Immunoglobulin G From Patients With Heparin-Induced Thrombocytopenia Binds To a Complex of Heparin and Platelet Factor 4

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Heparin-induced thrombocytopenia (HIT) is an important complication of heparin therapy. Although there is general agreement that platelet activation in vitro by the HIT IgG is mediated by the platelet Fc receptor, the interaction among the antibody, heparin, and platelet membrane components is uncertain and debated. In this report, we describe studies designed to address these interactions. We found, as others have noted, that a variety of other sulfated polysaccharides could substitute for heparin in the reaction. Using polysaccharides selected for both size and charge, we found that reactivity depended on two independent factors: a certain minimum degree of sulfation per saccharide unit and a certain minimum size. Hence, highly sulfated but small (<1,000 daltons) polysaccharides were not reactive nor were large but poorly sulfated polysaccharides. The ability of HIT IgG to recognize heparin by itself was tested by Ouchterlony gel diffusion, ammonium sulfate and polyethylene glycol precipitation, and equilibrium dialysis. No technique demonstrated reactivity. However, when platelet releasate was added to heparin and HIT IgG, a 50-fold increase in binding of radio-labeled heparin to HIT IgG was observed. The releasate was then depleted of proteins capable of binding to heparin by immunoadfinity chromatography. Only platelet factor 4-immunodepleted releasate lost its reactivity with HIT IgG and heparin. Finally, to determine whether the reaction occurred on the surface of platelets or in the fluid phase, washed platelets were incubated with HIT IgG or heparin and after a wash step, heparin or HIT IgG was added, respectively. Reactivity was only noted when platelets were preincubated with heparin. Consistent with these observations was the demonstration of the presence of PF4 on platelets using flow cytometry. These studies indicate that heparin and other large, highly sulfated polysaccharides bind to PF4 to form a reactive antigen on the platelet surface. HIT IgG then binds to this complex with activation of platelets through the platelet Fc receptors.

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HEPARIN-INDUCED thrombocytopenia (HIT) is probably the most important allergic reaction to a medication that physicians face today. Many patients with HIT have isolated thrombocytopenia that by itself causes minimal morbidity. However, those patients who develop the venous and arterial thrombotic complications of HIT can have major adverse effects. Some will die. Although the clinical consequences of the disorder are well known, its pathophysiology is less well understood. It is now acknowledged that the sequences of the disorder are well known, its pathophysiology is less well understood. It is now acknowledged that the sequences of the disorder are well understood. Its pathophysiological basis is uncertain and debated. In this report, we describe studies investigating the interaction of heparin, patient IgG, and platelet membrane components. We found that the IgG from patients with HIT binds to PF4 when the PF4 is complexed with heparin but does not bind to heparin alone. PF4 was found to be the only component in platelet releasate that could act as a cofactor in combining with heparin to form a complex to which the HIT antibodies bind. Additionally, other heparin analogues are able to substitute for heparin, although a high degree of sulfation and a certain minimum size are required.

MATERIALS AND METHODS

A variety of large and small sulfated compounds were tested for their ability to support the HIT-IgG-mediated platelet release. Heparan sulfate, chondroitin sulfates A, B, and C, pentosan polysulfate, and heparin disaccharide I-S were obtained from Sigma Chemical Co (St Louis, MO). Dextran sulfate was obtained from ICN Biochemicals (Cleveland, OH). The glucose 1,3,5-trisulfate was prepared by sulfuric acid esterification of α-D-glucose using the method Takiiura et al. Trans 1,2-cyclohexanediol and cis/trans (mixture) 1,4-cyclohexanediol (Aldrich Chemical Co, Milwaukee, WI) were converted to the disulfate esters using the method described by Eanor and Honeynan. IgG fractions of HIT and control sera were prepared by protein A-Sepharose (Pharmacia, Montreal, Quebec, Canada) affinity chromatography. Thrombospondin (TSP) was isolated by the method described by Lawler et al. and was further purified by immunoaffinity chromatography using a monoclonal antibody (MoAb) (CH-1) specific for TSP. von Willebrand factor (vWF) was isolated from cryoprecipitate using the method of Thorell and Blombäck and fibrinectin (Fn) was obtained from Sigma. PF4 was prepared by the method described by Levine and WoI using outdated platelet concentrates collected by the Canadian Red Cross Blood Transfusion Service. The PF4 ran on a single band at 9 kDa on an overloaded gel run under reducing conditions on sodium dodecyl sulfate-polyacryl-
amide gel electrophoresis (SDS-PAGE). PF4 from Sigma also ran as a single band and the two products reacted identically. Platelet releasate was prepared from washed platelets (10^9/mL) stimulated with trypsin-diumin (2 U/mL final, 10 minutes at 37°C) and then treated with phenylmethylsulphonyl fluoride (200 pmol/L). Platelet lysate was prepared from washed platelets (10^9/mL) lysed with Triton X-100 (1.0% final concentration) and centrifuged at 14,000g for 30 minutes. Platelet lysate and releasate gave identical results when used as a source of the cofactor, but for most experiments platelet releasate was used.

Plasma and sera were collected from patients who had isolated thrombocytopenia or thrombocytopenia plus thrombotic complications after exposure to heparin. Diagnosis in every patient was confirmed using the ^14C-serotonin release assay. Several strong positive and negative patient samples for which large volumes of sera or plasma were available were selected for further study; in general, there was no correlation between the degree of thrombocytopenia and the result of the release reaction.

**Platelet Serotonin Release Assay**

Some experiments were performed using the platelet serotonin release assay as described. In brief, test sera were inactivated by heating for 30 minutes at 56°C. Platelet-rich plasma (PRP) from two healthy donors who were receiving no medication was isolated and labeled with ^14C-serotonin (Amersham, Oakville, Ontario, Canada) using 0.1 μCi ^14C-serotonin/mL. The platelets were washed once in the presence of apyrase and resuspended in albumin-free Tyrode’s to a final concentration of 30 x 10^9/L. Twenty microliters of patient or control serum was added to 75 μL of ^14C-serotonin–labeled platelets (225 x 10^9/L final). To this solution was added 5 μL of porcine heparin (Hepalean; Organon-Teknika Canada, Ltd, Toronto, Ontario, Canada) or buffer to produce final heparin concentrations of 0, 0.1, and 100 U/mL. The platelet suspension was gently shaken for 1 hour at room temperature after which time 100 μL of 0.5% EDTA was added to terminate the reaction. The platelets were pelleted by centrifugation and the supernatant radioactivity measured. All tests were performed in triplicate and the platelet release of serotonin for each heparin concentration was reported as a percentage of the total platelet radioactivity after correction for background radioactivity. A positive test for HIT was defined as greater than 20% release at 0.1 U/mL of heparin and less than 20% release at 100 U/mL of heparin (Fig 1D). We have previously shown that this assay does not give positive results for other thrombocytopenic disorders.

**Interaction of Patient Sera and Heparin With Platelets**

**Interaction of Patient Sera With Heparin Analogues**

Various sulfated polysaccharides and sulfated analogues of the basic heparin unit were tested (using concentrations covering several orders of magnitude) for their ability to substitute for heparin in the serotonin release reaction using six different known positive sera.

**Preincubation of Platelets With Heparin or Sera**

We investigated the requirement for the simultaneous presence of heparin and patient IgG for interaction with the platelets. In the first experiments, the test platelets were preincubated with heparin then washed in calcium-albumin–free Tyrode’s buffer (CAF), pH 6.8, containing apyrase before being exposed to patient serum. In the second series of experiments, the test platelets were initially exposed to patient serum, then washed, (CAF plus apyrase) and then incubated with heparin. Both experiments used the platelet release reaction as the endpoint, with the FC-dependency of release confirmed by inhibition using MoAb (IV.3) (Medarex, West Lebanon, NH), which blocks FcRII-mediated activation.

**Platelets and heparin.** Washed platelets (CAF plus apyrase) were first incubated with varying concentrations of heparin (0 to 1,000 IU/mL final) for 30 minutes at 22°C in CAF, pH 6.8. After washing, the platelets were resuspended in albumin-free Tyrode’s (AFT), pH 7.4, and sera from patients with HIT or negative control sera were added along with 0, 0.1, or 100 IU/mL heparin. Serotonin release was measured as described.

**Platelets and sera.** In parallel studies, the test platelets were first incubated with sera from patients with HIT and after the wash step, (CAF plus apyrase) varying concentrations of heparin (0, 0.1, or 100 IU/mL final) were added and the release assay performed as described.

**Interaction of HIT Patient IgG and Platelet Proteins in a Platelet-Free System**

The interaction of patient IgG with heparin in the presence and absence of specific platelet proteins was investigated. In these experiments a platelet-free system was used, and heparin was radiolabeled with ^3H or ^14C. The ^3H-labeled heparin was a generous gift from Dr J. Hirsh and Dr E. Young (Henderson Civic Hospital Research Unit, Hamilton, Ontario, Canada) and had normal antithrombin III (ATIII) activity. The ^14C-heparin was obtained from NEN/Dupont (Boston, MA) and had normal anticoagulant activity (130 IU/mg). Identical results were obtained using either radiolabeled preparation of heparin.

**Interaction of Patient IgG With Heparin Alone**

Binding studies using patient IgG immobilized on protein A-Sepharose. The IgG from HIT patient sera (50 μL; n = 7) and negative control sera (n = 3) was adsorbed to protein A-Sepharose beads (50 μL of a 50% suspension), and the unbound proteins removed by two washes with phosphate-buffered saline (PBS) (2 x 1 mL). The beads were incubated for 1 hour at 22°C with ^3H-heparin (25 μL, 10 μg/mL, 440 μCi/mg). Unbound material was removed by washing four times, using PBS, and bound radioactivity eluted with 0.1 mol/L glycine, pH 3.0 (2 x 100 μL aliquots) and counted in a scintillation counter. All samples were run in duplicate.

**Ouchterlony analysis.** The purified IgG fraction (protein A-Sepharose) from two HIT sera and a negative control IgG fraction were tested for antiheparin reactivity by diffusion in agarose against heparin. The reactions were performed at different temperatures (4°C, 22°C, and 37°C) and at physiologic and low ionic strength (50 mmol/L).

**Equilibrium dialysis.** The purified IgG fraction (protein A-Sepharose) from two HIT sera and a negative control IgG fraction were concentrated to 30 mg/mL (200 μmol/L). One-milliliter aliquots of the IgG fractions or 1 mL of buffer were added to 50,000 molecular weight exclusion-limit dialysis bags (Spectra/Por 6; Fischer Scientific, Toronto, Ontario, Canada). The bags were submerged in 20 mL of PBS, pH 7.2, containing ^3H-heparin (40 μg, 0.25 μmol/L, specific activity 2.5 x 10^6 dpm/mg) and left to reach equilibrium for 72 hours at 4°C. The bags were removed and washed, and the contents were recovered. Aliquots of the contents and the dialysate were counted.

**Ammonium sulphate and polyethylene glycol (PEG 6000) precipitation analysis.** Initial experiments showed that ^3H-heparin was not precipitated by 50% saturated ammonium sulfate, (NH₄)₂SO₄, or by PEG 6000 (4%, 8%, or 12%). The IgG fractions (50 μL, 30 mg/mL) from two positive HIT sera and a negative serum were incubated with ^3H-heparin (25 μL, 4.25 μg/mL), in the presence of buffer (25 μL) or 100-fold excess of unlabeled "cold" heparin (25 μL, 425 μg/mL) for 2 hours at 22°C and a further 1 hour at 4°C.
Fig 1. Percent release of $^{3}C$ serotonin (ordinate) compared with the heparin concentration (abscissa) for the platelet release reaction. In (A), platelets were preincubated with increasing concentrations of heparin, washed, and then incubated with HIT or negative control sera without further addition of heparin. In (B), platelets were preincubated with heparin (100 IU/mL), washed, and then incubated with heparin (0, 0.1, 100 IU/mL) and HIT or negative control sera. The 0, 0.1, and 100 IU/mL heparin test samples were incubated also with the MoAb IV.3. In (C), platelets were preincubated with HIT or control sera, washed, and then incubated with heparin (0, 0.1, 100 IU/mL). Panel (D) shows the standard serotonin release assay using platelets, sera, and heparin incubated together. These graphs are representative of 10 different experiments all giving similar results.

An equal volume of saturated (NH$_4$)$_2$SO$_4$ was added and the mixture left for 15 minutes at 4°C. The samples were centrifuged, a sample of the supernatant was removed for counting of the free (unbound) heparin, and the precipitate was washed with cold, 50% saturated (NH$_4$)$_2$SO$_4$. The washed precipitates were dissolved in PBS and counted.

Similar experiments were performed using PEG 6000 solutions in place of the saturated ammonium sulphate solutions (12% final concentration) to precipitate the Igs.

**Interaction of Patient IgG With Heparin and Platelet Proteins**

**Binding studies using patient IgG immobilized on Protein A-Sepharose.** The IgG from HIT patient sera (n = 7) and negative control sera (n = 3) (50 μL) were adsorbed to protein A-Sepharose beads (50 μL of a 50%) suspension), and the unbound proteins removed by washes with PBS. The beads were incubated for 1 hour at 22°C with $^{3}H$-heparin (25 μL, 10 μg/mL, 440 μCi/mg) in the presence of platelet releasate (25 μL, from 2 U/mL thrombin-stimulated, washed platelets at 10$^{9}$/mL) or in the presence of purified platelet proteins (PF4, vWF, TSP, Fn, and albumin as a negative control, 25 μL at various concentrations covering the range 0.2, 2, and 20 μg/mL). Unbound material was removed by washing four times with PBS and bound radioactivity eluted with 0.1 mol/L glycine, pH 3.0 (2 × 100 μL aliquots) and counted in a scintillation counter. All tests were performed in duplicate.

The platelet proteins known to bind to heparin, including PF4, vWF, and TSP, were specifically removed from platelet releasate by immunodepletion using rabbit IgG against PF4 (American Diagnostica, Greenwich, CT), anti-TSP (MoAb prepared in our laboratory), and rabbit anti-vWF (Dako, Dimension Laboratories Inc, Mississauga, Ontario, Canada), each immobilized on Sepharose beads. Negative control beads were processed similarly, using IgG of anti-albumin (Dako, Dimension Laboratories Inc). The IgG fractions of the various antisera (250 μg) were coupled to 1 mL of CNBr-activated Sepharose beads (Pharmacia) using the manufacturer's directions. Platelet releasate (25 μL) was
Heparin-induced thrombocytopenia

<table>
<thead>
<tr>
<th>Compound</th>
<th>Degree of Sulfation</th>
<th>Molecular Weight</th>
<th>Concentration Range</th>
<th>Ability to Support the Release Reaction With HIT sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>1-1.2</td>
<td>12-15,000</td>
<td>0.05-50</td>
<td>+</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>0.3-0.6</td>
<td>12-15,000</td>
<td>0.05-50</td>
<td>+</td>
</tr>
<tr>
<td>Chondroitin sulfate A</td>
<td>0.5</td>
<td>25-50,000</td>
<td>0.02-20</td>
<td>-</td>
</tr>
<tr>
<td>Chondroitin sulfate B</td>
<td>0.5</td>
<td>25-50,000</td>
<td>0.02-20</td>
<td>-</td>
</tr>
<tr>
<td>Chondroitin sulfate C</td>
<td>0.5</td>
<td>25-50,000</td>
<td>0.02-20</td>
<td>-</td>
</tr>
<tr>
<td>Pentosan polysulfate</td>
<td>2</td>
<td>5,000</td>
<td>0.14-140</td>
<td>+</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>3</td>
<td>6-8,000</td>
<td>0.1-100</td>
<td>+</td>
</tr>
<tr>
<td>Glucose 1,3,6-trisulfate</td>
<td>3</td>
<td>420</td>
<td>0.1-1,000</td>
<td>-</td>
</tr>
<tr>
<td>1,2-Cyclohexaneol disulfate</td>
<td>2</td>
<td>276</td>
<td>0.1-1,000</td>
<td>-</td>
</tr>
<tr>
<td>1,4-Cyclohexaneol disulfate</td>
<td>2</td>
<td>276</td>
<td>0.1-1,000</td>
<td>-</td>
</tr>
<tr>
<td>Heparin disaccharide 1-S</td>
<td>1.5</td>
<td>665</td>
<td>0.1-1,000</td>
<td>-</td>
</tr>
</tbody>
</table>

The degree of sulfation is given as the average number of sulfate groups per saccharide unit (or equivalent unit). The low average degree of sulfation for heparan sulfate does not reflect the higher degree of sulfation that occurs in localized areas of the molecule. The analogues were tested using concentrations covering several orders of magnitude and the molarities shown are based on the mean molecular weight. Analogues that supported platelet release at low and intermediate concentrations (heparin, heparan sulfate, pentosan polysulfate, and dextran sulfate) caused high-dose inhibition of release.

Results are expressed as log platelet fluorescent intensity (arbitrary units).

**RESULTS**

**Platelet Release Assay**

The type of sulfated compound required to support the reaction of HIT IgG with platelets was relatively restricted. Only highly sulfated, polymeric saccharides were able to substitute for heparin in the HIT release reaction (Table 1). In contrast, low molecular weight highly sulfated compounds or large molecular weight but poorly sulfated polysaccharides were not able to substitute for heparin in the HIT release assay (Table 1).

Control platelets were preincubated with heparin in increasing concentrations from 0 to 1,000 IU/mL, then washed, and incubated with HIT sera. These platelets demonstrated a saturable dose-dependent platelet release. Serotonin release increased from 0% to 75% (0 to 1.0 IU/mL) (Fig 1A). Preincubation of the platelets with very high concentrations of heparin (>100 IU/mL) followed by washing away the bound heparin, resulted in a plateau of platelet release without any inhibition at the high concentrations of heparin. These results contrast to the results when high doses of heparin and patient sera are present together with platelets in the fluid phase (Fig 1D).

Because the standard serotonin release assay shows inhibition of release using 100 IU/mL heparin (Fig 1D), we tested whether the washed platelet-derived heparin would also behave in a similar fashion. Platelets, which had been preincubated with heparin (100 IU/mL) and then washed, were tested for release using patient or control sera in the presence of 0, 0.1, and 100 IU/mL heparin (Fig 1B). Positive serotonin release was observed with HIT patient sera only. This release was inhibited by high heparin concentrations and also by MoAb IV 3 (Fig 1B).

The same experiments were repeated, except that patient serum was preincubated with the platelets, the platelets were washed, and various heparin concentrations (0 to 100 IU/mL final) were added. No patient serum sample (three tested) produced platelet release (Fig 1C).

**Interaction of HIT IgG With Heparin and Platelet Proteins in a Platelet-Free System**

**Interaction of Patient IgG and Heparin**

There was no binding of 3H-heparin to IgG isolated from HIT sera using IgG immobilized on protein A-Sepharose beads (Fig 2). Large amounts of IgG were prepared using protein A-Sepharose affinity chromatography from two sera of patients with HIT. These IgG fractions gave positive results in the standard serotonin release assay. Each IgG was then tested for its ability to react with heparin. No precipitation lines were seen by Ouchterlony double-diffusion analysis using the IgG fractions and heparin despite using a variety of conditions of temperature and ionic strength. To investigate potential low-affinity interactions, high concentrations of IgG (30 mg/mL) from an HIT serum was analyzed by equilibrium dialysis. The amount of 125I-heparin bound to patient IgG in the dialysis bag (1.6% of the total counts added) was not different from that seen using control IgG at a similar concentration (1.8% of the total counts added). The specific activity of a dialyzed buffer sample was the...
same as the $^{125}$I-heparin dialysate, showing that equilibrium had been reached. From the Law of Mass Action it can be calculated that the concentration of IgG in the dialysis bag was sufficient to detect about 5% specific binding of the label by an antibody having an association constant ($K_a$) as low as $10^4$ L/mol and assuming only 1% of the total IgG was antigen specific. For higher-affinity antibodies or a higher percentage of specific antibody the binding of label would be expected to be even greater than 5%.

Further experiments also failed to show any binding of $^{125}$I-heparin by the HIT IgG serum using ammonium sulfate precipitation. Patient IgG had the same binding (2.3% of total counts precipitated) as a negative serum (2.0% of total counts precipitated). To exclude the possibility that ammonium sulfate interfered with the binding of heparin to antibodies present in the positive antiserum, the precipitation was repeated using PEG precipitation. Again, there was no difference in binding between the IgG of HIT sera and a negative control serum.

Interaction of Patient IgG With Heparin and Platelet Proteins

Binding of radiolabeled heparin to IgG immobilized on a solid phase. There was no binding of the $^3$H-heparin to IgG isolated from HIT sera, when the IgG was immobilized on protein A-Sepharose beads (Fig 2). However, when platelet releasate was added to the $^3$H-heparin and the IgG beads, heparin binding increased up to 50 times for the HIT IgG compared with the control IgG (Fig 2). Platelet lysate behaved identically to the platelet releasate (results not shown). Similar results were obtained when purified PF4 was substituted for the platelet releasate. In these studies, heparin binding occurred only with HIT sera and not with negative sera (Fig 3). Other heparin-binding platelet proteins could not substitute for PF4. No heparin binding by HIT IgG beads was seen when vWF, thrombospondin, fibronectin, or albumin (negative control) were substituted for PF4 and tested at several concentrations (Fig 3).

To confirm these results, platelet releasate was tested for its ability to support heparin-dependent binding to HIT patient IgG after removal of various heparin-binding proteins. The platelet releasate was immunodepleted of vWF, thrombospondin, albumin, and PF4 by immunoaffinity chromatography (Fig 4). Only the PF4-immunodepleted releasate lost its ability to serve as a cofactor for heparin in binding to HIT IgG. The specificity of the immunoaffinity beads was confirmed by eluting the bound material and analyzing it by SDS-PAGE. Only the expected protein for the specific antibody were bound by each of the beads.

Flow cytometry. Using FITC-conjugated IgG of rabbit antihuman PF4 (F/P 3.07) and FITC-conjugated normal rabbit IgG (F/P 2.90) as negative control gave platelet fluorescent intensities of (log scale) 47 ± 2.2 and 18 ± 0.9 U, respectively. These results are the means of three separate determinations and indicate a low level of PF4 on the platelet surface.

DISCUSSION

HIT is probably the most important allergic drug reaction that hematologists must manage. Most patients only have isolated thrombocytopenia, a complication which usually does not cause major morbidity. However, a subset of the thrombocytopenic patients (about 10%) will have thrombotic complications caused by the syndrome and can have lifelong disability from a stroke or ischemic damage to a limb that often requires amputation. Physicians now recognize the clinical importance of HIT. Yet, the pathophysiology of HIT remains uncertain and debated. In vitro studies from several laboratories have shown that HIT patient IgG activates the platelets through the platelet Fc receptor. Although this platelet interaction is generally accepted, many other aspects of the interaction of the HIT IgG with heparin and platelets remain uncertain. In particular, there is uncertainty about whether heparin is a specific or nonspecific participant in the reactions, whether the IgG reacts directly with the heparin or requires a platelet component, and finally whether the
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Fig 3. Binding of [H]-heparin by patient or control IgG immobilized on protein A-Sepharose in the presence of selected platelet proteins that bind heparin (PF4, vWF, TSP, Fn) or control protein (albumin) (results shown for 20 µg/mL final concentration). Seven different positive patient sera were used (1, 2, 3, 5, 7, 9, 10), as well as three different negative sera (4, 6, 8). This study showed that PF4 serves as a cofactor for the binding of HIT patient IgG with heparin.

interaction occurs in the fluid phase or on the platelet surface. In this report we describe studies investigating the various components of the interaction of HIT with heparin and the platelet membrane.

General Characteristics of the Sulfated Polysaccharides That Participate in the In Vitro Reaction

The interaction of HIT serum with platelet has been shown to be supported by both standard heparin and most low molecular weight heparins. In recent years, investigators demonstrated that other sulfated polysaccharides could support the reaction. These observations suggest that the reaction is more complex, or alternatively the heparin might be a nonspecific participant, which would function to increase the likelihood of platelet/platelet interaction, but might not directly participate in the reaction. We investigated this issue by testing the ability of a variety of large and small sulfated compounds and heparin analogues for their ability to interact with HIT IgG in the platelet-release assay. Consistent with the work of Wolf et al, Anderson, and Greinacher et al, we found that other highly sulfated polysaccharides could substitute for heparin in the reaction. Once the degree of sulfation had fallen below a certain critical level (approximately 0.5 sulfate per saccharide), the heparin analogue was no longer able to support the reaction. We also found that reactivity required a certain minimum size of a sulfated polysaccharide to participate in the reaction. Hence, highly sulfated larger compounds (pentosan polysulfate and dextran sulfate) were able to support the reaction, whereas equally sulfated, but smaller compounds (molecular weight <1,000) were unable to support the reaction. Together, these observations indicate that both polysaccharide chain length and degree of sulfation play a critical role in supporting the reaction. However, the ability of a number of different sulfated polysaccharides to substitute for heparin should not be interpreted to indicate that this is a nonspecific reaction. Rather, a variety of similar molecules can trigger the reaction both in vitro and in vivo, as has been shown by development of the syndrome in patients who receive heparin-like substances.

Reactivity of HIT-IgG With Heparin

Some investigators have suggested that serum from patients with HIT or the IgG fraction can react with heparin by itself. Others have not observed this reaction and suggested that a direct immunologic recognition of heparin by the HIT IgG did not occur. However, because of the methods used, it is possible that binding of the IgG to the heparin did occur, but could not be detected because it was below the limits of the sensitivity of the assay. Hence, we looked
for an HIT IgG interaction using Ouchterlony, ammonium sulfate, and PEG immunoprecipitation, and equilibrium dialysis in the cold. None of these techniques showed any interaction of HIT IgG with heparin above background levels, indicating that the antibodies in these patients do not recognize nor interact with heparin by itself.

Interaction of HIT/IgG With Heparin and a Platelet Component

Because we were unable to show any interaction of the HIT IgG with heparin by itself, we investigated whether a platelet component was required as a cofactor. A putative cofactor for binding with the heparin (and other sulfated polysaccharides) has also been the subject of some debate. Some investigators have indicated that HIT IgG can react with heparin by itself. However, Amiral et al have reported that PF4 in conjunction with heparin was the target for HIT IgG. These investigators used an enzyme-linked immunosorbent assay with PF4 and heparin on the plates to study the binding of HIT IgG. We approached this issue in a different fashion: Although the serotonin release assay for HIT is both a sensitive and specific assay, it uses intact platelets and one cannot dissect the components required for reactivity. We bound HIT IgG to a solid phase (protein A-Sepharose beads). We found that the immobilized HIT IgG was nonreactive with heparin. However, when platelet releasate was added, an approximately 100-fold increase in the binding of \(^{3}H\)-heparin to the HIT IgG was noted (Fig 2). In the next series of studies we investigated purified proteins and also immunodepleted the platelet releasate to identify that component which supported the reaction. Only platelet releasate immunodepleted of PF4 was no longer reactive (Fig 4). Hence, although several platelet proteins including TSP, vWF, and Fn all have been shown to bind heparin, none was capable of forming the reactive site for the HIT IgG.

Sequence of the Reaction

Patients with HIT were first shown to activate platelets using PRP and subsequently also using a washed platelet system. Our studies indicated that the target antigen of HIT-IgG was a complex of PF4 and heparin. However, they did not indicate whether the heparin/PF4 IgG reacted in solution, or whether the reaction occurred on the platelet membrane. To separate which of these possibilities could be responsible, we returned to the intact platelet system with serotonin release measured as the endpoint. Platelets were either mixed with HIT serum then washed and heparin added, or platelets were mixed with various concentrations of heparin and then after washing, HIT serum was added. As shown in Fig 1, the only reactive platelets were those that had heparin (at any concentration) added to it, then washed before the addition of HIT serum. These results indicate that the heparin/PF4 complex is formed on the platelet surface before interaction of the HIT IgG with this complex. Activation of platelets occurs via the platelet Fc receptor. As shown in the present study by flow cytometric analysis, PF4 is present on the surface of washed and resting platelets, and this presumably represents the binding site for the heparin. These latter observations are also consistent with earlier studies that noted PF4 on the surface of both resting and activated platelets.

Together, these studies suggest a potential model for HIT: Susceptible patients develop an antibody to a complex of
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heparin (or other large, polysulfated polysaccharides) and PF4. The HIT IgG/heparin PF4 immune complexes form on the surface of the platelets, allowing the Fc portion of the IgG to activate the platelets through their Fc receptors. It remains uncertain whether the actual recognition epitope represents a shared site between heparin and the PF4, or whether the heparin induces conformational changes in the platelet PF4.

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Immunoglobulin G from patients with heparin-induced thrombocytopenia binds to a complex of heparin and platelet factor 4

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