Platelet Glycoprotein IV (CD36) Deficiency Is Associated With the Absence (Type I) or the Presence (Type II) of Glycoprotein IV on Monocytes

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Platelet membrane glycoprotein (GP) IV (also called CD36 and GPIIb) deficiency is associated with Nak^a-negative platelets. Using flow-cytometric analysis of cells stained with the monoclonal anti-GPIV antibody OKM5, we have studied the expression of GPIV on the monocytes from 16 healthy Japanese individuals whose platelets were deficient in GPIV. GPIV was absent on the surface of monocytes from 2 platelet GPIV-negative donors (type I), whereas it was present on the monocytes from the remaining 14 platelet GPIV-negative donors (type II). The fluorescent intensity of OKM5-stained type II monocytes was significantly ($P < .05$) lower than that of normal monocytes derived from platelet GPIV-positive donors, suggesting that the expression of GPIV on the type II monocytes is also abnormally regulated as compared with that on normal monocytes. OKM5 induced an oxidative burst in the type II monocytes as well as in the normal monocytes, but it failed to induce it in the type I monocytes. Because the 2 individuals with the type I deficiency have been healthy and exhibited no immunologic problems, GPIV appears to be not essential for the normal physiologic functions of monocytes. An anti-GPIV antibody was detected in the serum from one of the type I GPIV-deficient women, who had never received any blood transfusions but had given birth to three apparently healthy children. These results suggest that type I GPIV-deficient individuals may be at risk of developing an anti-GPIV isoantibody upon blood transfusion or pregnancy.

PLATELET MEMBRANE glycoprotein (GP) IV (also referred to as GPIIIb) is also expressed on a number of other cells, such as monocytes,[1-4] endothelial cells,[4,5] and epithelial cells,[6] and on neoplastic cell lines.[4] Recently, we showed that platelet Nak^a antigen is located on the GPIV molecule[7] and that GPIV-deficient platelets correspond to Nak^a-negative platelets.[8] Several groups have reported that GPIV is a receptor for thrombospondin,[1,2,9-16] collagen,[17,18] and adhesion of *Plasmodium falciparum*-infected erythrocytes,[4,19-21] although two groups have shown that thrombospondin binds normally to Nak^a-negative platelets.[20,22] We have reported that the aggregation and secretion of GPIV-deficient platelets occur normally in response to several agonists, including collagen, and suggested that GPIV is not essential for platelet function.[23] There have been two different reports on the expression of GPIV on the monocytes from Nak^a-negative donors. Kashiwagi et al[24] detected GPIV on the monocytes from Nak^a-negative donors by flow-cytometric analysis with OKM5. In contrast, monocytes from a platelet GPIV-deficient donor (ARC-36, whom we had initially found in the United States,[8]) were subsequently found to lack GPIV.[20]

Anti-Nak^a antibody was first reported in a patient (S. Nak) who received multiple platelet transfusions, and this antibody induced refractoriness to HLA-matched platelet transfusions.[25] In this report, we describe two phenotypic variants of GPIV deficiency that can be distinguished by the absence (type I) or the presence (type II) of GPIV on monocytes. We suggest that type I GPIV-deficient individuals may possibly develop an anti-GPIV isoantibody when they receive transfusions of GPIV-positive platelets or when they give birth to GPIV-positive children.

MATERIALS AND METHODS

Antibodies. OKM5, a monoclonal antibody (MoAb) against CD36 differentiation antigen,[3] was a generous gift from Ortho Diagnostic Systems (Tokyo, Japan). MoAbs (RMO52 directed against CD14[26] and FA6-152 against CD36[27]) were purchased from Immunotech (Luminary, France). Fluorescein isothiocyanate (FITC)-labeled antimouse IgG antibody and FITC-labeled antihuman antibody were purchased from Coulter Electronics, Inc (Miami, FL). Mouse myeloma protein MOPC 21 was purchased from Cappel (West Chester, PA). Polyclonal anti-GPIV antibody[23] was produced in rabbits as previously described.

Materials. ^{125}I-Iodine was purchased from New England Nuclear (Boston, MA). FMLP was purchased from Sigma (St Louis, MO). Luminol was purchased from Kanto Chemical (Tokyo, Japan).

Subjects. Blood was voluntarily donated by the personnel of our Institute, the Tokyo Metropolitan Komagome Hospital, and the students of Tokyo Metropolitan Public Health and Nursing School; these samples were tested for the presence of GPIV as previously described.[8,23] Seven of the platelet GPIV-negative donors (Ta.K, Ka.Y, So.C, Ha.C, Ta.Y, Ha.Y, and Mo.R) have been previously reported.[23] Since then, our screening has identified a further 8 platelet GPIV-deficient donors (Su.K, Ch.T, Sa.H, Ka.S, Ki.K, Ki.F [father of Ki.K], Ki.M [mother of Ki.K], and Ki.S [sister of Ki.K]). CPD-anticoagulated blood from Na.S was kindly provided by Shoji Morita (Saitama Red Cross Blood Center, Saitama, Japan), who had previously found that Na.S's serum contained anti-Nak^a antibody as detected by serologic methods (unpublished data). Na.S was a 45-year-old healthy woman who had never received any blood transfusions but had given birth to three healthy children.

Preparation and ^{125}I-iodination of IgG from Na.S. IgG was precipitated at 40% ammonium sulfate saturation from the serum of Na.S and control individuals and washed twice with 40% saturated ammonium sulfate; the precipitate was solubilized in phosphate-buffered saline (PBS). After extensive dialysis against PBS, IgG was

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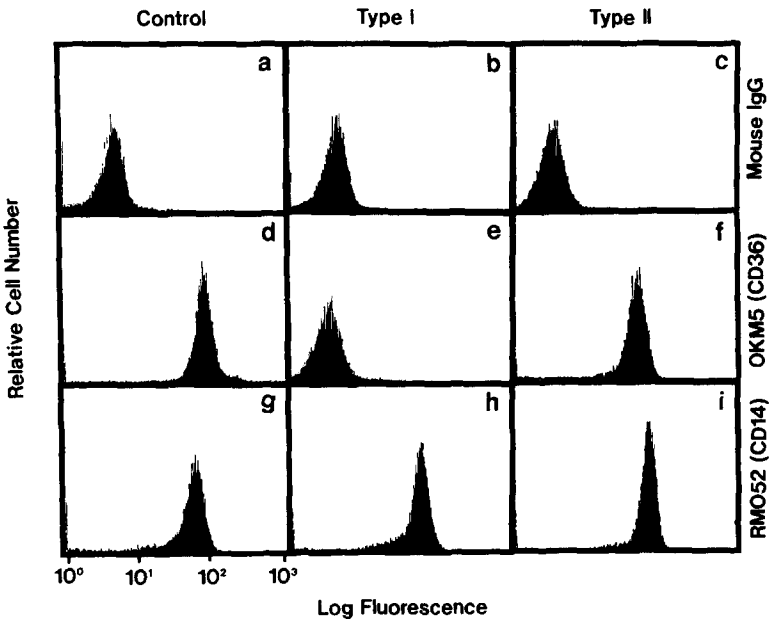

Fig 1. Flow cytometry of type I and type II GPIV-deficient monocytes. Mononuclear cells were incubated with control mouse IgG (a, b, and c), OKM5 (anti-GPIV; d, e, and f), or RMO52 (anti-CD14; h, i, and j) and stained with FITC-labeled antimouse IgG antibody. Fluorescence of 1 × 10^4 cells per sample was measured by flow cytometry as described in Materials and Methods. Profiles of control monocytes are shown in the left column (a, d, and g), type I monocytes in the middle column (b, e, and i), and type II monocytes in the right column (c, f, and j).

purified by protein A-sepharose chromatography. The purified IgG fraction was labeled with ^{125}I-iodine using iodobeads (Pierce Chemical, Rockford, IL),[8] and F(ab')$_2$ fragments were prepared as previously described.[23]

Preparation of washed platelets. Briefly, as described previously,[28] 18 mL of blood was drawn into a syringe containing 2 mL of 3.8% sodium citrate. Blood was then centrifuged (140*g* for 15 minutes) to prepare platelet-rich plasma. Platelets were washed in HEPES-albumin buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.42 mmol/L NaH_2PO_4, 4.2 mmol/L HEPES, 1 mmol/L $MgCl_2$, 5.5 mmol/L dextrose, 0.35% bovine serum albumin, pH 6.5) containing 1 U/mL apyrase.[23] Finally, the platelets were resuspended in apyrase-free HEPES-albumin buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.42 mmol/L NaH_2PO_4, 4.2 mmol/L HEPES, 1 mmol/L $MgCl_2$, 5.5 mmol/L dextrose, 0.35% bovine serum albumin, pH 7.4). Platelets to be studied by rocket immunoelectrophoresis were prepared and solubilized as previously described.[8]

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was performed according to the previous method[29] for crossed immunoelectrophoresis with minor modifications. Briefly, a low temperature melting agarose gel (NuSive GTG; FMC BioPreducts, Rockland, MA) in 1% Triton X-100 that contained polyclonal anti-GPIV antiserum (500 μL/mL of gel) was formed on the upper part (2.3 × 7 cm) of a glass plate (4.5 × 7 cm) and a lower gel without the antibody was formed on the lower part (2.2 × 7 cm) of the same plate. A 5-μL aliquot of platelet suspension (4 × 10^6/μL, 2 × 10^7 cell total) solubilized in a buffer (38 mmol/L Tris, 100 mmol/L glycine, pH 8.4) containing 1% Triton X-100 was subjected to rocket immunoelectrophoresis against anti-GPIV antibody. Electrophoresis was performed at constant voltage of 40 V for 18 hours. After electrophoresis, the plates were dried and stained with Coomassie blue. For autoradiography, the plates were placed on X-ray film and the film was exposed for 1 week at 80°C.

Identification of an anti-GPIV antibody in Na.S's IgG. The ^{125}I-iodinated samples of the IgG from Na.S were incubated with GPIV-positive platelets for 1 hour at room temperature. The suspension was layered on top of 0.1 mL of 15% sucrose solution in a centrifugation tube (SARSTEDT, Germany) and then centrifuged at 10,000*g* for 5 minutes. After centrifugation, the tube was cut off close to the bottom to facilitate removal of the pellet and the isolated pellet was then solubilized in a buffer (38 mmol/L Tris, 100 mmol/L glycine, pH 8.4) containing 1% Triton X-100. The solubilized platelets were analyzed by rocket immunoelectrophoresis.

Preparation of mononuclear cells. Citrated blood was centrifuged at 120*g* for 15 minutes at room temperature and then the platelet-rich plasma was removed. Buffy coat fractions (7 mL) containing white blood cells were each layered on 7 mL of Ficoll-Hypaque (Pharmacia LKB Biotechnology, Piscataway, NJ) and centrifuged at 650*g* for 15 minutes at room temperature. The cells at the interface were collected and washed twice with PBS.

Flow cytometry. Mononuclear cells were incubated with 10 μg/mL OKM5 for 1 hour at room temperature, washed three times in PBS, and then further incubated with FITC-labeled antimouse IgG antibody (5 μg/mL) for another 1 hour. After three washes with PBS, the cells were analyzed by a flow cytometer (Epics Profile; Coulter Electronics Inc). Ten thousand cells were analyzed in each sample after gating the area corresponding to monocytes in a plate of the forward scattering (transverse axis) versus the side scattering (longitudinal axis). To identify monocytes in the electric gate, normal mononuclear cells were stained with either the anti-CD36 antibody OKM5 or with RMO52, an anti-CD14 antibody specific for monocytes, and measured under gating conditions such that the gated cells were $CD14^+$ and $CD36^+$.

Oxidative bursts from mononuclear cells. Mononuclear cells (1 × 10^5/μL) were resuspended in HEPES-Tyrode's buffer containing 30 mmol/L luminol. Chemiluminescence of oxidative bursts were measured by a Lumiaggregometer (ChronoLog Corp, Malvern, PA). When the chemiluminescence of GPIV-deficient monocytes induced by OKM5 was measured, the gain was adjusted to 0.05 at 100 mV output.

RESULTS

Platelet GPIV deficiency. Platelet proteins from GPIV-deficient donors failed to form a radioactive immunoprecipitation arc because of bound ^{125}I-OKM5 on rocket immunoelectrophoresis. Examination of the platelets from 354 healthy Japanese donors (93 males and 261 females) by

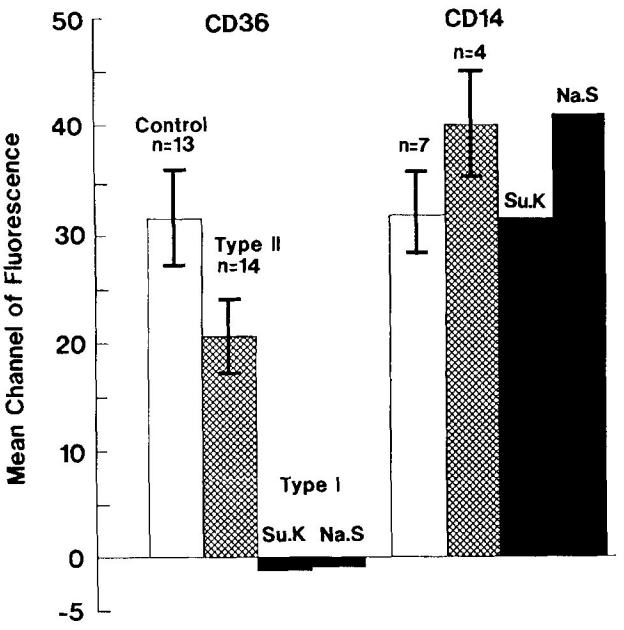

Fig 2. Flow cytometric mean channels of type I and type II monocytes stained with OKM5 and RMO52. Monocytes from type I (■) and type II (▨) deficiencies and controls (□) were stained with OKM5 (anti-CD36 antibody) (left) and RMO52 (anti-CD14 antibody) (right) and analyzed by a flow cytometer (Epics Profile). The average of mean channels was calculated (mean ± SEM).

rocket immunoelectrophoresis against a mixture of rabbit anti-GPIV antiserum and ^{125}I-OKM5 showed 16 (4.5%) platelet GPIV-deficient individuals (4 males and 12 females), including 4 members from the Ki. family and Na.S from the Saitama Red Cross Blood Center.

Platelet GPIV deficiency associated with GPIV-negative (type I) or positive (type II) monocytes. Monocytes were identified by their positive staining with RMO52, a monoclonal anti-CD14 antibody specific for monocytes, in flow cytometry (Fig 1g, h, and i). Monocytes from control (platelet GPIV-positive) donors stained positively with OKM5 (Fig 1d). However, monocytes from platelet GPIV-negative donors could be divided into two groups. One group comprised the monocytes from donors Su.K (Fig 1e) and Na.S (not shown), which exhibited only background fluorescence. This type of GPIV deficiency in both platelets and monocytes was classified as type I GPIV deficiency. The second group, the monocytes from the remaining 14 platelet GPIV-deficient donors, stained positively with OKM5. Figure 1f shows a typical flow cytometric profile of the OKM5-stained monocytes from a platelet GPIV-deficient donor (Ka.Y), indicating the presence of GPIV on the monocyte surface. The GPIV deficiency observed only in platelets but not in monocytes was classified as type II GPIV deficiency.

To estimate quantitatively the amount of GPIV expressed on monocytes, the mean channel values of the immunofluorescence of OKM5-stained monocytes were compared among controls and individuals with type I and II deficiencies (Fig 2). The fluorescence mean channel values of monocytes in normal controls (platelet CD36$^+$) was 32.4 ± 4.6 (mean ± SE, n = 13). Values for type II monocytes were 20.9 ± 2.7 (mean ± SE, n = 14) and were significantly lower ($P < .05$) than those given above for normal controls. The channel values for the type I monocytes from Su.K and Na.S were −3.06 (mean, n = 3) and −1.11 (n = 1), respectively, indicating the complete absence of GPIV. Similar results were obtained for detection of GPIV on type II monocytes using two other MoAbs against CD36, FA6-152 (Immunotech) and GS95, a new (unpublished) anti-CD36 MoAb developed by us.

Monocyte chemiluminescence. OKM5 (5 μg/mL) induced an oxidative burst in both normal and type II monocytes (Fig 3, left and right). However, it failed to induce an oxidative burst in type I monocytes of Su.K (Fig 3, middle) and Na.S. Similar results were obtained with FA6-152 and with the IgG antibody from donor Na.S (not shown). In contrast, FMLP (42 μg/mL) induced chemiluminescence of luminol from the monocytes of all these donors, verifying that the monocytes used in the OKM5 stimulation were all viable and that type I GPIV-deficient monocytes could generate oxidative bursts in response to FMLP to the same extent as normal monocytes.

Anti-GPIV antibody in the serum from Na.S. We used rocket immunoelectrophoresis to show the presence of anti-GPIV antibody in serum from Na.S. ^{125}I-labeled purified IgG from Na.S was incubated with GPIV-positive platelets (2×10^7). The platelets were then solubilized in 1% Triton X-100. Rocket immunoelectrophoresis against rabbit anti-GPIV antiserum showed an immunoprecipitation arc of GPIV (Fig 4, lane a) and an autoradiogram showed the incorporation of radioactivity into this arc (Fig 4, lane b).

DISCUSSION

The monocytes from platelet GPIV-positive donors so far examined have been found to express GPIV normally on their surfaces without exception. In this study, we have classified platelet GPIV deficiency into two types, type I or II, according to the absence or presence, respectively, of GPIV on the monocytes. The platelet GPIV-deficient individuals described by Kashiwagi et al[24] may belong to type II because they detected GPIV on the monocytes in all cases. Ockenhouse et al[18] described V.B. (ARC-36), whose platelets and monocytes were both deficient in GPIV and therefore can be classified as type I. Based on our screening, the incidence of type II GPIV deficiency is approximately 4.0% (14 of 354) of Japanese donors, whereas the type I GPIV deficiency was observed in 0.56% (2 of 354) of donors.

Although GPIV on the platelet membrane has been reported to play a role as a receptor for collagen[19,20] and thrombospondin,[1,2,9-16] we have previously observed that GPIV-negative platelets aggregate and secrete normally in response to several agonists, including collagen.[23] The normal binding of thrombospondin to Nak^a-negative platelets[20,22] suggests that molecules other than GPIV are also involved in the binding of thrombospondin. It may be noted that the two type I females (Su.K and Na.S) reported in the present study are healthy and they have had no abnormal bleeding episodes, chronic inflammation, or immunologic problems. The monocytes from both of these type I individ-

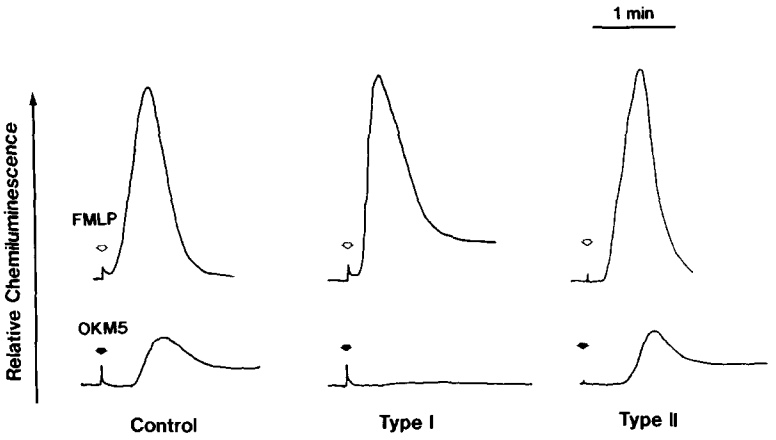

Fig 3. Oxidative bursts from monocytes by OKM5. Washed mononuclear cells were resuspended in HEPES-buffer containing 30 mmol/L luminol, and OKM5 (5 μg/mL) or FMLP (42 μg/mL) was added to the suspension. Luminescence was monitored by a Lumiaggregometer. The gain of the attenuation was adjusted to 0.05. Oxidative bursts from the mononuclear cells obtained from a control or type I or type II GPIV-deficient donor are shown in the left, middle, and right lanes, respectively.

uals normally elicited oxidative bursts in response to FMLP. Therefore, it appeared that the GPIV on monocytes is not essential for the physiologic functions of these cells. Differences, if any, in clinical profiles between GPIV-positive and -negative individuals need to be clarified.

GPIV has been considered to be a signalling molecule that is coupled to the *src* family of protein tyrosine kinases *fyn, lyn,* and *yes.*[30-32] In the present study, we have shown that the anti-GPIV antibody OKM5 induces an oxidative burst in control and type II monocytes that express GPIV, but not in type I monocytes that lack it. We have observed a similar distinction between type I and type II monocytes using FA6-152, GS95, and the IgG antibody from donor Na.S (not shown).

The incorporation of Na.S's IgG into the immunoprecipitation line of GPIV on rocket radioimmunoelectrophoresis indicates the presence of an anti-GPIV antibody. We have also found that, similar to OKM5, this antibody caused oxidative bursts in GPIV-positive monocytes and that it bound to milk fat globule membrane (MFGM) from GPIV-positive individuals (unpublished data). Although we could not analyze blood samples from other members of her family, it is most likely that Na.S's anti-GPIV antibody developed in association with her pregnancies and delivery of three children, because Na.S has never received a blood transfusion. Ikeda et al[25] have reported a patient (S. Nak) with acute myelogenous leukemia who developed an anti-GPIV antibody after receiving multiple platelet concentrates from random donors, further indicating the possible clinical significance of this antigen. No anti-GPIV antibody was detectable in the plasma of the other case of type I GPIV deficiency, Su.K, a 21-year-old woman who has neither been pregnant nor received blood transfusions.

The fluorescence intensity of the type II monocytes stained with OKM5 showed a single sharp peak, indicating that they were homogeneous in the expression of GPIV and thus excluding the possibility that they may consist of GPIV-negative and GPIV-positive populations. Interestingly, the mean channel fluorescence from type II monocytes stained with OKM5 was significantly lower than that in normal monocytes. We obtained similar results using the monoclonal anti-GPIV antibodies FA6-152 and GS95. These results suggest that the expression of GPIV in type II monocytes is abnormally regulated in comparison to normal monocytes.

Lipsky et al[33] initially found the presence of mRNA in Nak^a-negative platelets. Further investigation by Kashiwagi et al[24] showed the presence of mRNA in monocytes from Nak^a-negative individuals who appeared to be type II. However, it is not known which type of GPIV deficiency the individual reported by Lipsky et al[33] belongs to because they did not describe the expression of GPIV on the monocytes of the platelet GPIV-negative donor.

An analysis of CD36 cDNAs from Nak^a-negative individuals has been reported.[34] The results of Kashiwagi et al[34] are consistent with the notion that a type I deficiency leads to a lack of GPIV protein in all expressing cell types. It is easy to visualize that certain missense mutations and nonsense mutations located within the coding region would be predicted to give rise to the type I phenotype. Furthermore, mutations leading to increased CD36 mRNA instability would result in a lack of GPIV protein.[33]

The type II deficiency can be more readily explained by a posttranscriptional defect (such as aberrant tissue-specific CD36 pre-mRNA splicing or the creation of a translational block). It has recently been reported that alternative splicing occurs in the 5′ untranslated region of the human CD36 gene.[35] It will be of interest to examine Nak^a-negative individuals that have normal coding sequences for mutations in noncoding portions of the CD36 gene.

Although it is plausible that a generalized proteolysis of GPIV could account for either type I or type II deficiencies, our data argue against such a mechanism. GPIV in platelets is remarkably resistant to proteolysis[36] and we did not see a change in the levels or physical characteristics of the major platelet membrane GPs in membranes prepared from normal and Nak^a-negative individuals.[8]

A determination of the structure of the CD36 gene is an absolute requirement for understanding the role of specific mutations in each type of GPIV deficiency. Family studies will also need to be performed to determine if all mutations leading to the Nak^a-negative phenotype are linked to the CD36 gene locus.

In conclusion, we and others[37] have identified two types

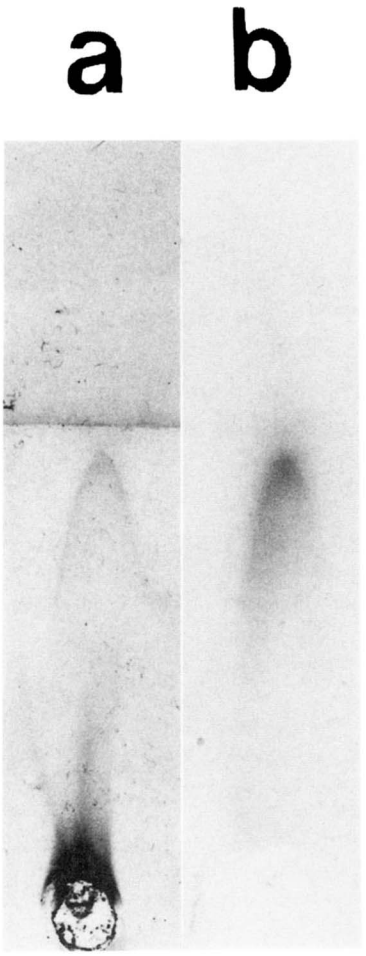

Fig 4. Identification of anti-GPIV antibody in the IgG from type I GPIV-deficient Na.S. GPIV-positive platelets were incubated with ^{125}I-labeled Na.S's IgG. After removing free antibody, platelets were solubilized with 1% Triton X-100 and subjected to rocket radioimmunoelectrophoresis. The plate was stained with Coomassie blue in (a) and its autoradiogram is shown in (b). The radioactivity was incorporated into the immunoprecipitation arc of GPIV.

of platelet GPIV deficiency. The type I deficiency is accompanied by GPIV deficiency in monocytes, whereas the type II deficiency is not. Type I GPIV-deficient individuals may be at additional risk of developing anti-GPIV isoantibodies after GPIV-mismatched platelet transfusion or during pregnancy, which may cause posttransfusion purpura and neonatal alloimmune thrombocytopenic purpura, respectively.

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