Gov*/*b Alloantigen System on Human Platelets

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In this report we describe a platelet alloantigen system that is carried on a novel platelet protein of 175 Kd. Antisera against the two alleles (Gov*/*Gov*) were found in two patients who had received large numbers of platelet transfusions. The anti-Gov alloantibodies could not be detected using a whole platelet solid phase enzyme immunoassay, or by a platelet glycoprotein capture enzyme immunoassay using monoclonal antibodies against glycoproteins Ib/IX, Ia/IIa, and Iib/IIia. Using radioimmunoprecipitation techniques, a protein was precipitated that migrated at 175 Kd (reduced). Under nonreduced conditions, a 150-Kd protein was detected with a minor component at 175 Kd. The detection of the alloantigens was not activation-dependent. Using immunodepletion studies, we demonstrated that each alloantiserum recognized an epitope on a discrete population of the 175-Kd platelet protein. Family studies demonstrated that the alloantigens designated as Gov* and Gov* were inherited in an autosomal codominant fashion. The phenotypic frequencies were Gov*/Gov*, 26%; Gov*/Gov*, 55%; Gov*/Gov*, 19%; giving gene frequencies of 0.532 and 0.468 for Gov* and Gov*, respectively (n = 33).

A NUMBER OF platelet alloantigens have been described. The P1, Bak, and Pen alloantigens are all on glycoproteins Ib or IIa. The Zav alloantigens (also known as Br or Hc) are on the platelet glycoprotein (GP) Ia/IIa complex. PL, is found on GPIb. In this report we describe a new alloantigen system on platelets that is not found on GP Ia/IIa, but on a GP having molecular weight (mol wt) 175/150 (reduced/nonreduced).

MATERIALS AND METHODS

Patient Studies

Patient 1. The index patient Gov had a long history of renal failure caused by glomerulonephritis. She had undergone renal transplantation and received platelet transfusion support because of pancytopenia caused by immunosuppressive drugs. Several years later she had hip surgery and received red blood cell transfusions. On the tenth postoperative day, she developed acute severe thrombocytopenia (platelet nadir 5 x 10^9/L). She did not respond to platelet transfusions, and bone marrow examination was normal. The diagnosis of posttransfusion purpura was made and she was treated with corticosteroids and plasmapheresis. She made a complete recovery with a return of the platelet count to normal by day 17. She died of unrelated causes 1 year later. The serologic investigation at the time of the posttransfusion purpura demonstrated the presence of strong antiplatelet reactivity against most donor platelets. Subsequent investigation demonstrated an anti-Bak antibody and an additional reactivity, designated as anti-Gov, which is the subject of this report.

Patient 2. This patient has had a life-long bleeding disorder with impaired platelet aggregation to adenosine diphosphate and epinephrine. The patient had received multiple platelet transfusions to which he had become refractory. Because of the severe nature of the bleeding disorder, the possibility of a congenital platelet glycoprotein deficiency was considered. However, the patient’s platelets showed the presence of platelet GPs Ib/IX, Ia/IIa, and Iib/IIia. Serologic investigations demonstrated an antibody that did not react with autologous platelets and was allelic to the platelet alloantibody observed in patient 1. It was designated anti-Gov. The mother and father of this patient were also platelet typed.

Serologic Techniques

Whole platelet, solid-phase enzyme immunoassay (EIA). A direct binding EIA using intact platelets and test serum was performed as described. In brief, 3 x 10^11 washed test platelets were layered onto microtiter plates (Immunon II; Dynatech Labs, Chantilly, VA) and incubated with 30 μL of patient serum for 60 minutes at 22°C. After washing, the bound immunoglobulin G (IgG) was detected using goat anti-human IgG conjugated to alkaline phosphatase.

Platelet GP capture EIA. A platelet GP capture EIA was used to characterize the protein specificity of the alloantiserum. The technique, performed as described, used the monoclonal antibodies (MoAbs) Raj-1 (anti-GP Ib/IIa), 12F1 (anti-GP Ia/IIa), Beb-1 (anti-GP Ib/IX), and PH-1 (anti-GP Ib/IX) (12F1 was a generous gift from Dr Virgil Woods, San Diego, CA). The MoAbs were bound to microtiter wells. Test serum was incubated with washed donor platelets, and after washing, the platelets were solubilized and added to the wells containing the MoAbs. The human anti-platelet IgG was detected using goat anti-human IgG conjugated to alkaline phosphatase.

Radioimmunoprecipitation studies. Platelets were prepared from whole blood drawn into acid citrate dextrose containing protamine E (PGE) (0.3 μmol/L), and theophylline (0.1 mmol/L). The platelets were radiolabeled with 125I-sodium iodide using lactoperoxidase. Immunoprecipitation of proteins from the lysate using patient and control sera was performed as described. In brief, 1 x 10^10 platelets were solubilized with 1% Triton X-100 (Bio Rad Labs, Richmond, CA) in Tris-buffered saline containing 10 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 0.02 mg/mL soybean trypsin inhibitor, and incubated with 40 μL of patient or control serum and 50 μL of protein A-Sepharose CL-4B (Pharmacia, Dorval, Quebec, Canada). After incubation at 22°C for 1 hour, the beads were washed five times and the complexes eluted and analyzed using 5% to 10% sodium dodecyl sulfate (SDS) polyacrylamide gradient gels and autoradiography. In some experiments, intact labeled platelets were first incubated with the sera and then washed, solubilized, and incubated with the protein A-Sepharose CL-4B beads. Unless otherwise stated, all immunoprecipitations were run without prior elution of proteins from the beads.

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under reducing conditions using 5% 2-mercaptoethanol in the sample buffer.

To investigate whether expression of the Gov alloantigens on the p175 protein was activation-dependent, radioimmunoprecipitation studies were performed using platelets isolated and washed using the technique of Berndt and Phillips to minimize activation. These platelets were collected into PGE<sub>1</sub>, theophylline, and N-ethylmaleimide before washing by gel filtration. A subsample of platelets (with and without activation by thrombin [0.5 U/mL]) were tested by radioimmunoprecipitation using the anti-Gov sera and MoAb Sl2 to the activation-dependent α granule protein GMP.140 (a generous gift from Dr R. McEver, University of Oklahoma).

Positive control sera included anti-P<sub>1α</sub><sup>1</sup> from the serum of a mother whose child had neonatal alloimmune thrombocytopenia and serum containing both anti-Zav&sup; and anti-Bak&sup; from a patient with posttransfusion purpura.

The human erythroleukemic cell line (HEL) was also investigated using this technique. Cells, 1 × 10<sup>6</sup>, were radiolabeled and solubilized as described for the platelet investigations, and radioimmunoprecipitation performed using both alloantisera.

**Immunodepletion studies.** Immunodepletion studies were performed to determine whether the alloantigens recognized by anti-Gov<sup>α</sup> (patient 1) and anti-Gov<sup>β</sup> (patient 2) were on discrete populations of molecules, as would be expected for an allelic system. Radiolabeled platelet lysate prepared from platelets heterozygous for Gov<sup>α</sup> and Gov<sup>β</sup> was sequentially immunodepleted (five times) with anti-Gov<sup>α</sup> alloantiserum and then reacted with the anti-Gov<sup>α</sup> antiserum. The resultant precipitate was compared with that obtained when the anti-Gov<sup>α</sup> was reacted with platelet lysate that had been sham-treated. Sham-treated lysate was incubated with normal serum as the primary antiserum.

**Family studies.** Two kindreds of individuals heterozygous for Gov<sup>αβ</sup> was studied to determine if Gov<sup>α</sup> and Gov<sup>β</sup> segregated independently, as would be expected for a codominant allelic system.

Fig 1. An autoradiogram using radiolabeled platelet lysate from donor A, who is heterozygous for Gov<sup>α</sup>/Gov<sup>β</sup>, and donor B, who is homozygous for Gov<sup>β</sup>. The alloantisera used were: lanes 1 and 6, anti-Gov<sup>α</sup> (patient 1); lanes 2 and 7, anti-Gov<sup>β</sup> (patient 2); lanes 3 and 8, anti-P<sub>1α</sub><sup>1</sup>; lanes 4 and 9, anti-Zav<sup>α</sup> plus anti-Bak<sup>α</sup>; lanes 5 and 10, control serum. This study demonstrates immunoprecipitation by the Gov antisera under reduced conditions of a band at 175 Kd (shown by the arrow). This protein has a different mobility than GPs Ia, IIa, Ib, and IIIa. This study also demonstrates that the two antisera recognize different alleles (lanes 1 and 2 compared with lanes 6 and 7). Serum from patient 1 also contains anti-Bak<sup>α</sup> reactivity, as shown by the immunoprecipitation of GPs IIb/IIIa (lanes 1 and 6).

**RESULTS**

When serum from patient 1 (Gov) was tested against donor panel platelets using the whole platelet solid-phase EIA, the only reactivity was against Bak<sup>α</sup> positive platelets. The serum was then investigated using the GP capture assay, utilizing MoAbs to capture the following GPs: Ia/IIa (12F1), IIb/IIIa (Raj-1), and Ib/IX (Beb-1 and PH-1).

When sensitized by anti-P<sub>1α</sub><sup>1</sup> antibodies and then solubilized, test platelets produced positive results in those wells containing MoAb to IIb/IIIa, and only when P<sub>1α</sub><sup>1</sup> positive platelet donors were used. Antisera containing reactivity to both Zav<sup>α</sup> and Bak<sup>α</sup> produced positive results in the wells containing MoAbs against GPIa/IIa and GPIIb/IIIa, respectively.

The serum from patient 1 demonstrated anti-IIb/IIIa reactivity, but only when Bak<sup>α</sup> positive platelets were used. Serum from patient 2 similarly was tested in both the whole platelet EIA and the GP capture assay, and did not demonstrate binding to any donor platelet sample (n = 8) using either assay.

Immunohistochemical analysis using the radioimmunoprecipitation assay confirmed the anti-Bak<sup>α</sup> reactivity in the serum of patient 1 (Fig 1, lanes 1 and 6). In addition, there was discrete reactivity with a major band at 175 Kd and a very minor band at 150 Kd (reduced) (Fig 1, lane 1). These bands were distinct from both the GPIIb/IIIa complex precipitated by the serum containing anti-P<sub>1α</sub><sup>1</sup> (Fig 1, lanes 3 and 8) and the GPIa/IIa complex precipitated by the serum containing anti-Zav<sup>α</sup> (Fig 1, lanes 4 and 9). The serum with anti-Zav<sup>α</sup> reactivity also contained weak anti-Bak<sup>α</sup> specificity as demon-
strated by the small amount of GPIIb/IIIa precipitated from the platelet donors, as previously described.2

The anti-Govb serum from patient 2 precipitated proteins that had identical migration patterns as those immunoprecipitated by the serum from patient 1 (Fig 1, lane 2). However, the serum from patient 1 and patient 2 demonstrated unique and distinct reactivity, suggesting that they were directed against different alleles (Fig 1). The anti-Gova antisera reacted only with donor A (lane 1), whereas the anti-Govb serum reacted against both donors A and B (lanes 2 and 7). Platelet donor A was designated as heterozygous for Govab, while platelet donor B was designated as homozygous for Govbb.

Family studies were performed to determine if the alloantigens segregated independently. As shown in Fig 2, the results of two family studies demonstrated that the Gova/Goib alloantigens were inherited in an autosomal codominant fashion, as expected by a codominant allelic system.

We also were able to test the platelets from patient 2 and his parents. As expected, the anti-Govb serum precipitated p175 from platelets of patient 2, while his own serum did not react. The mother and father typed Gov/a/Goib and Gov/a/Goib, respectively.

Immunodepletion studies were performed to confirm that anti-Govb and anti-Govb recognized antigens on discrete populations of the p175 platelet protein. Radiolabeled platelet lysate obtained from a donor heterozygous for Gova/Goib was immunodepleted sequentially using anti-Govb serum as shown in Fig 3 (lanes 1 through 5). After immunodepletion, the lysate was reacted with the anti-Govb serum (lane 6), and the resultant precipitate compared with p175 precipitated by anti-Govb from a sham-treated lysate (lane 12). No significant difference in the density of the p175 protein was observed, indicating that the two alleles reside on discrete populations of proteins. It should be noted that the depletion of the Govb antigen also had no effect on the density of the Bakb antigen precipitated in lanes 6 and 12, although the precipitated proteins are quite weak and have not reproduced well in the photograph in this publication. Using radioimmunoprecipitation techniques, the phenotypic frequencies were determined using unrelated platelet donors. The frequencies were: Gov/a/Goib, 26%; Gov/a/Goib, 55%; and Govb/Goib, 19%; giving gene frequencies of 0.532 and 0.468 for Gova and Govb, respectively (n = 33).

When cells from the HEL cell line were tested using the radioimmunoprecipitation assay, both the anti-Gova and anti-Govb antisera precipitated a major band at 175 Kd and a minor component at 150 Kd.

**Characterization of the Target Protein**

In some experiments, it was noted that an additional band was precipitated by the anti-Gov sera and that this protein had a mol wt of 130 Kd, which was slightly faster than GPIIb. The appearance of this 130-Kd band coincided with a decrease in density of the 175-Kd band. The addition of 10 mmol/L EDTA to the platelet washing and lysing buffers decreased the relative amount of the 130-Kd band to trace amounts, suggesting that it could be a degradation product due to a calcium-dependent enzyme.

To further study the migration characteristics of the protein precipitated by the anti-Gov sera, we used sepharose 2B gel-filtered platelets collected into PGE1, N-ethylmaleimide, and theophylline. These tests were performed under both reducing and nonreducing conditions. The reduced samples demonstrated a major band at 175 Kd with a very weak band at 150 Kd. Under nonreducing conditions, the major band migrated at 150 Kd with a minor band near 175 Kd. Neither band was observed when either normal serum or anti-P141a serum was incubated with the platelets (Fig 4). This suggests that the protein bearing the Gov antigen contains at least one intrachain disulfide bond and is susceptible to reduction during the experimental manipulation, or that the protein may exist in a nonreduced and a partially reduced form on the native platelet surface, or that the Gov antigens are present on a heterodimer of 175/150 Kd nonreduced. Further studies are now in progress to examine these possibilities.

To examine the effect of platelet activation on the surface expression of the protein carrying the Gov antigen, we prepared radiolabeled lysate from platelets gel-filtered with metabolic inhibitors and from platelets that had been activated with 0.5 U/mL thrombin. The surface expression of the p granule activation-dependent protein GMP-140 was markedly increased when the platelets had been activated by thrombin, as shown by the increased amount of labeled material precipitated by MoAb S12 (Fig 5, lanes 1 and 2). In contrast, the amount of surface-labeled protein precipitated by anti-Govb serum showed no difference when the lysate was prepared from unactivated or thrombin-stimulated platelets (Fig 5, lanes 3 and 4). Although it is possible that some stimulation of the unactivated platelets may have occurred during radiolabeling and caused expression of the Gov proteins, no difference was seen when the platelets were

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**Fig 2.** Two different family studies demonstrating the codominant inheritance of Gova/Goib.
stimulated with the strong agonist thrombin, in marked contrast to the dramatic increase of labeled GMP-140 detected.

**DISCUSSION**

In this report, we describe a diallelic alloantigen system, designated as Gov\(^a\)/Gov\(^b\), which is located on a novel platelet GP. Several aspects about these new platelet alloantigens make them unique. First, they could not be detected using either a whole platelet direct binding assay or a GP capture assay. The reason the intact platelet assay did not detect these antigens is uncertain, but it could indicate that the platelet GP carrying these antigens is present in low numbers on the platelet surface. Previously, we have shown that the \(7\alpha\) alloantigen system (also known as R\(\alpha\) or H\(\alpha\)) is difficult to detect using a standard whole platelet enzyme immunoassay because of the low number of GP la/IIa complexes on the platelet surface; ie, approximately 1,000 copies per platelet. Hence, our data suggest that the number of p175 molecules per platelet also is low. We did not detect the anti-Gov alloantibodies in our GP capture assay because we lack the appropriate captive MoAbs. However, the Gov\(^a\)/Gov\(^b\) alloantigen system was readily detectable using radioimmunoprecipitation techniques.

The second aspect of the Gov alloantigen system making it important is its presence on an apparently novel platelet protein. We found that under nonreducing conditions and using nonactivated platelets, a major band at mol wt 150 Kd was immunoprecipitated. Minor amounts of a 175-Kd band also were observed. However, on reduction, a 175-Kd band was immunoprecipitated almost exclusively, indicating the presence of at least one intrachain disulfide bond. The presence of small amounts of the 175-Kd band under nonreducing conditions suggests either a partial reduction of some of the protein occurred during platelet preparation or partial reduction of this protein takes place on the native platelet membrane. Alternatively, the protein could exist as a heterodimer.

The identity of this GP remains unknown. Previous reports that have characterized the surface proteins on human platelets have not demonstrated a protein with migration characteristics similar to p175.\(^\text{1,2}\) Although the reduced form of thrombospondin migrates similarly to the p175, its nonreduced form is quite different. Furthermore, using one of the

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**Fig 3.** An autoradiogram (reduced) using platelet lysate heterozygous for Gov (Gov\(^a\)/Gov\(^b\)). Immunodepletion studies were performed using anti-Gov\(^a\) (left) or normal human serum (NHS) (right). The platelet lysate was then reacted with anti-Gov\(^a\). This experiment shows that the Gov\(^a\) and Gov\(^b\) antigens reside on two discrete populations of the p175 protein. The GPIIb/IIIa bands indicated were very weak on the original autoradiogram and did not reproduce well in the photograph.

**Fig 4.** An autoradiogram investigating the mobility of the protein carrying the Gov antigens under nonreducing and reducing conditions. Normal serum was used in lanes 1 and 4. Under nonreducing conditions anti-Gov\(^a\) precipitated a major band at 150 Kd and a minor band at 175 Kd (lane 2). Under reducing conditions only a band at 175 Kd was observed with a trace amount of radiolabeled protein at 150 Kd (lane 6), which is barely detectable on the photographed autoradiogram. Lanes 3 and 6 illustrate the migration of GPIIla precipitated by serum containing anti-P1\(^\alpha\).
MoAb S-12 directed against thrombospondin in immunodepletion experiments, we demonstrated that the p175 protein that carries the Gov antigens is not thrombospondin (data not shown). The migration characteristics of the p175 protein is most similar to a heterodimeric protein described by Brashem-Stein et al using an MoAb that bound to activated platelets and activated T cells. However, the Gov antigens described in this report are expressed on the surface of unactivated resting platelets and do not demonstrate a relative increase when tested on thrombin-stimulated platelets. In contrast, the same platelets demonstrated a marked increase in the surface expression of the activation-dependent protein GMP-140. The clinical relevance of the Gov alloantibodies is unknown. In the one patient, refractoriness to platelet transfusion was noted. However, it is not known if the anti-Gov was responsible for the failure to respond to the transfused platelets. The other patient had posttransfusion purpura, but another alloantibody (Bak) was present.

In summary, we described a codominant diallelic platelet alloantigen system on a novel 175-Kd protein. Investigations into the structure and function of this GP are in progress.

REFERENCES
Gova/b alloantigen system on human platelets

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