

Sera From Patients With Heparin-Induced Thrombocytopenia Generate Platelet-Derived Microparticles With Procoagulant Activity: An Explanation for the Thrombotic Complications of Heparin-Induced Thrombocytopenia

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Heparin-induced thrombocytopenia is characterized by moderate thrombocytopenia and thrombotic complications, whereas quinine/quinidine-induced thrombocytopenia usually presents with severe thrombocytopenia and bleeding. Using flow cytometry and assays of procoagulant activity, we investigated whether sera from patients with these immune drug reactions could stimulate normal platelets to generate platelet-derived microparticles with procoagulant activity. Sera or purified IgG from patients with heparin-induced thrombocytopenia stimulated the formation of platelet-derived microparticles in a heparin-dependent fashion. Further studies showed that heparin-induced thrombocytopenia sera also produced a marked increase in procoagulant activity. In contrast, sera from patients with quinine- or quinidine-induced thrombocytopenia did not

generate platelet-derived microparticles nor generate increased procoagulant activity. However, quinine/quinidine-induced thrombocytopenia sera produced a significant increase in the binding of IgG to platelets in a drug-dependent fashion, whereas sera from patients with heparin-induced thrombocytopenia demonstrated no drug-dependent binding of IgG to platelets. We also observed increased levels of circulating microparticles in patients with acute heparin-induced thrombocytopenia compared with control patients. Our observations indicate that the generation of procoagulant platelet-derived microparticles in vivo is a plausible explanation for the thrombotic complications observed in some patients with heparin-induced thrombocytopenia.
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MANY DRUGS CAN cause idiosyncratic (immune-mediated) thrombocytopenia, including heparin, quinidine and quinine, penicillin, and sulfa antibiotics, among others.^{1,2} However, heparin-induced thrombocytopenia is unique for several reasons.³⁻⁵ First, heparin-induced thrombocytopenia is a frequent cause of drug-induced thrombocytopenia, occurring in approximately 1% to 3% of patients receiving therapeutic doses of heparin.³ Second, the severity of thrombocytopenia is usually moderate, with typical platelet counts ranging from 20 to 150 × 10⁹/L.³ Third, many patients with heparin-induced thrombocytopenia develop unexpected thrombotic complications, including arterial thrombosis, venous thrombosis, and disseminated intravascular coagulation.³⁻⁵ The clinical presentation of heparin-induced thrombocytopenia differs markedly from other drug-induced thrombocytopenic disorders. For example, the thrombocytopenia caused by quinine or quinidine is usually severe (typical platelet count nadir is less than 10 × 10⁹/L), and bleeding, rather than thrombosis, is observed.^{2,6} Although both heparin- and quinine/quinidine-induced thrombocytopenia are known to be caused by drug-dependent IgG binding to the platelet,^{1-4,7,8} there is a fundamental difference in clinical manifestations, which remains unexplained.

In this report, we describe studies investigating the basis for the clinical differences between heparin- and quinine/quinidine-induced thrombocytopenia. We found that sera or purified IgG from patients with heparin-induced thrombocytopenia generated platelet-derived microparticles that have procoagulant activity. In contrast, sera from patients with quinine- and quinidine-induced thrombocytopenia did not produce platelet-derived microparticles. Platelet microparticles were also observed in plasma collected from patients with acute heparin-induced thrombocytopenia. We suggest that the generation of the procoagulant activity contributes to the thrombotic complications of heparin-induced thrombocytopenia.

MATERIALS AND METHODS

Patient samples. Studies were performed using sera from patients with suspected drug-induced thrombocytopenia. Patients with

heparin-induced thrombocytopenia met the clinical criteria for diagnosis as described previously.⁹ In all patients with heparin-induced thrombocytopenia, the diagnosis was confirmed using the ¹⁴C-serotonin platelet release assay.^{9,10}

Patients were considered to have quinine- or quinidine-induced thrombocytopenia if they: (1) developed thrombocytopenia following ingestion of the particular drug; (2) the thrombocytopenia resolved following discontinuation of the drug; and (3) in vitro testing confirmed drug-dependent binding of IgG to platelets in the presence of patient serum.

Control sera were obtained from patients who tested negative for heparin-induced thrombocytopenia using the ¹⁴C-serotonin release assay, and from normal volunteers. Patient and control samples were collected according to university-approved Ethics Review Committee recommendations.

Materials. The platelet-specific monoclonal antibody used in these studies was anti-GPIIb (TW-1).¹¹ A monoclonal antibody (CAG-1) that reacts with all subclasses of IgG was used for measure-

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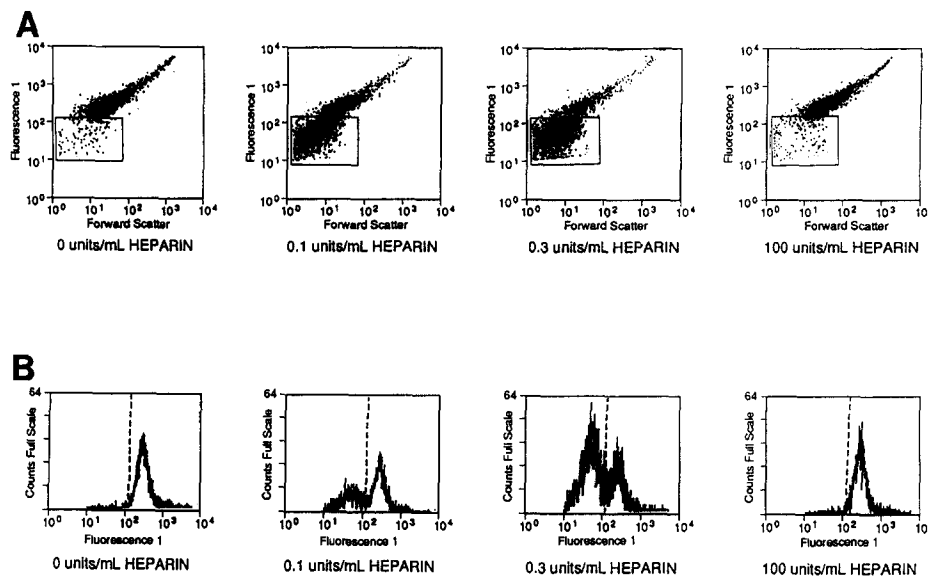


Fig 1. Platelet-derived microparticles generated by heparin-induced thrombocytopenia serum. (A) shows platelets and microparticles identified using fluorescence (FL1, FITC-anti-GPIb_α) and size (FSC, forward scatter) characteristics. The platelets had been incubated with heparin-induced thrombocytopenia serum in the presence of 0, 0.1, 0.3, or 100 U/mL heparin. The microparticles (insert box) were generated in the presence of 0.1 and 0.3 U/mL heparin, but not at 0 or 100 U/mL heparin. Results of a representative experiment are shown. (B) shows the corresponding fluorescence histograms. The microparticles (percent particles left of the dotted line) were 2%, 34%, 65%, and 3%, respectively, for this experiment.

ment of platelet-associated IgG. All antibodies were conjugated directly with fluorescein isothiocyanate (FITC). Monoclonal antibody IV.3, specific for the Fc_γ Receptor II, was purchased from Medarex (W. Lebanon, NH). This antibody blocks Fc-dependent platelet activation, including heparin-induced thrombocytopenia.⁷ Drug testing was performed using porcine mucosal heparin (Hepalean, Organon Teknika, Toronto, Canada), quinidine sulfate (Park-Davis, Scarborough, Ontario, Canada), and quinidine sulfate (Abbott, Montreal, Quebec, Canada). Filtering of buffers for flow cytometry, and of pooled plasma for use in the Russell's viper venom time (RVVT),

Table 1. Microparticle Generation by Heparin-Induced Thrombocytopenia Sera and Control Sera

Agonist	% Microparticles (mean ± SD)
Heparin-induced thrombocytopenia sera plus:	
0.1 U/mL heparin (n = 40)	48.1 ± 20.8
0.0 U/mL heparin (n = 40)	18.5 ± 19.6
0.1 U/mL heparin + IV.3 (n = 24)	5.0 ± 2.7
100 U/mL heparin (n = 40)	5.4 ± 3.0
0.1 U/mL heparin plus the following control sera:	
Patients receiving heparin without HIT (n = 60)	3.9 ± 2.1
Random hospitalized patients (n = 22)	3.2 ± 0.9
Patients with thrombosis without HIT (n = 11)	4.2 ± 1.7
Normal volunteers (n = 10)	4.0 ± 1.6

Heparin-induced thrombocytopenia sera generated greater numbers of microparticles in the presence of 0.1 U/mL heparin than in the absence of heparin or at high heparin concentrations. Monoclonal antibody IV.3 (which blocks the binding of IgG Fc to the platelet Fc receptors⁷) inhibited platelet activation by heparin-induced thrombocytopenia sera. All negative control sera tested negative for heparin-induced thrombocytopenia using the platelet ¹⁴C-serotonin release assay. Microparticle generation was significantly greater for heparin-induced thrombocytopenia sera at 0.1 U/mL heparin than all control groups ($P < .001$).

Abbreviations: SD, standard deviation; HIT, heparin-induced thrombocytopenia.

was performed using 0.22 μm filters (Millipore Corp, Bedford, MA). Recombinant hirudin ([Lys⁴⁷]-rHV2 variant), calcium ionophore A23187, and DiOC₆ were obtained from Sigma (St Louis, MO). Lyophilized, pooled normal rabbit serum was obtained from Cedarlane Laboratories (Hornby, Ontario) and was reconstituted before use as source of complement. Fresh human AB serum (clotted at 4°C) was also used as a source of complement for some experiments.

Platelet ¹⁴C-serotonin release assay. Diagnostic testing for heparin-induced thrombocytopenia was performed using the ¹⁴C-serotonin release assay.^{9,10} All tests were performed in triplicate.

Purification of IgG. IgG was purified from patient and control sera by affinity chromatography using Staphylococcal protein A beads (Pharmacia Biotech, Quebec, Canada). Purity was confirmed by analytical 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was greater than 95%.

Preparation of platelets for microparticle studies. For studies of microparticle generation by heparin-induced thrombocytopenia sera, platelets were prepared as described previously.^{9,10} Using 12 × 75 mm, round-bottom polystyrene tubes (Becton Dickinson, Lincoln Park, NJ), 35 μL of washed platelets (300 × 10⁹/L) were added to 10 μL of test serum, and 5 μL of (1) buffer, (2) heparin (0.1 to 100 U/mL, final), or (3) quinidine or quinidine sulfate (0 to 40 μmol/L, final). The sera used were heat-treated to inactivate residual thrombin, as described.^{9,10} Following a 1-hour incubation without stirring or shaking at room temperature, the reaction was stopped by the addition of sodium citrate (0.98% final) or monoclonal antibody IV.3 (anti-Fc_γ RII). At this time, 5 μL of FITC-labeled anti-GPIb_α was added, and after a 30-minute incubation, the specimen was diluted to 0.25 mL using filtered phosphate-buffered saline (PBS), pH 7.4. The specimens were kept in the dark until FACS analysis (Becton Dickinson, San Jose, CA). For some experiments, the washed platelets were preincubated with monoclonal antibody IV.3, human or rabbit complement, or hirudin before adding the test serum. In other experiments, we used a fluorescent membrane probe (DiOC₆) to label the platelets and microparticles.

Detection of platelet-derived particles. Platelet-derived microparticles were identified using a Becton-Dickinson FACSscan. The instrument was calibrated for fluorescence and light scatter using the manufacturer's standard beads (CaliBRITE, Becton Dickinson). FITC-fluorescence was detected using a 530 nm band pass filter.

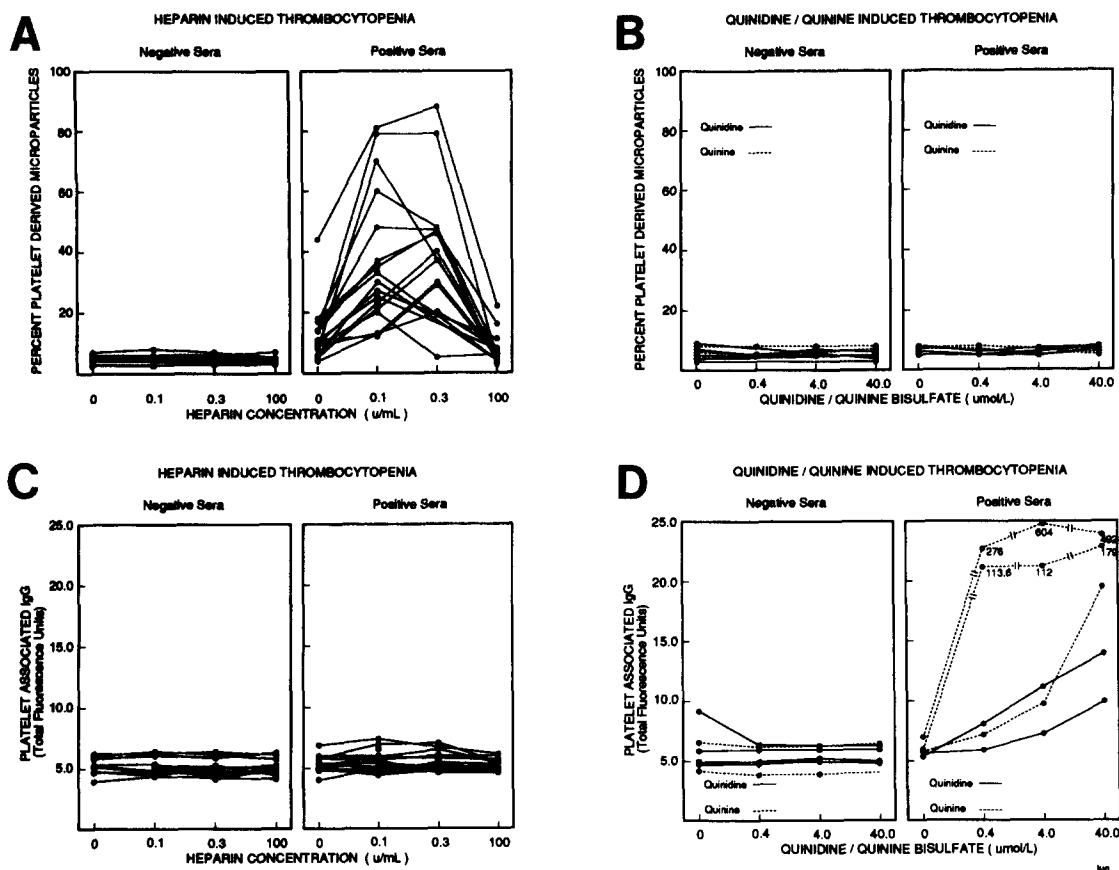


Fig 2. Drug-dependent generation of microparticles and platelet-associated IgG: comparison of heparin- and quinine/quinidine-induced thrombocytopenia sera. (A) Generation of platelet-derived microparticles by heparin-induced thrombocytopenia sera compared with control sera. All heparin-induced thrombocytopenia sera ($n = 17$) produced microparticles at 0.1 and/or 0.3 U/mL heparin (mean percentage microparticles generated = $38.2\% \pm 22.4\%$). Significantly fewer microparticles were generated in the absence of added heparin ($12.5\% \pm 9.2\%$), at high heparin concentrations ($7.2\% \pm 5.0\%$), or using sera negative for heparin-induced thrombocytopenia ($4.6\% \pm 1.3\%$) ($P < .001$ for each comparison). (B) Generation of platelet-derived microparticles by quinine/quinidine-induced thrombocytopenia sera. None of the quinine/quinidine-induced thrombocytopenia sera generated microparticles in the presence of corresponding drug (positive sera: percent microparticles in the absence of corresponding drug, $6.6\% \pm 1.1\%$, in the presence of corresponding drug, $6.0\% \pm 1.0\%$; control sera: percent microparticles in the absence of drug $5.8\% \pm 1.5\%$, in the presence of drug, $6.0\% \pm 2.2\%$). (C) Heparin-dependent binding of IgG to normal platelets. The heparin-induced thrombocytopenia sera did not produce significant drug-dependent binding of IgG when compared with control sera. For positive heparin-induced thrombocytopenia sera, the mean fluorescence at either 0.1 or 0.3 U/mL heparin was 5.5 ± 0.8 fluorescence units, which did not differ significantly from mean fluorescence at 0 U/mL heparin (5.3 ± 0.7) or 100 U/mL heparin (5.2 ± 0.5), or compared with control sera that tested negative for heparin-induced thrombocytopenia (5.3 and 5.4 ± 0.7 fluorescence units at 0.1 and 0.3 U/mL heparin, respectively). (D) Quinine/quinidine-dependent binding of IgG to normal platelets. All five quinine/quinidine sera produced drug-dependent binding of IgG to normal platelets compared with baseline (increase from 5.9 ± 0.6 mean fluorescence units in the absence of drug to 42.2 ± 47.2 mean fluorescence units in the presence of $40 \mu\text{mol/L}$ drug, $P = .020$ using paired *t*-test of log-transformed data); and compared with negative sera (5.3 ± 0.9 fluorescence units by negative sera at $40 \mu\text{mol/L}$ drug, $P = .002$ using *t*-test of log-transformed data).

Data for forward-angle scatter, side-angle scatter, and FITC-fluorescence were obtained with gain settings in the logarithmic mode. In most experiments, platelets and platelet-derived microparticles were identified using the FITC-labeled, platelet-specific monoclonal antibody TW-1 (anti-GPIb $_{\alpha}$). At least 5,000 FITC-positive events were collected for each sample. Microparticles were distinguished from platelets on the basis of their characteristic flow cytometric profile of forward light scatter (abscissa) versus FITC-fluorescence (ordinate). We defined microparticles as GPIb $_{\alpha}$ -positive events that exhibited less fluorescence than 95 to 99% of nonactivated, FITC-GPIb $_{\alpha}$ -labeled platelets (generally less than 100 arbitrary fluorescence units) (Fig 1). In preliminary experiments, we found that the FITC-labeled

anti-GPIb $_{\alpha}$ monoclonal antibody gave better separation of platelets from microparticles than did labeled anti-GPIX or anti-GPIIb/IIIa.

For additional investigations of platelet microparticle generation by heparin-induced thrombocytopenia sera, we used the fluorescent membrane probe, DiOC $_6$. DiOC $_6$ is a hydrophobic cyanine dye whose fluorescence has been used to measure membrane potential in cells and membranous vesicles.¹² This provided a platelet glycoprotein-independent detection method and permitted us to rule out GPIIb proteolysis as an explanation for an apparent increase in microparticle activity. The settings of the FACScan were adjusted to maximize total event counts by using side scatter as the trigger with a low threshold. Photomultiplier (PMT) voltages were set using 0.5

micron beads so that the peak of the beads was positioned in channel 400 on side scatter and channel 600 on 530 nm fluorescence. The threshold was optimized for each run by examining a series of platelet-rich plasma (PRP) samples treated with 0.2 $\mu\text{mol/L}$ DiOC₆, as previously described,¹³ so that at least 70% of the light scatter counts appeared to be positive.

Demonstration and quantitation of procoagulant activity of the platelet microparticles. The procoagulant activity of the platelet-derived microparticles was measured using a phospholipid-dependent coagulation assay, the RVVT, with a Diagnostica Stago 4 instrument (Wellmark Diagnostics, Guelph, Ontario, Canada). For these assays, 50 μL of filtered and pooled plasma (from 20 normal donors), 50 μL of Russell's viper venom solution, and 10 μL of microparticle-containing supernatant, were added together, before adding 50 μL of calcium-containing buffer (20 nmol/L, final) to perform the RVVT. To quantitate procoagulant activity, we generated a standard curve, plotting RVVT shortening (in seconds) versus μL of freeze-thawed platelets added.

A slow centrifugation step was used to remove platelets from the microparticle suspension (1000g for 10 minutes). A faster centrifugation (15,600g for 10 minutes) was used to precipitate the majority of the microparticles, as described.¹⁴ In some instances, the test platelet suspension was pretreated with monoclonal antibody IV.3 to prevent platelet activation by the heparin-induced thrombocytopenia IgG through the platelet Fc_{II} receptors. As a positive control, procoagulant microparticles with procoagulant activity were generated using calcium ionophore A23187, as described.¹⁵⁻¹⁸

In additional experiments, we also quantified procoagulant activity in the reaction suspension and supernatant using a chromogenic assay for prothrombinase activity.^{19,20} Briefly, 50 μL of sample was combined with 400 μL Tris buffer (pH 7.4) and 50 μL of normal citrated plasma, then activated with 50 μL of a solution containing V:CP and X:CP purified from crude Russell's viper venom. After 60 seconds at 37°C, calcium and the thrombin chromogenic substrate S-2238 were added and the absorbance at 405 nm was recorded in a Varian DMS-100 spectrophotometer (Varian Instrument Group, Sugar Land, TX). The maximum rate of change in absorbance was taken as the assay endpoint and quantified from a standard curve of lysed platelets, as described.²⁰

Measurement of drug-dependent platelet-associated IgG. Flow cytometry was used to measure drug-dependent platelet-associated IgG. Seventy μL of washed platelets ($300 \times 10^9\text{L}$) were obtained from normal volunteers and incubated with 20 μL of heat-inactivated test serum and 10 μL of varying concentrations of either heparin (0 to 100 U/mL, final) or quinine/quinidine (0 to 40 $\mu\text{mol/L}$, final) or buffer, for 60 minutes at room temperature. The concentration of drug was maintained throughout all of the wash steps, as described.²¹ After washing, FITC-labeled CAG-1 (monoclonal anti-IgG) was added to the platelet preparation. After a 1-hour incubation, the sample was diluted with 0.25 mL of PBS, pH 7.4, and analyzed in the FACScan.

Direct detection of platelet-derived microparticles in patient samples. Blood from patients with heparin-induced thrombocytopenia ($n = 15$), other thrombocytopenic disorders ($n = 15$), and acute thrombosis without thrombocytopenia ($n = 9$) was collected into acid citrate dextrose (pH 6.4), and platelet-rich plasma was obtained by sedimentation at 1g. Twenty μL of the platelet-rich plasma was added to 25 μL of PBS, after which 5 μL of FITC-labeled, platelet-specific monoclonal antibody TW-1 (anti-GPIIb₃) was added. Following a 1-hour incubation, 250 μL of PBS was added, and the sample was analyzed by the FACScan. The circulating microparticles were quantitated by relating the number of GPIIb₃-positive particles of less than 86 fluorescence units as a percentage of the total number of GPIIb₃-positive particles. This fluorescence value was determined

from the analyses of normal platelet-rich plasma samples demonstrating that the normal percentage of microparticles ranged from 1% to 5%, using this threshold.

Statistical analysis. Data were analyzed using the Student's *t*-test. Results were considered statistically significant at $P \leq .05$.

RESULTS

Generation of platelet-derived microparticles. Sera from 40 of 40 patients with heparin-induced thrombocytopenia generated platelet-derived microparticles in a heparin-dependent fashion (Table 1, Fig 1). Significantly fewer microparticles were generated by heparin-induced thrombocytopenia sera in the absence of added heparin, at high concentrations of heparin, and following preincubation with the monoclonal antibody IV.3, which blocks the binding of IgG to the platelet Fc receptors⁷ ($P < .001$ for each comparison). The formation of microparticles at therapeutic heparin concentrations closely paralleled the amount of ¹⁴C-serotonin release ($r = .85$). Microparticle generation by heparin-induced thrombocytopenia sera was also significantly greater than control sera, including sera from patients receiving heparin who did not develop heparin-induced thrombocytopenia, sera from randomly selected hospitalized patients, sera from patients with acute thrombosis, and sera from normal volunteers ($P < .001$ for each comparison).

Further studies were performed using sera from a subgroup of 17 patients with heparin-induced thrombocytopenia, representing a broad cross-section of clinical sequelae (venous thrombosis $n = 6$, arterial thrombosis $n = 5$, intracranial hemorrhage $n = 1$, and isolated thrombocytopenia $n = 5$). These patients had a median platelet count nadir of $52 \times 10^9\text{L}$ with a range of 15 to $309 \times 10^9\text{L}$. Sera from three patients with quinine-induced thrombocytopenia and two with quinidine-induced thrombocytopenia were also studied. These patients had a median platelet count nadir of $3 \times 10^9\text{L}$ with a range of 1 to $5 \times 10^9\text{L}$. All 17 heparin-

Table 2. Microparticle Generation by Purified Heparin-Induced Thrombocytopenia IgG

	Heparin 0 U/mL	Heparin 0.1 U/mL	Heparin 100 U/mL	Heparin 0.1 U/mL + IV.3
Purified IgG (positive for HIT)	31.5 ± 18.4	54.6 ± 23.0	16.3 ± 10.2	2.5 ± 0.7
Purified IgG (negative for HIT)	25.5 ± 8.8	26.2 ± 13.0	21.5 ± 10.2	3.5 ± 0.4

Data are mean ± standard deviation representing purified IgG obtained from 12 positive and 10 negative sera tested for heparin-induced thrombocytopenia. An increase in microparticles at 0.1 U/mL heparin, compared with 0 and 100 U/mL heparin, is seen with positive, but not negative, heparin-induced thrombocytopenia IgG ($P < .001$). High background microparticle generation in these experiments is attributed to formation of immune complexes during IgG purification, as microparticle generation is inhibited by IV.3 in both positive and negative heparin-induced thrombocytopenia sera ($P < .001$).

Abbreviations: HIT, Heparin-induced thrombocytopenia.

induced thrombocytopenia sera generated microparticles at 0.1 or 0.3 U/mL heparin, or both (Fig 2A); in contrast, none of five quinine/quinidine-induced thrombocytopenia sera generated microparticles in a drug-dependent fashion under identical experimental conditions (Fig 2B). The addition of fresh human or rabbit serum as a source of complement did not increase the number of microparticles, compared with baseline (not shown).

To further investigate the specificity of the reaction, we tested purified IgG from 12 patients with heparin-induced thrombocytopenia and IgG purified from the sera of 10 normal controls (Table 2). An increase in microparticle generation in the presence of 0.1 U/mL heparin was produced by purified IgG from heparin-induced thrombocytopenia, but not control, patients. Preincubation with IV.3 inhibited microparticle generation in these experiments.

We also investigated the ability of added hirudin (25 U/mL) to inhibit generation of microparticles. There was no decrease in microparticles generated at 0.1 U/mL heparin in the presence of hirudin (percent microparticles in the absence of hirudin = 43.5 ± 9.6 , percent microparticles in the presence of hirudin = 46.1 ± 10.0 , $n = 10$ positive heparin-induced thrombocytopenia sera tested), indicating that residual thrombin was not responsible for microparticle formation.

We also observed an increase in microparticle generation by five heparin-induced thrombocytopenia sera using the fluorescent membrane probe, DiOC₆. Only DiOC₆-positive events were quantified, with platelets distinguished from microparticles based on forward light scatter. The division between platelets and microparticles was set as the lower limit of the fresh platelet distribution in this method. Mean percent microparticle generation was 76.2% for the five heparin-induced thrombocytopenia sera, compared with 8.6% using the same five heparin-induced thrombocytopenia sera, pretreated with blocking antibody IV.3 (paired *t* test, $P < .001$). Two sera negative for heparin-induced thrombocytopenia and their corresponding IV.3 blocked samples generated a mean of only 7.8% and 4.0% microparticles, respectively.

Drug-dependent increase in platelet-associated IgG. Sera from patients with heparin-induced thrombocytopenia produced no increase in the binding of IgG to control platelets in the presence of varying heparin concentrations (Fig 2C). In contrast, sera from the patients with quinine- or quinidine-induced thrombocytopenia produced a significant increase in the drug-dependent binding of IgG to the control platelets (Fig 2D).

Demonstration that the platelet-derived microparticles have procoagulant activity. Platelet-derived microparticles were tested for procoagulant activity by measuring their ability to shorten the RVVT. This technique is quantitative, as shown by the linear relationship (semilog plot) when the shortening of the RVVT is compared with a standard using increasing volumes of freeze-thawed platelets (a procoagulant microparticle-rich source) (Fig 3). A microparticle-rich fraction was obtained using differential centrifugation following the incubation of normal platelets with heparin-induced thrombocytopenia or control sera in the presence of 0.1 U/mL heparin. In a parallel test for each patient or control

sample, monoclonal antibody IV.3 was added to block formation of microparticles. Significant shortening of the RVVT, compared with platelets incubated with buffer control, was observed only with heparin-induced thrombocytopenia sera and calcium ionophore (Table 3). Significant shortening was not observed with quinine- or quinidine-induced thrombocytopenia sera, even following addition of rabbit complement.

To estimate the relative amount of procoagulant activity generated by heparin-induced thrombocytopenia sera and ionophore compared with nonmicroparticle-generating conditions, the effect on RVVT shortening, either before or following low- and high-speed centrifugation, was expressed as microliters of freeze-thawed platelet equivalents (Table 3). A marked increase in procoagulant activity was observed following platelet activation by heparin-induced thrombocytopenia sera (approximately 100-fold greater than washed platelet buffer control) and calcium ionophore (approximately 600-fold greater than washed platelet buffer control). Following low-speed (platelet) centrifugation, approximately two-thirds of the procoagulant activity was lost. Following high-speed centrifugation, over 98% of the starting procoagulant activity was removed. In contrast, no significant procoagulant activity was generated by IV.3-pretreated platelets incubated with heparin-induced thrombocytopenia sera and heparin. Similarly, no procoagulant activity was generated by platelets incubated with quinine/quinidine-induced thrombocytopenia sera and corresponding drug, in the presence or absence of IV.3.

Simultaneous with the measurement of DiOC₆-positive microparticles, platelet reaction aliquots were also analyzed for procoagulant activity using the chromogenic assay for prothrombinase activity. Each reaction was assayed as a

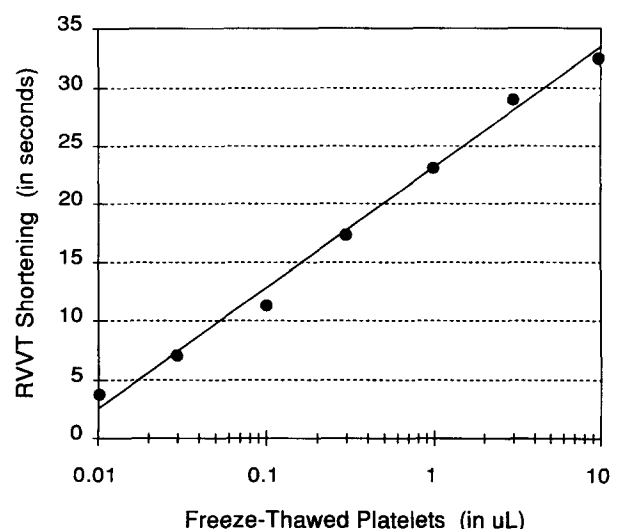


Fig 3. Standard curve of RVVT shortening versus freeze-thawed platelets added. A linear relationship was observed (semilog plot) between shortening of RVVT and the amount of freeze-thawed platelets added (μ L). The data points shown represent the mean of five separate experiments on five different days.

Table 3. Procoagulant Activity of Platelet-derived Microparticles Generated by Heparin-Induced Thrombocytopenia Sera

	Shortening of RVVT (sec) Before Centrifugation: Platelets and Microparticles Mean \pm SD (95% CI)	Shortening of RVVT (sec) After Low-speed Centrifugation: Microparticle-rich Supernatant Mean \pm SD (95% CI)	Shortening of RVVT (sec) After High-speed Centrifugation: Microparticle-deplete Supernatant Mean \pm SD (95% CI Seconds)
Positive control (ionophore) (n = 8)	37.2 \pm 3.9 (33.9-40.5)	32.5 \pm 5.2 (28.2-36.8)	18.0 \pm 4.3 (14.4-21.6)
μ L freeze-thawed platelet equivalents	25.2	8.7	0.33
Negative control (buffer) (n = 8)	8.6 \pm 4.3 (5.0-12.2)	4.5 \pm 3.2 (1.8-7.2)	5.1 \pm 5.1 (0.8-9.4)
μ L freeze-thawed platelet equivalents	0.040	0.016	0.018
HIT sera (n = 17)	29.1 \pm 6.5 (25.8-32.4)	23.9 \pm 6.5 (20.6-27.2)	11.5 \pm 5.2 (8.8-14.2)
μ L freeze-thawed platelet equivalents	4.05	1.25	0.076
HIT sera plus IV.3 (n = 17)	8.9 \pm 4.6 (6.5-11.3)	4.6 \pm 4.0 (2.5-6.7)	3.4 \pm 3.3 (1.7-5.1)
μ L freeze-thawed platelet equivalents	0.042	0.016	0.012
Sera negative for HIT (n = 4)	13.5 \pm 3.8 (7.5-19.6)	8.3 \pm 1.8 (5.4-11.2)	2.2 \pm 2.6 (0-6.3)
μ L freeze-thawed platelet equivalents	0.12	0.037	0.0093
Sera negative for HIT plus IV.3 (n = 4)	12.6 \pm 2.9 (8.0-17.2)	7.7 \pm 3.2 (2.6-12.8)	2.9 \pm 2.7 (0-7.2)
μ L freeze-thawed platelet equivalents	0.10	0.032	0.011
Quinine/quinidine-induced thrombocytopenia sera (n = 5)*	14.3 \pm 5.9 (7.0-21.6)	1.5 \pm 1.2 (0-3.0)	1.1 \pm 1.4 (0-2.8)
μ L freeze-thawed platelet equivalents	0.14	0.0080	0.0073
Quinine/quinidine-induced thrombocytopenia sera plus IV.3 (n = 5)	10.6 \pm 4.3 (5.3-15.9)	1.1 \pm 0.9 (0-2.2)	0.3 \pm 0.5 (0-0.9)
μ L freeze-thawed platelet equivalents	0.062	0.0073	0.0061

Procoagulant activity of the test samples was determined using shortening of the RVVT (in seconds, compared with baseline), a phospholipid-dependent coagulation assay. Samples were assayed either before centrifugation (containing platelets and microparticles, if generated), after low-speed centrifugation (microparticle-rich supernatant), and after high-speed centrifugation (removal of majority of microparticles). Test platelets were incubated either with ionophore (positive control), HIT sera, sera negative for HIT, or sera from patients with quinine/quinidine-induced thrombocytopenia, either with or without Fc receptor blocking monoclonal antibody IV.3. Note that only ionophore and HIT sera were able to shorten significantly the RVVT ($P < .001$ for each comparison with buffer control, negative HIT sera, positive or negative HIT sera pretreated with IV.3, and quinine/quinidine-induced thrombocytopenia sera). Pretreatment with Fc receptor blocking monoclonal antibody completely negated the effect of the HIT sera, but had no effect on ionophore (not shown). High-speed centrifugation removed the majority of the procoagulant activity generated by the HIT sera and ionophore. The procoagulant activity is also expressed as μ L equivalents of freeze-thawed platelets. A marked increase in procoagulant activity was observed after platelet activation by HIT sera (approximately 100-fold greater than washed platelet buffer control) and calcium ionophore (approximately 600-fold greater than washed platelet buffer control). After low-speed (platelet) centrifugation, approximately two-thirds of the procoagulant activity was lost. After high-speed centrifugation, over 98% of the starting procoagulant activity was removed. In contrast, no increase above washed platelet buffer control background procoagulant activity was observed using IV.3-pretreated platelets incubated with HIT sera and heparin or quinine/quinidine-induced thrombocytopenia sera and drug, or platelets incubated with quinine/quinidine-induced thrombocytopenia sera and drug alone.

Abbreviations: SD, standard deviation; CI, confidence interval; HIT, heparin-induced thrombocytopenia.

* Includes two experiments in which rabbit serum (as a source of complement) was added.

whole platelet suspension, and again following centrifugation at 15,000g for 10 minutes. The results (Table 4) show that platelets reacted with heparin-induced thrombocytopenia sera, and heparin 0.1 U/mL had a marked increase in prothrombinase activity in the suspension, the majority of which could be sedimented. Subsequent filtration of reaction supernatant through a 0.2- μ m Acrodisc membrane (Gelman Sciences, Ann Arbor, MI) produced a 94% reduction in prothrombinase activity compared with the initial suspension (not shown), confirming that the activity was associated with particulate material. The results are consistent with the RVVT assay, and together they indicate that procoagulant microparticles are generated as a result of the platelet stimulation by heparin-induced thrombocytopenia sera.

Direct demonstration of platelet-derived microparticles in patient platelet-rich plasma. The Becton Dickinson FACS-can was used to detect platelet-derived microparticles directly in platelet-rich plasma samples obtained from patients

with heparin-induced thrombocytopenia (n = 15), a group of patients with a variety of thrombocytopenic disorders (n = 15), and patients with acute thrombosis not related to heparin-induced thrombocytopenia (n = 9). Platelet-derived microparticles were observed in greater numbers in patients with heparin-induced thrombocytopenia during the acute episode (Fig 4A and B). In contrast, large numbers of microparticles were not observed in patients with other thrombocytopenic disorders. We also had the opportunity to test PRP from one patient with acute quinine-induced thrombocytopenia. Low levels of microparticles (3%) were observed in this patient despite severe thrombocytopenia ($20 \times 10^9/L$). Low levels of circulating microparticles were also observed in nine patients with acute thrombosis.

DISCUSSION

More than 100 drugs have been reported to cause idiosyncratic (immune-mediated) thrombocytopenia.¹ However,

Table 4. Prothrombinase Activity Levels Before and After Centrifugation of Platelet Reaction Mixtures Treated With HIT Sera or Controls \pm Blocking Monoclonal Antibody IV.3

Treatment Group	DiOC ₆ -positive % Microparticles	Prothrombinase Activity Units	
		Suspension	Supernatant
Control serum	10.5	0.099	0.016
Control serum + IV.3	5.4	0.116	0.016
HIT #1	87	0.385	0.146
HIT #2	81	0.439	0.175
HIT #3	67	0.256	0.113
HIT #1 + IV.3	5.4	0.023	0.016
HIT #2 + IV.3	17	0.029	0.014
HIT #3 + IV.3	3.2	0.035	0.013

Procoagulant activity, measured as prothrombinase activity units, was highly correlated with the amount of DiOC₆-positive microparticles generated ($r = 0.93$, $P < .001$, $n = 8$).

heparin-induced thrombocytopenia is unique; although the thrombocytopenia is only moderately severe, and bleeding is uncommon, many patients will develop life-threatening thrombotic sequelae as a complication of the syndrome.²⁻⁵ Consequently, although many different drugs can cause immune thrombocytopenia, serious and fatal outcomes are more frequently associated with heparin than any other drug.

The intent of this report was to look for a biologic explanation for the dramatic difference in clinical features between heparin- and quinine/quinidine-induced thrombocytopenia. The investigation focused on comparing these two distinct drug-induced immune thrombocytopenic syndromes with respect to two laboratory characteristics: the ability of these sera, in the presence of drug, to generate platelet-derived procoagulant microparticles; and the ability of the drug-dependent IgG to bind to platelets.

We observed distinct differences between heparin- and quinine/quinidine-induced thrombocytopenia sera in their effects on platelets in the presence of the respective drug. All of the heparin-induced thrombocytopenia sera (40 tested) generated platelet-derived microparticles in a heparin dose-dependent fashion (Figs 1 and 2). Maximal generation of microparticles occurred at concentrations of heparin that are achieved in patients (0.1 to 0.3 U/mL). In contrast, none of five quinine/quinidine-induced thrombocytopenia sera or other control sera produced microparticles. The microparticles were shown to have procoagulant activity, based on their ability to shorten the RVVT (Table 3) and to promote thrombin generation in a chromogenic assay (Table 4). Additionally, monoclonal antibody (IV.3) against the platelet Fc receptor, but not a thrombin inhibitor (hirudin), inhibited microparticle formation. Previous investigators have shown that a variety of strong platelet agonists, including thrombin and collagen, can induce generation of platelet-derived procoagulant microparticles.¹⁵⁻¹⁸ The studies described in this report indicate that activation of platelets via their Fc_γII receptors also leads to the generation of platelet-derived microparticles.

Although the quinine/quinidine-induced thrombocyto-

penia sera did not produce microparticles, they caused a significant binding of IgG to normal platelets in a drug-dependent fashion. Others have shown that large amounts of IgG (up to 160,000 molecules per platelet) can bind in the presence of quinine/quinidine-induced thrombocytopenia serum and the appropriate drug.²² This large amount of binding is caused by Fab binding of the IgG to a complex consisting of drug and either GPIIb/IIIa or GPIb/IX.²³⁻²⁶

In contrast, we did not observe heparin-dependent binding of IgG to normal platelets using sera from patients with heparin-induced thrombocytopenia. Using an enzyme-linked immunosorbent assay (ELISA) platelet-antibody technique, other investigators have shown that binding of heparin-dependent IgG to normal platelets is relatively low (approximately twice background).²⁷ This amount may be below the level of sensitivity of the technique we used. The type of interaction of the IgG with the platelet membrane may also explain the dramatic difference in the binding of drug-dependent IgG with the platelets: quinine/quinidine-dependent IgG bind to GPs IIb/IIIa and Ib/IX (50,000 and 20,000 copies per platelet, respectively), whereas heparin-dependent IgG binds to the platelet Fc receptor (1,000 copies per platelet).^{7,10,24,25}

We offer a cautious extrapolation of these in vitro observations to the in vivo characteristics of these two drug-induced immune thrombocytopenic syndromes. We suggest that quinine/quinidine-induced thrombocytopenia results in severe immune destructive thrombocytopenia because of the relatively large amounts of IgG bound to the platelet surface. Consequently, the IgG-sensitized platelets are cleared by the reticuloendothelial system. However, these antibodies do not activate platelets nor do they generate procoagulant microparticles. This is consistent with the clinical observations of severe thrombocytopenia and bleeding without thrombosis, similar to acute idiopathic thrombocytopenic purpura.

In contrast, sera from patients with heparin-induced thrombocytopenia proved to be potent activators of platelets causing the formation of procoagulant microparticles. Thus, even patients with relatively mild or moderate thrombocytopenia could develop thrombotic complications because the heparin-dependent IgG can induce platelet activation, with formation of platelet-derived procoagulant microparticles. The relatively small amounts of IgG bound to the platelet in heparin-induced thrombocytopenia may explain why severe thrombocytopenia is less common in this syndrome.

Our data suggest that generation of procoagulant microparticles in vivo might contribute to thrombosis in patients with heparin-induced thrombocytopenia. Previous investigators have demonstrated that the heparin-dependent IgG can activate endothelial cells, by interacting with endogenous glycosaminoglycans on endothelial cells.^{28,29} Our study indicates that the heparin-dependent IgG also activates platelets and causes them to produce microparticles that have procoagulant activity. These findings may explain the occurrence of thrombotic complications and disseminated intravascular coagulation in some patients with heparin-induced thrombocytopenia.

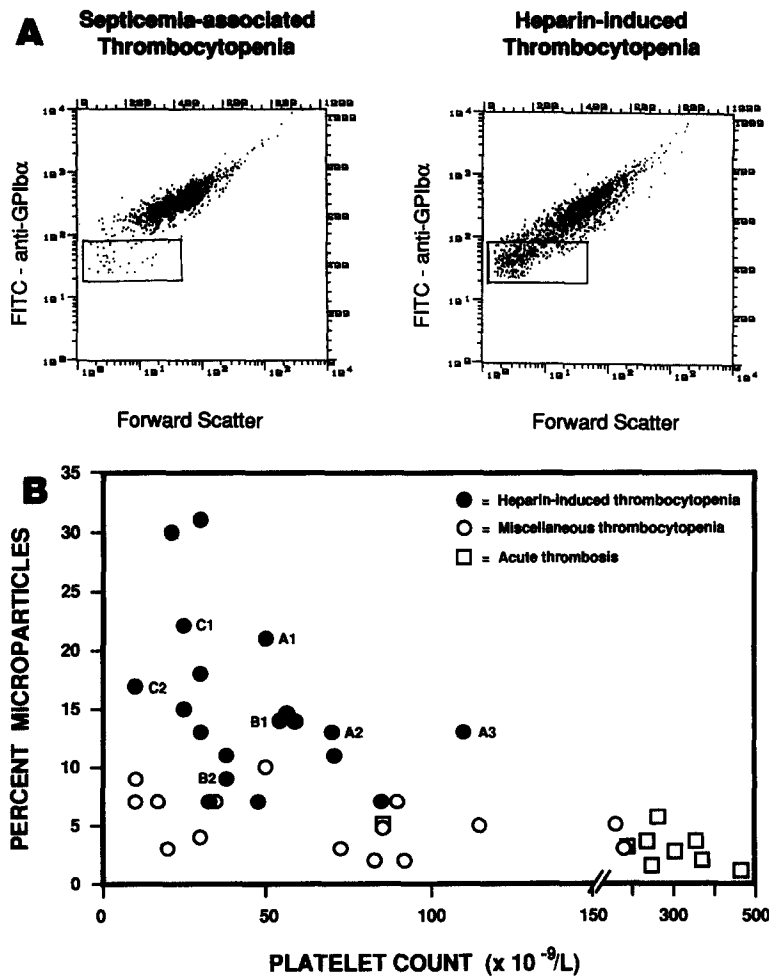


Fig 4. Demonstration of platelet-derived microparticles in PRP from patients with heparin-induced thrombocytopenia. (A) shows the FACScan profile, using FITC-anti-GPII_b (Y-axis, log scale) and particle size (X-axis, log scale), for a patient with heparin-induced thrombocytopenia and a patient with thrombocytopenia caused by septicemia. Both patients had a similar platelet count ($30 \times 10^9/L$) when studied. Platelet-derived microparticles (insert box) were observed in larger numbers in the patient with heparin-induced thrombocytopenia. (B) shows the percent platelet-derived microparticles (ordinate) in relation to the platelet count (abscissa) for a group of patients with heparin-induced thrombocytopenia (\bullet), thrombocytopenia caused by other factors (\circ), or patients with acute thrombosis (\square). Thrombocytopenic patients studied included idiopathic thrombocytopenic purpura ($n = 4$), septicemia-associated thrombocytopenia ($n = 3$), disseminated intravascular coagulation ($n = 3$), antiphospholipid antibody syndrome ($n = 1$), quinine-induced thrombocytopenia ($n = 1$), alcohol-induced thrombocytopenia ($n = 1$), and hemodilution ($n = 2$). Repeat samples were studied for three patients with heparin-induced thrombocytopenia (A1-3, B1-2, and C1-2). Increased numbers of percent microparticles were observed in the patients with heparin-induced thrombocytopenia ($15.1\% \pm 6.9\%$) compared with thrombocytopenia caused by other factors ($5.2\% \pm 2.5\%$) and in patients with acute thrombosis ($3.6\% \pm 1.3\%$) ($P < .001$ for each comparison).

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