Measurement of the activation of equine platelets by use of fluorescent-labeled annexin V, anti-human fibrinogen antibody, and anti-human thrombospondin antibody

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Objective—To investigate the potential use of fluorescent-labeled annexin V, anti-human fibrinogen antibody, and anti-human thrombospondin antibody for detection of the activation of equine platelets by use of flow cytometry.

Sample Population—Platelets obtained from 6 Thoroughbreds.

Procedure—Flow cytometry was used to assess platelet activation as indicated by detection of binding of fluorescent-labeled annexin V, anti-human fibrinogen antibody, and anti-thrombospondin antibody to unactivated and ADP-, collagen-, platelet activating factor (PAF)-, and A23187-activated equine platelets. Human platelets were used as control samples. Determination of ^{14}C-serotonin uptake and release was used to assess the extent of platelet secretion.

Results—Anti-human thrombospondin antibody failed to bind to equine platelets. Annexin V bound to platelets activated with PAF or A23187 when platelets had undergone secretion. Anti-human fibrinogen antibody bound to ADP-, PAF-, and A23817-activated platelets, but binding was not dependent on platelet secretion. The extent of binding of anti-fibrinogen antibody was less in equine platelets, compared with that for human platelets, despite maximal stimulation.

Conclusions and Clinical Relevance—Activation of equine platelets can be detected by use of fluorescent-labeled annexin V and anti-human fibrinogen antibody but not by use of anti-human thrombospondin antibody. These flow cytometric techniques have the potential for detection of in vivo platelet activation in horses at risk of developing thrombotic disorders. *(Am J Vet Res* 2002;63:513–519)

Platelets play a substantial role in thromboembolic disease and are implicated as important components in inflammation.[1] In horses, platelet activation has been associated with endotoxemia,[2] laminitis,[3] and equine infectious anemia.[4] In addition, altered platelet responses have been reported[5] in horses with chronic obstructive pulmonary disease.

Flow cytometry is widely used for evaluating platelet activation in humans. Numerous monoclonal antibodies have been developed against specific markers of platelet activation for use in this technique. Typically, whole blood samples are used, and platelets are initially identified by use of a platelet-specific antibody. Platelets then are further differentiated for activation status, using an activation-dependent molecular marker. Consequently, it is possible to detect as few as 0.8% of activated platelets in a blood sample.[6] A major problem in adapting this technique for use in the veterinary field is poor species cross-reactivity of many of the available molecular markers.[7]

Limited information has been published with regard to the measurement of platelet activation in horses. An anti-human fibrinogen antibody has been used to detect activation of equine platelets.[4,8] A potential problem with this method is that binding of fibrinogen to platelets can be reversible.[9-13] Therefore, in vitro assessment of platelet activation by use of an anti-fibrinogen antibody may not always reflect in vivo activation. In a study[12] that involved the use of human platelets, it was suggested that secretory activation markers such as P-selectin are better indicators of in vivo platelet activation. Unfortunately, to the authors' knowledge, antibodies that recognize equine P-selectin are currently not available. It recently has been reported[14] that an anti-human **thrombospondin (TSP)** antibody recognizes equine TSP on immunoblot analysis; thus, this may be an alternative marker of secretory activation that can be used to measure platelet activation in horses, because there is evidence of a good correlation between expression of P-selectin and TSP in activated human platelets.[15]

Activation of platelets also results in surface exposure of phosphatidylserine as part of the procoagulant process. Detection of surface expression of phosphatidylserine has been documented by use of fluorescent-labeled annexin V.[16,17] Annexin V is a calcium-binding protein that has a high affinity for phosphatidylserine.[16] Binding of fluorescent-labeled annexin V has been used to assess activation of human platelets.[18] This method has not been evaluat-

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ed in horses but may provide an additional means for assessing activation of equine platelets. It is unlikely that there would be problems with species cross-reactivity for binding of annexin V to phosphatidylserine.

Therefore, the objective of the study reported here was to evaluate 3 possible methods for the detection of activation of equine platelets. These methods involved the binding of fluorescent-labeled anti-human fibrinogen antibody, anti-human TSP antibody, and annexin V, respectively, to platelets. In addition, ^{14}C-serotonin uptake and release was used to assess the extent of platelet secretion and the effect of platelet secretion on binding of the 3 fluorescent-labeled markers.

Materials and Methods

Animals—Six healthy sedentary Thoroughbreds that ranged from 7 to 17 years old were used in the study. Blood samples were collected from each of the horses, and blood samples also were collected from 1 of the authors (JKK). All procedures were conducted in compliance with guidelines established by the Washington State University Animal Care and Use Committee and the Washington State University Institutional Board for the use of human subjects.

Collection of samples—Blood samples were collected from the horses by venipuncture, using an 18-gauge needle. Samples were collected from each horse on 2 separate days. Human blood was obtained by venipuncture, using a 20-gauge butterfly catheter.

Samples were collected by discarding the first 2 ml of blood and then collecting each sample into syringes containing 0.11*M* sodium citrate[a] (9 parts blood:1 part anticoagulant). The Hct for each sample was determined, using the microhematocrit method. After the Hct was determined, the sodium citrate concentration was adjusted so that the final concentration in all samples was equivalent to that found in a blood sample with a Hct of 45%. This was performed to adjust for differences in plasma-ionized calcium concentration that may have resulted from variations in plasma sodium citrate concentration.[13]

Preparation of platelet rich plasma—For samples to be used to assess platelet activation, **platelet-leukocyte-rich plasma (PLRP)** was prepared by allowing the samples to sit for 20 minutes to permit sedimentation of RBC. In an effort to minimize potential in vitro activation of platelets, samples were not centrifuged. Samples intended for radiolabeling with ^{14}C-serotonin were centrifuged at 150 × g for 10 minutes at room temperature (20 to 21 C) to provide **platelet-rich plasma (PRP)** and ensure minimal contamination with RBC and WBC.

Assessment of platelet activation—Following sedimentation, 10 µl of PLRP was added to 250 µl of 0.5% **bovine serum albumin (BSA)** in **HEPES-buffered Tyrode solution (HBTS**; 145 m*M* NaCl, 2.7 m*M* KCl, 0.42 m*M* $NaHPO_4$, 12 m*M* $NaHCO_3$, 5.5 m*M* dextrose, 5 m*M* HEPES, pH 7.4) containing saturating concentrations of **fluorescein isothiocyanate (FITC)**-conjugated anti-human fibrinogen antibody[b] (1 µl), **phycoerythrin-conjugated anti-human TSP (TSP-PE)** antibody[c] (10 µl), or matched control antibodies.[d,e] In addition, 20 µl of PLRP diluted 1:5 in PBS solution was added to 250 µl of **annexin binding buffer (ABB**; 140 m*M* NaCl, 2.5 m*M* $CaCl_2$, 10 m*M* HEPES-NaOH, pH 7.4)[f] containing 5 µl of FITC-annexin V.[g] Platelets were activated by the addition of ADP[h] (10 or 0.625 µ*M*), collagen[i] (8 or 2 µg/ml), **platelet activating factor (PAF**; 10 or 0.1 n*M*),[j] or A23187[k] (final concentrations of 10 or 2.5 µ*M*). Samples were mixed gently and incubated in the dark for 15 minutes. The reaction was stopped by the addition of 750 µl of HBTS, and samples were analyzed immediately.

For assessment of activation of human platelets, 10 µl of blood was added to 250 µl of 0.5% BSA in HBTS or ABB and incubated with agonists and antibodies as described previously. In addition, a specific platelet anti-human CD42b-FITC antibody[l] was used to label platelets; it was used in combination with TSP-PE antibody or its isotype control antibody.

Samples were analyzed with a cytometer,[m] using appropriate software.[n] The flow cytometer was calibrated daily, using standard beads provided by the manufacturer. Debris or machine noise was excluded from the analysis by setting the appropriate forward-scatter threshold. Data on light scatter and fluorescence were obtained by use of settings in the logarithmic mode, similar to the methods described by Weiss and Evanson.[8] The platelet population was identified on the basis of its forward- and side-scatter properties, which clearly distinguished it from particulate debris, RBC, and WBC. In preliminary studies, light-scatter properties of equine platelets were defined, using a mouse anti-sheep **glycoprotein (GP)**IIb-IIIa antibody[o] that cross-reacts with equine platelet GPIIb-IIIa. For analysis, a gate was drawn around the platelet population, and 10,000 gated events were collected for each sample. Platelet aggregates and platelet-leukocyte aggregates were not gated. The gate was set to display the mean channel fluorescence of the platelet population. Extent of platelet activation was determined by changes in mean total fluorescence.

Radiolabeling of platelets—The technique used for radiolabeling was similar to the method described by Boudreaux et al,[19] with some modifications. Briefly, PRP was incubated with ^{14}C-serotonin[p] at 20 to 21 C for 40 minutes. For each microliter of PRP, we added 6 µl of a stock solution consisting of 0.008 µCi ^{14}C-serotonin/µl of 70% ethanol. At the end of the 40-minute incubation, 3 µ*M* imipramine[q] (final concentration) was added to the platelets to prevent serotonin uptake during release experiments.

Assessment of platelet secretion—An aliquot (50 µl) of radiolabeled PRP was added to 200 µl of 0.5% BSA in HBTS or 200 µl of ABB containing 3 µ*M* imipramine (final concentration). After the addition of ADP (10 or 0.625 µ*M*), collagen (8 or 2 µg/ml), PAF (10 or 0.1 n*M*), or A23187 (final concentrations of 10 or 2.5 µ*M*), samples were gently mixed and incubated for 15 minutes. Control samples were incubated with an equivalent volume (5 µl) of isotonic saline (0.9% NaCl) solution. At the end of the 15-minute incubation, the reaction was stopped by the addition of 70 µl of cold formaldehyde (633 n*M* in 25 m*M* EDTA-0.15*M* NaCl) and the immersion of each sample in an ice bath. Samples then were immediately centrifuged for 1 minute at 14,000 × g, and duplicate 250-µl aliquots of supernatant were removed for counting in a scintillation counter.[r] Samples of diluted whole PRP and PRP supernatant also were used to determine total ^{14}C-serotonin uptake. All samples were solubilized with tissue solubilizer[s] and stored overnight prior to the addition of scintillation fluid[t] for counting.

Statistical analysis—Results were analyzed by use of an ANOVA for repeated measures.[u] When the value for the F statistic was significant, appropriate comparisons were

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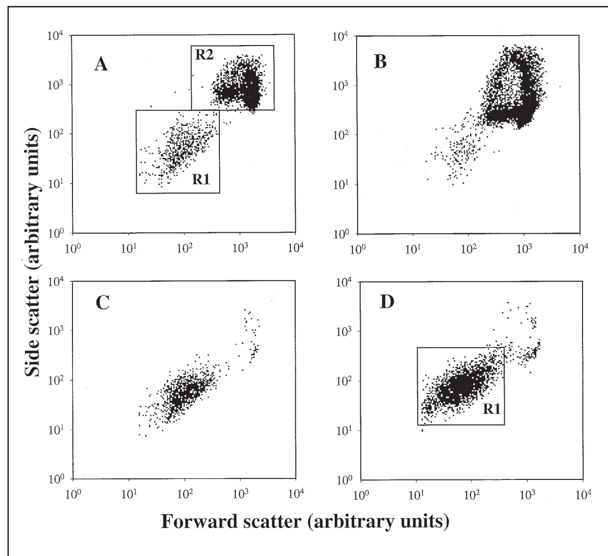

Figure 1—Scatter plots of light-scatter patterns measured by use of a flow cytometer for representative blood samples (panels A and B) and platelet-leukocyte rich plasma (panels C and D) of human (A and C) or equine (B and D) origin. Cells were separated on the basis of their size (logarithmic value of forward scatter) and granularity (logarithmic value of side scatter). R1 represent platelets, and R2 represents RBC and WBC.

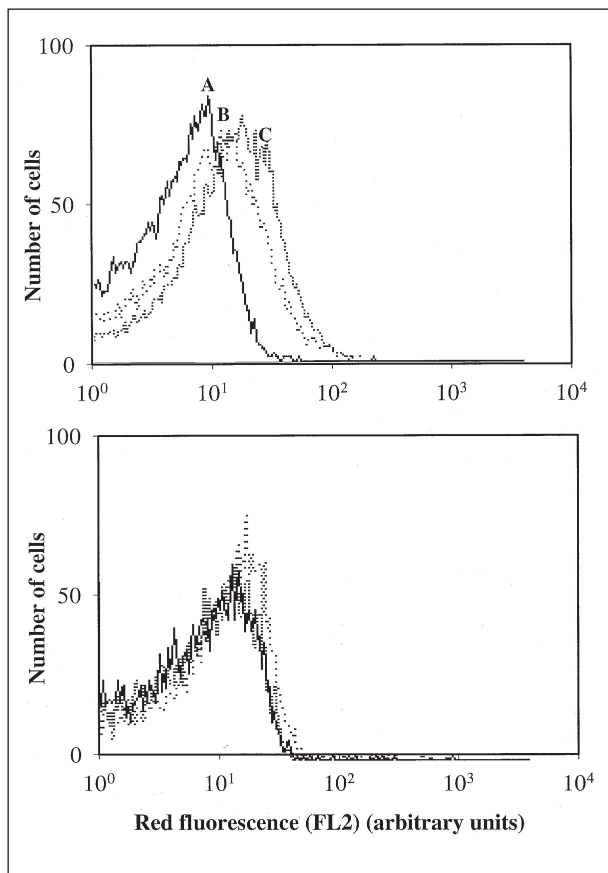

Figure 2—Graph depicting results of samples obtained from humans (top) or horses (bottom) by use of a 1-color method of flow cytometric analysis, using phycoerythrin-conjugated anti-human thrombospondin antibody, for unactivated platelets (A) or platelets activated with 10-n*M* platelet activating factor (PAF; B) or 10-µ*M* ADP (C).

made, using the Bonferroni post-hoc test. Differences were considered significant at values of $P < 0.05$. All results were reported as mean ± SEM.

Results

Platelet-leukocyte-rich-plasma—Following sedimentation, the average numbers of platelets, WBC, and RBC in PLRP were $235 \pm 34 \times 10^3/\mu l$, $4.2 \pm 1.5 \times 10^6/\mu l$, and $1.05 \pm 0.6 \times 10^6/\mu l$, respectively.

Platelet activation—Using light-scatter properties, it was difficult to differentiate platelets from leukocytes in equine blood samples, which was in contrast to the ability to differentiate these cells in human blood samples. The platelet population was easily identified in PLRP processed from equine and human samples **(Fig 1)**.

Platelets activated with ADP and PAF from 3 horses did not have increased binding of TSP-PE or control antibodies, whereas human platelet samples responded to all agonists with increased binding of TSP-PE antibody **(Fig 2)**. Activation with collagen resulted in increased fluorescence in samples containing TSP-PE and the isotype control antibody in equine and human platelets. Using anti-human CD42b-FITC antibody, an antibody that did not bind to equine platelets, in conjunction with human platelets and TSP-PE or control antibodies, the increase in fluorescence in collagen-activated samples was identified as not of platelet origin **(Fig 3)**. It was concluded that the TSP-PE antibody did not bind to activated equine platelets; therefore, additional samples were not evaluated.

Total fluorescence of activated platelets containing

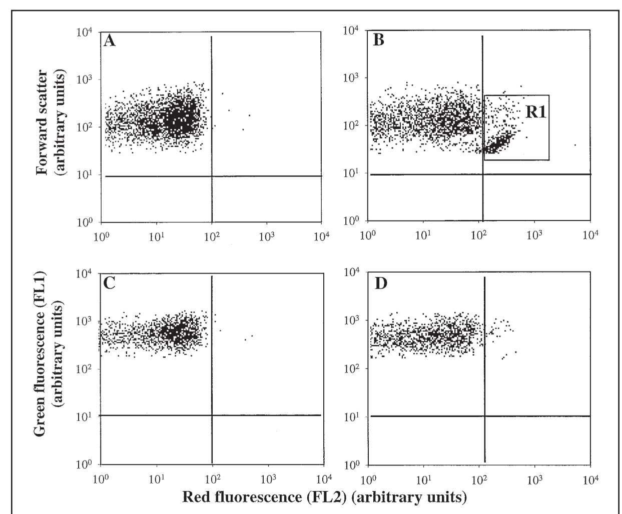

Figure 3—Scatter plots for a 1-color (panels A and B) or 2-color (panels C and D) analysis of a representative sample of human platelets containing fluorescein-labeled anti-CD42b and phycoerythrin-labeled IgG2 (isotype control antibody for anti-thrombospondin) antibodies. Samples were unactivated (A and C) or collagen-activated (B and D) platelets. The 1-color analysis identified platelets on the basis of light-scatter characteristics, and activation was determined on the basis of increased fluorescence (FL2) of that population. The 2-color analysis identified platelets on the basis of binding of the anti-CD42b antibody (FL1) and further differentiated the activated population on the basis of increased fluorescence (FL2). Platelet particles were not responsible for increased fluorescence (R1) seen with the 1-color method.

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the FITC-anti-human fibrinogen antibody was similar in equine and human blood samples. When equine platelets were activated with ADP, there was a small increase in fluorescence for samples activated with 10 µ*M* ADP but not in samples activated with 0.625 µ*M* ADP (**Fig 4 and 5**). However, human platelets had a much greater shift in fluorescence when activated with ADP. Activation with collagen resulted in increased binding of the control and FITC-anti-human fibrinogen antibody in equine and human samples. Equine and human samples activated with PAF and A23187 had dose-dependent increases in fluorescence in samples containing the FITC-anti-human fibrinogen antibody but not in samples containing the control antibody.

Samples containing FITC-annexin V had increased fluorescence in samples activated with PAF, collagen, and A23187 but not ADP in equine and human samples (Fig 5).

Platelet ^{14}C-serotonin uptake and release—Platelet uptake of ^{14}C-serotonin was ≥ 90% for samples obtained

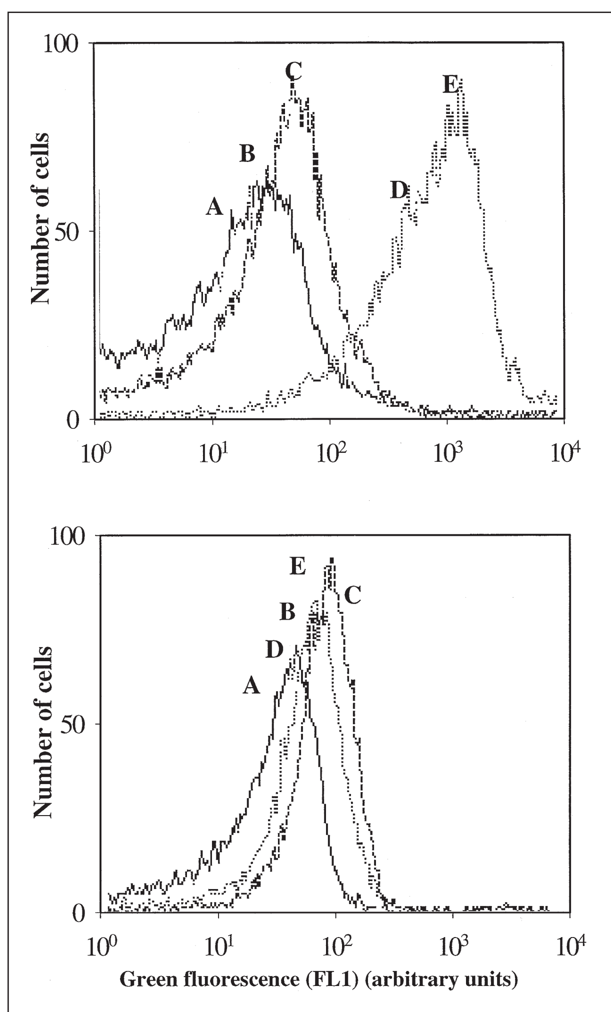

Figure 4—Representative fluorescence intensity (FL1) for fluorescein-labeled anti-fibrinogen antibody of human (top) and equine (bottom) origin that was incubated with unactivated platelets (A) and platelets activated with 0.1n*M* PAF (B), 10n*M* PAF (C), 0.625µ*M* ADP (D), or 10µ*M* ADP (E).

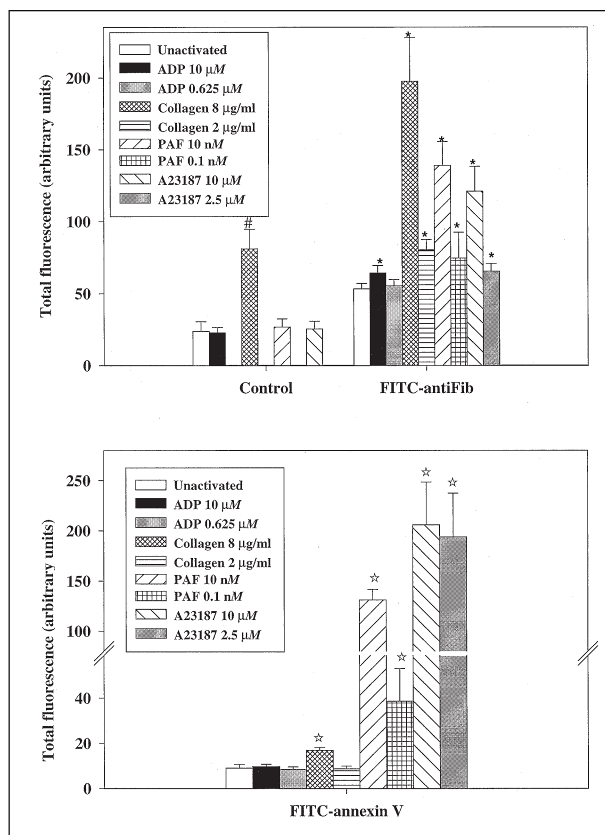

Figure 5—Mean ± SEM total fluorescence determined by use of flow cytometry of unactivated equine platelets and platelets stimulated with various agonists in samples obtained from 6 horses, using fluorescein isothiocyanate (FITC)-labeled anti-fibrinogen (FITC-antiFib) antibody or a control antibody (top) or FITC-labeled annexin V (FITC-annexin V; bottom). #Value differs significantly ($P < 0.05$) from value for unactivated platelets in samples containing control antibody. *Value differs significantly ($P < 0.05$) from value for unactivated platelets in samples containing FITC-labeled anti-fibrinogen antibody. Open star = Value differs significantly ($P < 0.05$) from value for unactivated platelets in samples containing FITC-labeled annexin V.

Table 1—Release of ^{14}C-serotonin from platelets obtained from 6 horses after the platelets were incubated with saline (0.9% NaCl; control) solution or various agonists for 15 minutes in 0.5% bovine serum albumin in HEPES-buffered Tyrode solution (HBTS) or annexin binding buffer (ABB)

	^{14}C-Serotonin release (%)	
Agonist	**HBTS**	**ABB**
Saline solution	0.9 ± 0.7	1.0 ± 0.9
ADP		
10 µ*M*	2.2 ± 0.8	1.9 ± 1.4
0.625 µ*M*	1.4 ± 0.5	1.6 ± 1.0
Collagen		
8 µg/ml	2.5 ± 1.2	2.2 ± 2.1
2 µg/ml	1.2 ± 1.1	2.0 ± 1.1
PAF		
10 n*M*	42 ± 9.4*	54 ± 11.0*
0.1 n*M*	25 ± 6.9*	35 ± 8.4*
A23187		
10 µ*M*	22 ± 13.9*	51 ± 18.3*†
2.5 µ*M*	7 ± 4.9*	28 ± 16.9*†

Values reported are mean ± SEM.
*Within a column, value differs significantly ($P < 0.05$) from corresponding value for saline solution. †Within a row, value differs significantly ($P < 0.05$) from corresponding value for HBTS buffer.

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from all 6 horses. There was minimal ^{14}C-serotonin release in response to the addition of ADP or collagen in both buffers. The ^{14}C-serotonin release was greatest in response to PAF in both buffers, whereas response to A23187 was significantly greater in ABB, compared with the response in 0.5% BSA in HBTS **(Table 1)**.

Discussion

Analysis of the results of the study reported here revealed that the identification of equine platelets on the basis of light-scatter properties is more difficult than for human platelets and that the light-scatter properties of human platelets in this study are similar to those described elsewhere (Fig 1).[6,10,20] There appeared to be a greater degree of overlap of light-scatter properties of RBC and WBC, compared with those in human blood samples.[6] Fortunately, with rouleaux formation, equine RBC sediment quickly in blood samples, eliminating the need for centrifugation to isolate PRP. Nonetheless, the efficiency of the flow cytometer is increased when platelets are identified by use of platelet-specific antibodies.[6] For such antibodies to be useful, they should be conjugated with a fluorescent label so that the washing and centrifuging needed for application of second-step fluorescent-labeled antibodies can be avoided. Although there is a commercially available antibody that recognizes equine platelet CD41/61,[8] it is currently not fluorescent-labeled. The ability to assess activation of equine platelets will be greatly enhanced when a fluorescent-labeled equine platelet antibody becomes readily available.

Assessment of the anti-human TSP antibody indicated that it bound to activated human platelets, but it did not bind to equine platelets (Fig 2). Thrombospondin is a platelet α-granule protein that has been identified in equine platelets.[14] Following secretion, part of the platelet TSP becomes associated with the surface of the cell.[21] Thrombospondin on the surface of platelets has been used as a marker of platelet activation in humans.[22,23] There is evidence of good correlation between surface expression of TSP and P-selectin on circulating activated platelets.[24] Because of the lack of an antibody that recognizes equine P-selectin and that would be suitable for use in flow cytometry, and because there is evidence that an anti-human TSP antibody cross-reacts with equine TSP,[14] we chose to evaluate a commercially available phycoerythrin-conjugated version of this antibody. We are not certain of the reason that anti-human TSP antibody failed to bind to activated equine platelets. An unconjugated version of the antibody has been used to detect equine TSP in prepared supernatants, using immunoblotting techniques.[14] It is possible that conjugation of the antibody to phycoerythrin resulted in alterations in its conformation, thereby rendering the antibody incapable of binding equine TSP through steric hindrance. In addition, the conformation of platelet-bound TSP may differ from platelet-released TSP. Finally, a less likely explanation is that TSP does not bind to activated equine platelets after it is released. Our ability to detect activation of human platelets by use of the same anti-TSP antibody rules out the likelihood of problems with the techniques used. However, we did not test the conjugated antibody on immunoblot preparations. Such an evaluation may have helped identify the reason that the conjugated version of the antibody failed to bind to activated equine platelets.

During evaluation of the anti-human TSP antibody, activation with collagen appeared to cause an increase in fluorescence in the equine platelets. However, a similar increase in fluorescence was seen with the control antibody. Evaluation of the light-scatter properties suggested there was a separate particle population that overlapped with the platelet population. This was evident in equine and human samples. Using a specific platelet antibody (anti-human CD42b) in conjunction with the control antibody in human samples, we were able to confirm that this separate population was not of platelet origin (Fig 3). Because this problem was only encountered when collagen was used, it is possible that the collagen precipitated out of solution and bound antibodies nonspecifically. It also is possible that the physiologic pH of the buffers we used resulted in precipitation of collagen, which was stored in an acidic solution. Given these problems, the results we obtained with collagen probably are invalid.

Anti-human fibrinogen antibody enables investigators to use flow cytometry to detect activated equine platelets.[4,8,13] Investigators often have used fixation of platelets and washing of samples. We were able to avoid washing and centrifugation as well as fixation of samples, and our results were comparable. Despite this, the degree of background fluorescence was much greater in samples with the anti-fibrinogen antibody, compared with values for the control antibody, which could imply activation of resting platelets (Fig 5). However, control antibodies can be of limited value in the quantitation of nonspecific binding of antibodies. The resulting fluorescence is dependent on the particular concentration of antibody as well as manufacturer-specific and clone-dependent fluorochrome conjugation and purification. It is recommended that when control antibodies are used that they be obtained from the same manufacturer as the matched isotype control antibody.[25] Such a matched control antibody was not available for the anti-human fibrinogen antibody, which was a polyclonal $F(Ab')_2$ fragment. Differences in concentration, conjugation, and purification may explain the lower amount of fluorescence in samples containing the control antibody. It also is possible that equine platelets have some degree of fibrinogen binding in the resting state. Russell et al[4] reported that 33% of normal unactivated equine platelets had increased fibrinogen binding. In contrast, results of studies[10,26] in humans indicate that only 1 to 2% of normal resting platelets have evidence of increased fibrinogen binding. Studies[27,28] with human platelets indicate that the GPIIb-IIIa receptor must undergo a conformational change during activation before fibrinogen will bind to platelets. However, this has not been evaluated in equine platelets. Additional studies are needed to better define the interaction of fibrinogen with quiescent equine platelets.

The extent of binding of the anti-human fibrinogen antibody was much less in activated equine

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platelets, compared with activated human platelets, even with maximal stimulation with PAF (Fig 4). The degree of fluorescent shift attributable to binding of the anti-human fibrinogen antibody with human platelets was similar to results reported elsewhere.[10,26] Even though 0.625 μ*M* ADP results in a weak aggregation response in horses,[13] we were unable to detect increased fibrinogen binding at this concentration with flow cytometry. In contrast, a study[26] of human platelets revealed increased fibrinogen binding at concentrations of ADP as low as 0.08 μ*M*. Although there are obvious species differences in platelet responses to ADP,[29] it is surprising that we were unable to document increased fibrinogen binding at ADP concentrations known to elicit an aggregation response. In our experience, ADP-activated equine platelets collected from an aggregometer have greater binding of fibrinogen than samples that are mixed gently. Therefore, platelet-to-platelet interactions induced by stirring may be an important component in ADP-induced fibrinogen binding and in vitro assessment of aggregation of equine platelets. It also is possible that aggregation responses of equine platelets are more dependent on extracellular calcium concentrations than human platelets, similar to the aggregation responses reported for platelets of rabbits.[30] The greater degree of fluorescence in activated human platelets may reflect greater specificity of the antibody for human fibrinogen, compared with the specificity for equine fibrinogen. Alternatively, human platelets may bind a larger number of fibrinogen molecules. Increased binding may be attributable to greater numbers of GPIIb-IIIa receptors on human platelets. Conversely, when compared to fibrinogen, a relatively greater proportion of other adhesion proteins such as TSP may bind to equine platelets. Additional comparative studies are needed to resolve this apparent species difference.

Annexin V is a protein that has high affinity and strict specificity for aminophospholipids such as phosphatidylserine, and it has been used to detect apoptotic cells.[31] When platelets are activated, they undergo a similar loss of membrane phospholipid asymmetry with resultant surface exposure of phosphatidylserine.[16] The surface-exposed phosphatidylserine provides a negatively charged catalytic surface for assembly of the prothrombinase complex that converts zymogen prothrombin into thrombin.[32] Binding of fluorescent-labeled annexin V to platelets has been used to detect platelet activation in human samples.[18] Analysis of our results indicated that in vitro activation of equine platelets by use of PAF or A23187 caused increased binding of annexin V. In contrast to results with human platelets,[17] collagen had minimal effect on binding of annexin V to equine platelets. It is possible that collagen precipitated out of solution and, thus, failed to bind and activate platelets. This possibility is supported by the data on platelet secretion in which collagen caused minimal release of serotonin. Results of another study[19] documented that collagen induces secretion in up to 45% of equine platelets. However, those studies were performed in conjunction with aggregometry, and the samples were continuously stirred. Samples in our study were mixed gently and allowed to sit undisturbed during a 15-minute incubation. Stirring of equine platelets may be necessary to stimulate a response to collagen, similar to the situation for ADP. On the basis of the results reported here, it appears that equine platelets need a stimulus sufficiently strong to induce secretion to enable membrane translocation of phosphatidylserine.

Analysis of results from our experiments on serotonin uptake and release indicated that for the conditions used in this experiment, ADP and collagen failed to cause substantial platelet release, whereas PAF and A23187 caused substantial serotonin secretion (Table 1). In addition, the degree of secretion was affected by the choice of buffer. The calcium ionophore, A23187, had a significantly greater effect on serotonin release in ABB, compared with the effect in 0.5% BSA in HBTS. Given that albumin strongly binds to A23187 and decreases its availability,[33] weaker responses in HBTS were likely attributable to the 0.5% BSA. Analysis of data on secretion and flow cytometry indicated that binding of fibrinogen was not dependent on platelet secretion, whereas annexin V binding paralleled platelet secretion. This suggests that fibrinogen binding should be a more sensitive indicator of platelet activation, preceding platelet secretion and translocation of phosphatidylserine to the surface membrane. However, if fibrinogen is bound reversibly, there is a possibility this method will not detect in vivo platelet activation.[12,13]

In the study reported here, activated equine platelets bound fluorescent-labeled anti-human fibrinogen antibody and annexin V, which can be detected by use of flow cytometry. Phycoerythrin-labeled anti-human TSP antibody failed to bind to activated equine platelets, and it is likely to prove unsuitable for flow cytometric assessment of activation of equine platelets. Binding of fibrinogen appears to be a more sensitive indicator of early activation (ie, activation preceding platelet secretion), whereas annexin V binding occurs in conjunction with platelet secretion. These methods have possible uses for assessing in vivo platelet activation and its potential role in the pathogenesis of diseases in horses. We anticipate that the sensitivity of these methods will be increased when used in conjunction with a fluorescent-labeled antibody specific for equine platelets and appropriate matched control antibodies.

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[a]Sodium citrate, JT Baker Inc, Phillipsburg, NJ.
[b]Fibrinogen polyclonal antibody, Cappel Research Products, ICN Biomedicals Inc, Aurora, Ohio.
[c]Thrombospondin monoclonal antibody, Immunotech, Beckman Coulter, Fullerton, Calif.
[d]IgG F(Ab')$_2$ polyclonal antibody, ICN Biomedicals Inc, Aurora, Ohio.
[e]Mouse IgG1 R-PE, Caltag Laboratories, Burlingame, Calif.
[f]Annexin binding buffer, BD PharMingen, San Diego, Calif.
[g]Annexin V-FITC, BD PharMingen, San Diego, Calif.
[h]ADP Grade I, Chrono-log Corp, Havertown, Pa.
[i]Collagen reagent, Chrono-log Corp, Havertown, Pa.
[j]PAF, Sigma Chemical Co, St Louis, Mo.
[k]A23187, Sigma Chemical Co, St Louis, Mo.
[l]Mouse anti-human CD42b:FITC, Serotec, Raleigh, NC.
[m]FACScan, Becton-Dickinson, San Jose, Calif.
[n]CellQuest, BD Immunocytometry Systems, San Jose, Calif
[o]Mouse anti-sheep GPIIb-IIIa, Serotec, Raleigh, NC.
[p]5-Hydroxytryptamine-beta-^{14}C creatinine sulfate complex, Sigma Chemical Co, St Louis, Mo.
[q]Imipramine hydrochloride, Sigma Chemical Co, St Louis, Mo.

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[r]Model 1600TR, Tri-Carb liquid scintillation analyzer, Packard Instrument Co, Meriden, Conn.
[s]NCS-II tissue solubilizer, Amersham Pharmacia Biotech Inc, Piscataway, NJ.
[t]BCS, Amersham Pharmacia Biotech Inc, Piscataway, NJ.
[u]NCSS 2000, NCSS Software, Kaysville, Utah.

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