Infusible Platelet Membranes Retain Partial Functionality of the Platelet GPIb/IX/V Receptor Complex

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Abstract

Infusible platelet membranes (IPMs) prepared from fresh or outdated human platelets have been shown to correct prolonged bleeding times in thrombocytopenic rabbits. In previous trials, IPMs did not seem to be immunogenic and lacked dose-limiting toxicity. The present study was undertaken to explore whether the platelet glycoprotein (GP) Ib/IX/V complex might retain functionality in the IPM preparation. IPMs did not spontaneously bind von Willebrand factor (vWF), but saturable binding could be induced by ristocetin, with a dissociation constant (Kd) of 0.31 ± 0.03 µg/mL at 1.0 mg/mL of ristocetin. Of 4 anti–GPIb-alpha monoclonal antibodies tested, AN-51 inhibited vWF binding 67.8% ± 5.8%, whereas AS-2, AS-7, and SZ-2 were ineffective. Maximal vWF binding induced by botrocetin was only 10% to 15% of that observed with ristocetin. Retention of partial functionality of the GPIb/IX/V receptor allowing vWF binding in a modulated manner seems to represent a critical mechanism by which IPMs may provide hemostatic efficacy.

Infusible platelet membranes (IPMs) are one of several new agents undergoing evaluation as possible adjuncts to traditional platelet transfusion therapy.[1] IPMs (Cyplex, Cypress Bioscience, San Diego, CA) are 0.3- to 1.1-µm diameter particles prepared from 10- to 19-day-old blood bank human platelets by lysis and differential centrifugation and treatment to inactivate blood-borne viruses.[2] IPMs, when administered to thrombocytopenic rabbits, demonstrate hemostatic activity by producing shortening of prolonged bleeding times without causing a clinically significant level of thrombogenicity.[2] Since IPMs recently have been shown to bind to subendothelial surfaces under high shear conditions,[3] the present study was undertaken to determine whether IPM might retain any functionality of the glycoprotein (GP) Ib/IX/V receptor, which mediates binding of von Willebrand factor (vWF) by platelets under conditions of high shear.[4]

Materials and Methods

IPMs were reconstituted to 2 mg/mL with distilled water. Unfiltered IPMs were washed twice and resuspended in Hanks buffered salt solution (Gibco BRL, Grand Island, NY) containing 1% bovine serum albumin and a 10-mmol/L concentration of EDTA. For study of vWF binding, 100 µL of IPM was added to tubes containing 25 µL of purified human vWF,[5] in the presence of ristocetin (Helena Laboratories, Beaumont, TX) or botrocetin (Centerchem, Stanford, CT) as a modulator. After 45 minutes' incubation at 22°C, the IPMs were washed and incubated for 30 minutes with rabbit antihuman vWF (DAKO, Carpinteria, CA). The IPMs were washed again and incubated for 30 minutes with fluorescein isothiocyanate–conjugated goat antirabbit IgG (Sigma

Chemical, St Louis, MO). After incubation, the IPMs were washed once more and fixed with 1.0% paraformaldehyde before analysis.

Ten thousand events from each specimen were analyzed for fluorescein isothiocyanate fluorescence on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The settings for the flow cytometer were forward scatter (FSC), log; side scatter (SSC), log, photomultiplier tube (PMT) voltage, 288; and fluorescence channel 1, log, PMT voltage, 487. The instrument was calibrated daily, and, for the entire study period, machine drift was less than 5%. A forward light scatter threshold setting of 112 permitted measurement of IPMs or similarly sized latex particles, but background noise was excluded effectively when IPM-free control buffer was sampled. For data analysis, the gate was drawn around the main population on an FSC vs SSC dot blot. This gate included particles having low FSC and SSC distribution, but it excluded particles with high FSC and SSC distribution representing larger particles. The particles included in this analysis represented more than 95% of the entire population collected.

Studies also were undertaken with murine monoclonal antibodies (Mabs) directed against epitopes within platelet GPIb-alpha. Commercially available Mabs SZ-2 (Immunotech, Marseilles, France) and AN-51 (DAKO) or the AS-2 and AS-7 Mabs developed in our laboratory,[6] were added at 10 µg/mL or 20 µg/mL. Following a 30-minute preincubation of IPMs with Mabs or phosphate-buffered saline (PBS), vWF and modulator were added, and analysis of binding was performed as described in the preceding text. Statistical significance was determined by paired *t*-test of the values obtained with each antibody, as compared with PBS.

Results

There was no fluorescence above background observed when vWF and IPMs were incubated in the absence of a modulator. Similarly, there was no appreciable fluorescence above background when flow cytometry was performed on samples devoid of IPMs but containing vWF with or without a modulator.

When IPMs were incubated with 1.5 µg/mL of vWF in the presence of ristocetin, half-maximal binding occurred around 1.0 mg/mL, with maximal binding achieved at a ristocetin concentration of 1.5 mg/mL **Figure 1A**. Virtually no binding was observed at ristocetin concentrations of 0.5 mg/mL or less. At a constant ristocetin concentration of 1.0 mg/mL, increasing concentrations of vWF produced a hyperbolic binding isotherm **Figure 1B**, with maximal binding achieved at approximately 1.5 µg/mL vWF and a mean ± SEM dissociation constant (Kd) of 0.31 ± 0.03 µg/mL of vWF (n = 3). Binding of vWF induced by the snake venom botrocetin never exceeded 10% to 15% of the magnitude observed with ristocetin, even when very high botrocetin concentrations (20-30 µg/mL) were used.

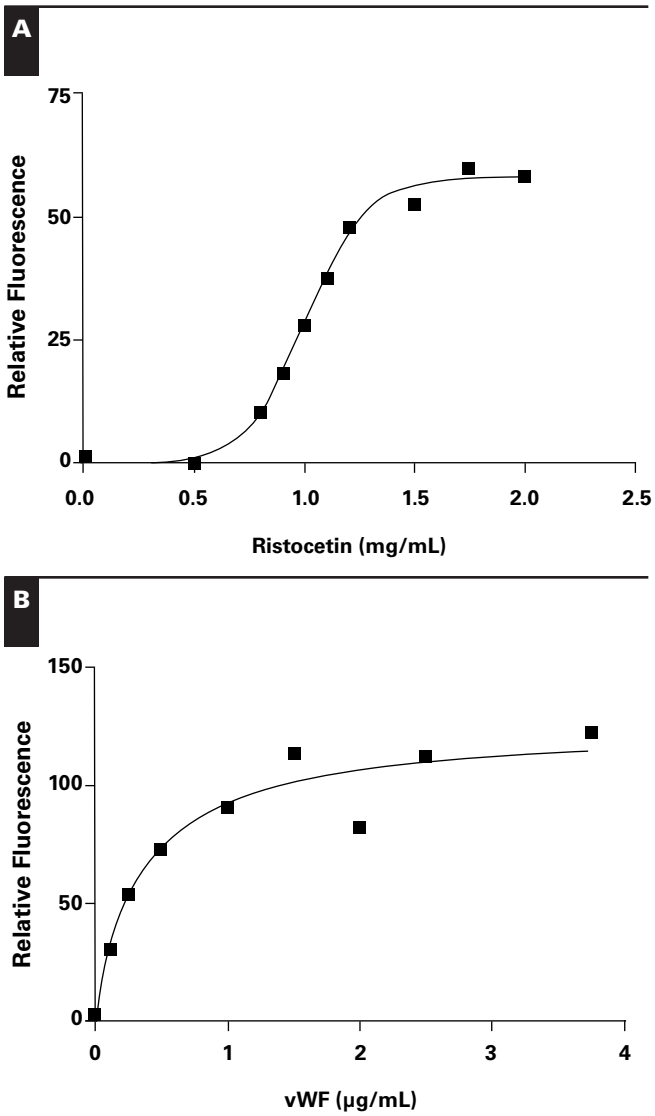

Figure 1 Ristocetin-induced binding of von Willebrand factor (vWF) to infusible platelet membranes (IPMs). Data shown are representative of 3 separate experiments. A, For flow cytometric studies of ristocetin sensitivity, IPMs were incubated with 1.5 µg/mL vWF at varying ristocetin concentrations. No significant binding was observed at ristocetin concentrations at or below 0.5 mg/mL. Half-maximal binding occurred around 1.0 mg/mL, with maximal binding achieved at a ristocetin concentration of 1.5 mg/mL. B, For studies of the concentration dependence of vWF binding to IPMs, IPMs were incubated in the presence of increasing concentrations of vWF, with 1.0 mg/mL of ristocetin as modulator. The binding isotherms were hyperbolic, with an observed mean ± SEM dissociation constant of 0.31 ± 0.03 µg/mL.

A panel of anti–GPIb-alpha Mabs was used to assess specificity of vWF binding to this platelet receptor. Mab AS-2 failed to inhibit vWF binding to IPMs **Figure 2A**. Mabs AS-7 and SZ-2 also did not inhibit the binding of vWF to IPMs in the presence of ristocetin **Table 1**. In contrast, Mab AN-51 produced strong and statistically significant inhibition of vWF binding to the IPMs **Figure 2B** (Table 1).

Discussion

The present study was undertaken to determine whether one of the key attributes of platelet function, the regulated binding of vWF, is retained in the Cyplex IPMs. By using the chemical modulator ristocetin as a surrogate for in vivo platelet stimulation, we demonstrated a ristocetin dependency for vWF binding virtually identical to that well known to exist for platelets. The ability of AN-51 to inhibit ristocetin-induced vWF binding by approximately 70% indicates that this binding is mediated by GPIb-alpha.[6,7] However, the loss of effect of 3 anti–GPIb-alpha Mabs known to be inhibitory with platelets and only minimal responsiveness to the modulator botrocetin strongly suggest that GPIb-alpha persisting through the IPM production process is likely far from its native state. The recent study by Shen et al[8] using a series of canine-human chimeras of GPIb-alpha suggested possible regions within GPIb-alpha for epitope localization of at least 2 of the Mabs used in the present study: the epitope for AN-51 was mapped within the first 35 amino acids at the N-terminal region of GPIb-alpha, while the epitope for SZ-2 was mapped within the Asp_{269}-Glu_{282} anionic region just carboxyl to the double-disulfide loop of GPIb-alpha. Based on these proposed epitope assignments, our results showing relative preservation of the inhibitory activity of AN-51 would suggest that the most N-terminal region of GPIb-alpha actually may be less denatured than regions carboxyl to it in the IPM manufacturing process.

Botrocetin is believed to bind to vWF in such a manner as to make the botrocetin-vWF complex competent to bind spontaneously to GPIb-alpha.[9] In contrast, ristocetin is thought to bind to motifs within the ligand vWF and within the receptor GPIb-alpha.[10,11] Since ristocetin-induced binding of vWF to the IPM is preserved, it seems likely that the manufacturing process left enough of the ristocetin-dependent sites within the receptor still reasonably functional. The "resting" GPIb-alpha conformation within the IPM may, however, be altered sufficiently so as not to permit the botrocetin-vWF complex to bind as well as in the native platelet.

The indirect immunofluorescence approach developed to study vWF binding to IPMs yielded apparent Kd values for ristocetin-induced binding that were close to those previously reported for platelets. For example, using a direct radioligand approach, Kao et al[12] determined the dissociation constant for iodine 125–labeled vWF binding to washed human platelets to be 0.5 µg/mL when 0.5 or 1.0 mg/mL of ristocetin was used as the modulator. A direct comparison with platelets using the flow cytometric method with the IPMs could not, however, be made, owing to the large aggregate formations that occurred when platelets were similarly incubated with vWF in the presence of ristocetin.

In addition to the loss of surface receptor proteins that may be anticipated in the initial platelet storage phase,[13,14] the subsequent lysis, centrifugation, and exposure to heat

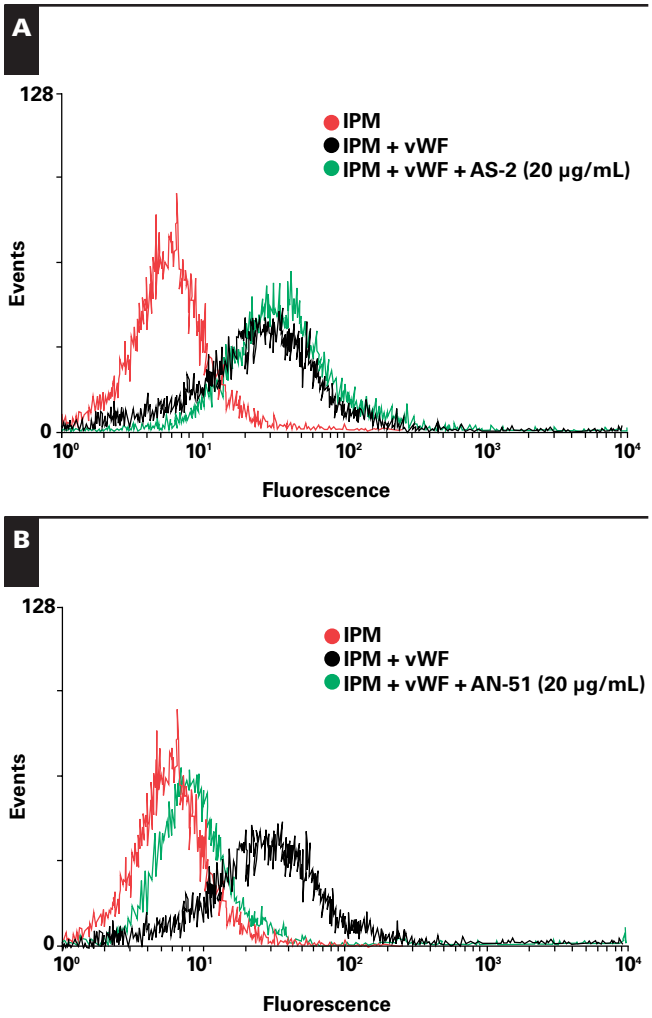

Figure 2 Monoclonal antibody inhibition of ristocetin-induced binding of von Willebrand factor (vWF) to infusible platelet membranes (IPMs). A, Monoclonal antibody AS-2 added to IPM 30 minutes before the addition of vWF (1.5 µg/mL) to IPM in the presence of 1.0 mg/mL of ristocetin produced no inhibition of vWF binding. Data shown are representative of 2 separate experiments. B, Monoclonal antibody AN-51 added to IPM as described for Figure 2A produced strong inhibition of vWF binding. Data shown are representative of 4 separate experiments.

Table 1
Inhibition of von Willebrand Factor (vWF) Binding by Anti–GPIb-alpha Monoclonal Antibodies

Monoclonal Antibody	Concentration (µg/mL)	Inhibition of vWF Binding*
AS-2	10	0.0
	20	0.0
AS-7	10	0.0
	20	0.0
SZ-2	10	6.4
	20	0.0
AN-51	10	45.1 ± 7.0 (n = 3)†
	20	67.8 ± 5.8 (n = 4)‡

* Inhibition is expressed as the percentage inhibition of vWF binding to infusible platelet membranes in the absence of monoclonal antibodies; mean ± SEM for AN-51.
† $P < .015$.
‡ $P < .001$.

that occurs during processing would be anticipated to result in further alteration of the platelet surface glycoprotein receptors. In fact, HLA antigens and GPIIb/IIIa epitopes seem to be lost from platelets during the production of IPMs,[2] and this would certainly be anticipated to make a major contribution to the loss of antigenicity. In addition, since GPIIb/IIIa is implicated in the final common pathway for platelet aggregation, lack of aggregation of IPMs in vitro is further consistent with the effective loss of this receptor complex during IPM preparation. In contrast, a significant presence of GPIb retaining at least partial functionality is demonstrable in the IPMs. This residual functionality seems sufficient at least to permit IPMs to bind vWF in a modulated fashion in the presence of ristocetin and conceivably could also be a mechanism underlying the observed binding of IPMs to denuded vascular surfaces observed under flow conditions.[3] This mechanism also may explain, at least partially, the observed hemostatic effectiveness of IPMs after transfusion into thrombocytopenic animals[2] or into human patients.[15]

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