Identification of Bak\textsuperscript{b}, a New Platelet-Specific Antigen Associated With Posttransfusion Purpura

By T.S. Kickler, J.H. Herman, K. Furihata, T.J. Kunicki, and R.H. Aster

Bak\textsuperscript{a} is a platelet alloantigen whose putative allele, Bak\textsuperscript{b}, has not been identified previously. By using a serum, "Har," obtained from a patient with posttransfusion purpura, we describe the platelet alloantigen Bak\textsuperscript{a}. The Har serum reacted with an NP-40-extractable platelet membrane protein of 142 kd with mobility similar to platelet glycoprotein Ib\textsubscript{a}. We found that the antigen recognized by the Har serum is inherited in an autosomal dominant mode with an apparent gene frequency of .39. Chi-square analysis of observed and expected phenotype frequencies indicated that serum Har recognizes Bak\textsuperscript{a}, the anticipated allele of Bak\textsuperscript{b}. Our findings provide new evidence for polymorphism of glycoprotein Ib\textsubscript{a} and for the association of posttransfusion purpura with alloimmunization to determinants on this glycoprotein.

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The detailed clinical history of the patient was previously reported.\textsuperscript{4} Briefly, Mrs "Har" is a 46-year-old multiparous woman who received blood transfusions at the time of a hysterectomy. She had been transfused 20 years earlier. Two weeks after surgery, she returned to the hospital because of bleeding due to a platelet count of 1,000/\mu L. Spontaneously, her platelet count recovered during her seven-day hospitalization.

Methods

Case Report

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Methods

Antiserum. Platelet typing serum used were anti-\textsuperscript{PlA}\textsuperscript{1} and \textsuperscript{PlA}\textsuperscript{2}, -Bak\textsuperscript{a}, -Pl\textsuperscript{b}, and Pen in use at the Southeastern Minnesota Blood Center or at the Johns Hopkins Hospital. The anti-\textsuperscript{PlA}\textsuperscript{1} and anti-\textsuperscript{PlA}\textsuperscript{2} were gifts from Drs S. Slichter and N.R. Shulman, respectively.

Platelets. Type 1 Glanzmann's platelets were a gift of Dr Margaret Johnson (Wilmington, DE). Serological studies. The patient’s platelets were phenotyped by using either a radiolabeled or immunofluorescent technique.\textsuperscript{10} Electrophoresis methods. Platelets were obtained from EDTA-anticoagulated blood and washed in 0.15 mol/L NaCl and 10 mmol/L Tris HCl, pH 7.4, with 10 mmol/L EDTA. Platelet counts were adjusted to 1 x 10\textsuperscript{9} platelets/\mu L. The platelets were solubilized in 2% sodium dodecyl sulfate (SDS) under nonreduced conditions. Platelet membrane proteins were extracted in 1% NP-40 and spun at 15,000 g for ten minutes and the supernatants lyophilized and solubilized as before.

Platelet protein separation and transfer. Electrophoretic separation of platelet proteins by using 7% polyacrylamide gels containing 0.1% SDS was performed according to the method of Laemmli.\textsuperscript{11} Electrophoretic transfer of platelet proteins from polyacrylamide gels to nitrocellulose paper was performed according to the method of Towbin et al.\textsuperscript{12} Modifications for use were described previously.\textsuperscript{13} Fifty micrograms of platelet protein was applied to each lane. Completeness of transfer was assessed by fast green staining of the nitrocellulose paper and Coomassie blue staining of postblot gels. Molecular weight markers (Sigma Chemical Co, St Louis) were included in each gel and consisted of myosin (205 kd), \beta-galactosidase (116.25 kd), bovine serum albumin (66.2 kd), and ovalbumin (45 kd).

Immunoblotting. The patient’s serum was partially purified by adsorption and elution from reactive platelets by the method of Shulman et al.\textsuperscript{1} Five milliliters of the Har serum was incubated with 5 x 10\textsuperscript{9} platelets for 15 minutes at 37\degree C. After four adsorptions, the antibody was completely removed. After adsorption, the cells were sedimented at 50,000 g for 15 minutes to prevent trapping of serum. The supernatant was removed, and the cell buttons were rinsed with phosphate-buffered saline (PBS) maintained at 4\degree C; after three rinses, the volume of platelets was adjusted to 5 mL with PBS. The saline cell suspension was brought to pH 3.0 by adding 0.1 N HCl and allowed to stand for ten minutes at room temperature. The mixture was then centrifuged at 1,000 g for 15 minutes. The supernatant was decanted and the pH adjusted to 7.4 with 0.02 N NaOH. The eluate was then lyophilized for storage at 4\degree C. Before use in immunoblotting, the lyophilized antibody was rehydrated with distilled water to yield a protein concentration of 10 \mu g/mL. The semipurified antibody was then diluted 1:20 in 10 mmol/L Tris, pH 7.4, and 0.1% Tween 20 buffer with 3% bovine serum albumin. The diluted antibody was then incubated for one hour at room temperature with the nitrocellulose strips. After five washes with 10 mmol/L Tris-saline buffer, the blots were incubated with affinity-purified, goat antihuman IgG (Kirkegaard-Perry Laboratories, Gaithersburg, MD) radiolabeled by the chloramine-T method with \textsuperscript{125}I. This second antibody was diluted in 10 mmol/L Tris, pH 7.4, and 0.1% Tween 20 with 3% bovine serum albumin to give approximately
300,000 cpm/mL. After an incubation of one hour at room temperature, the blot was repeatedly washed with Tris-saline buffer. Finally, the nitrocellulose paper was dried and autoradiography carried out by using Kodak Xomatic XRP-6 film for 24 hours as previously described.13

Two-dimensional electrophoretic analysis. Platelet membrane extracts were analyzed by the method of O’Farrell under nonreduced conditions in the first dimension.14 Fifty micrograms platelet protein was electrophoresed. This was followed by electrophoresis into the second dimension by using SDS-polyacrylamide gel electrophoresis (PAGE) under reduced conditions as described by Phillips and Poh A.15 The lane at the left margin of the slab gel was used to electrophorese the platelet lysate in only one dimension under the same conditions as the lysate electrophoresed two-dimensionally. This one-dimensional lane serves as a reference for interpreting the two-dimensional blot. Immunoblots of these two-dimensional gels were then prepared as described in the immunoblotting section.

Solid-phase enzyme-linked immunosorbent assay. To perform the population and family studies, a solid-phase immunobilized antigen assay was used.16 This permitted using the Har serum that contained not only the Bakb antibody but also multispecific HLA antibody. The principle of the assay is that AP2 (monoclonal antibody specific to IIb-IIIa) is bound to a microtiter well under alkaline conditions. Platelet membrane antigens solubilized in Triton X-100 are then added to the wells, which allows GPIIb-IIIa complex to bind to the AP-2 monoclonal. Approximately 5 μg of total platelet protein is added per well. After washing, antiserum is added to each well, and antibody binding is determined by using a biotin-conjugated murine monoclonal antibody. Approximately 50,000 cpm/mL. After an incubation of one hour at room temperature, the blot was repeatedly washed with Tris-saline buffer. Finally, the nitrocellulose paper was dried and autoradiography carried out by using Kodak Xomatic XRP-6 film for 24 hours as previously described.16

RESULTS

Serological Studies

The patient’s platelets phenotyped as PI A1, PI A2, Bakb, and P1 E2, PenA-positive, which suggests that the patient’s serum contained an alloantibody to an antigen other than to these known platelet-specific antigens.

On lymphocytotoxic testing of the patient’s serum, it reacted with all 20 of a lymphocyte panel. Testing of the serum against platelets HLA identical to the patient (HLA-A1, -25, -B8, -18) showed reactivity with six of eight panels. The serum did not react with the patient’s autologous platelets or with Glanzmann’s (Type I thrombasthenic) platelets. These results suggested that the serum contained an antibody to a platelet-specific antigen associated with glycoprotein IIb (GPIIb) or GPIIIa and was not an autoantibody.

Western Blotting

To define the antigen against which this antibody was directed, we used SDS-PAGE under nonreduced conditions followed by Western blotting (Fig 1). The Har serum was partially purified by absorption and elution from platelets.3 We found that the Har antibody reacted strongly at 142 kd with a protein that was not detected on Glanzmann’s plate-
lets and Bak$^{A/B}$-positive platelets. This band comigrated with a protein recognized by anti-Bak of Baka$^{A/B}$ platelets. This indicates that GPIIb is normally present and migrates identically in both Baka$^{A/A}$ and Baka$^{A/B}$ platelets. Some fainter bands were present with both antisera. The significance of these bands is unclear, but it has been reported that patients’ sera may react with many determinants both on membranes and internal components.

Two-Dimensional Immunoblots

Further evidence that the antigen of interest is on GPIIb was obtained by blotting the patient’s antibody against an NP-40 membrane extract of platelets after two-dimensional gel electrophoresis under nonreduced and then reduced conditions (Fig 2). In this procedure, any protein that does not have a disulfide bond will be on the diagonal line because its mobility will be the same in both directions. Any shift above or below the line indicates that the protein contains disulfide bonds. As described by Phillips and Poh Agin, GPIIbα characteristically falls below the diagonal line and GPIIla above. In the blot shown in Fig 2, reactivity below the diagonal line at approximately 125 kd is seen showing that the antigen is on IIbα rather than IIla.

Immunogenetic Studies

Family studies. When the solid-phase ELISA assay was used, a pattern consistent with codominant inheritance of Bak$^a$ and the antigen recognized by Har serum was seen.

Population study. To determine the phenotypic frequency and estimated gene frequency of the antigen recognized by serum Har and to determine whether the gene determining this antigen is in genetic equilibrium with the one determining Bak$^a$, platelets from 91 randomly selected, unrelated blood donors were tested with anti-Bak$^a$ and serum Har in the solid-phase ELISA assay.

This approach was first validated by using the well-characterized PI$^{A1}$/PI$^{A2}$ system. Figure 3 shows the results of testing anti-PI$^{A1}$ and anti-PI$^{A2}$ against immunofinity-purified GPIIb-IIIa from 35 random donors. With anti-PI$^{A1}$, the strongest reactions were obtained with donors positive for PI$^{A1}$, ie, PI$^{A1}$-homozygous donors. The single PI$^{A1}$-negative subject, ie, PI$^{A1}$ homozygous, tested gave the strongest reaction with anti-PI$^{A2}$. Preparations from donors positive for both PI$^{A1}$ and PI$^{A2}$, ie, heterozygotes, gave reactions of intermediate strength with both antisera. Of 91 unrelated white donors tested, there was a close fit between the observed and expected PI$^{A}$ phenotype (based on Hardy-Weinberg calculations), with values similar to those obtained by serological techniques. The observed phenotype frequencies for PI$^{A1}$-positive and PI$^{A2}$-negative were .98 and .2, respectively, values similar to other reported population studies.

Initial studies of 34 donors by using anti-Bak$^a$ are shown in Fig 4. It is apparent that preparations reacting most strongly with anti-Bak$^a$ gave negative reactions with Har and that those negative for Bak$^a$ gave the strongest reactions with Har. Preparations positive with both antisera gave intermediate reactions.

A chi-square analysis for a Hardy-Weinberg fit between the genes coding for the antigens recognized by the anti-Bak$^a$ and Har serum is shown in Table 1 for 91 random donors. We found a close fit between the observed frequencies of phenotypes Bak$^{A/A}$, Bak$^{A/B}$, and Bak$^{B/B}$ if it is assumed that
Hypothesis if x strength, which is helpful in determining homozygosity or antibodies. To date, laborious serological procedures involving absorption, elution, or serial dilution of antibodies have been used for this purpose. Recently, selective destruction of antibodies complicates the identification of platelet-specific antigens. Since the original description of posttransfusion purpura and the implication of the PI\textsuperscript{A} immunogen in the pathogenesis of this disorder by Shulman et al,\textsuperscript{1} other alloantigens have been shown to be capable of inciting posttransfusion purpura. Ziegler and coworkers first suggested that posttransfusion purpura may be caused by platelet-specific antigens other than PI\textsuperscript{A}.\textsuperscript{24} Others subsequently implicated Bak,\textsuperscript{7} PI\textsuperscript{A},\textsuperscript{25} and Pen.\textsuperscript{26} Our report shows that posttransfusion purpura can occur in association with alloimmunization against Bak. The cause of posttransfusion purpura is not completely understood. Investigations of its pathogenesis should take into consideration that this condition can apparently be induced by numerous platelet-specific alloantigens.

### DISCUSSION

Patients transfused with blood may make anti-HLA antibodies and/or antibodies to platelet-specific antigens. The simultaneous development of both types of antibodies complicates the identification of platelet-specific antibodies. To date, laborious serological procedures involving absorption, elution, or serial dilution of antibodies have been used for this purpose. Recently, selective destruction of HLA antibodies by chloroquine has been used. With intact platelets, these approaches are not always reliable and generally do not permit quantitative analysis of antigen strength, which is helpful in determining homozygosity or heterozygosity. The solid-phase, antigen-capture ELISA assay permits accurate phenotyping of platelets and allows immunogenetic studies to be performed even when sera are contaminated with HLA-specific antibodies.

To date, at least five well-characterized platelet-specific antigen systems have been described. These include PI\textsuperscript{A} (Zw), PI\textsuperscript{E}, Bak (Lek), Ko, and Pen (Yuk).\textsuperscript{1,4} These antigens are only now beginning to be characterized biochemically. The PI\textsuperscript{A} antigen has been localized to an epitope on GPIIIa.\textsuperscript{21} PI\textsuperscript{E} on GPIb\textsubscript{a},\textsuperscript{22} Bak\textsuperscript{a} on IIb,\textsuperscript{23} and Pen on GPIIIa.\textsuperscript{16} Our studies demonstrate that the new antigen we describe, Bak\textsuperscript{b}, is on GPIIIa as would be expected of an allele of Bak\textsuperscript{a}.

These studies also demonstrate the genetic polymorphism of platelet GPIIb. In addition to this report, three other previously uncharacterized antigens have been described within the last 2 years. These antigens include Pen,\textsuperscript{5} Yuk,\textsuperscript{6} which may be identical to Pen,\textsuperscript{16} and Yuk.\textsuperscript{6} All these antigen systems allow for at least four genetically determined variants of GPIIIa and two variants of GPIIb.

Since the original description of posttransfusion purpura and the implication of the PI\textsuperscript{A} immunogen in the pathogenesis of this disorder by Shulman et al,\textsuperscript{1} other alloantigens have been shown to be capable of inciting posttransfusion purpura. Ziegler and coworkers first suggested that posttransfusion purpura may be caused by platelet-specific antigens other than PI\textsuperscript{A}.\textsuperscript{24} Others subsequently implicated Bak,\textsuperscript{7} PI\textsuperscript{A},\textsuperscript{25} and Pen.\textsuperscript{26} Our report shows that posttransfusion purpura can occur in association with alloimmunization against Bak. The cause of posttransfusion purpura is not completely understood. Investigations of its pathogenesis should take into consideration that this condition can apparently be induced by numerous platelet-specific alloantigens.

### REFERENCES

6. Shibata Y, Miyati T, Ichikawa Y, Matsuda I: A new platelet antigen system, Yuk\textsuperscript{a}/Yuk\textsuperscript{b}. Vox Sang 51:334, 1986

### Table 1. Bak Phenotypes of 91 Unrelated White Donors

<table>
<thead>
<tr>
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<th>Bak\textsuperscript{a}</th>
<th>Bak\textsuperscript{b}</th>
<th>Bak\textsuperscript{b}</th>
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<tbody>
<tr>
<td>Observed</td>
<td>38</td>
<td>42</td>
<td>11</td>
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<tr>
<td>Expected</td>
<td>33.67</td>
<td>43.68</td>
<td>13.65</td>
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\[ \chi^2 = 1.14; P = .30 \text{ to } .20; \chi^2 (1.074 \text{ to } 1.642). \text{ Reject the hypothesis if } \chi^2 \geq 3.841 \text{ at } P = .05. \]


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