio were normal at the time of her first hospital admission and a serum electrophoretic pattern was normal 3 months later. A patch test (1) done 3 months after her first admission by placing a gauze pad soaked in a saturated aqueous solution of quinidine gluconate on the forearm showed no petechiae after 32 hours, and a capillary fragility test did not produce petechiae over the area of the patch. Fig. 1 shows the platelet counts obtained on the patient during and after quinidine purpura.

Case 2, R. A. K., a white female, age 62, was admitted to the Clinical Center, National Institutes of Health, Bethesda, Maryland, on August 20, 1956, for evaluation of digitalis therapy in controlling recurrent episodes of paroxysmal auricular tachycardia. She had taken quinidine for paroxysmal tachycardia intermittently over a period of several years up to 3 years prior to admission, at which time she had developed extensive ecchymoses and petechiae which cleared spontaneously over a period of several days after the drug was stopped. Quinidine sensitivity was suspected but not proven and she had not taken quinidine for 3 years. When admitted she had no detectable antibody in her serum by complement fixation, platelet agglutination, or inhibition of clot retraction tests.

Results of Tests in Vivo

Case 1.—

(*a*) *Antibody concentration during 2 year period of observation.* Fig. 2 shows the concentration of antibody in units/milliliter determined by complement fixation and the titer determined by platelet agglutination over a 2 year period beginning with the initial episode of quinidine purpura. Antibody concentration determined on serum obtained the morning purpura was first noticed was 50 units/ml., and it rose within a 3 week period to levels in the order of 300 units/ml. The subsequent decline of antibody concentration and the antibody response to test doses are charted.

(*b*) *Test doses of quinidine and quinine during the 7th month of observation.* The patient was readmitted to the hospital 7 months after quinidine purpura had occurred to determine the *in vivo* effects of quinidine. Antibody concentration in the patient's plasma at this time was 40 units/ml.

It was known that complement fixation, the most sensitive test of antibody activity, would occur minimally with this concentration of antibody when quinidine concentration was in the order of 10^{-6} M (see Paper I). It was considered that *in vivo* administration of quinidine in amounts sufficient to produce approximately this concentration of quinidine in the patient's plasma might provide a safe procedure for determining the effects of quinidine on the level of circulating platelets. If the *in vivo* concentration of administered quinidine were

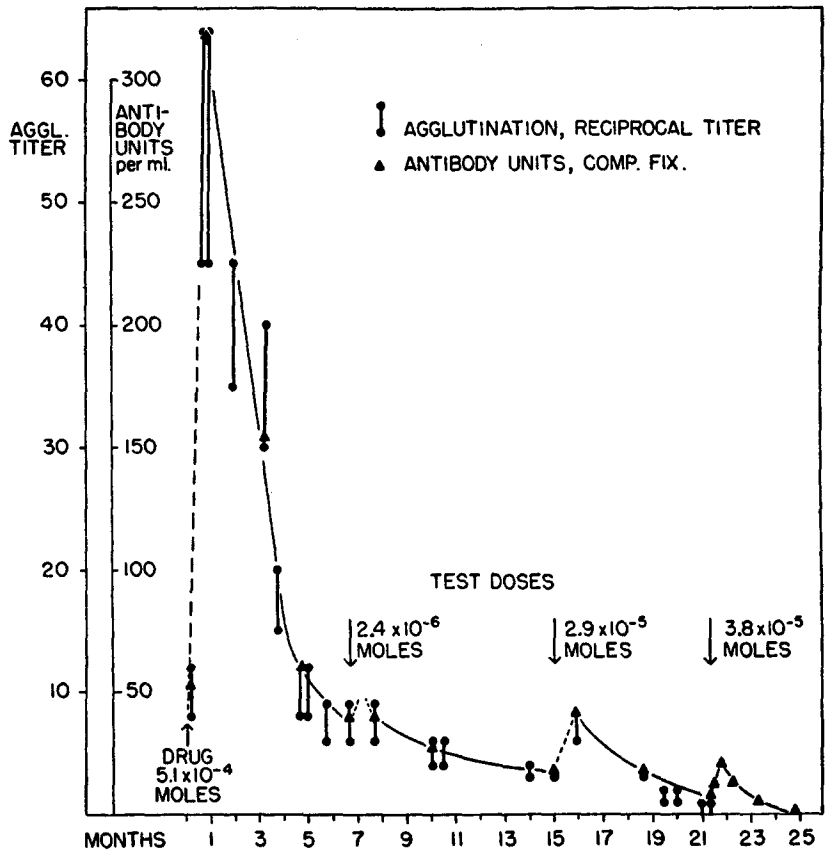

FIG. 2. Case 1, antibody concentration in patient's serum over a 2 year period. Antibody concentration in units/milliliter determined by complement fixation as described in Paper I. Agglutination titers show the range of the reciprocal of serum dilutions producing 1+ platelet agglutination in 1 hour in the standard agglutination mixture described in Paper III. The drug dose which initiated purpura was 5.1×10^{-4} moles of quinidine sulfate, $(C_{20}H_{24}O_2N_2)_2 \cdot H_2SO_4 \cdot 2H_2O$, or 10.2×10^{-4} moles of quinidine. First test dose during 7th month of observation, 2.4×10^{-6} moles of quinidine; second test dose beginning of 16th month, 1.2×10^{-5} moles of cinchonine + 1.7×10^{-5} moles of quinidine; third test dose, 22nd month, 3.8×10^{-5} moles of quinidine.

determined only by dilution in the patient's plasma volume (assumed to be 2 liters), approximately 1 mg. of quinidine gluconate would have to be given to produce a 10^{-6} M concentration.

Quinidine gluconate was given intravenously at a concentration of 35 γ/ml. in 5 per cent glucose in water ($10^{-4.18}$ M quinidine) and at a rate of 1.5 ml./minute (10^{-4} millimols/minute). After the patient had received 0.63 mg. of quinidine gluconate over a period of 12 minutes, the platelet count had fallen from the control value of 3.2×10^5/mm.^{3} to 1.75×10^5/mm.^{3}

(Fig. 3). The infusion was stopped after it had been running for a total of 24 minutes during which time 1.26 mg. of quinidine gluconate had been given. Platelets were at the lowest level, 0.77×10^5/mm.^{3}, when measured one-half hour after the infusion was stopped, and had returned to 2.0×10^5/mm.^{3} within the next 5 hours. One day later platelets were still lower than control values.

White counts done at the same time as the platelet counts showed no significant variation. The patient did not develop symptoms and a capillary

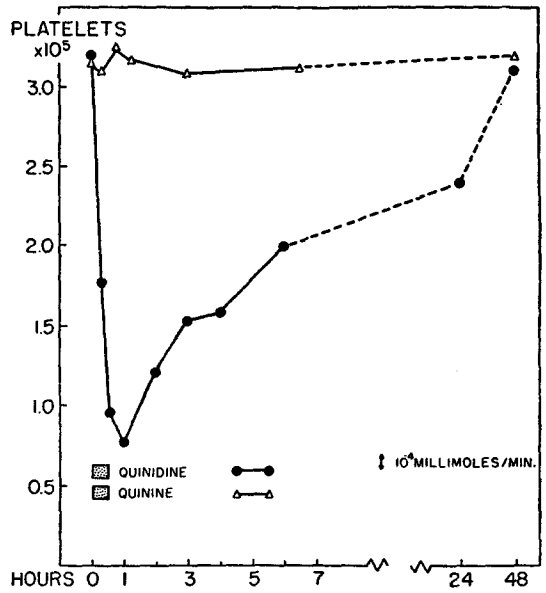

FIG. 3. Case 1, changes in platelet count during 7th month tests. See text. Platelet counts were taken from freely flowing blood from a finger puncture using 4 pipettes and 8 chambers for each count. Three individuals each counted several chambers, and the average of the results of all counts is plotted. Control values were obtained from two 8 chamber counts performed within 1 hour before the infusion was started. The rate of intravenous flow was regulated with a calibrated mechanical pump.

fragility test and bleeding time done when platelets were at the lowest level were normal. A clot retraction test done as in Paper III, but without adding quinidine, gave 22 per cent free fluid when platelets were at the lowest level, and 45 per cent free fluid 1 hour later. Platelets observed in the patient's plasma taken at the same time as samples for clot retraction appeared normal in morphology. They did not agglutinate in 16 hours without added quinidine but showed rapid agglutination on addition of an optimal concentration of quinidine (see Paper III). The concentration of antibody in serum samples taken at the same time as platelet counts on the day of the infusion did not vary significantly from the pre-infusion value of 40 units/ml.

Quinidine dihydrochloride given 2 days after the test dose of quinidine at the same molar concentration and rate of administration produced no effect on the platelet count.

(*c*) *Test doses of cinchonine, quinidine, and cinchonidine during the 16th month of observation.* When antibody concentration in the patient's serum had decreased to 18 units/ml., the effects of intravenous cinchona alkaloids were observed again (Fig. 4). Cinchonine, which was found to be as effective as qui-

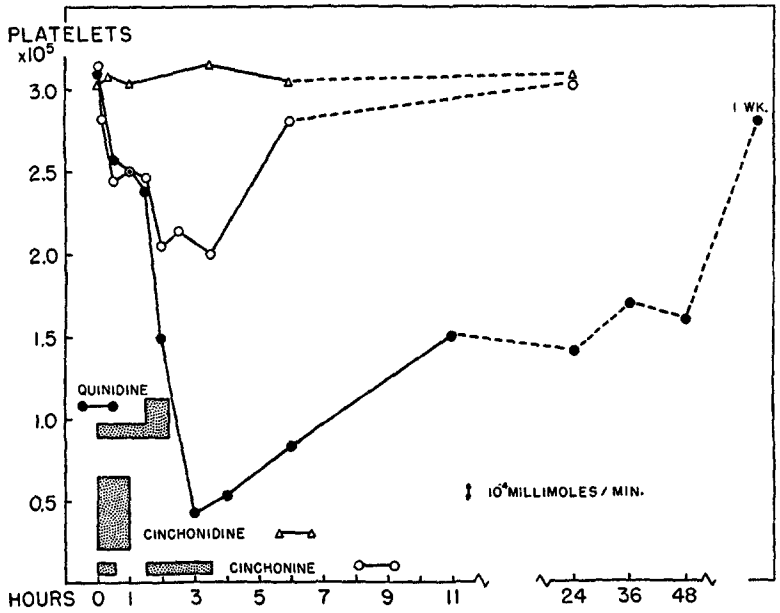

FIG. 4. Case 1, changes in platelet count during 16th month tests. See text and legend Fig. 3. The height of the stippled block is proportional to the rate of drug administration

nidine in *in vitro* tests (see Papers I and III) was given intravenously at a concentration of 35 γ of cinchonine sulfate/ml. in 5 per cent glucose in water ($10^{-4.31}$ M cinchonine sulfate, 10^{-4} M cinchonine) and at a rate of 0.8 ml./minute (0.8×10^{-4} millimols cinchonine/minute).

After the patient had received 0.84 mg. of cinchonine sulfate over a period of 30 minutes, the platelet count had fallen from a control value of 3.11×10^5/mm.^{3} to 2.44×10^5/mm.^{3}, a very significant difference with the method of platelet counting used (2). The infusion was stopped and the platelet count remained at approximately the same level for 1 hour. At this time the infusion was started again at the same rate of administration. Within 30 minutes after the infusion was restarted, the platelet count had fallen further to 2.0×10^5/mm.^{3}. It remained at approximately that level during the 2 hour period in which an additional 4 mg. of cinchonine sulfate was given. After the infusion was stopped, the platelet count returned to 2.8×10^5/mm.^{3} within 2½ hours.

There was no significant change in antibody concentration in serum samples taken at the same time as platelet counts and no significant variation in white cell count. The patient had no symptoms.

One day after cinchonine was given, the patient was given a total of 14.5 mg. of cinchonidine sulfate intravenously over a 1 hour period (6.5×10^{-4} millimols cinchonidine/minute) without effect on the platelet count.

Two days after cinchonine was given, at which time antibody concentration was still approximately 18 units/ml., quinidine gluconate at a concentration of 40 γ/ml. ($10^{-4.12}$ M) was given intravenously at a rate of 1.2 ml./minute (0.9×10^{-4} millimols/minute).

During continuous administration at this rate for 1½ hours, the platelet count decreased in approximately the same manner as it had when a similar amount of cinchonine was given. The rate of infusion was then increased approximately threefold to 2.5×10^{-4} millimols/minute, and within 30 minutes the platelets had fallen to 1.5×10^5/mm.^{3}. The infusion was given at the increased rate for a total of 37 minutes before being stopped, and 40 minutes after it was stopped, the platelet count was 0.43×10^5/mm.^{3}. Within 1½ hours after the infusion was stopped, platelets had begun to rise and continued to rise for the next 7 hours, but then remained stable for at least 3 days at a level between 1.4 and 1.7×10^5/mm.^{3} before returning to normal.

The patient had no symptoms, and a capillary fragility test and bleeding time done when the platelet count was at its lowest level were normal. Platelets observed in the patient's plasma taken at the same time as platelet counts had normal morphology and did not agglutinate when incubated for 16 hours without added quinidine but agglutinated in all samples on addition of quinidine at optimal concentration. The serum sample taken at the time the quinidine infusion was stopped, when the *in vivo* concentration of quinidine would have been highest, did not fix complement in the presence of an optimal concentration of platelets from a normal donor unless quinidine was added. The complement content of this serum sample did not differ significantly from that of a serum sample taken just prior to the test dose. Antibody concentration measured in all samples taken at the same time as platelet counts on the day of the test dose did not vary significantly from 18 units/ml. Two weeks after this series of tests antibody concentration in the patient's serum had risen to 42 units/ml.

(*d*) *Test dose of quinidine, 22nd month of observation.*—At this time antibody concentration in the patient's serum was 8 units/ml. *In vitro* tests showed complete inhibition of clot retraction on addition of an optimal concentration of quinidine to the patient's blood and 1+ agglutination when optimal quinidine was added to the patient's platelet-rich plasma or to mixtures of platelets resuspended in the patient's undiluted serum (see Paper III). No platelet agglutination occurred within 1 hour in agglutination mixtures in which the patient's serum was diluted as much as twofold.

Quinidine gluconate at a concentration of 35 γ/ml. was given at a rate of 1.6 ml./minute (1.1×10^{-4} millimols/minute) continuously for a period of 3½ hours except for the 12 minute interruption indicated in Fig. 5. The platelet count fell within 30 minutes from the control value of 2.8×10^5/mm.^{3} to approximately 2.25×10^5/mm.^{3} at which point it remained stable.

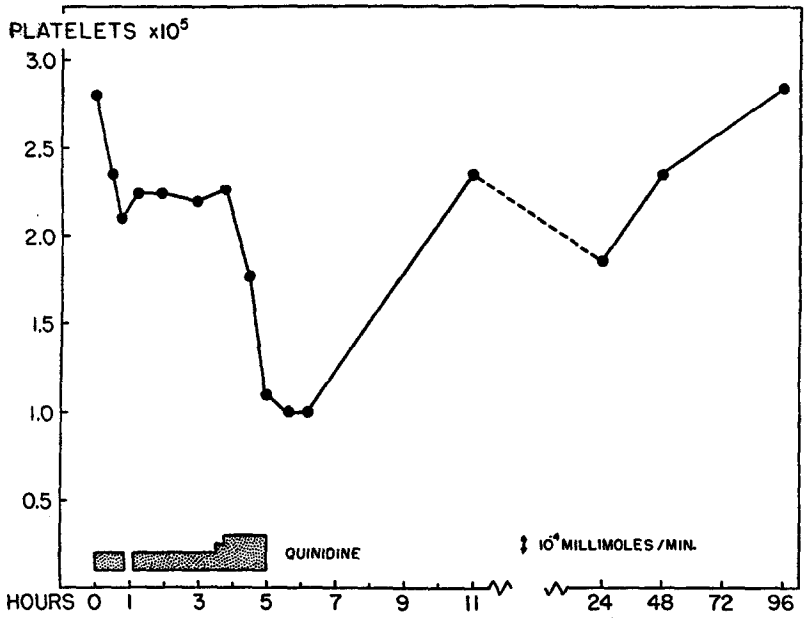

FIG. 5. Case 1, changes in platelet count during 22nd month test. See text and legend, Fig. 3. After the infusion had been running for 50 minutes, it was stopped for a 12 minute period because it appeared, from cursory inspection of a freshly flooded counting chamber, that the platelet count was going to be much lower than it proved to be after platelets had settled.

The rate of infusion of quinidine was then increased to 2.4 ml./minute for 15 minutes, and to 3.2 ml./minute (2.2×10^{-4} millimols/minute) for 70 minutes. With the increased rate of infusion the platelet count decreased further to approximately 10^5/mm.^{3} and remained at that level for approximately 1 hour after the infusion was stopped. There was a biphasic return of platelets to the control level over a period of 3 to 4 days.

Clot retraction tests without added quinidine gave a value of 59 ± 4 per cent free fluid on 4 pre-infusion samples, 49 ± 7 per cent on 6 samples taken in the 2½ to 3½ hour interval during the infusion, 31 ± 3 per cent on 3 samples taken at the time the infusion was stopped, and 36 ± 3 per cent on samples taken 1 hour later. The concentration of antibody in sera obtained at the same time as the platelet counts on the day quinidine was given did not vary significantly from 8 units/ml. Quinidine content in the serum sample taken at the time the

infusion was stopped was insufficient to cause complement fixation in the presence of an optimal concentration of added platelets.

Case 2.—

(*a*) *First test dose of quinidine.* The patient had no detectable antibody activity in her serum at the time of the first test dose. Quinidine gluconate at a concentration of 1.4 mg./ml. in 5 per cent glucose in water was given intravenously at a rate of 2.0 ml./minute (53.4×10^{-4} millimols/minute) for 78 minutes, the total amount received being 218 mg. The plasma quinidine concentration had become sufficiently high to produce a 0.06 second prolongation

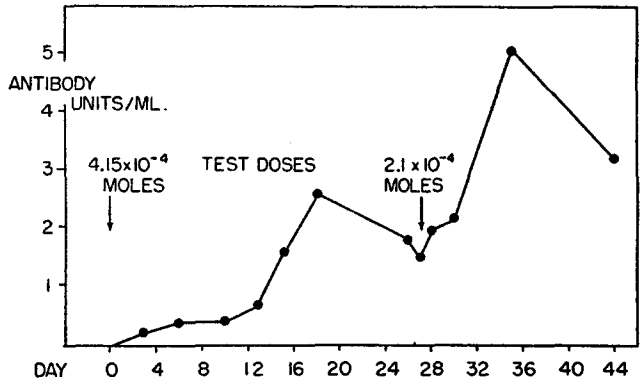

FIG. 6. Case 2, antibody concentration in patient's serum following test doses of quinidine. See text. Patient had taken no quinidine for 3 years prior to the first test dose.

of the *Q-T* interval of the electrocardiogram by the time the infusion was stopped. The patient had no symptoms and there was no signficant variation in platelet count during or after the infusion.

There was no detectable antibody in the patient's serum for 2 days after the infusion, but by the 3rd day antibody was measurable at a concentration of 0.2 units/ml. (see Fig. 6). Antibody concentration continued to rise over the subsequent 2 weeks and by the 18th postinfusion day was 2.6 units/ml. Prior to the 18th postinfusion day inhibition of clot retraction did not occur when an optimal concentration of quinidine was added to the patient's whole blood; and agglutination of platelets did not take place in the patient's platelet-rich plasma over a period of 16 hours in the presence of an optimal quinidine concentration. The same tests done on the 18th day gave partial inhibition of clot retraction (26 per cent free fluid); and ± to 1+ platelet agglutination in the patient's platelet-rich plasma after an incubation period of 16 hours, but no agglutination within 1 hour.

(*b*) *Second test dose of quinidine.* Twenty-seven days after the first test dose

(see Fig. 6), when antibody concentration was 1.5 units/ml. and platelet agglutination and inhibition of clot retraction could no longer be detected by *in vitro* tests, quinidine gluconate at a concentration of 0.48 mg./ml. was given intravenously at an initial rate of 0.7 ml./minute (6.5×10^{-4} millimols/minute), the rate being increased in a stepwise fashion shown in Fig. 7.

Within the 1st hour, during which time the patient received 40 mg. of quinidine gluconate, the platelet count fell from a control value of 2.9×10^5/mm.^{3} to 1.95×10^5/mm.^{3} and remained at approximately that same level in spite of the fact that an additional 70 mg. o

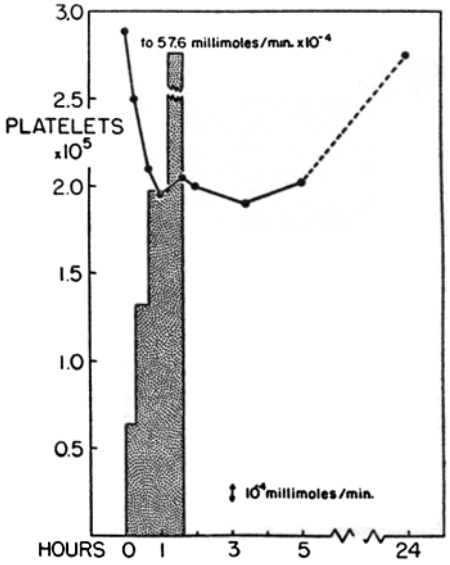

FIG. 7. Case 2, changes in platelet count during second test dose of quinidine. See text and legend, Fig. 3.

quinidine gluconate was given in the next 40 minutes. The platelet count was still approximately 2.0×10^5/mm.^{3} 3½ hours after the infusion was stopped, and the following morning it was at a normal level. The patient did not have any symptoms and the maximum prolongation of the *Q-T* interval of the electrocardiogram was 0.02 second at the time the infusion was stopped.

Eight days after the second test dose, antibody concentration in the patient's serum was 5 units/ml. At this time complete inhibition of clot retraction (6.7 per cent free fluid) occurred when quinidine was added to her whole blood and 1+ platelet agglutination occurred within 1 hour in the patient's platelet-rich plasma with added quinidine. Within 17 days after the second test dose antibody concentration had begun to decline. There was only partial inhibition of clot retraction (23 per cent free fluid) when quinidine was added to her whole

blood, and platelet agglutination was not evident within 1 hour in the patient's platelet-rich plasma with added quinidine.

Measurement of in vivo Quinidine Concentration.—

The lowest rate of quinidine administration used to produce thrombocytopenia in Figs. 3 to 5 was approximately 10^{-4} millimols/minute. The plasma quinidine concentrations produced at this dose rate are shown in Table I.

TABLE I

Infusions of quinidine gluconate at a concentration of 35 γ/ml. in 5 per cent dextrose in water were given to two normal subjects at the dose rates indicated in the table. In each case the infusion was stopped after 30 minutes. The average value of duplicate determinations of plasma quinidine concentration done at each time interval are recorded. The variation was no greater than ±8 per cent of the average.

The method of measuring quinidine was described by Udenfriend *et al.* (3). The drug was extracted from 7 ml. of alkalinized platelet-rich plasma with 15 ml. of benzene. Fluorescent impurities in reagent grade benzene had been removed by adsorption on silica gel columns and by washing with sulfuric acid. A 10 ml. aliquot of the benzene extract was then extracted with 1.5 ml. of 0.1 N H_2SO_4, and the concentration of quinidine in the acid extract was determined using an Aminco-Bowman spectrophotofluorometer at an activation wavelength of 340 mμ and a fluorescence wavelength of 440 mμ. Recovery of quinidine gluconate from plasma and whole blood was 87 ± 10 per cent when the amounts added *in vitro* produced plasma concentrations ranging between 0.005 to 0.14 γ/ml. (9.6×10^{-9} to 2.7×10^{-8} M).

Min. after infusion started	Plasma quinidine concentration	
	Dose rate: 0.92×10^{-4} millimols/min.	Dose rate: 1.12×10^{-4} millimols/min.
5	3.44×10^{-9} M	
10	9.77×10^{-9} M	2.18×10^{-8} M
20	2.48×10^{-8} M	2.54×10^{-8} M
30	2.62×10^{-8} M	3.33×10^{-8} M
40		2.74×10^{-8} M
50	9.20×10^{-9} M	

DISCUSSION

1. Factors of Significance in the Development of Thrombocytopenia Based on Observations of Quinidine Purpura.—Observations made on the patient who developed quinidine purpura suggested that capillary abnormalities which lead to hemorrhagic manifestation are a consequence of thrombocytopenia, and that thrombocytopenia may exist for a period of time before abnormal capillary permeability develops.

In view of the high concentration of antibody in the serum of the patient the morning after admission (50 units/ml.) and the large amount of quinidine (200 mg. quinidine sulfate) which the patient had taken approximately 7 hours and again 3

hours prior to admission, marked thrombocytopenia had no doubt been present the evening of admission in spite of the fact that purpuric manifestations were not yet evident at that time. Antibody concentration in the patient's plasma at the time quinidine was taken therapeutically must have been approximately the same as that measured in serum taken the following morning because an induction period of 2 to 3 days was observed before antibody concentration rose following all subsequent test doses. Even if the concentration of antibody had been as low as 8 units/ml. at the time quinidine was taken therapeutically profound thrombocytopenia would have developed within an hour after taking quinidine in view of the rapidity of quinidine absorption (4) and the rapidity of development of thrombocytopenia following test doses of quinidine (*e.g.*, Fig. 5).

It would appear, therefore, that thrombocytopenia preceded the development of purpura by a number of hours. In other reported cases of purpura following therapeutic or test doses of quinidine it appears that thrombocytopenia preceded hemorrhagic manifestations by a similar time interval (5). Furthermore, profound effects on the level of circulating platelets were observed in the present series of tests without effects on capillaries as judged by the tourniquet test, bleeding time, and occurrence of spontaneous hemorrhage. In these instances thrombocytopenia was apparently insufficient or too transient to result in capillary damage. It does not seem necessary to consider that antibody damages blood vessels or endothelium by a direct reaction, and attempts to demonstrate such a reaction *in vitro* have been unsuccessful (see Paper III).

A question remains as to whether megakaryocytes as well as platelets are affected by the antibody. In the patient with quinidine purpura there were practically no platelets in the circulation for 4 days before a gradual rise in platelets occurred (Fig. 1); and in view of the rate at which quinidine is excreted (4), the stimulus for thrombocytopenia had no doubt persisted for the entire 1st day. Hemorrhagic manifestations were clearing at least 2 days before there was a significant rise in platelets, and it may be considered that platelets were being utilized for hemostasis during this period. Craddock *et al.* (6) observed a 3 to 4 day period of delay before circulating platelets increased following production of thrombocytopenia in dogs by a technique involving mechanical removal of platelets extracorporeally. They interpreted the delay as reflecting the period of time required for platelets to develop from megakaryocytes after all available platelets had been released from mature megakaryocytes and other platelet stores. In view of the observations of Craddock *et al.*, the observed delay in return of platelets in this patient would be expected even if megakaryocytes had been functioning at maximum capacity during the course of the disease. Bone marrow samples taken in this case and a number of other reported cases (review by Bolton and Dameshek (7)) at the height of drug purpura or shortly thereafter have contained at least a normal number, if not an

increased number, of megakaryocytes; and any morphologic changes observed in megakaryocytes have been consistent with immaturity of hyperplastic cells described by Craddock *et al.* Moreover, it has been observed *in vitro* that antibodies which affect platelets do not necessarily affect megakaryocytes (8). It therefore appears that megakaryocytes were not destroyed and that their function was not impaired during drug purpura. Any changes in the level of circulating platelets produced by the antibody in the presence of the drug may be interpreted in this light.

2. Factors of Significance in the Development of Thrombocytopenia Based on Observations during Test Doses.—The amount of quinidine administered during the first test dose in Case 1 was chosen to assure that the plasma concentration would not exceed 10^{-6} M, the concentration at which minimal complement fixation occurred *in vitro*. After 0.63 mg. of quinidine gluconate had been given over a period of 12 minutes, a decrease of 1.45×10^5 platelets/mm.^{3} was observed (Fig. 3), but it is evident that platelets had begun to decrease before this time. The concentration of quinidine present in plasma at the time platelets first began to decrease in the circulation was less than 10^{-8} M (see Table I) and did not reach levels higher than 3.3×10^{-8} M during the entire test. Thus the *in vivo* concentration of quinidine which caused thrombocytopenia was much too low to cause complement fixation or platelet agglutination. Blood samples taken during three of the tests at the time of marked thrombocytopenia when *in vivo* quinidine concentration was highest contained insufficient quinidine to produce measurable *in vitro* complement fixation, platelet agglutination, or inhibition of clot retraction (other than that consistent with the degree of thrombocytopenia). This is further evidence that decreases in circulating platelets occur *in vivo* when quinidine concentration is too low to cause the various *in vitro* effects which have been considered to be of importance in the pathogenesis of thrombocytopenia.

The observation that no significant decrease in antibody concentration occurred in the patient's plasma during the tests suggested that only minimum amounts of antibody were attached to platelets at the time thrombocytopenia occurred. Considering the amount of antibody which platelets adsorb even when quinidine concentration is too low to cause complement fixation (Paper I, Fig. 6), and the number of platelets available for reaction with antibody during the tests, if sufficient antibody had been adsorbed to cause complement fixation during these tests, a decrease in antibody concentration would have been measurable. It was unlikely that constant antibody concentration was the result of continual release of antibody from sequestrated or destroyed platelets, for it was shown in Paper I that platelet fractions adsorb antibody as well as intact platelets. The amounts of antibody attached to platelets when thrombocytopenia occurred were evidently less than the measurable amounts attached to platelets *in vitro* even at quinidine concentrations too low to cause complement

fixation. The fact that platelets adsorbed very little antibody does not imply that platelets were not affected by the minimal amount of antibody which must have attached *in vivo*. Although not measurable, the amount of adsorbed antibody nevertheless could have been consistent with the presence of many antibody molecules per platelet; and it has been shown that the attachment of as few as 60 antibody molecules per cell may alter properties of cell membranes sufficiently to cause reactions such as agglutination (9). The development of thrombocytopenia was no doubt due to alteration of platelets by attachment of antibody, but the mechanism of removal of platelets from the circulation may only be surmised. It is possible that changes in the surface properties of platelets produced by attachment of antibody may have made platelets more susceptible to sequestration by physiologic surface phenomena such as phagocytosis (10) in capillary beds or sinusoids of such organs as the spleen, liver, and lungs.

The initial *in vivo* decrease in platelets during other tests in Case 1 (Figs. 4 and 5) occurred when quinidine was given at approximately the same rate and for a similar period of time as during the first test, but decreases in platelets were less when plasma antibody concentrations were lower. A marked fall in platelets resembling that which occurred when antibody concentration was 40 units/ml. (Fig. 3), occurred in the presence of 18 (Fig. 4) and 8 (Fig. 5) units of antibody/ml. only when the rate of administration of quinidine was increased two- or threefold. The test dose given to Case 2, when antibody concentration was 1.5 units/ml., produced an initial decrease in platelets only when a comparatively high rate of quinidine administration was used, but then produced no further decrease in platelets even when quinidine was administered at greatly increased rates. In view of the electrocardiographic changes at the end of this test, the maximum plasma quinidine concentration attained was in the order of 10^{-6} M (4). Thus there was an inverse relationship between antibody concentration and the amount of quinidine necessary to produce thrombocytopenia. At concentrations of antibody as low as 8 units/ml. complete removal of circulating platelets appeared to be possible if sufficient quinidine were given; but when antibody concentration was quite low (1.5 units/ml.), the degree of decrease in platelets which could be produced was limited by antibody concentration rather than quinidine concentration.

At appropriate rates of administration of quinidine the platelet count fell but then became stable at a lower level in the face of continued administration of quinidine at the same rate (Figs. 4, 5, and 7). The level of circulating platelets during test doses therefore appeared to reflect a balance between the rate of platelet removal and supply. When antibody and quinidine were present in sufficient concentration, the rate of removal of platelets could completely overcome the rate of supply (Figs. 4 and 5). The rate of return of platelets to the circulation appeared to be related not only to the severity of the thrombocytopenia but also to its duration (compare Figs. 3 to 5). After slight thrombocytopenia there

was a comparatively rapid return to normal levels; *e.g.*, Fig. 4 (cinchonine curve) and Fig. 7. Part of the delay in return of platelets in Fig. 7 was no doubt related to persistence of a significant concentration of quinidine after a comparatively high dose. Following more marked or more prolonged thrombocytopenia, there was a prolonged and biphasic recovery of platelets as seen in Fig. 3, the quinidine curve of Fig. 4, and Fig. 5. Biphasic recovery consisted of an initial rapid increase in platelets within several hours followed by a period of stabilization at a level lower than normal for 1 to 2 days before a return to pre-infusion values. It is probable that platelets reappearing in the immediate postinfusion period were supplied mainly from pre-existing stores in spleen and lung and from megakaryocytes still capable of releasing platelets, as was suggested by Craddock (6). There may also have been a contribution by platelets released from sequestration after detachment of antibody when quinidine concentration declined. The second phase of recovery may have been due to the period required for maturation of hyperplastic megakaryocytes after the ability of mature megakaryocytes to release platelets and platelet stores were exhausted (6). The extent of delay before platelets returned to normal probably reflects the over-all number of platelets destroyed as a result of sequestration during test doses. Further observations of effects of test doses will be necessary before more precise interpretations can be made.

3. A Suggested Method of Performing Test Doses in Patients with the Antibody of Drug Purpura.—

Because observations of responses to test doses will be made for their diagnostic as well as their experimental value, the experience gained in performing the current series of tests requires further comment. When antibody concentration was quite low (in the order of 1.5 units/ml.), no platelet agglutination or inhibition of clot retraction was detected *in vitro*, and an amount of quinidine equivalent to a therapeutic dose produced only a moderate thrombocytopenia of no clinical significance. When antibody concentration was sufficiently high to produce detectable platelet agglutination and inhibition of clot retraction, severe thrombocytopenia could be caused by much lower doses of quinidine.

If the concentration of antibody were known, the amount of quinidine necessary to produce a desired degree of thrombocytopenia could be chosen with reference to the tests described above. If antibody concentration were not known, 10^{-4} M quinidine given at a rate of 1 ml./minute would be a safe initial test dose. If there were no evidence of thrombocytopenia developing within 10 to 15 minutes, the amount of quinidine given could be doubled each 15 minute period until as much as 3×10^{-3} millimols/minute were given. Failure of platelets to decrease at this rate of administration of quinidine would be evidence that no clinically significant amount of antibody was present. A single negative test would not rule out the existence of sensitivity (see Fig. 6). A negative test should be followed by at least one repeated test 2 to 3 weeks later. In order to assure an adequate anamnestic antibody response within 2 to 3 weeks an additional oral dose of 200 mg. quinidine sulfate could be

given to the patient with impunity several hours after the initial negative intravenous test; for the degree of antibody response appeared to be related to the amount of quinidine given.

Performance of test doses with intravenous quinidine as described appears to be a safe procedure. Even if marked thrombocytopenia is produced with low doses of quinidine when antibody concentration is high, the quinidine is rapidly dissipated *in vivo*, and thrombocytopenia is so transient that significant capillary fragility does not develop. The suggested technique for performing *in vivo* tests is meant only as a guide, for responses of different individuals may not be the same. Quantitative relationships between *in vitro* and *in vivo* reactions may be similar in the case of other drugs causing thrombocytopenia.

4. Implications of Observation on Drug Purpura in ITP.—

The syndrome of ITP is thoroughly described and current interpretations of its pathogenesis presented in references 11–13. ITP is characterized by a variable clinical course with spontaneous remissions and relapses, an inconstant response to splenectomy or the administration of adrenosteroids and adrenocorticotropic hormone (ACTH), and discrepancies between degree of hemorrhagic manifestations and the level of circulating platelets and between the severity of the disease and the presence of an *in vitro* platelet agglutinin or an *in vivo* thrombocytopenic factor. Hypotheses concerning the role of megakaryocytes, platelets, capillaries, spleen, antibodies, hormones, and their interactions in the pathogenesis of ITP have included the following possibilities: (*a*) direct destruction of platelets by a "lytic" antibody, (*b*) removal of "sensitized" platelets by the spleen, (*c*) destruction of megakaryocytes by antibody, (*d*) inhibition of megakaryocyte maturation by a splenic hormone, (*e*) production of antiplatelet antibodies by the spleen, (*f*) production of a capillary-fragility factor by the spleen, (*g*) independent effects on capillaries and platelets by antibodies or hormones, and (*h*) combination of several of these possibilities.

If it is assumed that in ITP an antibody attaches to platelets and leads to thrombocytopenia in a manner similar to that described in the present work, then a more detailed interpretation of the pathogenesis of ITP and its puzzling attributes is possible.

Antigenic stimuli in ITP could be simple molecules (haptenes) which are ingested, formed metabolically, or produced by infectious agents, in which case the antibody-haptene-platelet complex formation might be similar to that in drug purpura (see Paper I). It would be possible, however, for the antigen to be a protein molecule without an associated haptene, the antigen being adsorbed by platelets in combination with the antibody it provokes. This possibility is suggested by the observations of Bounameaux and Lecomte (14) concerning the agglutination of platelets by nonspecific antigen-antibody complexes. Variations in degree and persistence of antibody response or in relative concentration of haptene (or other types of antigen) could account for the variable picture of ITP just as the variable responses to test doses were related to relative antibody and haptene concentrations. In chronic ITP effective antigenic stimulus would be persistent and in acute ITP, transient. Antigen

and antibody complexes with platelets would no doubt differ in individual cases; and it would be impossible to predict in each case whether amounts of adsorbed antibody, although sufficient to produce thrombocytopenia, would necessarily cause platelet agglutination, complement fixation, or inhibition of clot retraction *in vitro*; or for that matter whether measurable *in vitro* effects would necessarily be associated with thrombocytopenia.

If in ITP the mechanism of production of thrombocytopenia and capillary permeability were the same as it appears to be in the case of quinidine purpura, clinical manifestations of ITP could be interpreted solely in terms of factors which affect the rate of sequestration of platelets and the rate of production of platelets by megakaryocytes. Beneficial effects following splenectomy would be due to removal of a very active site of sequestration and would not affect the underlying basis of the disease, just as splenectomy benefits hemolytic anemia due to hereditary spherocytosis. If no improvement followed splenectomy, it may be considered that the spleen was not the only important site of sequestration. It is possible that the susceptibility of platelets to sequestration in organs other than the spleen may be related to the degree of platelet abnormality which in turn would be dependent on the amount or type of antibody adsorbed on platelets. This interpretation would account for exacerbations of ITP after an initial good response to splenectomy and would weaken the rationale for the usually disappointing search for accessory spleens in recurrences.

Following treatment with steroid hormones or ACTH or during spontaneous remissions in ITP, disappearance of hemorrhagic manifestations frequently antedates elevation of circulating platelets by several days to a week. This has been interpreted as indicating that the disease affects platelets and capillaries separately. However, complete amelioration of symptoms without some subsequent rise in platelets does not occur frequently and is not so well documented. Furthermore, it appears that increased production of platelets following any thrombocytopenic state may be evidenced first by cessation of hemorrhage and only later by a rise in circulating platelets (*e.g.*, Fig. 1). The lag in rise of circulating platelets may be due in part to increased utilization of platelets for hemostasis after a hemorrhagic diathesis has existed. If this were the case, benefit from platelet transfusions might be expected without rise in the level of circulating platelets, and we have occasionally observed such responses. Since ACTH or steroid hormones are capable of increasing the production of other blood cells in some disease states as well as in normal individuals (11), beneficial results of hormone therapy in ITP may be due simply to increased platelet production. Increased platelet production may be manifested only by cessation of hemorrhage if it is just sufficient to match requirements for both hemostasis and continued abnormal sequestration, or manifested by a concomitant rise in platelets if increased production is sufficient to overcome the rates of utilization and sequestration. If it is considered that hormone therapy is only a moderate stimulus for platelet production then significant rises in circulating platelets following such treatment may indicate that the susceptibility of platelets to sequestration was only slightly increased, in which case successful results following splenectomy might be more likely. Direct effects on the antigen-antibody system resulting in decreased antibody formation (15) or in interference with cell-antibody union (16) may also be considered in accounting for benefits of hormone therapy. The assump-

tion that a direct effect on capillaries alone accounts for cessation of hemorrhage in the face of persistent thrombocytopenia does not appear to be necessary.

It has frequently been considered that the morphologic changes in megakaryocytes observed in association with ITP are indicative of cellular damage. However, these morphologic changes are the same as those observed by Craddock *et al.* when megakaryocytes were hyperplastic (6); and the same as those seen in drug purpura, a disease which does not appear to be associated with megakaryocyte damage (see above discussion). These considerations, in addition to the observation that a striking rise in platelets frequently occurs immediately after splenectomy, support the conclusion that damage to megakaryocytes is not an integral part of ITP.

The frequency with which initial episodes of ITP follow acute upper respiratory infections and with which exacerbations of the disease follow intercurrent infections has implied that sensitization is related in some way to infectious agents. It is possible, however, that incidental acute infections lead to temporary suppression of bone marrow function and a thrombocytopenic crisis similar to the hemolytic crisis of hereditary spherocytosis (17). This possibility is supported by the occurrence of exacerbations of ITP after trauma as well as after infections.

Platelets altered sufficiently by attachment of antibody to be sequestered abnormally might still function effectively in hemostasis during their period of circulation. The possibility exists, however, that attachment of special antibodies to platelets may affect their hemostatic function primarily and have relatively little effect on their susceptibility to sequestration. This may occur in certain diseases in the categories of "thrombocytopathic purpuras" and "pseudohemophilia" (11) in which capillary abnormalities usually associated with thrombocytopenia can occur in the presence of a normal or only slightly reduced platelet level.

These hypotheses offer a unitary concept for the pathogenesis of a group of diseases characterized by thrombocytopenia and capillary dysfunction, and lend themselves readily to further experimental evaluation both *in vivo* and *in vitro*. Similar mechanisms may play a role in diseases of sensitivity involving other types of blood cells.

SUMMARY

Regulated intravenous doses of quinidine were given to patients with the antibody of quinidine purpura to produce controlled thrombocytopenia without clinical sequelae. The degree of thrombocytopenia and the rate at which it developed were dependent on the relative plasma concentration of quinidine and antibody. By relating *in vivo* changes in platelet levels to concurrent *in vitro* tests for antibody activity and to quantitative relationships between reactants determined in Papers I and III of this series, it was concluded that the amount of antibody which attaches to platelets when thrombocytopenia develops is insufficient to cause complement fixation or platelet agglutination. Platelets do not appear to be destroyed directly by reaction with antibody *in vivo*. The minimal amount of antibody which does attach to platelets *in vivo* appears to increase their susceptibility to the usual mechanisms of sequestration.

Megakaryocytes and blood vessels do not appear to be affected directly by the antibody which causes quinidine purpura, and hemorrhagic manifestations of the disease appear to be consequent to changes in platelets alone.

A safe method of performing *in vivo* tests for the presence of an antibody of drug purpura is described.

The implications of the present work in idiopathic thrombocytopenic purpura are discussed.

I wish to express my appreciation to William G. Clutter, Hospital Corpsman First Class, United States Navy, Jefferson W. Hamby, Hospital Corpsman First Class, United States Navy, and Miss Mildred L. Garrison for their assistance in this work, and to Dr. Gerald I. Plitman and Dr. John D. Davidson for referring the patients who were studied.

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