FLOW CYTOMETRIC DETECTION OF THE REDISTRIBUTION OF THE GLYCOPROTEIN Ib-IX COMPLEX ON THROMBIN-STIMULATED PLATELETS IS DEPENDENT ON THE TYPE OF ANTIBODY CONJUGATE USED

To the Editor:

Flow cytometric measurement of changes in platelet surface membrane antigens is gaining acceptance as a method for assessing platelet activation status both in vitro and in clinical studies.1 One effect of the strong agonist, thrombin, is the reciprocal up-regulation of the glycoprotein (GP) IIb-IIIa complex from intracellular membrane stores, and the loss of around 50% of the GPIb-IX complex from the platelet surface. Michelson et al, in this Journal, have reported the detection of such changes, by flow cytometry, in washed platelets2 and in platelets analysed in a whole blood assay.3 Loss of the GPIb-IX complex may be a useful marker of platelet changes in vivo. Reduced expression of GPIb-IX has been reported in blood emerging from bleeding time wounds1 and after exercise.4 Whole blood flow cytometry, which analyzes diluted samples of unseparated blood, has advantages over fixed, washed platelet methods in that it is rapid, and it avoids manipulations that might introduce artefactual activation of the platelets, as for example during centrifugation or separation procedures.5 Platelet response to agonists can be monitored in these assays, including the response to thrombin. Aggregation of the platelets can be prevented by addition of glycyl-L-prolyl-l-arginyl-L-proline-acetate peptide (GPRP) to inhibit cross-linking of fibrin.3 In such assays, directly conjugated antibodies are often used to minimize assay time and sample processing.

When using a monoclonal antibody (MoAb) to GPIb (RFGP37), raised in this laboratory3 and conjugated to fluorescein isothiocyanate (FITC), we were surprised to find no reduction in GPIb-IX expression on thrombin-stimulated platelets, despite increases in GPIIb-IIIa expression and the appearance of the P-selectin membrane antigen, GMP-140 (Fig 1). We had previously seen downregulation of GPIb in washed, thrombin-activated, fixed platelets using the same MoAb in an indirect flow cytometric assay.

Epitope variability was not suspected. Michelson et al's studies clearly demonstrated that a number of MoAbs to different epitopes on the GPIb-IX complex gave identical results.2,3 Therefore, we looked at the methods used. Both Michelson and ourselves used essentially identical whole blood assays, but while we used an MoAb directly conjugated to FITC, Michelson's study employed biotinylated MoAbs, visualized with a streptavidin-phycocerythrin (ST-PE) conjugate. When we repeated our investigations with biotinylated RFGP37 and ST-PE, a decrease in GPIb was clearly seen, concomitant with an increase in the expression of GPIIb-IIIa and the neo-expression of GMP-140 as illustrated in Fig 1.

We postulate that the reason for the discrepancy in the results obtained with these different reagents lies in the location of the internalized GPIb-IX complexes that are lost from the platelet surface. Electron microscope studies have demonstrated that they become translocated to the surface connecting canalicular system (SCCS).6 GPIb-IX complexes in the SCCS may be accessible to the MoAb-
FITC conjugates but inaccessible to ST-PE or to polyclonal second antibody conjugates. While we have not determined whether this different pattern, between directly conjugated MoAb and an indirect assay, is seen with all MoAbs to the GPIb-IX complex, a second, directly conjugated, commercially available anti-GPIb MoAb (AN51: Dako Ltd, High Wycombe, UK) gave the same results as directly conjugated RFGP37.

These observations have implications for the use of such reagents for the detection of changes in GPIb-IX expression in clinical studies. We propose that these changes should be analyzed using an indirect flow cytometric assay and that the behavior of the particular antibody preparation being used should first be checked on platelets stimulated with thrombin in vitro. However, directly conjugated anti-GPIb-IX MoAbs do provide useful pan-platelet markers for flow cytometric studies as the level of expression of this antigen, detected with such reagents, would not be altered by the activation status of the platelets.

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RESPONSE

Thrombin results in a decrease in the platelet surface expression of the glycoprotein (GP) Ib-IX complex,1,2 as a consequence of a redistribution of GPIb-IX complexes to the membranes of the open canalicular system.3

In our whole blood flow cytometric assay described in Blood,3 we incubated with a biotinylated GPIb-specific monoclonal antibody (MoAb) before the addition of thrombin. Phycoerythrin-streptavidin was added to the assay after the addition of the thrombin. The thrombin-induced redistribution of the GPIb-IX complex was observed because only the biotinylated GPIb-specific MoAb molecules still on the platelet surface were accessible to the phycoerythrin-streptavidin fluorescent probe.

Goodall et al, as described in their letter above, modified our whole blood flow cytometric assay in only one way. Before the addition of thrombin, Goodall et al incubated with a fluorescein isothiocyanate (FITC)-conjugated GPIb-specific MoAb rather than a biotinylated GPIb-specific MoAb. Under these conditions, the thrombin-induced redistribution of the GPIb-IX complexes to the open canalicular system would not be expected to have any effect on platelet fluorescence because a flow cytometer can detect FITC fluorescence irrespective of whether the conjugated antibody is on the surface or the interior of the platelet.

However, we have previously published experiments demonstrating that FITC-conjugated GPIb-specific MoAbs can be used to detect changes in the platelet surface expression of the GPIb-IX complex (Fig 4 of ref 2). The critical difference in methodology is that we added the FITC-conjugated GPIb-specific MoAb to the assay after the addition of thrombin.2

This effect is illustrated in Fig 1. In panel (A), the FITC-conjugated GPIb-specific MoAb 6D1 (provided by Dr Barry S. Coller, SUNY, Stony Brook, NY) was added to the assay before the addition of purified human α-thrombin (provided by Dr John W. Fenton II, New York Department of Health, Albany). As expected, and as shown by Goodall et al, no thrombin-induced decrease in platelet GP Ib expression could be detected (Fig 1A). In panel (B), the experiment was identical except that the FITC-conjugated 6D1 was added to the assay 10 minutes after the addition of thrombin. A decrease in platelet surface GP Ib expression was then detected (Fig 1B), because only the GPIb molecules on the platelet surface were accessible to the FITC-conjugated 6D1. When the sample incubated with control buffer only (no added thrombin) was assigned 100 linear units of fluorescence (derived from Elite software version 3.8; Coulter Cytometry, Hialeah, FL), the sample with added thrombin had 108 units of fluorescence in Fig 1A and 34 units of fluorescence in Fig 1B. Similar results were obtained in experiments using different thrombin concentrations and different MoAbs directed against various epitopes on the GPIb-IX complex.

In summary, the experiments shown in Fig 1 clearly demonstrate that, contrary to the conclusions of Goodall et al, flow cytometric detection of the redistribution of the GPIb-IX complex on thrombin-stimulated platelets is not dependent on the type of antibody conjugate used, but is dependent on the timing of the addition of the antibody conjugate. To detect the thrombin-induced redistribution of the GPIb-IX complex, antibodies that are directly conjugated (eg, with FITC) must be added to the assay after the addition of thrombin. In contrast, antibodies that require an additional (indirect) detection reagent (eg, phycoerythrin-streptavidin3 or FITC-conjugated polyclonal goat anti-mouse antibody4) can be added to the assay before or after the addition of thrombin, provided that the additional detection reagent is added to the assay after the addition of thrombin.

Provided these guidelines are followed, either biotinylated or FITC-conjugated GPIb-IX-specific MoAbs can be used to detect changes in the GPIb-IX complex in clinical or in vitro studies. Polyclonal goat anti-mouse antibodies should not be used in whole blood assays.

REFERENCES

Fig 1. Whole blood flow cytomteric analysis of the effect of thrombin 1 U/mL on platelet surface GPIb-IX complex, as determined by FITC-conjugated MoAb 6D1 (GPIb-specific). (A) FITC-6D1 added before addition of thrombin or control buffer. (B) FITC-6D1 added 10 minutes after the addition of thrombin or control buffer. Background refers to assays in which FITC-conjugated mouse IgG was substituted for FITC-6D1. The peptide glycyl-L-prolyl-L-arginyll-L-proline was included in the assay to prevent thrombin-induced fibrin polymerization and platelet aggregation. The experiment was performed at 22°C. Samples were analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL). The experiment is representative of six so performed.

because they cannot differentiate between the binding of the test murine MoAb (eg, GPIb-IX-specific) and the binding of the murine MoAb (eg, GPIb-IIIa-specific or GPIV-specific) used to identify platelets.  

REFERENCES
Flow cytometric detection of the redistribution of the glycoprotein Ib-IX complex on thrombin-stimulated platelets is dependent on the type of antibody conjugate used [letter; comment]

AH Goodall, M de Oliveira Domingos, N Chronos, SL Janes and DJ Wilson