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 immunoblot 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.

PLATELET ANTIBODY has been demonstrated in patients with heparin-associated thrombocytopenia (HAT). In the presence of exogenous heparin, immunoglobulin in HAT sera binds to platelets and may induce platelet aggregation and release. Several theoretical models have been proposed to describe the clinical syndrome and immunologic mechanism; however, the interaction and binding specificities of HAT antibody and heparin to platelet membrane antigen have remained obscure. In this study, the quantitative binding of HAT antibody to platelet surface was determined using an enzyme-linked immunosorbent assay (ELISA) procedure. The binding specificities of the antibody and heparin to platelet fractions were investigated using Western blotting and immunoprecipitation.

MATERIALS AND METHODS

Patients and control groups. Group 1 consisted of four patients with severe delayed HAT. Medical histories included a normal platelet count (150,000 to 400,000 platelets per microliter) prior to heparin therapy, a decrease in platelet count below 80,000 per microliter during heparin administration, an increase in platelet count within ten days after discontinuing heparin administration, and the exclusion of other causes of thrombocytopenia.

Group 2 consisted of 13 normal nonthrombocytopenic volunteers.

Group 3 consisted of ten nonthrombocytopenic patients one week after intravenous (IV) heparin therapy.

Group 4 consisted of ten patients with autoimmune thrombocytopenic purpura (ATP) following the criteria of Lacey and Penner. Group 5 consisted of ten patients with nonimmune thrombocytopenia (4,000 to 60,000 per microliter) attributed to lymphoma, chemotherapy, sepsis, leukemia, or increased peripheral platelet destruction.

Specimens. Patients were evaluated after we obtained informed consent in accordance with our institution’s Committee on Human Experimentation. Specimens consisted of serum or plasma obtained by venipuncture using siliconized clot tubes or 0.11 mol/L sodium citrate (Becton Dickinson Vacutainer Systems, Rutherford, NJ). At the time the sera were obtained, no patients in these groups were being treated with heparin. To prevent possible immunoglobulin loss, sera was not heat-inactivated to destroy residual thrombin. Whole blood was centrifuged at 1,200 g for ten minutes. Plasma and sera were separated, tested immediately, or stored frozen at −70 °C. Sera were collected during the thrombocytopenic phase and prior to corticosteroid treatment in ATP patients. Platelet counts and routine coagulation values were obtained from the clinical laboratory. Heparin-induced platelet aggregations were performed independently in the clinical laboratory, as described by Kapsch and Silver. All chemicals were purchased from Sigma Chemical Company (St Louis) unless otherwise indicated.

ELISA for heparin-induced platelet-bindable immunoglobulin. A modification of an ELISA for serum platelet-bindable immunoglobulin (S-PBIg) was performed as previously described. Brieﬂy, the assay was as follows: pooled human platelets were washed in phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde. These platelets were added in amounts of 107 cells to wells of a Nunc Immunoplate I (Vanguard International, Neptune, NJ) and centrifuged at 1,000 g for ten minutes to adhere the platelets to the wells. A standard curve was prepared with quantitated chromatographically purified human IgG (Cappel Laboratories, Malvern, Pa) and cells adsorbed to the plate wells overnight at room temperature to obtain maximum binding. All wells were “blocked” with 0.1% gelatin (Difco Laboratories, Detroit) in PBS–0.05% Tween-20 for 90 minutes at room temperature. The plates were washed twice with PBS–0.05% Tween-20 using a Dynadrop SR Dispenser Unit (Dynatech Laboratories, Alexandria, Va). Triplicate aliquots of 100 μL of undiluted sera were added to microtiter wells as follows: (1) sera alone and (2) sera with 5 U/mL (final concentration) beef lung heparin (Organon, Orange, NJ) coincubated in the wells. Beef lung heparin was selected for use in this assay, as it provided the most pronounced platelet aggregations in the two patients positive for heparin-induced platelet aggregations. The ELISA plate was incubated for 60 minutes at 37 °C, drained, bloated, and incubated with PBS–Tween–gelatin for ten minutes. The gelatin incubation was followed.
by three washes with PBS-Tween. Optimally titrated (1:500) horse
radish peroxidase-conjugated goat F(ab')2 anti-human immunoglo-
bulin (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 dihydro-
chloride 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 Na2PO4, 50 mL
of H2O], 0.15 mL of 30% H2O2 added prior to use) was added in
20 minutes with the addition of 100 μL of 0.2 mol/L H2SO4, and
the absorbance was read at 490 nm with an EIA Reader (Bio-tek,
Burlington, Vt). S-PB1g 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 × 10^5
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
reaction was terminated with a tap water wash and the strips
were allowed to dry in the dark.

Immuno precipitation. Immunoprecipitation was performed us-
ing 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 × 10^11 per milliliter in PBS with 0.2 mmol/L EGTA. To this
suspension 200 μg/mL leupeptin and 0.5 mmol/L phenylmethyl-
sulfonyl fluoride was added and solubilized with a final concen-
tration 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 incuba-
tion, 50 μL of a 1% solution of protein A-CB Sepharose (Pharma-
cia, 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 twice with PBS-EGTA with
2% (wt/vol) bovine serum albumin, and once with PBS-EGTA
alone, with centrifugation at 18,000 g for 3 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.

SDS-PAGE and 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 into 100 mL distilled H2O). The
nonreduced lysate was adjusted to a final concentration of 10^6
platelets per milliliter. The platelet lysate and molecular weight
standards (Bio-Rad Laboratories, Richmond, Calif) were separated
with a 10-mA current for 18 hours on 8.5% polyacrylamide gels.
Prior to Western blotting, gel-separated proteins were stained with
Coomassie Blue (1.4 g Coomassie Blue G-250, 908 mL methanol,
908 mL H2O, and 184 mL glacial acetic acid) for three hours at
37°C and destained with three changes of 10% vol/vol acetic acid
and 10% vol/vol 2-propanol.

The proteins separated in the SDS-PAGE system were trans-
ferred using the basic method of Towbin and associates from the
gels to nitrocellulose strips (Schleicher and Schuell Inc, Keene, NH)
using a lateral electrical field (Trans-Blot Cell, Bio-Rad) of 280 mA
for four hours at 4°C. Transferred proteins were stained with 0.1%
Amido Black. Immunoglobulin bound to the transferred proteins
was detected using the following immunoperoxidase method. The
nitrocellulose strips were washed once in Western blot saline
([WBS] 9 g NaCl, 10 mmol/L Tris, and 0.05% nonidet P-40, 0.01% SDS,
in 1 L distilled H2O, pH 7.0). The washed strips were then
incubated with WBS-0.05% Tween-3% albumin (EIA grade bovine
albumin) for 60 minutes. All incubations were carried out at 37°C
with constant rocking. Following this "blocking" incubation, test
sera were diluted 1:10 in WBS-3% albumin, with and without
heparin (final concentration, heparin 5 U/mL) and were incubated with
the nitrocellulose strips for 60 minutes. The strips were washed in
WBS after incubation for a minimum of three changes over three
hours. Peroxidase-conjugated goat anti-human F(ab')2 immuno-
globulins (Cappel) at 1:300 was added to the washed strips and
incubated for 90 minutes. The strips were washed as above, then
added to substrate (1 vol of 4-chloro-1-naphthol [3 mg/mL metha-
nol]) and 0.018% (vol/vol) H2O2 and maintained at room tempera-
ture for visualization of protein bands, usually for about ten minutes.

The reaction was terminated with a tap water wash and the strips
were allowed to dry in the dark.

RESULTS

Patients. Clinical data for the HAT patients are pre-
sented 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 hepa-
rin, and the fourth showed severe retroperitoneal hemor-
rhage. 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 pulmo-
nary emboli and this was attributed to clot lysis. Two of four
HAT serum samples aggregated test platelets when coincu-
bated 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-PBlg (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-PBlg, with a mean increase of 20.9 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-PBlg 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-PBlg 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 1. Laboratory and Clinical Data for Patients With HAT

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>No. of Platelets on Admission</th>
<th>Platelet Nadir</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>Retropertoneal 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 inadequate.

Table 2. Comparison of S-PBlg 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>
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<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>
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<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>10.0</td>
<td>26.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Values are expressed as femtograms per platelet.
*Four HAT patient sera.
†Thirty 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-PBlg with heparin over base.
| Range of differential increases seen in the groups.

Fig 1. Differential binding of S-PBlg 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. Antibodies 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>2</td>
<td>124</td>
<td>124+++</td>
</tr>
<tr>
<td>3</td>
<td>82</td>
<td>82+++</td>
</tr>
<tr>
<td>4</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>
</tbody>
</table>

Values are expressed as apparent molecular weights (kilodaltons). +, increased; ++, 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. 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...
detect significant immunoprecipitation of those determinants at 180 and 124 kd, and therefore could not substantiate 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 coincubations 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
HAT PLATELET ANTIBODY

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.

ACKNOWLEDGMENT

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