Heparin-Associated Thrombocytopenia: Antibody Binding Specificity to Platelet Antigens

By Dona M. Lynch and Stephen E. Howe

Sera from four patients with heparin-associated thrombocytopenia (HAT) were evaluated by a quantitative enzyme-linked immunosorbent assay (ELISA) to detect heparin-dependent serum platelet-bindable immunoglobulin (S-PBIg) and by Western blotting and immunoprecipitation to investigate the specificity of the antibody binding. All HAT sera showed mildly increased S-PBIg (mean: 7.8 fg per platelet; normal: <6.0 fg per platelet) to intact target platelets in the ELISA, which was markedly increased in the presence of heparin (mean: 20.9 fg per platelet). This increase was 20-fold greater than normal control sera, which showed a mean differential increase of only 0.5 fg per platelet. Immunoglobulin binding specificity to platelet antigens was investigated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis of platelet lysate with transfer of the platelet fractions onto nitrocellulose strips (Western blotting) and subsequent immunosassay using HAT and normal sera. In the presence of heparin, the four HAT patients demonstrated increased binding of immunoglobulin to platelet antigens of apparent molecular weights of 180, 124, and 82 kd. Radiolabeled heparin when incubated with HAT sera, normal sera, or albumin blanks bound to platelet proteins of the same apparent molecular weights. These observations are consistent with current hypotheses suggesting that HAT antibody is directed to heparin-platelet complexes or, alternatively, that heparin induces conformational change of antigenic sites on the platelet membrane.

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by three washes with PBS-Tween. Optimally tethered (1:500) horse radish peroxidase-conjugated goat F(ab')2, anti-human immunoglobulin (Cappel), in 100-μL volumes, was added to all wells and incubated at room temperature for 90 minutes, followed by a ten-minute PBS-Tween-gelatin incubation and two washes with PBS-Tween. OPD substrate (40 mg o-phenylenediamine dihydrochloride dissolved in 100 mL of phosphate citrate buffer [24.3 mL of 0.1 mol/L citric acid, 25.7 mL of 0.2 mol/L Na₂HPO₄, 50 mL of H₂O], 0.15 mL of 30% H₂O₂ added prior to use) was added in 20 minutes with the addition of 100 mL of 0.2 mol/L H₂SO₄, and the absorbance was read at 490 nm with an EIA Reader (Bio-tek, Burlington, Vi). S-PBlg in femtograms per platelet was calculated using previously standardized binding percentages of the platelets and standards. In the lot of microtiter plates tested, 1.4 × 10⁶ platelets remained in the well at the conclusion of the assay. After incubation with patient sera, absorbances were compared to a standard reference curve to obtain nanograms of Ig per well. This value was then divided by the number of platelets per well to obtain S-PBlg in femtograms per platelet.

**Iodination of heparin.** Beef lung heparin, 0.5 mL in a concentration of 10⁸ U/mL, was iodinated with ¹²⁵I (New England Nuclear, Boston) using the iodogen method of Markwell. After iodination, the heparin was extensively diazylated with a Spectrapor 40 membrane (Spectrum Medical, Los Angeles) against PBS with 1% (wt/vol) KI. The extent and quality of iodination was determined by the modified thin-layer chromatography method of Rosenberg and Teare using ITLC-SG paper (Gelman Sciences, Ann Arbor, Mich). The specific activity of the diazylated heparin was approximately 10⁶ dpm/μL. The biological activity of the iodinated heparin was not evaluated.

**Iodination of platelet surface proteins.** One milliliter of a washed suspension of platelets at 10⁹ cells per milliliter in PBS was iodinated with ¹²⁵I (New England Nuclear) using the iodogen method. After iodination, the suspension was dialyzed as above, centrifuged, and washed once with PBS. The dialysate was centrifuged at 1,200 g for five minutes to pellet the platelets. The labeled platelet pellet was solubilized in 3% SDS for SDS-PAGE electrophoresis and autoradiography or solubilized in Triton X-100 for immunoprecipitation.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/Western blotting.** SDS-PAGE was performed using the method of Laemmli. Pooled PBS (with 0.2 mmol/L EGTA as a protease inhibitor)-washed platelets were solubilized with sample buffer (12.5 mL of 0.5 mol/L Tris base, pH 6.8, 10 mL glycerol, 3 g SDS, and 0.003 g bromphenol blue in 100 mL distilled H₂O). The nonreduced lysate was adjusted to a final concentration of 10⁶ platelets per milliliter. The platelet lysate and molecular weight standards (Bio-Rad Laboratories, Richmond, Calif) were separated using a lateral electrical field (Trans-Blot Cell, Bio-Rad) of 280 mA for four hours at 4 °C. Transferred proteins were stained with Coomassie Brilliant Blue, the gels were dried, and autoradiography was performed.

**Immunoprecipitation.** Immunoprecipitation was performed using a modification of the method of Chong et al. Fresh washed platelets were surface-labeled with ¹²⁵I by the iodogen method described. After dialysis, the labeled platelets were resuspended to 5 × 10⁸ per milliliter in PBS with 0.2 mmol/L EGTA. To this suspension 200 μg/mL leupeptin and 0.5 mmol/L phenylmethylsulfonyl fluoride was added and solubilized with a final concentration of 2% (vol/vol) Triton X-100 (Bio-Rad). The solubilized surface-labeled platelets were centrifuged at 1,200 g for ten minutes to sediment the insoluble platelet fractions. A 50-μL aliquot of solubilized platelets was added to 50 μL of patient serum with or without beef lung heparin (final concentration, 5 U/mL). The mixture was incubated at 37 °C for 90 minutes. Following incubation, 50 μL of a 1% suspension of protein A-CB Sepharose (Pharmacia, Uppsala, Sweden), in PBS-EGTA with 1% Triton X-100, was added and allowed to equilibrate for 30 minutes at 4 °C. The Sepharose sediment was washed two times with PBS-EGTA with 2% (wt/vol) bovine serum albumin, and once with PBS-EGTA alone, with centrifugation at 18,000 g for three minutes between washes. Following the last wash, the pellet was solubilized in 3% SDS sample buffer, boiling for ten minutes. The solubilized sample was centrifuged as above and loaded into wells of an 8.5% SDS-PAGE slab gel, electrophoresis performed as described. The proteins were stained with Coomassie Brilliant Blue, the gels were dried, and autoradiography was performed.

**Western blotting.** Nitrocellulose strips incubated with radiolabeled heparin and the dried immunoprecipitate gel were autoradiographed using X-Omat RP film (Eastman Kodak Company, Rochester, NY) with Cronex Lightening Plus intensifying screens (DuPont, Wilmington, Del) at −70 °C for up to one month, depending on radioactivity levels.

**RESULTS**

**Patients.** Clinical data for the HAT patients are presented in Table 1. On admission, all HAT patients had normal platelet counts, which decreased to 12,000 to 70,000 per microliter after nine to 12 days of heparin therapy. Three patients developed pulmonary emboli while receiving heparin, and the fourth showed severe retroperitoneal hemorrhage. Prior to heparin therapy, all coagulation tests were normal. There was no evidence of disseminated intravascular coagulation, although the fibrin split products (FSPs) became slightly elevated in the three patients with pulmonary emboli and this was attributed to clot lysis. Two of four HAT serum samples aggregated test platelets when incubated with beef lung heparin. Plasma from patient 3 induced spontaneous platelet aggregation in the absence of exogenous heparin. The specimen tested was obtained three days after...
cessation of heparin therapy. Although no active heparin was detected using a thrombin time assay, no attempt was made to exclude low levels of heparin not detected by this assay or the presence of inactive antigenic heparin.

**ELISA.** Four HAT patient serum samples, 13 normal control serum samples, and combined control groups (N = 43) were tested with and without heparin in the ELISA procedure. The results are presented in Table 2. All HAT patient sera demonstrated mildly elevated baseline S-PBIg (no heparin), with a mean value of 7.8 fg per platelet (normal, <6.0 fg per platelet). Coincubations of heparin, serum, and target platelets produced a markedly increased S-PBIg (no heparin), with a mean value of 7.8 fg per platelet, a differential increase of 168%. Normal control sera showed a mean increase of only 0.5 fg per platelet, a 16% differential increase. The increase demonstrated in the HAT group was 20-fold greater than that of the normal controls. To further evaluate this elevation of S-PBIg after heparin coincubations, we similarly tested ten patients with ATP, ten patients with nonimmune thrombocytopenia, and ten nonthrombocytopenic patients following therapeutic IV heparin therapy. For the combined control groups, there was only a relatively slight increase of 0.7 fg per platelet, or 12% above baseline S-PBIg with heparin coincubations (Fig 1).

**Western blot.** Western blots performed on four HAT serum samples in the presence of heparin showed increased binding of immunoglobulin to platelet membrane antigens with apparent molecular weights of 180, 124, and 82 kd (Table 3). Representative nitrocellulose strips with and without heparin coincubations from one HAT patient and one normal control are presented in Fig 2. These platelet antigens were determined to be surface components by iodination of intact platelets. In general, as described in Table 3, there were low but detectable levels of immunoglobulin bound to these antigens in the absence of added exogenous heparin. However, in the presence of heparin, binding to these antigens was greatly enhanced, paralleling the ELISA results. Western blots performed on ten normal and ten ATP patients showed no enhancement of immunoglobulin binding to any platelet antigen with heparin coincubation.

**Autoradiography.** Binding of heparin to platelet fractions in the Western blot were studied using radiolabeled heparin. Nitrocellulose strips containing transferred platelet proteins were incubated with iodinated heparin and test sera. After Western blotting was completed with localization of precipitated immunoglobulin bands, these strips were autoradiographed. In the presence of HAT sera, normal control sera, and bovine serum albumin blank, 125I-labeled heparin bound most strongly to platelet fractions of approximate molecular weights of 180, 124, and 82 kd. The iodinated heparin bound to these antigens independent of immunoglobulin detected in the immunoperoxidase system. Heparin

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>No. of Platelets on Admission</th>
<th>Platelet No.</th>
<th>Onset of HAT*</th>
<th>Average Heparin†</th>
<th>PTT (s)</th>
<th>Fibrinogen (mg/dL)</th>
<th>FSP (µg/mL)</th>
<th>Aggregation Platelet</th>
<th>Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>250,000</td>
<td>17,000</td>
<td>12</td>
<td>1,600</td>
<td>30</td>
<td>294</td>
<td>40–80</td>
<td>Positive</td>
<td>Pulmonary embolus‡</td>
</tr>
<tr>
<td>2</td>
<td>252,000</td>
<td>19,000</td>
<td>8</td>
<td>28,000</td>
<td>31</td>
<td>320</td>
<td>40–80</td>
<td>Positive</td>
<td>Pulmonary embolus‡</td>
</tr>
<tr>
<td>3</td>
<td>280,000</td>
<td>12,000</td>
<td>7</td>
<td>5,000</td>
<td>31</td>
<td>400</td>
<td>40–80</td>
<td>Spontaneous</td>
<td>Pulmonary embolus‡</td>
</tr>
<tr>
<td>4</td>
<td>Adequate§</td>
<td>70,000</td>
<td>9</td>
<td>3,000</td>
<td>26</td>
<td>190</td>
<td>&lt;10</td>
<td>Negative</td>
<td>Retropertioneal hemorrhage</td>
</tr>
</tbody>
</table>

Normal (150–400,000) — — (25–35) (180–400) (<10) Negative —

*No. of days after heparin therapy was initiated.
†Average dose of heparin infused (U/d).
‡Patient expired.
§Admission slide was estimated to be adequate.

Table 2. Comparison of S-PBIg From HAT, Normal, and Combined Control Sera Using an ELISA Platelet Antibody Procedure to Quantitate the Increase of Immunoglobulin Binding in the Presence of Heparin

<table>
<thead>
<tr>
<th></th>
<th>HAT*</th>
<th>Normal†</th>
<th>All Controls‡</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Base</td>
<td>Heparin§</td>
<td>Base</td>
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<tr>
<td>Mean</td>
<td>7.8</td>
<td>20.9</td>
<td>3.1</td>
</tr>
<tr>
<td>SD</td>
<td>2.1</td>
<td>9.6</td>
<td>1.3</td>
</tr>
<tr>
<td>SEM</td>
<td>1.2</td>
<td>5.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Increase†</td>
<td>13.1</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Range‡</td>
<td>(7.8–26.1)</td>
<td>(0.3–2.8)</td>
<td>(1.2–2.8)</td>
</tr>
</tbody>
</table>

Values are expressed as femtograms per platelet.
*Four HAT patient sera.
†Thirteen normal control sera.
‡Forty-three patients in all control groups (see text).
§Heparin co-incubations (5 U/mL, final concentration).
†Mean differential increase of S-PBIg with heparin over base.
‡Range of differential increases seen in the groups.

Fig 1. Differential binding of S-PBIg from the platelet antibody ELISA in the absence (C) or presence (B) of heparin. The groups are: (1) four HAT patients, (2) 13 normal individuals, (3) ten non-thrombocytopenic patients after IV heparin therapy, (4) ten patients with autoimmune thrombocytopenia purpura, and (5) ten patients with nonimmune thrombocytopenia. Bars indicate the SEM.
Table 3. Antibody From HAT Patients to Platelet Glycoproteins as Identified by Western Blotting

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Baseline Serum Bands</th>
<th>Heparin and Serum Bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>180</td>
<td>180 ++</td>
</tr>
<tr>
<td></td>
<td>124</td>
<td>124 ++</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>82 ++</td>
</tr>
<tr>
<td>2</td>
<td>180</td>
<td>180 ++</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>124 ++</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>82 ++</td>
</tr>
<tr>
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<tr>
<td>3</td>
<td>180</td>
<td>180 +</td>
</tr>
<tr>
<td></td>
<td>124 +</td>
<td>124 ++</td>
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<tr>
<td></td>
<td>82</td>
<td>82 +</td>
</tr>
<tr>
<td>4</td>
<td>180</td>
<td>180 + +</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>124 + +</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>82 +</td>
</tr>
</tbody>
</table>

Values are expressed as apparent molecular weights (kilodaltons). +, +++, increased or markedly increased antibody binding, respectively.

qualitatively appeared to bind strongest at the 180- and 82-kd fractions (Fig 3). The intensity of the bound labeled heparin did not necessarily correlate with the intensity of the bound immunoglobulin detected by Western blotting.

Immunoprecipitation of radiolabeled surface platelet components with heparin-enhanced platelet antibody. Im-

Fig 2. Nitrocellulose strips from the Western blot procedure comparing a representative HAT patient with a pooled normal control. Lane 1 is native HAT serum with faintly detectable immunoglobulin binding to platelet proteins at apparent molecular weights of 180 and 82 kd. Lane 2 is HAT serum from the patient in lane 1 with 5 U/mL of heparin coincubated with the nitrocellulose strips and shows increased immunoglobulin binding to these same antigenic sites plus a prominent band appearing at 124 kd. Lane 3 is normal pooled sera. Lane 4 is normal pooled sera with 5 U/mL of heparin coincubated. Lanes 1, 3, and 4 show only minimal background binding to platelet fractions. Molecular weight determinations were based on molecular weight standards electrophoresed along with the patients and controls, and consisted of: myosin (200 kd), β-galactosidase (116 kd), phosphorylase B (92.5 kd), bovine serum albumin (66 kd), and ovalbumin (45 kd).

Fig 3. An autoradiograph of 125I-labeled heparin bound to platelet fractions separated on 8.5% SDS-PAGE and transferred to nitrocellulose strips. Faint binding of heparin was concentrated primarily to antigens of apparent molecular weights of 180, 124, and 82 kd as indicated.

Immunoprecipitation of surface-labeled Triton X-100-solubilized platelets was performed with HAT, ATP, and normal sera using protein A bound to Sepharose. Precipitated fractions were electrophoresed and autoradiography was performed (Fig 4). Weak precipitation was demonstrated by the HAT sera of a protein fraction migrating at 82 kd, which appeared qualitatively to be enhanced in two HAT patients in the presence of added heparin. These bands were not precipitated by control sera. Following the albumin fraction at approximately 64 kd, a protein fraction was precipitated by HAT sera. This precipitated protein was not detected in the Western blotting procedure and may represent a product of proteolysis.

DISCUSSION

Immune mechanisms have been described by several investigators in studies of HAT. A variety of techniques have been used to demonstrate heparin-dependent platelet antibodies in HAT patients' sera. The classes of immunoglobulin identified in these studies have included IgG, IgM, and IgG-IgA, and the complement component C3. In the present study, sera from HAT patients and sera from four control groups were evaluated using an ELISA procedure to determine the effect
of heparin on immunoglobulin binding to intact platelets. To determine the binding specificities of heparin and immunoglobulin to platelet fractions, Western blotting was performed. Immunoprecipitation of surface-labeled platelet fractions was then attempted to evaluate the significance of these binding specificities.

Using the ELISA platelet antibody procedure, HAT patient sera demonstrated elevated levels of S-PBlg (mean, 7.8 fg per platelet), comparable to ATP patients tested (mean S-PBlg, 9.0 fg per platelet), substantiating the primary immunologic nature of the syndrome. Normal controls and nonimmune thrombocytopenic patients had S-PBlg values within the normal range of <6.0 fg per platelet (Fig 1). In the baseline measurements of S-PBlg, no attempt was made to eliminate the effect of endogenous heparin. In the presence of heparin added in vitro, HAT sera showed enhancement of immunoglobulin binding (Table 2). The importance of additional exogenous heparin in the interaction of the HAT antibody with platelet antigen was evident in the 20-fold increase of bound immunoglobulin in the HAT group, as compared with the indicated control groups of non-HAT individuals.

The specificity of the heparin-enhanced immunoglobulin binding to platelet antigen was assessed using SDS-PAGE, Western blotting, and immunoprecipitation. In the presence of heparin, all HAT patient sera showed the appearance and/or augmentation of immunoglobulin bound to three platelet antigens of apparent molecular weights of 180, 124, and 82 kd. We were able to confirm only the antigenic fraction migrating at 82 kd to be surface-associated by immunoprecipitation with HAT sera. We were not able to detect significant immunoprecipitation of those determinants at 180 and 124 kd, and therefore could not substantiate that these antigens were in fact surface-associated. However, this does not exclude the possibility that these antigens are surface-associated, as the sensitivity of the technique may not be adequate or the antibody may be lacking in avidity. Another band of apparent molecular weight of approximately 60 kd was also detected by immunoprecipitation, but was not seen on the Western blots. This precipitation product may represent a product of proteolysis, and binding to this fraction was not appreciably augmented by added heparin. Patient 2 (Table 3) also showed immune binding to platelet fractions at 98 and 50 kd. These bands were not enhanced with heparin incubations and were perhaps a consequence of platelet transfusions received prior to testing. The four control groups described were tested in parallel and did not show increased immune binding by Western blotting to platelet fractions in the presence or absence of heparin.

Using 125I-labeled heparin in the Western blot procedure, specific binding of heparin occurred primarily to platelet fractions with electrophoretic migration of approximately 180, 124, and 82 kd, platelet fractions with the same apparent molecular weights as those bound by antibody in the HAT sera. A fainter band was also detected in the autoradiograph at approximately 145,000 daltons, which was not detected in the patients with HAT syndrome. The binding of heparin 125I to specific platelet fractions occurred in the presence of HAT sera, control sera, and albumin blanks.

We have not attempted to specifically identify the platelet constituents migrating with apparent molecular weights of 180, 124, and 82 kd that complex with heparin and HAT antibodies. Several platelet components, both surface glycoproteins, and α-granule proteins have been reported to bind heparin. Alpha-granule proteins such as thrombospondin (TSP) may become surface-associated after release and could function as a surface antigen. Of the known heparin-binding platelet proteins, TSP, with a molecular weight of 180 kd, a digestion product of TSP at 120 kd, and glycoprotein V (thrombin substrate) at 80 kd are provocative candidates as antigen for HAT antibody. Possible involvement of the thrombin substrate may explain the strong association in HAT patients with irreversible platelet aggregation, thrombosis, and thrombocytopenia.

Various mechanisms of heparin interaction in immune-mediated HAT have been proposed. Heparin has been suggested to function as a hapten, an antigen, a heparin–plasma protein complex, a platelet–heparin antigenic complex, or as an intermediate of an independent immune reaction. Although the data presented do not exclude theoretical models involving antibody directed to a hapten or heparin–platelet complex, the possibility of heparin and platelet–protein interaction involving charge relationships and conformational change of antigenic binding sites is suggestive. In the absence of exogenous heparin, platelet-directed antibody was detected by ELISA, Western blotting, and immunoprecipitation in HAT patient sera. These observations suggest that the antigenic sites were present on platelets in the absence or with low concentration of heparin. Immune binding to these platelet proteins was greatly enhanced when heparin was added to the system. One mode of action postulated for highly negatively charged preparations such as heparin involves the alteration of the
protein configuration of platelet membranes after binding, resulting in new antigenic sites, or making existing sites on the platelet surface more accessible. Although we did not test the effects of other polyglycosaminoglycans, Wolf and associates used heparin and the polysulfated glycosaminoglycan, Artepon, to compare the effect on platelet aggregation and platelet IgG binding when coincubated with suspected HAT sera. They found that heparin and Artepon caused platelet aggregation and positive indirect immunofluorescence reactions using whole IgG, F(ab')2, or Fc fractions from these patients. Singer and Nicholson's fluid mosaic model for biological membranes is consistent with the theory of altered antigenic sites. With highly charged molecules such as heparin bound to one surface receptor, alteration of the binding kinetics of another receptor may occur due to the dynamic nature of the hydrophobic bilayer arrangement of phospholipids. By binding to opposite charges on the exposed platelet membrane proteins, heparin may induce conformational changes of antigenic sites and effectively expose or access a sequestered amino acid sequence recognized by the immune system of a patient with HAT.

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