The activity and surface antigenicity of \( \alpha_2\beta_1 \) on platelets from 27 normal subjects were found to vary significantly. A fourfold range of surface antigen correlates with a 20-fold variation in the ability of nonactivated, washed platelets to adhere to type I collagen and a fivefold variation in the adhesion of platelets to type III collagen. These differences in surface receptor are reflected in significant variation in the lag time required for type I collagen-induced platelet aggregation in platelet-rich plasma. Among the same individuals, no difference was observed in surface levels or activities of two other platelet integrins, the fibronectin receptor \( \alpha_5\beta_1 \) and the fibrinogen receptor \( \alpha_m\beta_3 \). In all cases studied, we observed complimentary differences in the incorporation of \(^{125}\)I into surface \( \alpha_2\beta_1 \), in quantity of surface \( \alpha_2\beta_1 \) antigens, and in \( \alpha_2\beta_1 \) collagen receptor activity. Despite variations in these parameters, there was no difference in the electrophoretic mobility or isoelectric point of either integrin subunit among the individuals studied. The wide range of activity and antigenicity of this platelet collagen receptor may result from polymorphism(s) in the \( \alpha_2\beta_1 \) genes, or the activity of \( \alpha_2\beta_1 \), may be variably regulated by another gene product. The heterogeneity of platelet \( \alpha_2\beta_1 \) that we describe in this report certainly explains previous discrepancies concerning the contributions of this integrin to platelet adhesion to collagen.

COLLAGEN SUPPORTS platelet adhesion in both static and flowing systems.\(^{14}\) A number of receptors for collagen, including the integrin \( \alpha_2\beta_1 \),\(^{15}\) have been proposed to mediate this adhesion. Platelet adhesion mediated by \( \alpha_2\beta_1 \) can be supported by types I, II, III, IV, or VI collagens,\(^{1-3} \) and each of these collagen types, except IV, has been shown to induce platelet aggregation.\(^{4,5} \) The strongest evidence that fluid-phase, collagen-induced platelet aggregation can be initiated via binding of collagens to the \( \alpha_2\beta_1 \) collagen receptor comes from patient studies. On the one hand, it has been reported that patients with quantitative abnormalities of platelet \( \alpha_2 \) present with both defective adhesion to collagen and absent collagen-induced aggregation.\(^{9,10} \) On the other hand, it has been reported that human serum containing autoantibodies that bind to \( \alpha_2 \) will block platelet adhesion to collagen and collagen-induced aggregation.\(^{11} \) A second integrin, \( \alpha_5\beta_1 \), may contribute to adhesion to collagen under certain circumstances,\(^{12} \) particularly in relation to the expression of ligand-induced binding sites (LIBS) on the \( \alpha_5\beta_3 \) receptor.\(^{13,14} \) However, one can eliminate the contribution, if any, of \( \alpha_5\beta_3 \) to adhesion in vitro by inhibiting platelet activation, for example, with prostaglandin E\(_2\) (PGE\(_2\)).\(^{15,16} \)

In this study, we conduct an extensive analysis of the adhesion of platelets from normal individuals to types I and III human collagen and have compared this to adhesion with human plasma fibronectin or fibrinogen. We find that there is significant heterogeneity in adhesion to collagens, but not to fibronectin or fibrinogen, that correlates with levels of surface \( \alpha_2\beta_1 \) antigens and with the extent to which this surface receptor incorporates radiolabel. This heterogeneity may help to explain certain discrepancies reported by different laboratories regarding the relative contribution of \( \alpha_2\beta_1 \) to platelet adhesion to purified collagens and extracellular matrices. Most importantly, variation in levels of platelet \( \alpha_2\beta_1 \) among normal individuals may represent an important contributing factor to a predisposition toward stroke, thrombosis, or cardiovascular disease.

**Materials and Methods**

Reagents. Human collagens type I and type III were purified from placenta as described by Miller and Rhodes.\(^{17} \) Human fibronectin was purified from plasma of normal donors as described by Vuento and Valher.\(^{18} \) Purified human Peak-I fibronectin,\(^{19} \) containing only platelet-reactive \( \gamma \) chains, was a generous gift from Dr. David Amran (University of Wisconsin-Milwaukee, Milwaukee, WI). The purity of each of these proteins was assessed to be at least 99% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Based on lack of reactivity with specific murine monoclonal antibodies (MoAbs) in enzyme-linked immunosorbent assay, purified fibronectin was free of detectable fibrinogen, vitronectin, or von Willebrand factor (vWF), and purified fibronogen contained no detectable fibronectin, vitronectin, or vWF. The murine MoAbs P1H5 (anti-\( \alpha_2\beta_1 \) complex) and P1F8 (anti-\( \alpha_2\beta_1 \) complex) were a gift from Dr. William Carter (Fred Hutchinson Cancer Research Foundation, Seattle, WA) and Dr. Elizabeth Wayner (University of Minnesota, St Paul). Murine MoAb 6F1 (anti-\( \alpha_2\beta_1 \) complex) was a gift of Dr Barry Coller (SUNY-Stony Brook, Stony Brook, NY). Murine MoAb 12F1 (anti-\( \alpha_2\beta_1 \) complex) was a gift of Dr Virgil Woods (University of California-San Diego, San Diego, CA).

Adhesion. Assays measuring the adhesion of washed platelets to fibronectin-coated, collagen-coated, or fibronogen-coated microtiter wells were performed as previously described.\(^{16} \) Platelets were harvested from whole blood drawn in one-seventh volume of acidcitrate dextrose-NIH formula A (ACD-A) containing 140 ng/mL PGE\(_2\) (Sigma Chemical Co, St Louis, MO) and washed by differential centrifugation in Ringer’s citrate-dextrose, pH 6.5, plus 20 ng/mL PGE\(_2\) (RCD-PGE\(_2\)). After washing, the platelets were labeled with sodium \(^{31}\)chromate, as described,\(^{16} \) and washed 3 additional times in RCD-PGE\(_2\). Platelets were resuspended at a concentration...
of $1 \times 10^7$/mL in the appropriate buffer. The assays were performed in detachable microtiter wells (Immulon 1; Dynatech Lab Inc, Chantilly, VA) that had been coated with adhesive proteins by overnight incubation at 4°C with 50 μL per well of a solution of human plasma fibronectin (5 μg/mL in phosphate-buffered saline [PBS]), human placental type-I collagen (5 μg/mL in PBS), human placental type-III collagen (5 μg/mL in PBS), or human plasma fibrinogen (5 μg/mL in PBS). Wells coated with bovine serum albumin (BSA; 5 mg/mL in PBS) served as controls. After precoating, the remaining surface of the wells was blocked by the addition of 1% BSA to completely fill each well, and the wells were incubated for 1 hour at room temperature. Platelets (1 × 10^7 in 100 μL of buffer) were allowed to adhere to the coated wells for 90 minutes at 37°C. Nonadherent platelets were removed by 6 washes of each well without centrifugation. The number of adherent platelets in each well was determined from the amount of radioactivity remaining in the wells and from the specific activity of the platelets. All assays were performed in triplicate.

**Scanning electron microscopy (SEM).** After the final washing of the adhesion procedure and in anticipation of subsequent analysis by SEM, adherent platelets in selected wells of microtiter plates were fixed by flooding the wells with 1% glutaraldehyde (Polysciences, Inc, Warrington, PA) in 0.1 mol/L phosphate buffer, pH 7.4. After a 1-hour incubation at ambient temperature and then a 12- to 18-hour incubation at 4°C, the glutaraldehyde solution was aspirated, and the wells were washed with 0.1 mol/L phosphate buffer, pH 7.4. The fixed platelets were then dehydrated by sequential incubation for 5 minutes each in 25%, 50%, 70%, 80%, 85%, 90%, and 95% ethanol, followed by three changes, 8 minutes each, in 100% ethanol. Platelet samples were critical-point dried (Ladd Industries, Burlington, VT), sputter coated with gold-palladium (Hhummer VI; Anatech, Ltd, Alexandria, VA), and examined with a Hitachi S-520 scanning electron microscope (Hitachi, Inc, Tokyo, Japan).

**Aggregation.** Platelet aggregation was monitored using a BioData Model PAP-4 platelet aggregometer (BioData Inc, Hatboro, PA) at 37°C with a stirring rate of 1000 rpm as previously described. Platelet-rich plasma (PRP) was obtained from blood anticoagulated with one-tenth part of 3.8% trisodium citrate, and the final concentration of platelets was adjusted to 300,000/μL by adding platelet-poor plasma. Aggregation was induced by addition of adenosine diphosphate (ADP; 8 μmol/L final concentration), acid-soluble collagen (80 μg/mL), purified type-I human collagen (20 to 115 μg/mL), or purified type-III human collagen (1 to 10 μg/mL). In inhibition assays, MoAb IgG in PBS was preincubated with PRP for 15 minutes at 37°C before the addition of agonists. Final antibody IgG concentrations ranged from 5 to 30 μg/mL.

**Flow cytometry.** Murine monoclonal IgG was purified from ascites fluid by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ) and used at a final concentration ranging from 1 to 30 μg/mL. Washed platelets were prepared as described above for the adhesion studies except that platelets were finally resuspended at a concentration of 5 × 10^8/mL in RCD-PGE_2_. Flow cytometry was performed as described with slight modifications. One-hundred-microliter aliquots of washed platelet suspension (4 × 10^7/mL) were added to tubes containing IgG or Fab fragments of MoAbs in RCD-PGE_2_, and the platelets were incubated in a total volume of 200 μL for 120 minutes at ambient temperature. Platelets were then pelleted at 500g for 7 minutes, supernatants were aspirated, and the platelets were resuspended in 100 μL of RCD-PGE_2_, containing fluorescein isothiocyanate-conjugated secondary antibody (Fab fragments of goat antirabbit IgG; Zymed, Inc, San Francisco, CA). After a 30-minute incubation at ambient temperature, platelets were diluted to 1.0 mL with RCD-PGE_2_, and analyzed in a flow cytometer (FACS Star plus, Becton Dickinson, Mountain View, CA). The data were analyzed using the software program Lyssys (Becton Dickinson) on an HP9000 personal computer.

**Direct quantitation of platelet-bound antibody.** Binding assays were performed as previously described. Washed platelets were resuspended in 15 mmol/L HEPES, 150 mmol/L NaCl, 1% (wt/vol) BSA, 1 mg/mL glucose, pH 7.4, containing 20 ng/mL of PGI_2_. Various concentrations of MoAb IgG were added to the platelet suspension, whereas the final platelet concentration was maintained at 5 × 10^9/mL. After a 60-minute incubation, diluted 100 μL samples were layered onto 200 μL of 30% sucrose in the same buffer in 400-μL microcentrifuge, polypropylene tubes. Tubes were centrifuged at 7,000g for 10 minutes. The supernatants were aspirated, and the radioactivity of both supernatants and pellets was measured in a γ scintillation counter.

**Two-dimensional electrophoresis.** Platelets, harvested from ACD-A anticoagulated whole blood and washed 3 times in RCD-PGE_2_, as described above, were surface-labeled with [3H] by the lactoperoxidase-catalyzed method, as described. Labeled platelets were lysed by the addition of SDS to a final concentration of 2% (vol/vol) plus N-ethylmaleimide to a final concentration of 10 mmol/L, and soluble protein was analyzed by two-dimensional nonreduced-reduced SDS-PAGE, as described. For two-dimensional isoelectric focusing (IEF)-PAGE, according to the procedure of O’Farrell, aliquote of the same labeled platelets was lysed by addition of SDS to 2% plus 2-mercaptoethanol (2-ME) to a final concentration of 5% (vol/vol). To 100 μL of the lysate was added 200 μL of 9.5 mol/L urea, 8% (vol/vol) NP-40, 5% (vol/vol) 2-ME, 5% (vol/vol) ammonium solution (pH range 4 to 8; Pharmacia). Separation in the first dimension was achieved by focusing in the presence of 9.5 mol/L urea and ammonium at 400 V for 12 hours and then 600 V for 1 hour in 4% acrylamide cylinder gels. Cylinder gels were then fixed to the top edge of a 7% acrylamide-slab gel for separation in the second dimension. Proteins were detected by silver-staining, and labeled proteins were detected by autoradiography.

**RESULTS**

Platelets from 27 healthy individuals (15 males and 12 females) were analyzed during the course of this study; each individual was studied on at least three occasions. More extensive analyses were performed with platelets from 14 individuals (7 males and 7 females), measuring adhesion every 3 to 5 days for 12 weeks, and these findings are summarized in Fig 1. The maximum number of platelets (mean ± SD) that adhered to collagen type I (Fig 1A), collagen type III (Fig 1B), fibronectin (Fig 1C), or fibrinogen (Fig 1D) is shown. Individuals are ranked from left to right with respect to increasing mean number of platelets adherent to type I collagen; mean values ranged from 1.1 to 24.2 × 10^5 adherent platelets per well. The ability of platelets from individual donors to adhere to type I and type III collagens was similar over the 90-day period during which these measurements were taken. When adhesion to type III collagen is compared with adhesion to type I collagen, the same relative differences between donors are apparent, and mean values ranged from 5.4 to 28.4 × 10^5 adherent platelets per well (Fig 1B). These differences in adhesion to collagens become more striking when one compares the ability of platelets from the same subjects to adhere to fibronectin (Fig 1C) or...
INTEGRIN α2β1, HETEROGENEITY

Fig 1. Platelet adhesion. The number of 35Cr-labeled platelets bound to microtiter wells coated with (A) human type I collagen; (B) human type III collagen; (C) human plasma fibronectin; or (D) human plasma Peak 1 fibrinogen was measured. The mean number of platelets bound per well (X 10^-5) plus or minus two standard deviations is depicted. The identity of the normal donor is indicated on the abscissa, as is the number of observations (n). In (A), the donors are arbitrarily ranked from left to right as a function of increasing mean adhesion. To facilitate comparisons, the same order of donors is maintained in (B) through (D) irrespective of the actual mean adhesion observed for the respective ligand.

fibrinogen (Fig 1D). With respect to fibronectin or fibrinogen, there is very little difference in the observed mean numbers of adherent platelets among these same individuals. For fibronectin, mean values ranged from 12.3 to 15.6 X 10^5 adherent platelets per well; for fibrinogen, 18.7 to 23.8 X 10^5 adherent platelets per well. The specificity of adhesion was monitored in each experiment by prior incubation with the inhibitory antibodies, P1H5 or 6F1 (anti-αβ1 complex), P1F8 (anti-αβ1 complex), and AP2 (anti-αIIbβ3 complex). Adhesion of nonactivated platelets to type I or type III collagen was inhibited by P1H5 or 6F1 but not by P1F8 or AP2; adhesion to fibronectin was inhibited only by P1F8; and adhesion to fibrinogen was inhibited only by AP2.

We sought to distinguish adhesion from microaggregate formation, because, once they are activated, platelets can form microaggregates (or microthrombi) on platelet monolayers that have adhered to surfaces coated with collagen or fibronectin. In our hands, this increase in numbers of platelets bound to the surface caused by microaggregate formation is mediated by αIIbβ3 and can be inhibited completely by RGD peptides or antibodies that block the function of that integrin, eg, AP2. Adhesion of nonactivated platelets in
This study was constantly monitored by SEM to confirm that observed increases in adhesion to collagens typical of higher activity donors were not a result of microaggregate formation. Representative SEM are shown comparing adherent platelets from a lower-activity donor (no. 4), where $1.8 \times 10^5$ platelets are bound per well (Fig 2A), with a higher-activity donor (no. 14), where $36.9 \times 10^5$ platelets are bound per well (Fig 2B). Regardless of the number of adherent platelets, microaggregate formation was not observed in any sample analyzed. What differs between samples from these donors is the total number of adherent platelets. Further evidence that microaggregate formation does not contribute to differences in adhesion to collagens is the fact that the numbers of adherent platelets observed with any of the donors studied could not be decreased by preincubation with antagonists of $\alpha_{IIb}\beta_3$ function, such as RGD peptides or AP2 (data not shown).

Variation in $\alpha_{IIb}\beta_3$-mediated adhesion correlates with differences in in vitro platelet aggregation induced by purified collagens (Fig 3). When stimulated with an equivalent dose of human type I collagen, the lag time before onset of aggregation was significantly longer with PRP from lower-activity donors compared with PRP from higher-activity donors. For example, in Fig 3, panel 1, the lag time for PRP from donor no. 2 (tracing D) was 12.1 minutes, and that for PRP from donor no. 13 (tracing B) was 7.7 minutes. The lag time of any donor, regardless of basal activity, was always prolonged by prior incubation with 6F1 or PIH5. In Fig 3, panel 1, prior incubation with 6F1 resulted in increases in the lag times to 18.1 minutes and 15.7 minutes for donors no. 2 (tracing C) and no. 13 (tracing A), respectively. 12F1, which binds to $\alpha_{IIb}\beta_3$ but does not inhibit function, fails to increase the lag time in response to type I collagen, as shown for PRP from donor no. 13 in Fig 3, panel 2. Human type-III collagen-induced lag times did not differ between higher- and lower-activity donors (Fig 3, panel 3). At very low doses of type III collagen ($\geq 1 \mu g/mL$), prior incubation with 6F1 actually caused inhibition of aggregation (Fig 3, panel 4). However, no inhibition could be observed at type III collagen doses $\geq 2 \mu g/mL$ (not shown). The differences in lag times seen with purified type I collagen were not observed when aggregation was induced by a crude collagen preparation, i.e., bovine tendon collagen.

Using flow cytometry and any one of three murine MoAbs specific for $\alpha_{IIb}\beta_3$ (6F1, PIH5, or 12F1), a direct correlation is found between the expression of this surface collagen receptor and the aforementioned ability of the same platelets to adhere to collagens type I or type III (Fig 4). Data obtained with PIH5 are shown, but identical results were obtained with 6F1 and 12F1. A comparison of forward and side scatter for each platelet sample bound by PIH5, indicates that only one population of antibody-binding platelets is present, regardless of the amount of antibody bound per platelet (Fig 5). In marked contrast, one sees less variation in platelet adhesion to fibronectin and in surface content of the fibronectin receptor $\alpha_{IIb}\beta_3$ (Fig 6A). No correlation was observed between the surface content of $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}\beta_1$ (Fig 6B).

The level of $\alpha_{IIb}\beta_1$ determined by flow cytometry using PIH5 correlated with the number of receptor molecules determined by direct binding assay using $^{125}$I-labeled 6F1 (Table 1), where a range of 968 to 2,874 molecules bound per platelet was observed. The threefold range in surface receptor determinants determined in this direct binding assay correlates well with the threefold to fourfold range in surface antigen content observed by flow cytometry (see Fig 4A and B). As shown for those 2 subjects with the lowest baseline collagen-receptor activity (nos. 1 and 2) in Table 1, the level of surface $\alpha_{IIb}\beta_1$ (6F1 antigen) did not increase by greater than 5% after platelets were preincubated with 10 $\mu$mol/L phorbol myristate acetate or 8 $\mu$mol/L thrombin receptor peptide SFLLRNPDKY for 15 minutes at ambient temperature in the presence of 10 $\mu$g/mL unlabeled AP2 Fab fragments (to inhibit aggregation). Platelets treated in this manner were fully activated and, in the absence of AP2 Fab fragments, would immediately aggregate even on gentle agitation. The observed differences in collagen receptor antigen or activity do not correlate with the sex of the individual, the...
INTEGRIN αβ1, HETEROGENEITY

Fig 3. Platelet aggregation. Panel 1: Aggregation in PRP was induced by purified type I collagen (116 µg/mL, final concentration) under the following conditions: (A) PRP from donor no. 13 preincubated with 30 µg/mL 6F1; (B) PRP from donor no. 13 in absence of antibody; (C) PRP from donor no. 2 preincubated with 6F1; and (D) PRP from donor no. 2 in the absence of antibody. Panel 2: (A) PRP from donor no. 13; (B) PRP from donor no. 13 preincubated with 30 µg/mL 6F1; and (C) PRP from donor no. 13 preincubated with 30 µg/mL 12F1. Panel 3: Aggregation of PRP from donor no. 2 (A and C) or donor no. 13 (B and D), induced by 10 µmol/L ADP (A and B) or 2 µg/mL type I11 collagen (C and D). Panel 4: Aggregation induced by 1 µg/mL type III collagen. PRP from donor no. 13 with (B) or without (A) prior incubation with 30 µg/mL 6F1. Bars in each panel indicate 1 minute. Arrows with lowercase letter designations indicate the point of addition of collagen to the corresponding PRP samples, as described above.

age (range, 22 to 45 years) of the individual, the time of day that blood is drawn, or the whole-blood platelet count. Levels of some integrins of the human endometrium, particularly αβ3, may be regulated in synchrony with the menstrual cycle in women,4 and levels of αγβ5 in human osteoclasts can be upregulated by the synergistic effects of estrogen or retinoic acid and vitamin D.25 However, the differences in platelet collagen receptor activity noted here occurred independently of the menstrual cycle in women. Despite the threefold variance in levels of surface azPl, the surface content of αIIbβ3, determined by the binding of 125I-AP2 showed a variance of only 470, ranging from 48,644 to 52,868 molecules per platelet (Table 1).

Additional studies were conducted to confirm the quantitative difference in surface αβ1 using a technique that does not rely on antigenicity of this receptor. Total proteins in SDS lysates of surface-radioiodinated platelets were separated by two-dimensional nonreduced/reduced SDS-PAGE (Fig 7), and the 125I content of 8 distinct protein spots, including those given by the α2, α5, and β1 integrin subunits, was determined. Platelet proteins from 3 high-activity and 3 low-activity donors were compared. No difference in the mobility of α2 or β1 was noted. Interestingly, two radiolabeled spots were distinguishable in the position of α3. The more intensely labeled, faster mobility protein (with respect to the first dimension) was designated α3, and the less intensely labeled, slower mobility protein was designated protein 7. However, both forms were present in platelets from each donor regardless of receptor activity. As shown below, any quantitative differences observed for α2 alone also applied to protein 7 alone or to the combination of α2 and protein 7. Although we have not confirmed it, we suspect, that the slower mobility protein actually represents a minor portion of α3 (hence, the decreased radiolabel content) that is already partially or completely reduced at the time of platelet solubilization.

In two-dimensional IEF-PAGE (Fig 8), there was no apparent difference in the isoelectric point (pI) of either α2 or β1 obtained from lower-activity or higher-activity platelets. Heterogeneity of both proteins, unrelated to receptor activity, was noted giving rise to double protein spots for both α2 and β1. As was the case in the nonreduced-reduced SDS-PAGE system, the levels of both α2 spots coordinately increased or decreased in correlation with receptor activity; however, the intensity of the β1 spots did not differ.

Visual inspection of two-dimensional electrophoretic gels
certainly provides suggestive evidence that quantitative differences in $\alpha_2$ exist among normal donors. To obtain a more precise quantitation, the regions of acrylamide gel encompassing 8 representative radiolabeled protein spots in two-dimensional, nonreduced-reduced gels derived from each platelet preparation were excised, and the amount of $^{125}$I incorporated into each protein spot was determined. As shown in Table 2, a significant difference was observed in $^{125}$I content of the $\alpha_2$ but not the $\beta_2$ subunits. This difference was observed whether one expressed the amount of glycoprotein as a percentage of total cpm present in all 8 proteins or as the ratio of $\alpha_2$ cpm to cpm incorporated into any one of the other 7 protein spots. The $^{125}$I content of the $\alpha_{IIb} + \alpha_5$ spot was selected in Table 2 for this calculation because this spot showed the smallest variance among the 6 platelet preparations. The two spots of the $\alpha_2$ cluster ($\alpha_2$ or protein 7) showed an identical quantitative relationship to the other labeled proteins. The level of $\beta_2$ or any of the 5 remaining labeled proteins did not correlate with differences in collagen-receptor activity. These findings confirm that, of the 8 surface-labeled proteins analyzed by this approach, the level of $\alpha_2$ correlates best with the observed differences in platelet adhesion to collagen.

**Fig 4.** Relationship between surface $\alpha_2\beta_1$ content and platelet adhesion to type I (A) or type III (B) collagens. The number of platelets bound per well (abscissa) is plotted against the surface level of P1H5 antigen (ordinate). Surface antigen was determined by flow cytometry on separate samples and is represented by mean fluorescence (linear scale). For type I collagen ($n = 60$), there is a significant correlation between surface antigen and adhesive ability ($r = .7423; P < .01$). A significant correlation was also observed in the case of type III collagen ($n = 60$; $r = .6359; P < .01$).

**Fig 5.** Flow cytometry: Side scatter (SSC) versus forward scatter (FSC). For each platelet sample analyzed by flow cytometry, regardless of the observed mean fluorescence or the number of adherent platelets obtained in a corresponding sample, a single population of platelets was observed, as determined by the relationship between FSC and SSC. The measured parameters for each of the depicted samples: (A) mean F = 8.79, type I collagen adhesion (CI) = $36.7 \times 10^5$ platelets, donor = no. 14; (B) mean F = 6.51, CI = $22.3 \times 5$ platelets, donor = no. 11; (C) mean F = 4.82, CI = $6.8 \times 10^5$ platelets, donor = no. 9; (D) mean F = 2.45, CI = $0.8 \times 5$ platelets, donor = no. 1.

**DISCUSSION**

The integrins are present on nearly all human cells and can mediate cell attachment to the extracellular matrix, cell spreading on the extracellular matrix and cell-to-cell interactions. Messenger RNAs specific for a number of the integrin subunits, including $\alpha_2$ and $\beta_1$,26,27 have been cloned,
and the corresponding amino acid sequences of the protein subunits have been deduced. These important accomplishments have enabled investigators to begin to localize functional sites on the integrin subunits and to identify specific mutations in either subunit that may affect expression or activity of the receptor.

Our results show that two characteristics of surface α2β1 differ markedly from one individual to the next, the expression of specific monoclonal antigen epitopes and the incorporation of radiolabel into this surface protein. The most important finding of our study is that this heterogeneity of the surface integrin correlates precisely with significant differences in the activity of this platelet receptor.

The heterogeneity that we report here helps to explain previously reported differences in the quantity of surface α2β1 on platelets. Using 12F1, Pischel et al. reported that there are about 1,800 molecules of α2β1 per platelet. Using 6F1, Coller et al. then estimated the number of surface receptors to be less than this or approximately 800 molecules per platelet. Both of these estimates are within the range of values that we have observed for surface α2β1 using either one of these antibodies. In side-by-side comparisons of these antibodies, Coller et al. found that the number of molecules of either antibody bound per platelet for any one individual were essentially identical. Our results concur with those findings and show that identical results are obtained with a third antibody, P1H5. The basis for the range of values is not differences in methods used by each laboratory, but rather it is an inherent variation in the content of antigen among platelets from different individuals.

We confirm and extend the observation of Coller et al. that the binding of 6F1 can induce an increase in the lag time before onset of aggregation induced by collagen. In this study, we observe the same effect of 6F1 on aggregation induced by purified type I human collagen. Moreover, we show that differences in the lag time in the absence of inhibitory antibody correlate with the ability of platelets from the same individual to adhere to type I collagen. Both the increase in lag time before aggregation and the decreased numbers of platelets bound to the collagen surface can be explained by identical molecular differences in α2β1, that are reflected in the antigen variability described above.

This study has focused on the collagen receptor α2β1, and our results clearly show that variations in the level of this integrin alone on the platelet surface correlate with observed variations in platelet adhesion to collagen. These findings
strongly support the role of $\alpha_2\beta_1$ as an important platelet collagen receptor. Other platelet receptors for collagens, notably glycoprotein IV (or CD36) and a 62-kD surface protein, presumably glycoprotein VI, have been described. In our hands, the Mg$^{2+}$-dependent adhesion of platelets to types I and III collagen in a static system and in the presence of the platelet inhibitor PGE$_1$ is completely inhibited by murine MoAb P1H5 or 6F1, which are specific only for $\alpha_2\beta_1$. Collier et al have arrived at an equivalent conclusion. It is particularly relevant that the surface levels of glycoprotein IV on platelets from 3 lower-activity donors and 3 higher-activity donors, determined by direct surface-labeling of platelets with $^{125}$I, did not show a correlation with the activity of platelets from these same donors in the adhesion assay.

We have been very careful to monitor and eliminate the potential contribution of another platelet integrin, $\alpha_{IIb}\beta_3$, to adhesion to collagens. The reasons for this are obvious. The surface content of $\alpha_{IIb}\beta_3$ (at least 40,000 copies per platelet) far exceeds that of $\alpha_2\beta_1$. Consequently, the participation of even a portion of $\alpha_{IIb}\beta_3$ can influence the findings, provided that it is truly a mediator of adhesion to collagen. It has been reported that $\alpha_{IIb}\beta_3$ may mediate adhesion to collagen under certain circumstances, and this interaction is related to the expression of LIBS on the $\alpha_{IIb}\beta_3$ receptor. Although it has been well documented that ligand binding, particularly RGD-dependent ligand binding, to $\alpha_{IIb}\beta_3$ will induce the expression of LIBS, like PMI, like LIBS 1, it has now been reported that certain antibodies that recognize such LIBS can themselves induce ligand binding to $\alpha_{IIb}\beta_3$, presumably by shifting a conformational equilibrium between an “inactivated” and “activated” state of $\alpha_{IIb}\beta_3$.

We attribute the differences in platelet adhesion to type I or type III collagen seen in this study to subtle differences in the mechanisms whereby platelets interact with these collagen molecules. In adhesion assays, normal subjects differed about 20-fold with respect to adhesion to type I collagen but only fivefold with respect to adhesion to collagen type III. Moreover, we have observed that MoAbs such as P1H5 can completely abolish platelet adhesion to type I collagen but can only inhibit about 90% to 95% of the adhesion to type III collagen. The residual adhesion may be mediated by yet another platelet receptor or by alternate recognition sites on the same receptor. The distinction between collagen types was particularly apparent in aggregation assays in which differences in aggregation induced by type III collagen were subtle, whereas differences in aggregation induced by type I collagen were obvious. Moreover, at limiting agonist dilutions, 6F1 would markedly prolong the lag time associated with aggregation induced by type I collagen but would completely inhibit aggregation induced by type III collagen. These findings clearly indicate that more needs to be learned about the variety of mechanisms that are involved in platelet binding to different collagen types. There is already evidence that suggests the existence of diverse mechanisms of receptor-collagen interaction. Staatz et al and Santoro et al recently concluded that a tetrapeptide sequence from the $\alpha_1(I)$ chain, Asp-Gly-Glu-Ala (DGEA), blocks platelet adhesion mediated by the platelet collagen receptor $\alpha_2\beta_1$. DGEA is not present in type III collagen, and Karnigian et al reported that another peptide sequence, KPGEPGPK, located once in $\alpha_1$(III), inhibits type III collagen-induced platelet aggregation but does not inhibit adhesion.

A granule-associated protein with electrophoretic characteristics similar to $\alpha_2$ and designated GPIa* has been described. If this glycoprotein was expressed on the surface of activated platelets in our study, it certainly did not contribute appreciably to surface 6F1 or 12F1 antigen content. In addition, adhesion to collagen observed in our study does not either require or result in $\alpha$-granule content release and is completely blocked by 6F1. Because of this, we conclude that GPIa* is an entity distinct from $\alpha_2$ that is not affected.
**INTEGRIN α2β1 HETEROGENEITY**

**Fig 8.** Two-dimensional IEF/SDS-PAGE. Proteins (200 pg) from radioiodinated platelets that had been lysed in SDS were separated in the first dimension by isoelectric focusing and in the second dimension by PAGE under reduced conditions (top to bottom). The pl of acrylamide gel samples is indicated across the top of the gel. The position of molecular weight markers in the second dimension is indicated to the left with the corresponding molecular weights (in kilodaltons). (A) A sample from donor no. 14; (B) a sample from donor no. 1. The protein spots given by selected integrin subunits, namely, α2*, α3β1, and β3, are labeled.

by and does not contribute to the heterogeneity that we describe in this study.

Sequence or structural polymorphisms of α2 or β1 could be responsible for the functional heterogeneity that we de-

**Table 2. Quantitative Analysis of Radioiodinated Membrane Glycoproteins in Platelets From Normal Subjects**

<table>
<thead>
<tr>
<th></th>
<th>Subject No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>11</th>
<th>12</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of total cpm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2</td>
<td></td>
<td>0.7</td>
<td>1.8</td>
<td>1.7</td>
<td>2.1</td>
<td>4.2</td>
<td>3.5</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>1.0</td>
<td>0.7</td>
<td>1.1</td>
<td>1.3</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>αm + αg*</td>
<td></td>
<td>16.3</td>
<td>17.3</td>
<td>16.4</td>
<td>16.8</td>
<td>15.6</td>
<td>16.1</td>
</tr>
<tr>
<td>β1</td>
<td></td>
<td>7.2</td>
<td>7.1</td>
<td>8.1</td>
<td>6.0</td>
<td>8.3</td>
<td>7.9</td>
</tr>
<tr>
<td>β3</td>
<td></td>
<td>63.2</td>
<td>55.8</td>
<td>62.4</td>
<td>61.1</td>
<td>60.1</td>
<td>57.4</td>
</tr>
<tr>
<td>Ib†</td>
<td></td>
<td>3.0</td>
<td>4.9</td>
<td>3.1</td>
<td>3.7</td>
<td>2.9</td>
<td>4.2</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>7.5</td>
<td>10.9</td>
<td>6.4</td>
<td>7.5</td>
<td>6.3</td>
<td>7.4</td>
</tr>
<tr>
<td>PECAM-1</td>
<td></td>
<td>0.6</td>
<td>0.9</td>
<td>0.5</td>
<td>0.8</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.6</td>
<td>0.5</td>
<td>0.4</td>
<td>0.8</td>
<td>0.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of x/αm + αg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2</td>
<td></td>
<td>0.043</td>
<td>0.104</td>
<td>0.104</td>
<td>0.125</td>
<td>0.269</td>
<td>0.217</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.061</td>
<td>0.040</td>
<td>0.067</td>
<td>0.077</td>
<td>0.096</td>
<td>0.112</td>
</tr>
<tr>
<td>αm + αg*</td>
<td></td>
<td>0.442</td>
<td>0.410</td>
<td>0.494</td>
<td>0.357</td>
<td>0.532</td>
<td>0.491</td>
</tr>
<tr>
<td>β3</td>
<td></td>
<td>0.184</td>
<td>0.283</td>
<td>0.189</td>
<td>0.220</td>
<td>0.186</td>
<td>0.261</td>
</tr>
<tr>
<td>Ib†</td>
<td></td>
<td>0.460</td>
<td>0.630</td>
<td>0.390</td>
<td>0.446</td>
<td>0.404</td>
<td>0.460</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>0.037</td>
<td>0.052</td>
<td>0.030</td>
<td>0.048</td>
<td>0.038</td>
<td>0.050</td>
</tr>
<tr>
<td>PECAM-1</td>
<td></td>
<td>0.037</td>
<td>0.029</td>
<td>0.024</td>
<td>0.048</td>
<td>0.032</td>
<td>0.043</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.037</td>
<td>0.029</td>
<td>0.024</td>
<td>0.048</td>
<td>0.032</td>
<td>0.043</td>
</tr>
</tbody>
</table>

* Combined heavy chains of αm and αg.
† Glycoprotein Ib heavy chain, ie, Ibα.

scribe in this report. One polymorphism in human α2 that is described serologically but has not yet been identified biochemically is responsible for the alloantigen alleles Brα and Brβ. A number of the normal subjects studied in this report have been independently typed for Br phenotype. We found no correlation between serologic Br phenotype and level of platelet α2β1 or extent of platelet adhesion to collagen in 9 subjects whose Br genotype is known. Consequently, the Br polymorphism is not associated with the functional heterogeneity that we observe. We are unaware of any other reported polymorphisms in the α2 subunit. Although our data indicate that levels of α2 vary in normal individuals, whereas levels of β1 do not, it is conceivable that a polymorphism in β1 might be responsible for decreased surface expression of the complex or might alter the structure and, consequently, the function of the α2 subunit. Two variants of the cytoplasmic domain of β1, that are caused by alternate splicing have been reported. The first variant, β1S, expressed in HEL and K562 erythroleukemia cell lines, results from a 116-nucleotide insert that produces a frame shift in the 3' end of β1S and codes for a unique 48-amino acid COOH-terminal sequence. A murine MoAb raised against this unique amino acid sequence binds to an HEL cell surface molecule that comigrates in SDS gels with native β1, although β1S constitutes a minor portion of total HEL cell β1. Only the β1 sequence was amplified from platelet mRNA when primers common to β1 and β1S were used in polymerase chain reaction, and it was concluded that β1S mRNA must be present in insignificant amounts in platelets. In this study, we searched for this variant β1S protein in platelets from individual donors, categorized by vir-
tue of platelet $\alpha_2\beta_1$ activity, using the same murine MoAb (a gift from Dr L. Languino, La Jolla, CA). We confirmed that platelets contain no detectable variant $\beta_{12}$ protein using an immunoblot assay (data not shown). Platelets or megakaryocytes have not yet been tested for the presence of the second variant $\beta_{13}^{\alpha_2}$. Unfortunately, neither the $\beta_{13}^{\alpha_2}$ protein nor the $\beta_b$ protein can be distinguished from native $\beta_1$, protein based on electrophoretic mobility in SDS-PAGE.

It is unlikely that differences in carbohydrate composition of the $\alpha_3$ subunit, particularly differences in sialic acid content, account for the heterogeneity that we observe in this study, because we have observed identical electrophoretic properties of the $\alpha_3$ subunit from all subjects studied, including higher- and lower-activity donors, in two-dimensional IEF-PAGE. On the other hand, the heterogeneity of platelet $\alpha_2\beta_1$, that we describe may be related to the proposed structural polymorphism of $\alpha_3$ recently reported by Chan and Hemler.54 In that report, it was shown that transfected $\alpha_3$ cDNA gives rise to three distinct forms of $\alpha_3\beta_1$, which differ in reactivity toward collagen or laminin but show no detectable differences in glycosylation or phosphorylation. It was concluded that $\alpha_2\beta_1$ can exist in multiple stable but interconvertible conformations. The relationship of this finding to the platelet $\alpha_2\beta_1$ heterogeneity that we describe here will be explored in subsequent studies.

Whatever the molecular basis for the $\alpha_2\beta_1$ heterogeneity that we observe, it is certain to have an impact on the ability of platelets to adhere to collagen and on platelet function in vivo. Before we can intelligently assess the importance of this collagen receptor and other integrins to hemostasis and cell adhesion, in general, we must first learn more about the general mechanisms by which integrins on platelets and other adhesive cells are regulated. The identification of the heterogeneity in $\alpha_2\beta_1$, that we describe in this report is an important first step toward such an understanding.

ACKNOWLEDGMENT

We thank Dr Barry Coller (SUNY-Stonybrook) and Dr Virgil Woods (UCSD) for their helpful discussions and suggestions as well as gifts of the antibodies 6F1 and 12F1, respectively. We thank Dr L. Languino (RW Johnson Pharmaceutical Research Institute, La Jolla, CA) for her gift of the antibody reactive with $\beta_{12}$. We also thank Dr William Carter (Fred Hutchinson Cancer Research Center) and Dr Elizabeth Wayner (University of Minnesota, St Paul, MN) for their MoAbs P1H5 and P1F8.

REFERENCES

2. Pischel KD, Bluestein HG, Woods VL Jr: Platelet glycoproteins Ia, Ic and Ila are physiocochemically indistinguishable from the very late activation antigens adhesion-related proteins of lymphocytes and other cell types. J Clin Invest 81:505, 1988
INTEGRIN αβ₁ HETEROGENEITY


41. Chan BMC, Hemler ME: Multiple forms of the integrin VLA-2 can be derived from a single α₂ cDNA clone: Interconversion of forms induced by an anti-β₁ antibody. J Cell Biol 120:537, 1993
Variability of integrin alpha 2 beta 1 activity on human platelets

TJ Kunicki, R Orzechowski, D Annis and Y Honda

Updated information and services can be found at:
http://www.bloodjournal.org/content/82/9/2693.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml