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Antibodies Against Human Cell Receptors, CD36, CD41a, and CD62P Crossreact With Porcine Platelets

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Background: A limitation in platelet study has been the availability of platelet function–specific membrane receptor antibodies for use in the various animal species that are currently used in the study of hemostasis and other phenomena.

Methods: Platelets were isolated from human and porcine blood. Resting and activated platelets were incubated with antibodies against the human cell surface receptors CD36 (clone CB38), CD41a (clone HIP8), CD62P (clone AK4), and CD63 (clone H5C6). Antibody titration and ligand blocking studies also were performed.

Results: Binding of anti-CD41a and anti-CD62P were similar for human and porcine platelets in percentage of platelets labeled and in number of receptors per cell. Binding of anti-CD36 was similar between species, with fewer receptors present in porcine cells. Anti-CD63 and anti-CD107a did not bind specifically to porcine platelets.

Conclusion: The anti-CD36, anti-CD41a, and anti-CD62P antibodies studied crossreact with porcine platelets and will be useful in the investigation of platelet function in porcine models. Cytometry Part B (Clin. Cytometry) 56B:62–67, 2003. Published 2003 Wiley-Liss, Inc.†

Key terms: platelet; antibody; human; swine

Platelets are an anucleate cellular component of blood. In circulation, normal platelet levels are $2\text{–}4 \times 10^5/\mu l$ (1). The primary function of platelets in circulation is to aid in the formation of clots after a vascular injury. Platelet activation occurs as part of the hemostatic mechanism or as part of the inflammatory response. When activated, platelets undergo physical shape change, release contents of cytoplasmic granules, exhibit a change in surface phospholipid configuration, and express a specific population of cell surface receptors. The expression of these receptors may be detected by flow cytometric techniques.

Flow cytometry has assumed an important role in platelet function evaluation and diagnosis in the past 10 years. A limitation in platelet study has been the availability of platelet function–specific membrane receptor antibodies for use in the various animal species that are currently used in the study of hemostasis and other phenomena. A variety of antibodies against human platelet membrane glycoproteins have been characterized, but those available for other species are limited.

Four commonly studied platelet glycoprotein receptors are: CD36, CD41a, CD62P, and CD63. CD36, also known as glycoprotein (GP) IV, is the receptor for the extracellular matrix proteins thrombospondin and collagen (2). In humans, CD36 is involved in cell-to-cell adhesion and aggregation and is also a scavenger receptor for oxidized low-density lipoproteins and shed photoreceptor outer segments (3). Activation of CD36 receptors on platelets induces degranulation, release of adenosine triphosphate and serotonin, increase in intracellular calcium, and activation of tyrosine kinase activity (4). CD41a is a calcium-dependent component of the GPIIb/IIIa complex expressed in normal platelets and megakaryocytes (5). The GPIIb/IIIa complex is the receptor for fibrinogen and von Willebrand factor. This receptor mediates platelet adhesion and aggregation. Antibodies to CD41a inhibit platelet aggregation and adenosine triphosphate secretion induced by adenosine monophosphate, thrombin, or collagen in human platelets (6). CD62P is the platelet-specific

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selectin protein, which is expressed on activated platelets, endothelial cells, and megakaryocytes (7). This receptor mediates tethering and rolling of platelets on the surface of activated endothelial cells. Upon activation, CD62P is upregulated by mobilization from storage granules to the cell surface of platelets and is later shed from the cellular membrane (8). CD63 is the receptor for a lysosomal glycoprotein of the tetraspan transmembrane 4 superfamily (9) and is expressed in activated platelets (10). It is observed in pathologic specimens from patients with primary melanoma, atherosclerotic plaques, and Hermansky-Pudlak syndrome (11).

The pig is often the preferred species for experimental models related to hemorrhage and hemostasis. The importance of understanding the role of platelet function in these animal models is clear. The need for porcine antibodies in research is an important one, but not one that will likely be considered cost effective by commercial antibody manufacturers. The exact binding epitope configuration might create a significant difference in the recognition of the functional receptor in question. Identification of well-characterized human antibodies that crossreact in swine would add valuable tools for hemostasis research. Crossreactivity charts for specific antibody clones are available for some species, but many species have not been tested. This study was performed to determine the crossreactivity of four commercially available antibodies with porcine platelet glycoproteins.

MATERIALS AND METHODS

Experimental Approach

Binding of each antibody was determined for human and porcine platelets in the resting and activated states. Next, antibody concentrations were titrated from undiluted to 1:640, and binding was determined for human and porcine platelets. The undiluted concentration was the highest concentration supplied by the manufacturer. This concentration was not high enough to demonstrate saturation in the case of CD62P and CD41a (Fig. 3a and 3c). Antibodies that displayed evidence of binding to porcine platelets in the first two experiments were further studied by determining binding to resting porcine platelets in the presence or absence of a blocking receptor ligand.

Sample Collection

Ten normal crossbred pigs weighing 40–45 kg were used for this study. Animals were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. This study was approved by the Institutional Animal Care and Use Committee of the U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas. Animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23, revised 1996). Animals used for this study underwent surgery as part of a separate study. Blood was collected for the present study before any other experimental procedures were performed. Each animal was fasted for 24 h before the procedure and then anesthetized, catheterized, and prepared for surgery. A flexible Tygon catheter (2.0 mm outer diameter, 1.25 mm inner diameter, 100 cm long) was placed in the femoral vein and inserted 10 cm into the vessel. Blood samples were collected through a four-way stopcock. Catheter patency was maintained with 0.25 mM sodium citrate. At the time of sample collection, 3.0 ml of blood was collected and discarded. Immediately thereafter, a syringe containing 3.0 ml of 0.25 M sodium citrate was used to collect 30 ml of blood at a rate of 400 μl/s.

Blood was collected from volunteer adult human subjects with prior informed consent, in accordance with the guidelines of the institutional Human Use Review Committee. Volunteer human subjects were normal male (n = 3) or nongravid female (n = 3) and had not been administered any medications for at least 1 week before the study. Human blood was collected by medial cubital venipuncture directly into vacuum collection tubes containing sodium citrate.

Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 200*g* for 10 min. After centrifugation, PRP preparations were divided into two aliquots. Aliquot 1 was labeled "resting" and aliquot 2 was labeled "activated." The resting PRP aliquot was immediately fixed by the addition of an equal volume of 3% methanol-free formaldehyde solution (Polysciences Inc., Warrington, PA). The activated PRP aliquot was incubated for 5 min with 10 μg/ml collagen (Helena Laboratories, Beaumont, TX) and then fixed as described for the resting group. All samples were stored at 4°C for 48 h and then stained.

Direct Immunofluorescence Staining for CD36, CD41a, CD63, and CD107a Receptors

Antibodies to CD36 (clone CB38), CD41a (clone HIP8), CD63 (clone H5C6), and CD107a (clone H4A3) were mouse anti-human monoclonal antibodies (mAb) and were purchased from BD Pharmingen (San Diego, CA). Antibodies against receptors CD36 and CD41a were conjugated to fluorescein isothiocyanate (FITC). The antibody to CD63 was conjugated to R-phycoerythrin. Platelets were washed twice with phosphate buffered saline (PBS; ValTech Diagnostics, Brackenridge, PA) and reconstituted in the original volume. An aliquot of 100 μl containing 2.5×10^6 platelets was incubated with 20 μl containing 1 μg of mAb for 15 min at room temperature. Cells were washed with 2 ml of PBS. Samples were centrifuged at 1,000*g* for 10 min, and the supernatant was discarded. Cells were resuspended in 500 μl of 2% paraformaldehyde. Samples were stored at 4°C in the dark until flow cytometric acquisition.

Indirect Immunofluorescence Staining of P-Selectin (CD62P) Receptor

An aliquot of 100 μl containing 2.5×10^6 washed platelets was incubated with 1 μg of purified rabbit anti-CD62P (clone AK4) polyclonal antibody (BD Pharmingen) for 15 min at room temperature. Cells were washed with 2 ml of PBS and centrifuged at 1,000*g* for 10 min, and the

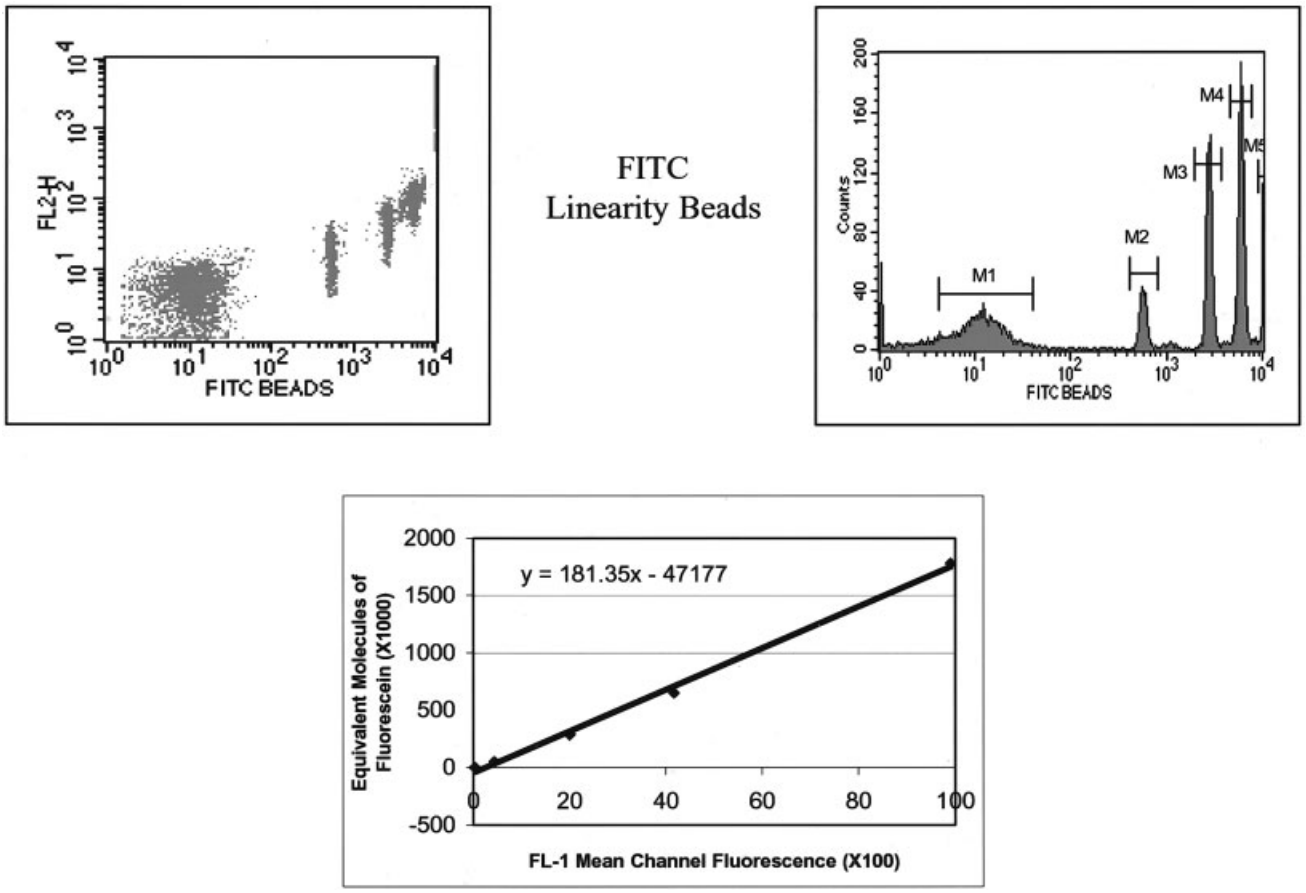

FIG. 1. Representative plots and standard curve of commercial fluorescein isothiocyanate (FITC) linearity beads used in the conversion of mean fluorescence channel intensity to equivalent molecules of fluorescein. Regression analysis from the standard curve resulted in an R^2 of 0.997. All data files are the result of 10,000 events.

supernatant was discarded. Next, cells were incubated for 15 min at room temperature with 50 μl of FITC-labeled goat anti-rabbit immunoglobulin (Ig) G (1:10 dilution) obtained from Caltag Laboratories (Burlingame, CA). Cells were washed with 2 ml of PBS and centrifuged at 1,000*g* for 10 min, and the supernatant was discarded. Cells were resuspended in 500 μl of 2% paraformaldehyde. Samples were stored at 4°C in the dark until flow cytometric acquisition.

Receptor-Specificity Blocking Assay

Cells were incubated with 50 μl containing 1 μg of purified antibody for 30 min at room temperature. CD36, CD41a, and CD62P bindings to antibodies were blocked with soluble forms of thrombospondin (Calbiochem, La Jolla, CA), fibrinogen (Sigma, St. Louis, MO), and P-selectin (R&D Systems, Minneapolis, MN), respectively. Cells were washed with 2 ml of PBS and centrifuged at 1,000*g* for 10 min. The supernatant was discarded, cells were resuspended in 100 μl of PBS, and staining was performed as indicated above.

Flow Cytometry: Controls, Acquisition, and Analysis

For all staining the proper isotypic (IgG1, IgG2a, IgG2b, IgM), negative (autofluorescence, background, nonspecific G∝R-FITC binding), and positive (stimulation) controls were performed for each sample stained. All antibodies were titrated for optimal fluorescence concentrations. The optimal concentration was defined as the lowest concentration of antibody that generated the highest fluorescent intensity. Paired correlation between porcine and human platelets was performed to assess initial binding similarities. Cells were acquired on a FACSCalibur flow cytometer and analyzed with CELLQuest software (Becton Dickinson, San Jose, CA). Flow cytometer calibration was verified for linearity, sensitivity, compensation, and photo multiplier tube adjustment. No instrument drift was observed because all stains were acquired with identical instrument settings on multiple days. Figure 1 illustrates the data for the conversion of mean fluorescence channel values to equivalent molecules of fluorescein by performing standard curves using commercially available FITC-labeled beads (Flow Cytometry Standards, San Juan, PR) of known fluorescent intensity. The fluorescein-to-antibody

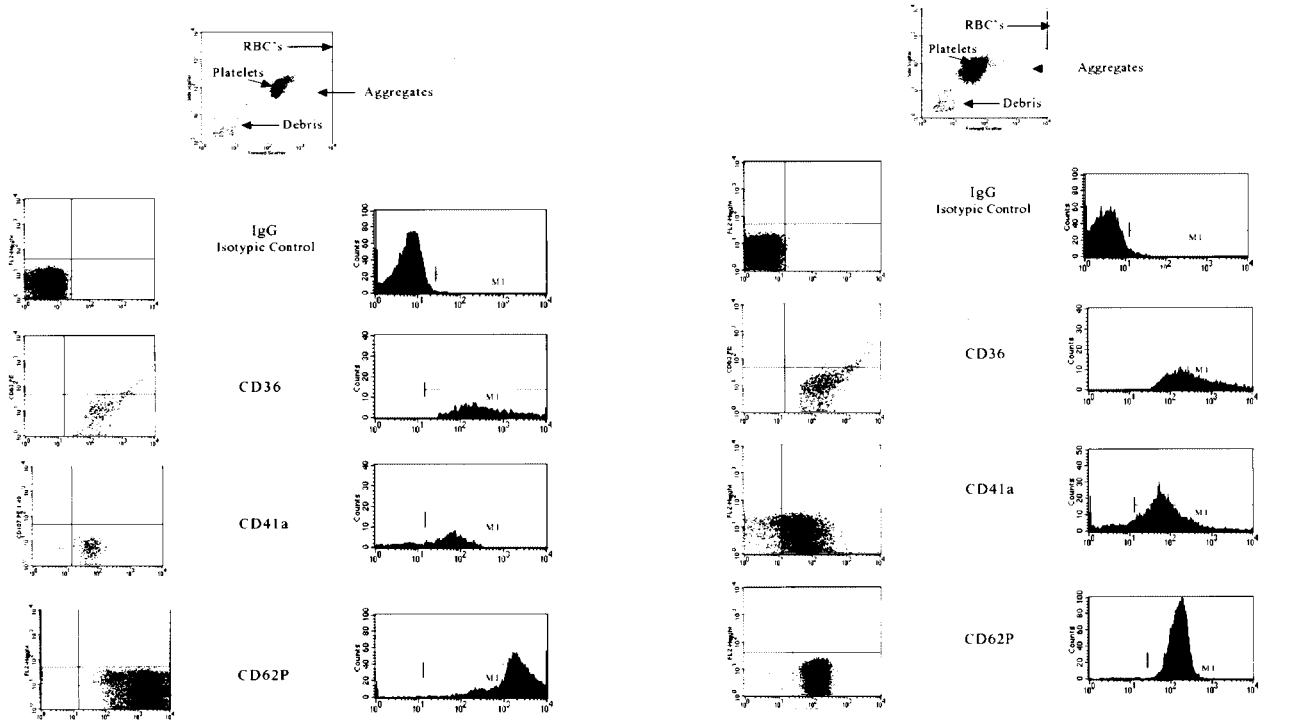

FIG. 2. Representative flow cytometry dot plots and histograms of resting (left) swine and (right) human platelet analyses. Data were analyzed with an inclusive gate of platelets and aggregates. All files are the result of 10,000 total events. IgG, immunoglobulin G; RBC, red blood cell.

ratio was provided by the manufacturer for all antibodies used in this study. The number of receptors was determined by dividing the equivalent molecules of fluorescein by the fluorescein-to-antibody ratio of the mAb. All fluorescent bead standard curves were generated with instrument settings identical to those used for sample acquisition. All values are the mean of 10,000 events (Fig. 2).

Statistical Analysis

Data were analyzed by analysis of variance. Differences were considered significant at $P \leq 0.05$. Results are presented as means ± standard error of the mean. All statistical analyses were performed with SAS/STAT software (SAS Institute, Cary, NC).

RESULTS

Labeling of resting platelets with the anti-CD36 antibody was similar ($P > 0.05$) for human and pig platelets (95.3 ± 1.4% vs. 99.8 ± 1.7%, respectively). Percentage of binding was reduced in activated platelets from both species ($P < 0.01$; Fig. 4). The reduction in binding was 95.8 ± 1.3% in human platelets, which was a greater ($P = 0.04$) reduction than the 90.2 ± 1.0% observed for porcine platelets. The number of labeled receptors per cell was affected by anti-CD36 titration in both species, with a different pattern observed between the two ($P < 0.01$). Under undiluted and 1:5 diluted conditions, larger ($P < 0.01$) numbers of receptors were labeled in human platelets (Fig. 3b). Addition of thrombospondin reduced by greater than 99% the percentage of labeled porcine platelets (Table 1).

There was no difference between species in the percentage of resting or activated platelets labeled with anti-CD41a. There was no change in either species in the percentage of platelets labeled after activation (Fig. 4). The numbers of receptors labeled per cell declined with increasing antibody dilution ($P < 0.01$) (Fig. 3c). The pattern of change did not differ between the human and porcine species. Fibrinogen reduced the percentage of labeled porcine platelets by greater than 99% (Table 1).

The percentages of resting and activated platelets that were labeled with anti-CD62P were similar in human and pig platelets. Platelets of both species displayed reduced binding in the activated state compared with those of resting platelets ($P < 0.01$). Numbers of receptors labeled per cell declined with increasing antibody dilution in both species ($P < 0.01$), with similar patterns of change for each species (Fig. 3a). Anti-CD62P binding was reduced by over 98% in the presence of P-selectin (Table 1).

In resting human platelets, 55.4 ± 1.7% of platelets bound anti-CD63. Binding in resting porcine platelets was lower ($P < 0.01$). Binding increased to 98.4 ± 1.7% after activation of human platelets ($P < 0.01$). Binding in the resting pig platelets was 11.8 ± 1.4% and declined ($P = .03$) to 6.2 ± 1.4% in the activated platelets. Between the resting and active states, binding increased 78.6 ± 9.8% in human platelets, which was greater than the 46.7 ± 8.0%

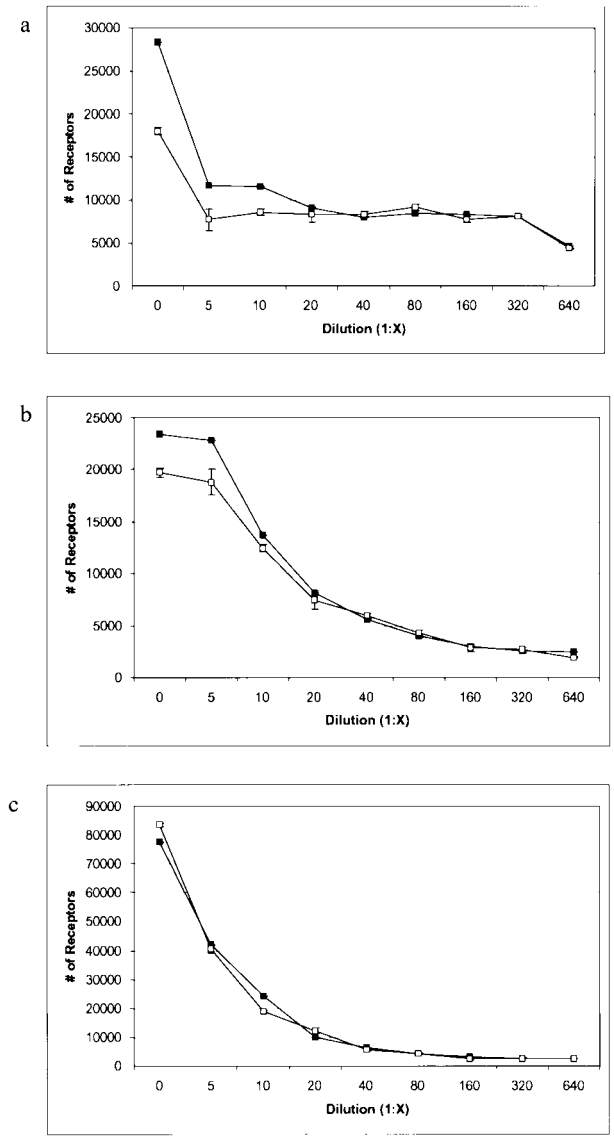

FIG. 3. Titration curves of (**a**) CD62P, (**b**) CD36, and (**c**) CD41a on human and swine platelets under resting conditions. Open squares represent the porcine platelets, and solid squares represent the human platelets. Each point represents the mean ± standard error of the mean (n = 3).

decline in pig platelets ($P < 0.01$). Titration of anti-CD63 resulted in a steady decline in the number of receptors labeled for human platelets ($P < 0.01$) but no pattern of change in porcine platelets. The number of receptors bound by porcine platelets was indistinguishable from zero at each dilution (data not shown).

Results from the experiments performed with anti-CD107a showed binding to activated human platelets only; no binding was observed with porcine platelets (data not shown).

DISCUSSION

Previous studies have documented the similarity and homogeneity of various cellular proteins between human and porcine systems. Saalmuller et al. (12) found that crossreactivity of human mAb to porcine cells exists for a variety of T-cell markers. Zhang et al. (13) reported a group of mouse anti-human mAbs against α/β integrins that crossreacts with pig cells. Homogeneity of human and porcine interferon-γ was determined by the identification of a shared antigenic domain (14). Evolutionarily, there is a high degree of conservation across mammalian species for a variety of blood proteins. This suggests that platelet proteins may exhibit homogeneity.

Antibody binding to CD36 was similar for human and porcine platelets in resting and activated states. Binding response to antibody titration was different for the two species at the highest antibody titers but was similar at dilutions greater than 1:5. Blocking with thrombospondin resulted in a greater than 99% reduction in binding. Taken together, these data indicated a high degree of crossreactivity for the anti-CD36 antibody with porcine platelets. The observed differences in the numbers of labeled receptors suggested that fewer CD36 receptors may be present on porcine than on human platelets. Because numbers of receptors were similar at more dilute antibody preparations, below the saturation level, it does not appear likely that the differences noted were due to a significant difference in binding affinity.

Binding of anti-CD41 was similar for human and swine platelets in terms of percentage of platelets labeled and in terms of the change in the number of labeled receptors over the titration range. Fibrinogen almost completely abolished binding, confirming that binding was specific. The present data are consistent the previously published finding that porcine surface glycoprotein GPIIb can be immunoprecipitated by a mouse anti-human CD41 antibody (15). Based on currently available data, it appears that the CD41 antibody studied here has a high degree of crossreactivity in porcine platelets. A similar situation exists with anti-CD62. Binding to resting and activated platelets and the response to antibody titration were similar for human and porcine platelets. Specificity of binding was confirmed by the greater than 98% abolition of binding in the presence of thrombospondin. These findings are consistent with a previous study, which reported that the porcine P-selectin primary sequence contains an open reading frame encoding 646 amino acids with 82% identity to human P-selectin (16).

The antibodies used against CD63 showed no evidence of crossreactivity between human and porcine platelets. Binding by porcine platelets was much lower than that

Table 1
Normal Swine Platelet Percentage Binding of Blocked and Control (Unblocked) Receptors[a]

	Control	Blocked
CD36	97.25 ± 0.69	0.96 ± 0.49
CD62P	98.46 ± 1.26	1.76 ± 0.90
CD41a	99.16 ± 0.49	0.69 ± 0.35

[a]Data presented are mean ± standard error of the mean (n = 2).

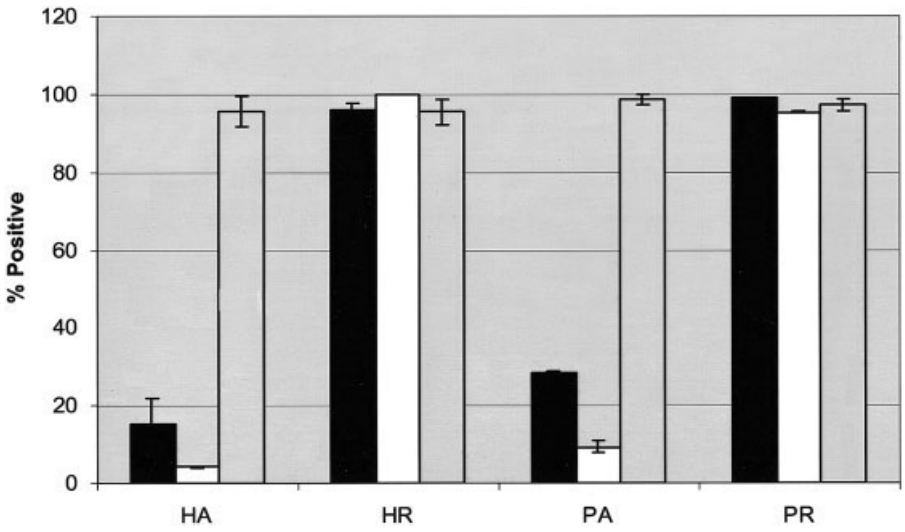

FIG. 4. Responses of human swine platelets to collagen (10 μg/ml). Black bars, CD62P; white bars, CD36; gray bars, CD41a. Each point represents the mean ± standard error of the mean (n = 3). HA, human activated; HR, human resting; PA, pig activated; PR, pig resting.

observed for human platelets and was unaltered by antibody titration. The lack of binding to porcine platelets may have been due to a change in protein conformation during handling of pig platelets. It has been proposed that fixation interferes with the epitope conformation on cell surface proteins, which would affect binding properties to mAb. However, Shuberth et al. (17) demonstrated that paraformaldehyde concentrations lower than 5% do not modify binding. In addition, binding to human platelets, which were handled identically, was not ablated.

The present data demonstrate that the anti-CD36, anti-CD41a, and anti-CD62P antibodies studied crossreact with porcine platelets. These antibodies will be useful in the investigation of platelet function in porcine models. We also identified that anti-CD63 and anti-CD107a antibodies does not crossreact with porcine receptors. Therefore, the H5C6 and H4A3 antibody clones should not be used for swine.

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