Rapid Platelet-Function Assay
An Automated and Quantitative Cartridge-Based Method

Jeffrey W. Smith, PhD; Steven R. Steinhubl, MD; A. Michael Lincoff, MD; Jacqueline C. Coleman, PhD; Theodore T. Lee, PhD; Robert S. Hillman, PhD; Barry S. Coller, MD

Background—The platelet glycoprotein (GP) IIb/IIIa receptor is important in mediating platelet thrombus formation, and the GP IIb/IIIa antagonist abciximab (c7E3 Fab; ReoPro) is effective in preventing thrombotic ischemic cardiovascular complications of unstable angina and percutaneous coronary interventions. Small-molecule antagonists of GP IIb/IIIa based on the Arg-Gly-Asp (RGD) sequence show similar benefit, and some of these agents are orally active. However, there may be significant interindividual variation in response to such antagonists, especially with chronic oral therapy. It will be essential to balance the beneficial antithrombotic effect of these drugs with their potential for causing bleeding. In response to this need, we have developed a rapid platelet-function assay (RPFA), a point-of-care system that provides a quantitative measure of the competence of the GP IIb/IIIa receptor as reflected in the ability of platelets to agglutinate fibrinogen-coated beads.

Methods and Results—Polystyrene beads were coated with fibrinogen and placed in a cartridge along with a lyophilized peptide that activates the thrombin receptor. Anticoagulated whole blood was added to the cartridge, and then a microprocessor-controlled operation mixed the reagents and detected agglutination between platelets and coated beads. Quantitative digital results were displayed within 3 minutes. Because there is no dilution of the blood, the assay can be used to measure platelet activity in samples that have been treated with GP IIb/IIIa antagonists with high dissociation rates. RPFA results of whole-blood samples treated with different GP IIb/IIIa antagonists correlated well with both conventional turbidimetric platelet aggregation ($r^2=0.95$) and the percentage of free GP IIb/IIIa molecules in the sample ($r^2=0.96$). The mean difference in measurements between RPFA and aggregometry was $-4\%$ (±4% SD), and the mean difference in measurements between RPFA and free GP IIb/IIIa receptors was $-2\%$ (±6% SD).

Conclusions—The RPFA provides rapid information on platelet function that mirrors turbidimetric platelet aggregation and reflects GP IIb/IIIa receptor blockade. (Circulation. 1999;99:620-625.)

Key Words: platelets glycoproteins drugs
by a thrombin receptor–activating peptide. This assay, however, required the user to interpret a semiquantitative endpoint. Here, we describe a microprocessor-controlled, whole-blood, cartridge-based, automated version of the assay that provides a quantitative digital display of the results within minutes. Results with this rapid platelet-function assay (RPFA) correlate well with results of traditional turbidimetric platelet aggregometry using citrated platelet-rich plasma (PRP) and radiometric binding assays of GP IIb/IIIa receptor occupancy.

Methods

Materials

Human fibrinogen (Enzyme Research Laboratories) was >95% pure, as judged by Coomassie blue staining of SDS polyacrylamide gels. Abciximab was the generous gift of Centocor (Malvern, Pa). [125I]Nal was purchased from Amersham. A modified thrombin receptor–activating peptide (iso-TRAP), iso-SFLLRN, which is resistant to plasma aminopeptidase M, was purchased from Multiple Peptide Systems. Vacutainer tubes containing 3.8% sodium citrate were purchased from Becton Dickinson. Polystyrene beads (6 μm in diameter) with surface carboxyl groups were purchased from Poly-sciences. Chemicals used for buffers and assays were purchased from Sigma Chemical Co. The GP IIb/IIIa antagonist TAK-029 was the generous gift of TAP Holdings (Deerfield, Ill).

Fibrinogen Coating of Beads

Polystyrene beads are coated with fibrinogen by passive adsorption and then dyed with an infrared (IR) dye to facilitate measurements of bead agglutination in whole blood. A 3-step procedure is used to make IR-dyed beads. First, fibrinogen (330 μg/mL) is passively adsorbed to white polystyrene beads (6 μm in diameter) in 20 mmol/L NaPO₄, pH 7.5, for 2 hours at room temperature. Unbound fibrinogen is removed by washing 3 times in the same buffer. Protein assays indicate that ~15% to 20% of the total fibrinogen is coupled to the beads under these conditions. Then the beads are dyed for 5 minutes with IR-140 (Aldrich) in 20 mmol/L NaPO₄/methylene chloride/2-propanol (1:4:6). The dye is removed and replaced with fresh dye in the same solution for an additional 5 minutes. Finally, the beads are washed extensively with 10 mmol/L HEPES, pH 7.5, containing 1 mg/mL of BSA and 0.02% sodium azide. The absorbance properties and the ability of the beads to be agglutinated are standardized for each lot of beads.

Blood Collection and Preparation

For studies in which platelets were treated ex vivo with a GP IIb/IIIa antagonist, human blood (40.5 mL) was collected into 5-mL citrated Vacutainer tubes with a Vacutainer device and a 21-gauge needle. Blood was obtained from nonsmoking volunteers who had not ingested aspirin for at least 3 days. The first tube was discarded, and the remaining blood was pooled into a polypropylene container and maintained at room temperature. A 1.5-mL sample of untreated blood was removed for the RPFA, and the remainder was divided into 5-mL aliquots and transferred to 75-mm polypropylene tubes. For studies in which platelets were treated ex vivo with a GP IIb/IIIa antagonist, human blood (40.5 mL) was collected into 5-mL citrated Vacutainer tubes with a Vacutainer device and a 21-gauge needle. Blood was used for the RPFA. All of the patients were taking aspirin, but none were on heparin or taking ticlopidine; 78% were male, 20% had recent myocardial infarctions, 61% had unstable angina, and 19% had stable angina. Separate blood samples were obtained for platelet count, mean platelet volume, and hematocrit.

Rapid Platelet-Function Assay

This assay is conducted in an integrated device, attached to a personal computer, that houses a cylindrical warming chamber designed to accommodate a 4.5-mL Vacutainer tube and a cartridge port to accommodate a dual-chamber cartridge (Figure 1). The device also contains a light source and optical detectors, as well as magnets to control mixing by the steel balls in the reaction chambers. Each reaction chamber contains lyophilized iso-TRAP and fibrinogen-coated beads. The assay is initiated by addition of 160 μL of whole blood to each channel, which reconstitutes the reagents such that the final iso-TRAP concentration is 4 μmol/L. The sample is mixed back and forth by the movement of the steel ball running the length of the sample chamber for 70 seconds, and the absorbance of the sample is measured 16 times per second by the automated detector. Agglutination of fibrinogen-coated beads by platelets is quantified by use of a microprocessor algorithm that takes into account both the rate and extent of agglutination. The data are reported as a rate of platelet function in millivolts per 10 seconds (mV/10 s) ±95% CI based on goodness of fit of the model to the data used to calculate the slope. The instrument is programmed to reject the data (ie, a slope will not be reported) if the interchannel variability is >15% or if the intrachannel noise relative to the baseline is >7.5%. Experiments were also conducted with cartridges containing ADP (20 μmol/L final concentration) instead of iso-TRAP.
Platelet Aggregometry

PRP (400 μL) was pipetted into a siliconized cuvette containing a Teflon-coated stir bar. PPP was used to establish maximal light transmission. The cuvette containing PRP was placed into a Chrono-Log model 490D turbidimetric platelet aggregometer, and a baseline tracing was established for 15 seconds. To stimulate aggregation, ADP in 0.01 mol/L HEPES saline, 0.15 mol/L NaCl, pH 7.4, was added to achieve a final concentration of 20 μmol/L. Aggregation was monitored for 2 minutes, and the maximal aggregation rate was recorded.

Binding of 125I-Abciximab to Platelets

Blockade of GP IIb/IIIa receptors on the platelet surface by abciximab was assessed by use of a modification of our previously described binding assay. Briefly, a 200-μL aliquot of PRP prepared as above was incubated with 20 nmol/L 125I-abciximab for 10 minutes at 22°C. Then, two 100-μL aliquots of the mixture were layered onto 30% sucrose cushions and centrifuged at 12 000g for 3 minutes at 22°C to pellet the platelets. Platelet pellets were separated from the supernatant and counted in a gamma counter (Iso-Data 100). GP IIb/IIIa occupancy by abciximab was calculated by comparison of the amount of 125I-abciximab bound to the platelet surface in the presence of different concentrations of unlabeled abciximab with the amount of 125I-abciximab bound to untreated platelets.

Binding of 14C-TAK-029 to Platelets

TAK-029 is a small-molecule GP IIb/IIIa antagonist patterned on the Arg-Gly-Asp (RGD) cell adhesion motif. It was also tested for its effects on platelet aggregometry and RPFA, and the results were correlated with those obtained with an assay to measure GP IIb/IIIa receptor occupancy by TAK-029. 14C-TAK-029, diluted with 10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4, was added to 200 μL of PRP to yield the concentrations noted in the figure legends. After a 20-minute incubation period, 100 μL of each mixture was placed into each of 2 polypropylene microcentrifuge tubes and centrifuged to obtain platelet pellets. Each platelet pellet was subject to scintillation counting in a Packard model 1500 Liquid Scintillation Analyzer. Nonspecific binding was determined by competition with a 300-fold excess of TAK-029. Specific binding of the compound was then calculated by subtraction of nonspecific binding from the total binding. The data were analyzed according to the method of Scatchard.

Statistical Analysis

Although correlation coefficients are commonly used to assess the agreement between 2 methods, Bland and Altman demonstrated that this is statistically inappropriate, because the correlation coefficient measures the strength of the relationship between 2 variables, not the agreement between them. They proposed a method that involves analyzing the differences between the methods and assessing whether the differences are similar at all assay values. The mean difference and the SD of the differences are valuable measures that, when judged in the context of the medical significance of the differences, allow one to assess the agreement between the methods. We are reporting the data according to Bland and Altman as our primary analysis. We are, however, also providing the correlation coefficients to permit comparison of our data with those in the literature.

Results

Rapid Platelet-Function Assay

Mixing citrated whole blood with iso-TRAP and fibrinogen-coated beads in the cartridge reaction chamber results in progressive bead and platelet agglutination as the platelets interact with the fibrinogen-coated beads and with each other. This agglutination is detected as an increase in the transmission of infrared light through the sample (Figure 2A). Both EDTA (Figure 2B) and abciximab 3 μg/mL (Figure 2C) prevent the increase in transmission, indicating that the agglutination is divalent cation– and GP IIb/IIIa–dependent, respectively.

Day-to-Day and Cartridge Lot-to-Lot Variations in RPFA

Day-to-day variation in the RPFA of a single individual was measured by testing 1 person’s blood on 5 separate occasions over a period of 5 months. The results (mean±SD) were 591±22 mV/10 s, indicating relatively little variation. Five separate lots of cartridges were used for this experiment, indicating that different lots of cartridges give similar results.
RPFA Values of Patients Undergoing Coronary Intervention

In 50 patients presenting for elective percutaneous coronary intervention, the mean±SD values were 464±149 mV/10 s, with similar values for men (578±20 mV/10 s, n=39) and women (478±142 mV/10 s, n=11). The rates were not significantly correlated with donor platelet count ($r^2 = 0.002$), platelet density (platelet count/1−hematocrit) ($r^2 = 0.014$), mean platelet volume ($r^2 = 0.064$), platelet mass (platelet count × platelet volume) ($r^2 = 0.16$), or platelet mass density (platelet mass/1−hematocrit) ($r^2 = 0.014$). The rates, however, correlated moderately well with the inverse of the hematocrit ($r^2 = 0.43$). We suspect that this relationship is most likely the result of the increased absorbance of erythrocytes at higher hematocrits, but we cannot rigorously exclude a potential effect of erythrocytes on platelet function or rheology in the assay.

Comparison of the Effect of Abciximab and TAK-029 on GP IIb/IIIa Receptor Blockade and Platelet Function Measured by RPFA and Turbidimetric Aggregometry

The in vitro addition of increasing concentrations of abciximab to citrated whole blood produced increasing inhibition of both the RPFA and turbidimetric aggregometry (Figure 3). When normalized to baseline control values, the percentage inhibition of the RPFA correlated well with both the percentage inhibition of turbidimetric platelet aggregometry and GP IIb/IIIa receptor blockade (Figure 3). In blood obtained from 4 separate donors, the correlation between RPFA and turbidimetric aggregometry was $r^2 = 0.98$, and that between RPFA and unblocked GP IIb/IIIa receptors was $r^2 = 0.96$ (Figure 4). The mean difference in measurements between RPFA and aggregometry was −4%, and the SD of the differences was 4%. The mean difference between RPFA and unblocked GP IIb/IIIa receptors was −2%, and the SD of the differences was 6%. IC_{50}s determined on 4 separate patients with all 3 methods gave very similar results (Table 1). Moreover, the interindividual differences in IC_{50}s were small, differing from the group means by <30%.

An in vitro study was performed to assess the effect of aspirin on the ability of the RPFA to detect the effect of abciximab. Aspirin or buffer was added to the blood from 10 different volunteers (150 $\mu$mol/L final concentration), and then a 7-point abciximab dose-response curve was performed. The regression analysis comparing the 2 dose-response

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<th>TABLE 1. Comparison of IC_{50}s for Abciximab by Turbidimetric Aggregometry, Receptor-Binding Assay, and RPFA</th>
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<td>Method</td>
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<td>Patient</td>
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<td>Mean</td>
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<td>SD</td>
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curves showed $r^2=0.97$, demonstrating that aspirin did not diminish the sensitivity of RPFA to the effect of abciximab. Similarly, substituting heparin for citrate as the anticoagulant did not diminish the sensitivity of the RPFA to the effect of abciximab ($r^2=0.98$).

TAK-029 was also tested for its ability to block GP IIb/IIIa receptors and to inhibit platelet function measured by aggregometry and RPFA. Our preliminary studies with TAK-029 and other low-molecular-weight inhibitors of GP IIb/IIIa demonstrated that even modest dilution of the blood sample immediately before testing resulted in an underestimation of the degree of platelet-function inhibition because the inhibitors dissociate from GP IIb/IIIa very rapidly. To overcome this problem and to make the RPFA potentially compatible with all GP IIb/IIIa antagonists, the reagents and beads were lyophilized so that there is no dilution in the assay beyond the anticoagulation step. Figure 5A shows the binding curve of $^{14}$C-TAK-029 to platelets, and Figure 5B shows the data expressed by the method of Scatchard, indicating a $B_{\text{max}}$ of $\approx 137,000$ molecules per platelet and a $K_D$ of 37 to 42 nmol/L. These data were then used to calculate the percentage of receptors blocked by the different concentrations of TAK-029 in each sample tested with the RPFA. The results shown in Figure 5C demonstrate a close correlation ($r^2=0.92$) between the RPFA results and the GP IIb/IIIa receptor blockade assay. The mean difference in measurement between the RPFA and GP IIb/IIIa receptor blockade assay was $-0.2\%$, and the SD of the differences was $8\%$.

Comparison of ADP and iso-TRAP as Agonists in the RPFA

The RPFA results on blood obtained from 6 donors by use of cartridges containing either lyophilized iso-TRAP or ADP 20 $\mu$mol/L are shown in Table 2. The correlation between the values obtained with the different agonists was very close (<3% difference for 4 donors, and 8% and 12% differences for the other 2). The addition of abciximab to whole blood at increasing doses resulted in progressive inhibition of RPFA values with both the iso-TRAP and ADP cartridges ($r^2=0.87$) (Figure 6).

Discussion

The RPFA measures platelet function using a small, undiluted sample of anticoagulated whole blood. The advantages of RPFA over other methods of assessing platelet function are (1) the speed with which results can be obtained, (2) the need for only a small sample of blood, (3) the semiautomated format in which processing of the blood is not required, (4) the microprocessor-controlled digital readout, and (5) its compatibility with a variety of anticoagulants and platelet agonists. The results from the RPFA can be reported as a percentage of baseline aggregation or as an absolute rate of aggregation. The results from the RPFA correlate well with results obtained from turbidimetric platelet aggregometry and with the percentage of free GP IIb/IIIa receptors. Thus, the mean differences between the assays range from $-4\%$ to $2\%$, and the SDs of the differences were $4\%$ to $8\%$. The assay differs, however, from in vivo conditions in that it is not conducted under flowing conditions.

One potential use of the RPFA is to monitor GP IIb/IIIa receptor blockade by GP IIb/IIIa antagonists. It may, in fact, be applicable to both acute and chronic therapy. The dosing strategy with abciximab, which is administered as an intravenous bolus and 12-hour infusion, is to reach or exceed an 80% threshold of blockade and rely on the short exposure time to limit the hemorrhagic risk. Monitoring of abciximab treatment with RPFA may be beneficial in identifying unusual patients who do not obtain the desired response, for example, those with severe thrombocytosis, and ensuring...
that the blockade is sustained during the infusion; it may also be used to define when platelet function returns to baseline after therapy is completed. Chronic oral GP IIb/IIIa antagonist therapy may benefit from drug monitoring and dose adjustment because (1) the therapeutic window is likely to be relatively narrow, given the steepness of the dose-response curves, and (2) there are likely to be significant interindividual differences in bioavailability, pharmacokinetics, and functional effects, especially because renal and hepatic function may be variably compromised in the target population. It will be important, therefore, to assess the extent of interindividual variability in response to the agents, whether the variability correlates with outcome, and finally, whether dose adjustments made on the basis of the RPFA improve outcomes. Many other assays are being developed to monitor GP IIb/IIIa receptor blockade, some of which assess receptor blockade directly (reviewed in Reference 9). A functional assay such as the RPFA provides information on the total effect of the agents on platelet function, independent of the agent, and so provides a measure that is likely to be most biologically relevant. It can also be used when 2 different antagonists are present, as may occur during a transition from an intravenous agent to an oral agent.11

The RPFA may also be valuable in diagnosing the inherited platelet disorder Glanzmann thrombasthenia, because patients with this disorder lack functional GP IIb/IIIa receptors. Modifications to the RPFA with different agonists may also be useful to diagnose other platelet disorders or even to monitor the response of therapy with aspirin or other antiplatelet agents.

To further increase the automation of the RPFA, work is underway on a system that does not require an interface with a personal computer and that will require only the insertion of the Vacutainer tube into the cartridge. Finally, the cartridge will contain additional sample channels that can be formatted for other assays and to adjust for differences in optical properties among blood samples.

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References


Table 2. Comparison of RPFA Values With iso-TRAP or ADP Used as the Agonist

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<thead>
<tr>
<th>Patient</th>
<th>iso-TRAP</th>
<th>ADP</th>
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<tbody>
<tr>
<td>1</td>
<td>103±4</td>
<td>101±5</td>
</tr>
<tr>
<td>2</td>
<td>299±11</td>
<td>293±24</td>
</tr>
<tr>
<td>3</td>
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<td>287±2</td>
</tr>
<tr>
<td>4</td>
<td>98±11</td>
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<tr>
<td>5</td>
<td>663±68</td>
<td>664±51</td>
</tr>
<tr>
<td>6</td>
<td>693±66</td>
<td>612±63</td>
</tr>
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</table>

Values are mV/10 s, mean±95% CI of triplicate determinations.

Figure 6. Effect of abciximab on RPFA using ADP or iso-TRAP. RPFA cartridges containing either iso-TRAP (●) or ADP (□) were used to measure platelet function in blood treated with abciximab at indicated doses. Results shown are from 1 of 3 separate, similar experiments.
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