A Murine Antiglycoprotein Ib Complex Monoclonal Antibody, SZ 2, Inhibits Platelet Aggregation Induced by Both Ristocetin and Collagen

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A new monoclonal antibody (MoAb), SZ 2, reactive with the human platelet glycoprotein Ib complex has been produced by the hybridoma technique. SZ 2 immunoprecipitated the components of the glycoprotein Ib complex, glycoprotein Ib and glycoprotein IX, from Triton-X-100-solubilized, periodate-labeled platelets. Western blot analysis indicated that the epitope for SZ 2 was on the α-subunit of glycoprotein Ib. Scatchard analysis of SZ 2 binding to formaldehyde-fixed, washed platelets revealed a single class of binding sites with \( K_d = 6.6 \pm 3.3 \times 10^{-10} \) mol/L and 15,200 ± 4,100 binding sites per platelet (mean ± SD, n = 10). Intact antibody and its purified (Fab’)_2 fragments not only inhibited the ristocetin-dependent binding of von Willebrand factor to platelets and ristocetin-induced platelet agglutination but also inhibited platelet aggregation induced by Type I collagen and platelet-activating factor (PAF). SZ 2 inhibited platelet serotonin and \( \beta \)-thromboglobulin release in response to these stimuli and also platelet thromboxane A\(_2\) formation in response to ristocetin and collagen. SZ 2 was without effect on platelet aggregation or release in response to other platelet stimuli such as ADP, thrombin, or arachidonic acid. The inhibition by SZ 2 of collagen- and PAF-induced platelet aggregation is surprising in that Bernard-Soulier syndrome platelets, which lack the glycoprotein Ib complex, respond normally to both these stimuli. SZ 2 was unreactive toward Bernard-Soulier syndrome platelets, as evaluated by fluorescence-associated cell sorting, and had no effect on the collagen- and PAF-induced aggregation of Bernard-Soulier syndrome platelets. The combined results suggest that the inhibition by SZ 2 of collagen- and PAF-induced aggregation of normal platelets is steric and are consistent with the glycoprotein Ib complex and the platelet collagen and PAF receptor(s) being adjacent in the human platelet plasma membrane.

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MATERIALS AND METHODS

Materials. Bovine serum albumin (Fraction V), adenosine diphosphate, hypoxanthine, aminopterin, thymidine, A 23187, platelet-activating factor, and arachidonic acid were purchased from Sigma, St. Louis; sodium periodate (Univar) from Ajax Chemicals, Sydney, Australia; pristane from Aldrich, Milwaukee, WI; polyethylene glycol 4000 from Merck, Darmstadt, West Germany; ristocetin sulfate from Lundbeck, Copenhagen, Denmark; epinephrine...
from Wuxi No. 4 Pharmaceutical Plant, Wuxi, China; equine tendon collagen from Horn, Munich, West Germany; bovine von Willebrand factor from Speywood, England; bovine α-thrombin from Biochemical Products, Tianjin, China; RPMI 1640 medium from Gibco, Grand Island, NY; pepsin from Worthington, Freehold, NJ; protein-A-Sepharose CL-4B from Pharmacia, Sydney, Australia; Na+ 1.2% from New England Nuclear, Boston; and H-sodium borohydride from Amersham, Sydney, Australia. Platelet-activating factor (PAF), type I calf skin collagen, and human α-thrombin were the generous gifts of Dr Vargafiq, Paris; Dr Y. Legrand, Paris; and Dr J. W. Fenton, II, Albany, NY, respectively. Fluorescein-tagged goat antimouse IgG was from Cappel, Philadelphia; peroxidase-conjugated goat antimouse IgG was from Kirkegaard and Perry Laboratories, Gaithersburg, MD. Human von Willebrand factor was purified from commercial Factor VIII concentrate (CNTS, Paris, France) essentially as previously described14 and labeled by lactoperoxidase-catalysed radiodination.

**Platelet preparation and labeling.** Platelet preparation from freshly drawn blood, platelet washing, and the periodate-labeling of platelets were each performed as previously described.15 For fluorescence-associated cell sorting and the analysis of SZ 2 binding to platelets, the platelets were washed and then fixed with 1% (wt/vol) paraformaldehyde essentially as previously described.15 Blood was also obtained from two patients, RS and LS, with the Bernard-Soulier syndrome.7 Platelets were isolated from these patients essentially as previously described.7

**Monoclonal antibodies.** The SZ 2 antibody (IgG,) was prepared according to the method of Kohler and Milstein16 using 8-week-old BALB/C mice and washed platelets as the immunogen. Clones were assessed for antibody secretion into supernatant culture fluid by radioimmunooassay (RIA) using 125I-labeled affinity-purified rabbit antimouse IgG and washed platelets essentially according to our previously described method.17 Selected hybridomas were grown to confluence in 2-mL Costar plates and subsequently in 25-cm² plastic flasks. Cloning was performed by the limiting dilution method using 96-well microtiter plates with mouse peritoneal macrophage feeder layers. For SZ 2 this procedure was repeated a total of six times, prior to injection (10⁶ cells) intraperitoneally (IP) into BALB/C mice previously primed with 0.5 mL of pristane. Resulting ascitic fluid were drained, centrifuged, and stored at −20°C.

IgG was purified from SZ 2 ascites fluid by chromatography on protein-A-Sepharose CL-4B as described by Eyt et al18 and was dialysed against either 0.01 mol/L sodium phosphate, 0.15 mol/L sodium chloride, pH 7.4 (PBS buffer) or 0.01 mol/L Tris, 0.15 mol/L sodium chloride, pH 7.4 prior to storage at −20°C. The purified IgG gave a single band at 150,000 molecular weight as assessed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. SZ 2 (Fab’), fragments were prepared from SZ 2 IgG by pepsin digestion as described by Collier.19 On SDS-polyacrylamide gel electrophoresis under nonreducing conditions, the SZ 2 (Fab’), gave a single band with a molecular weight of 100,000. SZ 2 IgG was radiiodinated by the chloramine T method; >95% of the incorporated radioactivity was precipitable by 10% (wt/vol) trichloroacetic acid. Other MoAbs against the human platelet glycoprotein Ib complex have been previously reported: FMC 25 (IgG3),20 AN 51 (IgG3);21 WM 23 (IgG1);21 and AP 1.22 AP 1 was the generous gift of Dr T. J. Kunicki, Milwaukie. In some experiments SZ 2 (IgG), and WM 17 (IgG3) directed against the human platelet glycoprotein IIb/IIIa complex and FMC 18 (IgG3) directed against an antigen on Toxoplasma gondii23 were used as negative controls. IgG was purified from these MoAbs by chromatography on protein-A-Sepharose CL-4B essentially as described above.

**Aggregation studies.** The effect of MoAb IgG or (Fab’), fragments on platelet aggregation response was evaluated essentially as previously described.14 Platelet aggregation was followed in PRP (3 × 10⁸ platelets/mL, final concentration) stirred at 1,000 rpm at 37°C using either a Payton (Scarborough, Ontario) lumaggregometer (model 1000) or a PAM–2 aggregometer (Danyang, China). Monoclonal antibody IgG (10 to 50 μg/mL, final concentration) or (Fab’), fragments (10 μg/mL) were added three minutes before the addition of stimuli at the following final concentrations: ADP, 2 μmol/L; epinephrine, 2.5 μmol/L; type I calf skin collagen, 10 to 20 μg/mL; equine tendon collagen, 0.5 to 1 μg/mL; ristocetin, 1.25 mg/mL; bovine vWF, 0.75 U/mL; A23187, 16 μmol/L; PAF, 0.3 μmol/L; and arachidonic acid, 200 μg/mL. Platelet aggregation in response to α-thrombin was performed using washed platelets suspended in Tyrode’s solution (3 × 10⁸ platelets/mL, final concentration; human α-thrombin, 0.05 to 0.1 U/mL; bovine α-thrombin, 0.125 U/mL). To evaluate platelet release the contents of the aggregometer cuvette were centrifuged five minutes after the addition of the platelet stimulus. Released serotonin was measured using the fluorescence method of Curzon et al.24 β-Thromboglobulin was measured by RIA (the β-thromboglobulin assay kit was supplied by Shanghai 2nd Medical College, Shanghai, China). The formation of thromboxane B₂ was measured according to the procedure of Fitzpatrick,25 using a radiolabeled tracer of thromboxane B₂. The anti-thromboxane B₂ antibody used for this assay was the generous gift of Dr J. Maclouf (Unité 150 INSERM, Hopital Lariboisière, Paris).

**Inhibition of binding of von Willebrand factor to platelets.** Binding of von Willebrand factor (vWF) to washed platelets was determined as previously described15 with minor modification. The binding assay for 125I-labeled vWF to platelets was performed at room temperature in a final volume of 0.25 mL. Each tube contained 200 μL of washed platelets (2 × 10⁸), 20 μL of PBS buffer or MoAb IgG (10 to 40 μg/mL, final concentration) in PBS buffer, 20 μL of 125I-labeled vWF (25,000 cpm, 10 μg/mL final concentration), and 10 μL of ristocetin (1 mg/mL, final concentration). After 30 minutes duplicate 100-μL aliquots of the reaction mixture were overlayed on 50 μL of dibutyl phthalate/dioctyl phthalate (2:1) in a narrow bore eppendorf tube and centrifuged at 12,000 g for three minutes. After careful aspiration of the supernatant, the pellet was counted for radioactivity in a gamma counter. Control experiments with this assay system confirmed that >95% of the binding of 125I-labeled vWF to platelets was ristocetin-dependent.

**Monoclonal antibody binding to platelets.** Binding of SZ 2 IgG to formaldehyde-fixed, washed human platelets was assessed by equilibrium saturation analysis of 125I-labeled SZ 2 IgG binding essentially as described by Powell-Jones et al.26 Preliminary experiments established that equilibrium binding of SZ 2 IgG to platelets was achieved within 30 minutes under the assay conditions and remained at a constant level until 60 minutes. For the equilibrium saturation analysis of binding, 0.01 to 100 μg/mL, final concentration, of unlabeled SZ 2 IgG in PBS buffer was mixed with 10,000 cpm of 125I-labeled SZ 2 in the same buffer and added to the platelet suspension (10⁸ platelets/tube) in PBS buffer containing 0.3% (wt/vol) bovine serum albumin. The final volume was 200 μL/tube. After incubation at 37°C for 60 minutes, the platelets were separated by centrifugation, washed briefly in PBS buffer containing 0.1% (wt/vol) bovine serum albumin, and the pellet counted for radioactivity in a gamma counter. Control experiments confirmed that no dissociation of bound SZ 2 occurred within the time period of the platelet washing step. Nonspecific binding under the assay condition was <2%. Binding data was analyzed according to the method of Scatchard.27 The effect of a 100-fold excess of the antigycoliprotein Ib complex MoAbs, FMC 25, WM 23, AN 51, and AP 1, on the equilibrium binding of SZ 2 to platelets was performed using platelet-rich plasma prepared from venous blood collected into 3.2% sodium citrate as anticoagulant essentially as described by Collier et al.28
Platelet immunofluorescence and examination by flow cytometry. Normal or Bernard-Soulier syndrome platelets (0.5 to 1.0 x 10^6) were incubated with saturating concentrations of SZ 2 IgG or WM 17 IgG at 20°C for ten minutes with intermittent agitation and then washed twice with Hanks' buffered saline solution (HBSS) containing 8 mmol/L potassium EDTA. As a control, first layer MoAb was substituted with an "irrelevant" MoAb, FMC 18, which reacts only with T gondii. The washed platelets were then incubated with fluorescein-conjugated goat antimouse IgG (previously optimized for titre) at 20°C for ten minutes and then washed twice as described above. The amount of platelet-bound antibody was analyzed on a FACS 440 flow cytometer (Becton Dickinson, Mountain View, CA) using an argon ion laser at 488 nm and 200 mW and a 530 ± 15 nm bandpass filter for log fluorescence determination. Data was stored and analyzed as LIST-mode files on a Becton Dickinson Consort 40 computer essentially as described by Adelman et al.28

Immunoprecipitation. Immunoprecipitation using the mouse MoAb, SZ 2, employed the Triton X-100 lysate of periodate-labeled platelets and was performed as previously described.11,14 A mouse MoAb, WM 21, directed against human CALLa, was used as the negative control.

Electrophoresis. SDS-polyacrylamide gel electrophoresis, protein staining, fluorography, western blot analysis, and crossed immunoelectrophoresis were all performed as previously described.11,14

RESULTS

Immune studies: Demonstration that SZ 2 is directed against the human platelet glycoprotein Ib complex. Immunization of mice with washed human platelets led to the establishment of a number of hybridoma cell lines secreting MoAbs to human platelets. One of these MoAbs, SZ 2, proved to be of special interest and was the subject of further detailed studies. The results of immunoprecipitation experiments using Triton-X-100-solubilized, periodate-labeled platelets are shown in Fig 1. SZ 2 immunoprecipitated the components of the glycoprotein Ib complex (Fig 1 lane 3—nonreduced; lane 6—reduced). The glycoprotein assignments are based on the apparent molecular weights under nonreducing and reducing conditions and their correspondence to the platelet glycoprotein profile in the platelet lysate: glycoprotein Ib, nonreduced mol wt = 170,000, reduced mol wt = 135,000 and 25,000, most intensely labeled glycoprotein by the sialic-acid-specific periodate labeling procedure;29 glycoprotein IX, nonreduced and reduced mol wt = 22,000. In addition, SZ 2 gave the same immunoprecipitation pattern when directly compared with other defined antiglycoprotein Ib complex MoAbs, FMC 25, WM 23, AN 51, and AP 1 (data not shown) and the same pattern of minor bands previously observed in immunoprecipitation experiments with FMC 25, AN 51, and quinine/quinidine drug-dependent antibodies.14 These comprise two minor bands of slightly lower apparent molecular weight than glycoprotein Ib in the nonreduced gel (lane 3) and a band of slightly higher molecular weight than glycoprotein Ib (lane 3) and glycoprotein Ib (lane 6) in the nonreduced and reduced gels, respectively. As previously discussed in detail,14 these bands probably derive from partial reduction of glycoprotein Ib and the co-precipitation of glycoprotein Ia with the glycoprotein Ib complex.

Since both the glycoprotein Ib complex and glycoprotein Ia co-immunoprecipitate with antiglycoprotein Ib complex MoAbs,14,30,31 presumably because of their mutual association with actin-binding protein,31,32 western blot analysis and crossed immunoelectrophoresis were performed to further characterize the reactivity of SZ 2. By western blot analysis, SZ 2 reacted with a single protein band of molecular weight 170,000, nonreduced, and 135,000, reduced (Fig 2), consis-
tent with SZ 2 recognizing an epitope on the α-chain of glycoprotein Ib. In contrast, glycoprotein Ia has an apparent molecular weight of 153,000 under nonreducing conditions and 167,000 under reducing conditions.33 The reactivity of and glycoprotein lb. In contrast, glycoprotein Ia has an apparent

ectrophoresis with polyspecific rabbit antihuman class saturation within 30 minutes. Figure 3 shows the relationship shown to contain the immuneprecipitation arc for glycoprotein Ia33 that SZ 2 recognizing an

epitope on the glycoprotein lb complex MoAb, WM 23.11 There was no binding in the region of the gel previously shown to contain the immuneprecipitation arc for glycoprotein Ia33 (data not shown).

Binding of 125I-SZ 2 to fixed, washed platelets was rapid, being ≈50% complete within ≈five minutes and reached saturation within 30 minutes. Figure 3 shows the relationship between the amount of SZ 2 added and the amount bound. Binding of SZ 2 reached saturation with ≈0.3 μg of SZ 2 bound per 10^9 platelets. Scatchard analysis revealed a single class of binding sites (Fig 3) with Kd = 6.6 ± 3.3 x 10^-10 mol/L and 15,200 ± 4,100 binding sites per platelet (mean ± SD, n = 10). The results are comparable with the number of binding sites determined under identical conditions for the antiglycoprotein lb complex MoAb, AN 51:12,800 ± 3,000 (mean ± SD, n = 10)34 and similar to the number of binding sites found for other antiglycoprotein lb complex MoAbs with platelets in platelet-rich plasma.11,20 The binding of 125I-SZ 2 (1 μg/mL) to platelets in platelet-rich plasma (10^9/mL) was inhibited by ≈98% by a 100-fold excess of unlabeled SZ 2, by ≈35% by 100 μg/mL of AP 1, by ≈25% by 100 μg/mL of AN 51, and by <10% by 100 μg/mL of WM 23 or FMC 25 (data not shown), indicating that SZ 2 bound to a unique epitope on the glycoprotein lb complex relative to the other antiglycoprotein lb complex MoAbs.

Effect of SZ 2 on platelet function. SZ 2 IgG (10 to 50 μg/mL) had no effect on platelet aggregation induced by adenosine diphosphate, ADP (2 μmol/L), epinephrine (2.5 μmol/L), calcium ionophore, A23187 (16 μmol/L), arachidonic acid (200 μg/mL), human α-thrombin (0.05 to 0.1 U/mL), or bovine α-thrombin (0.125 U/mL) (data not shown). In contrast, SZ 2 IgG (10 μg/mL) was a potent inhibitor of ristocetin-induced, von Willebrand factor-depen-

dent platelet agglutination (Fig 4A) and bovine von Willebrand factor-dependent platelet agglutination (Fig 4B). SZ 2 IgG directly inhibited ristocetin-induced platelet agglutination, since at saturating concentrations it blocked the ristocetin-dependent binding of human von Willebrand factor to washed human platelets by ≈80%. In this regard, it was less effective than AN 51 IgG, which completely blocked ristocetin-dependent von Willebrand factor binding under the same conditions (data not shown). SZ 21, directed against the human platelet glycoprotein IIb/IIIa complex, inhibited ristocetin-dependent von Willebrand factor binding by <20% (data not shown). Unexpectedly, SZ 2 IgG (10 μg/mL) was also a potent inhibitor of platelet aggregation induced by type I calf skin collagen (20 μg/mL) (Fig 4C) and by platelet-activating factor, PAF (0.3 μmol/L) (Fig 4D). SZ 2 IgG (10 to 50 μg/mL) was also a strong inhibitor of platelet aggregation induced by the more potent collagen stimulus, equine tendon collagen (0.5 to 1.0 μg/mL) (data not shown, cf Fig 6). Higher concentrations of equine tendon collagen (2 to 10 μg/mL) partially overcame the inhibitory effect of SZ 2 IgG. However, the lag time before the onset of platelet shape change and aggregation was always increased in the presence of SZ 2 IgG, and the rate of aggregation was always slower than the rate in the absence of SZ 2. Since there is some evidence that the platelet Fc-receptor and the glycoprotein lb complex may be sterically related in the human platelet membrane,35 the aggregation experiments were repeated with SZ 2 (Fab')2 fragments to eliminate the possibility that the Fc-region of the mouse immunoglobulin had a compounding effect on the observed results. The SZ 2 (Fab')2 fragments, however, at 10 μg/mL gave similar inhibitory effects with respect to the intact immunoglobulin for platelet aggregation induced by ristocetin, by type I calf skin collagen, and by PAF (Fig 4). Early events in platelet activation in response to ristocetin, collagen, and PAF as defined by the platelet release reaction (Table 1) and thromboxane B2 formation (Table 2) were also inhibited by SZ 2 IgG. In contrast, SZ 2 IgG had little or no effect on the release reaction or thromboxane B2 formation in response to

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**Fig 3.** Effect of increasing concentration of 125I-labeled SZ 2 on its steady-state binding to formaldehyde-fixed platelets. The insert shows a Scatchard plot of the same data with the Kd = 4.4 x 10^-10 mol/L and the maximum number of sites ≈1.3 x 10^9/platelet. The binding analysis was performed as described under Materials and Methods.

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**Fig 4.** Effect of SZ 2 and its (Fab')2 fragments on platelet aggregation. Trace 1, buffer control; trace 2, 10 μg/mL SZ 2; trace 3, 10 μg/mL SZ 2 (Fab')2 fragments. Panel A, ristocetin (1.25 mg/mL); panel B, bovine von Willebrand factor (0.75 U/mL); panel C, type I calf skin collagen (20 μg/mL); panel D, platelet activating factor (0.3 μmol/L). The platelets were pre-incubated with buffer, SZ 2, or SZ 2 (Fab')2 fragments for three minutes before the addition of the appropriate stimulus.
a-thrombin or arachidonic acid. In addition, SZ 21, directed against the human platelet glycoprotein Ib/IIa complex, had no significant effect on platelet thromboxane B2 formation in response to either ristocetin or collagen (Table 2).

A series of experiments were also performed to contrast the effects of other antiglycoprotein Ib complex MoAbs (at 50 μg/mL, final concentration) on platelet aggregation induced by ristocetin (1.5 mg/mL, final concentration), equine tendon collagen (1 μg/mL, final concentration), PAF (0.1 μmol/L), and ADP (5 μmol/L). None of the antiglycoprotein Ib complex antibodies had any effect on ADP-induced platelet aggregation. Neither FMC 25 (epitope on glycoprotein IX) nor WM 23, FMC 25, or AP 1, directed against a distinct epitope on the α-chain of glycoprotein Ib, inhibited platelet aggregation induced by either ristocetin or collagen. Consistent with the report of Ruan et al., AN 51 (epitope on the α-chain of glycoprotein Ib) inhibited aggregation induced by either ristocetin or collagen. Similar to SZ 2, AP 1 (epitope on the α-chain of glycoprotein Ib) also completely inhibited platelet aggregation induced by ristocetin and collagen (data not shown). The inhibition of collagen-induced platelet aggregation by AP 1 was less pronounced at higher collagen concentrations in similar manner to that observed with SZ 2. PAF-induced platelet aggregation was not inhibited by AN 51, WM 23, FMC 25, or AP 1, or FMC 18 (directed against T. gondii).

**Effect of SZ 2 on Bernard-Soulier syndrome platelets.** The inhibition by SZ 2 of collagen-induced aggregation is inexplicable in the sense that Bernard-Soulier syndrome platelets, which lack the components of the glycoprotein Ib complex, aggregate normally in response to collagen. There are two likely possibilities consistent with these results. One possibility is that SZ 2 is directed against an epitope present on more than one platelet surface membrane protein. SZ 2 could be directed against both the α-chain of glycoprotein Ib and a platelet collagen receptor. Alternatively, the inhibition of collagen-induced aggregation by SZ 2 could be steric with the binding of SZ 2 to the glycoprotein Ib complex affecting the normal interaction of collagen with a proximal collagen receptor. The first possibility seems unlikely since immunoprecipitation, western blot analysis, and crossed immunoelectrophoresis strongly suggest that SZ 2 is directed against an epitope present only on the glycoprotein Ib complex. Furthermore, another antiglycoprotein Ib complex antibody, AP 1, directed against a distinct epitope to SZ 2 on the glycoprotein Ib complex, was also found to inhibit collagen-induced platelet aggregation. To help further distinguish between these two possibilities we examined the reactivity of SZ 2 toward Bernard-Soulier syndrome platelets. The reactivity of SZ 2 toward normal and Bernard-Soulier syndrome platelets as evaluated by fluorescence-associated cell sorting is shown in Fig 5. The upper panels show the reactivity of normal and Bernard-Soulier syndrome platelets toward FMC 18, directed against T. gondii, and establish the negative controls for background fluorescence by this method. The middle panels show the reactivity of normal and Bernard-Soulier syndrome platelets toward WM 17, directed against the human platelet glycoprotein Ib/IIIa complex and establish the controls for positive platelet

| Table 1. Inhibition (%) of Platelet Function by SZ 2 IgG |
| Stimulus | Serotonin Release (% inhibition) | β-Thromboglobulin Release (% inhibition) |
| Ristocetin (1.25 mg/mL) | 85 | 78 |
| Collagen (20 μg/mL) | 72 | 47 |
| PAF (0.3 μmol/L) | 73 | 78 |
| Thrombin (1 U/mL) | <10 | <10 |
| Arachidonic Acid (200 μg/mL) | <10 | <10 |

Platelet-rich plasma or washed platelets were pre-incubated with SZ 2 IgG (10 μg/mL) final concentration at 37 °C for three minutes before the addition of the appropriate stimulus. Measurement of serotonin and β-thromboglobulin release were as described under Materials and Methods. The results are the mean of three separate experiments.

| Table 2. Effect of SZ 2 and SZ 21 on the Formation of Thromboxane B2 |
| Stimulus | Thromboxane B2 (ng/3 × 10⁸ Platelets, ± SD) |
| | SZ 2 (10 μg/mL) | SZ 21 (10 μg/mL) |
| Arachidonic Acid (330 μmol/L) | 1210 ± 640 | 1060 ± 380 | 1610 ± 420 |
| Thrombin (0.125 U/mL) | 151 ± 68 | 169 ± 72 | 203 ± 95 |
| Collagen (25 μg/mL) | 42 ± 15 | 9 ± 3* | 30 ± 19 |
| Ristocetin (1.25 mg/mL) | 27 ± 13 | 9 ± 3* | 22 ± 9 |

Platelet-rich plasma or washed platelets were pre-incubated with SZ 2 IgG or SZ 21 IgG at 37 °C for three minutes before the addition of the appropriate stimulus. Measurement of thromboxane B2 formation was as described under Materials and Methods. The results are the mean of six separate experiments.

* p < 0.01 for SZ 2 IgG compared to buffer control or SZ 21 IgG by Student’s t test.

![Fig 5. Immunofluorescence profiles of normal (control) and Bernard-Soulier syndrome platelets (BSS) stained with FMC 18 (against Toxoplasma gondii), WM 17 (against glycoprotein IIb/IIIa), and SZ 2 (against glycoprotein Ib complex). Formaldehyde-fixed platelets incubated with the respective MoAb and fluorescein-labeled second antibody were analyzed by fluorescence flow cytometry. Each single parameter histogram, cell number v log fluorescence (arbitrary units), was derived from the analysis of 10,000 platelets. The experimental procedure and data analysis were performed as described under "Materials and Methods."](http://www.bloodjournal.org/content/full/90/11/574#supplemental)
immunofluorescence. Although SZ 2 was positive against normal platelets, the immunofluorescence seen with Bernard-Soulier syndrome platelets was indistinguishable from that observed with the negative control, confirming that SZ 2 was solely directed against an epitope present on the glycoprotein Ib complex. Furthermore, although SZ 2 inhibited collagen-induced platelet aggregation with control platelets, it was without effect on the collagen-induced aggregation of Bernard-Soulier syndrome platelets at any collagen concentration (Fig 6). The latter result strongly suggests that SZ 2 does not bind directly to a platelet-collagen receptor and supports the concept that the human platelet glycoprotein Ib complex and the platelet-collagen receptor are proximal in the human platelet membrane. Similar results were obtained with PAF-induced platelet aggregation. Although SZ 2 (50 µg/mL) was a potent inhibitor of the aggregation of normal platelets by PAF (40 nmol/L), SZ 2 had no effect on the PAF-induced aggregation of Bernard-Soulier syndrome platelets at any PAF concentration (data not shown).

**DISCUSSION**

In response to vascular injury, platelets rapidly adhere to the exposed vascular subendothelium. At high shear flow, one adhesion mechanism appears to involve von Willebrand factor and a specific von Willebrand factor receptor on the human platelet membrane surface, the glycoprotein Ib complex. For example, Bernard-Soulier syndrome platelets, which apparently genetically lack the components of the glycoprotein Ib complex, glycoprotein Ib and glycoprotein IX, fail to bind von Willebrand factor, to agglutinate in the presence of ristocetin, and to adhere normally to subendothelium at high shear flow. In recent years a number of antiglycoprotein Ib murine MoAbs have been described (AN 51, 6D1, and AP 1, SZ 2) that block the ristocetin-dependent binding of von Willebrand factor to human platelets. The inhibition of von Willebrand factor binding to platelets correlates well with the failure of MoAb-treated platelets to agglutinate with ristocetin or to adhere normally to exposed vascular subendothelium at high shear flow. In this study we have described a new murine MoAb, SZ 2, directed against an epitope on the α-subunit of glycoprotein Ib (Fig 2). Like the previously reported murine MoAbs, AN 51, 6D1, and AP 1, SZ 2 and its (Fab')2 fragments were potent inhibitors of both ristocetin-induced, human von Willebrand factor-dependent and bovine von Willebrand factor-dependent platelet agglutination (Fig 4) and inhibited the ristocetin-dependent binding of human von Willebrand factor to platelets by 80%. SZ 2 had no effect on platelet aggregation induced by ADP, epinephrine, A23187, arachidonic acid, and by human or bovine α-thrombin. Unexpectedly, however, SZ 2 and its (Fab')2 fragments were potent inhibitors of platelet aggregation induced by collagen and by PAF (Fig 4). SZ 2 appeared to interfere with an early event in collagen-platelet interaction. SZ 2 inhibited the platelet release reaction in response to collagen as measured by the extent of secretion of dense body serotonin or α-granule β-thromboglobulin (Table 1) and also inhibited the mobilization of arachidonic acid from platelet membrane phospholipid as measured by thromboxane B2 formation (Table 2). With high concentrations of equine tendon collagen (2 to 10 µg/mL), SZ 2 consistently prolonged the lag time between the addition of the collagen fibrils and the onset of platelet shape change. Although there is some evidence that collagen-induced platelet aggregation may be partially von Willebrand factor-dependent at threshold concentrations of collagen, the inhibition of the binding of von Willebrand factor to platelets by SZ 2 is unlikely to represent the mechanism by which SZ 2 inhibits collagen-dependent platelet aggregation. AN 51, which completely blocks the binding of von Willebrand factor to platelets, has no effect on collagen-induced platelet aggregation (this study). Similarly, 3F8, a MoAb against human von Willebrand factor, completely blocks ristocetin-induced, human von Willebrand factor-dependent platelet agglutination but has no effect on collagen-induced platelet aggregation at collagen concentrations at which SZ 2 completely inhibits platelet aggregation (Berndt and Gregory, unpublished observations).

The inhibition of collagen-induced platelet aggregation by SZ 2 is surprising in the sense that Bernard-Soulier syndrome platelets, which lack the glycoprotein Ib complex, aggregate normally in response to collagen (this study, Fig 6). One possible explanation for this apparent discrepancy is that SZ 2 is directed against a binding site on more than one platelet membrane surface protein, ie, SZ 2 could be directed against an epitope present on a platelet collagen receptor as well as the platelet membrane glycoprotein Ib complex. The available evidence, however, is inconsistent, with this being the probable explanation for the observed results since detailed immunochemical analysis indicated that SZ 2 only recognized the glycoprotein Ib complex and since SZ 2 did not inhibit the collagen-induced aggregation of Bernard-Soulier syndrome platelets (Fig 6). An alternate possibility to explain the inhibition of collagen-induced aggregation by SZ 2 is that the glycoprotein Ib complex and the platelet collagen receptor are proximal in the platelet membrane, ie, the binding of SZ 2 or its (Fab')2, fragments to the α-subunit of glycoprotein Ib sterically interferes with the normal interaction of collagen with an adjacent collagen receptor or membrane protein required for normal collagen-dependent platelet activation. There are three lines of evi-
dence consistent with this possibility. First, consistent with the preliminary observations of Pidard et al., AP 1, directed against a distinct epitope on the glycoprotein Ib complex from that of SZ 2 (since AP 1 and SZ 2 only partially crossblock), also inhibited collagen-induced platelet aggregation. Second, SZ 2 only inhibited collagen-induced platelet aggregation when bound to glycoprotein Ib, since SZ 2 had no effect on the collagen-induced aggregation of Bernard-Soulier syndrome platelets. A third line of evidence derives from the recent data of Nieuwenhuis et al. and of Fox.1, 12 Nieuwenhuis et al have described a patient whose platelets failed to react with collagen and lacked a specific membrane protein, glycoprotein Ia. Fox has recently shown that in unstimulated platelets at least one subunit of the glycoprotein Ib complex spans the platelet membrane and is bound to a platelet membrane endoskeleton via actin-binding protein.1, 12 Glycoprotein Ia also apparently spans the platelet membrane and, like glycoprotein Ib, is also bound to the platelet endoskeleton via actin-binding protein.1, 12 These results suggest that glycoprotein Ia and the glycoprotein Ib complex may be normally associated in the human platelet membrane because of their mutual interaction with actin-binding protein and provide an explanation for the inhibition by SZ 2 of both ristocetin-induced, human von Willebrand factor-dependent platelet agglutination and collagen-induced platelet aggregation. Similar arguments apply for the inhibition of PAF-induced platelet aggregation by SZ 2. SZ 2 was found to be a potent inhibitor of both the PAF-induced platelet release reaction (Table 1) and PAF-induced platelet aggregation (Fig. 4). However, Bernard-Soulier syndrome platelets aggregated normally in response to platelet activating factor, and this aggregation was unaffected by SZ 2. This suggests that, similar to the glycoprotein Ib complex and the platelet collagen receptor, the glycoprotein Ib complex and the platelet PAF receptor (or a membrane protein required for normal PAF-dependent platelet activation) may also be proximal in the plane of the platelet membrane.

The combined evidence suggests that the antiglycoprotein Ib complex MoAb, SZ 2, inhibits platelet aggregation induced by both ristocetin and collagen because the platelet glycoprotein Ib complex and the platelet collagen receptor may be sterically adjacent in the human platelet plasma membrane. Houdijk et al have recently demonstrated that the adhesion of platelets to collagen substrata at high shear flow is via Willebrand factor-dependent.4 It is interesting to speculate that the possible steric relationship between the platelet von Willebrand receptor and the platelet collagen receptor suggested by the current study may be relevant to these observations. Future studies should help resolve this question and help delineate the precise molecular role of glycoprotein Ia in collagen-induced platelet aggregation.

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