Effects of OKM5, a Monoclonal Antibody to Glycoprotein IV, on Platelet Aggregation and Thrombospondin Surface Expression

By Martha L. Aiken, Mark H. Ginsberg, Vicky Byers-Ward, and Edward F. Plow

The monoclonal antibody, OKM5, recognizes an 88-Kd monocyte membrane protein and also binds to the platelet membrane protein, GPIV (GPIIb, CD36). In this study, we have found that the OKM5 target epitope is present at approximately 12,000 copies per platelet and that interaction with the antibody has both stimulatory and inhibitory effects on platelet function. In the absence of other stimuli, OKM5 induced platelet aggregation, secretion, and expression of fibrinogen receptors. These stimulatory responses required intact antibody as F(ab)'2 fragments were not active but blocked the stimulatory activity of the intact antibody. In contrast, exposure of platelets to OKM5 followed by another strong stimulus such as thrombin resulted in a marked suppression of fibrinogen, fibronectin, and von Willebrand factor binding to the cells. This effect was not noted when a weak stimulus, adenosine diphosphate, was the second agonist. At OKM5 concentrations that interfered with fibrinogen binding to thrombin-stimulated platelets by 80% to 90%, platelet binding of exogenous thrombospondin, or surface expression of endogenous thrombospondin was not affected. The inhibitory effect of OKM5 on fibrinogen binding to thrombin-stimulated platelets was related to the formation of massive platelet aggregates in the samples. These results show that interaction of OKM5 with its target antigen on platelets can elicit diverse functional responses from the cells.

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MATERIALS AND METHODS

OKM5 purification and characterization. OKM5 ascites was obtained from Ortho Diagnostics Systems, Inc (Raritan, NJ), and three separate batches of this reagent were used during the course of these studies, including 10 mL of ascites (350S-15) provided by Dr Peter Kramer of Johnson and Johnson (Raritan, NJ). The MoAb was purified essentially as described by Ey et al.14 In brief, 3 mL of ascites fluid, diluted 1:1 with 0.1 mol/L phosphate buffer, pH 8.0, was incubated for 18 hours with 6 mL of Protein-A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) with gentle agitation at 4°C. The resin was then washed with phosphate-buffered saline (PBS) at pH 7.3 to remove unbound material. The MoAb was eluted with 0.1 mol/L Na citrate, pH 4.5, and collected in 4-mL aliquots into 0.5 mL of 1.0 mol/L Tris, pH 8.0. The fractions containing the MoAb were pooled, treated with 0.5 mmol/L phenylmethylsulfonyl fluoride (Calbiochem, La Jolla, CA), and dialyzed against PBS. It was our experience that purified OKM5 frequently lost activity during storage due to aggregation and that a high concentration of bovine serum albumin (BSA) helped to stabilize the responses of platelets and other cells. In the present study, we have examined the stoichiometry and functional consequences of the interaction of OKM5 with platelets. The results indicate that this MoAb exerts multiple effects on platelets, suggesting a multifunctional role of its target antigen in platelet responses.

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2501
reagent. Therefore, most of the antibody was stored at -80°C
in 10% BSA, and only a portion was frozen without BSA for
radioiodination. The activity of OKM5 was routinely tested on the day
of its use in a solid phase radiometric assay in which binding of
OKM5 to platelets coated onto microwells was assessed as previously
described for other platelet MoAbs.17 OKM5 was detected by the
binding of 125I-amontiusrase immunoglobulin G (IgG) at 0.5 µg/mL.
Active OKM5 gave a signal of at least threefold above background
in this assay at concentrations of 20 fmol/L.

F(ab')2 fragments of OKM5 were prepared by pepsin digestion of
the antibody. OKM5, at 140 to 220 µg/mL in 1% BSA (spiked with
a small amount of 125I-OKM5 to permit polyclaracylamide analysis of
the preparations), was dialyzed overnight into 0.2 mol/L NaCl, 0.2 mol/L sodium acetate, pH 4.0, and the pH was then adjusted to 4.0
with acetic acid. Pepsin (Sigma Chemical Co, St Louis, MO) was
added to a final concentration of 30 µg/mL, and the mixture was
incubated for 3 hours at 37°C, at which time the pH was adjusted to
7.0.

Other proteins. Fibrinogen (Fg), fibronectin (Fn), and von
Willebrand factor (vWF) were purified from fresh human plasma.
The purifications involved differential ethanol or ether precipitation
for Fg, gelatin-Sepharose affinity chromatography for Fn, and gel
filtration of cryo-precipitates for vWF. TSP was purified from fresh
platelet concentrates. The physical and platelet binding characteristics
of the radiiodinated and nonlabeled forms of each of these proteins were
as prepared by our laboratories have been previously
described.18,19 The preparation of the polyclonal anti-Fg and the
production and purification of F(ab')2 fragments of this antibody
have also been previously reported in detail.20 The MoAbs, TSP-1
(a TSP antibody), and PMI-1 (a GPIIb antibody), were produced
and isolated as previously reported.21 These MoAbs were purified
by the same protocol as described for OKM5, but they did not
exhibit the instability of OKM5, even in the absence of BSA.

Radiolabeling of proteins. Proteins were radiolabeled by a
modified chloramine-T procedure to specific activities of 0.5 to 1.5
µCi/µg. Residual free iodine was removed from the adhesive
proteins by dialysis against PBS. To remove the residual free iodine
from the radiolabeled antibodies, including OKM5, the samples
were gel filtered on PD-10 columns (Pharmacia), equilibrated in
and eluted with PBS. The protein concentrations of the radiolabeled
antibodies was determined from the original protein concentration of
the unlabeled protein, based on Lowry protein determinations or by
their absorbance at 280 nm, and the precipitability of the radioactive-
ity in 15% trichloracetic acid. The precipitability in 15% trichloro-
acetic acid of radiolabeled OKM5, as well as of the other radiola-
belabeled proteins, exceeded 95%, and protein recoveries were typically
greater than 90%.

Platelets. Washed human platelets were isolated from the blood
of healthy adult donors with the approval of our local Human
Investigations Committee and the Department of Health and
Human Services. In brief, 90 mL of fresh human blood, collected
into acid-citrate-dextrose, was centrifuged in two steps, first to
obtain platelet-rich plasma (PRP) and then to obtain a platelet
Counter Model Zf (Coulter Electronics, Inc, Hialeah, FL) or
microscopically in a hemacytometer. In some experiments, the
cells were fixed with paraformaldehyde before microscopic counting.
For this purpose, paraformaldehyde was added to the platelet suspension
at a final concentration of 0.5%. After 30 minutes at 22°C, an equal
volume of 0.02 mol/L NH4Cl in 0.15 mol/L Tris, pH 7.2, was
added.22-25

Ligand binding assays. 125I-radiolabeled antibodies or adhesive
proteins were incubated with washed human platelets at cell
concentrations ranging from 1 x 10^8 to 4 x 10^8/mL in Tyrode's-
BSA buffer, pH 7.4, supplemented with selected divalent ions as
previously described.18-25 Following 30 to 60 minutes at 22°C, 50-µL
aliquots of the cell suspension were layered on 20% sucrose, and
the platelets were pelleted by a 2.5-minute centrifugation in a Beckman
microfuge B (Beckman, Palo Alto, CA). The tips of the tubes were
amputated, and the molecules associated per cell calculated based on
the determined specific activities of the ligands. Binding isotherms
were analyzed with the Ligand Computer Program26 as previously
described in studies from our laboratory.22-23

Platelet aggregation. Washed human platelets at a final concentra-
tion of 1 x 10^8/mL in Tyrode's BSA buffer containing 1 mmol/L Ca2+
and Mg2+ were incubated at 37°C at a constant stirring rate of
1,000 rpm in a dual channel platelet aggregometer (Science, Inc,
Morrisen, CO). Exogenous Fg was added at 0.1 mg/mL. Aggrega-
tion was initiated by addition of the stimulus. Aggregation studies
were also performed in a 1/5 dilution of PRP with cells at 1 to 2 x
10^9/mL.

Platelet secretion. To quantitate the release of serotonin, PRP
(-20 mL) was incubated with 150 µCi of [3H]-serotonin (27.3
Ci/mmol/L; NEN Research Products, Boston, MA) for 60 minutes
at 37°C. The platelets were then isolated by gel filtration and
suspended at 1 x 10^6/mL in Tyrode's buffer containing 2 mmol/L Ca2+,
2 mmol/L imipramine, 300 mmol/L Fg, and the selected
stimulus. After 30 minutes at 22°C, conditions which paralleled the
ligand binding assays used in this study, aliquots were centrifuged
through 20% sucrose. The pellet cells were solubilized with 10 mL
of Aquasol (NEN) and counted with a Beckman LS 7500
B-counter (Beckman Instruments, Inc, Fullerton, CA). 3-thromo-
globulin secretion was measured with a commercial radioimmunoas-
say (Amersham, Arlington Heights, IL). Washed human platelets
at 2 x 10^6/mL were incubated with or without 20 mlol/L OKM5 for
5 minutes followed by the addition of another platelet stimulii to
the samples. After 5 minutes, D-phenylalanyl-L-prolyl-L-arginine chlo-
romethylketone (PPACK from Calbiochem), was added to the cells
stimulated with thrombin. Following a 10-minute incubation at
22°C, the cells were placed on ice and fixed with cold 2% paraform-
aldehyde. After 30 minutes, the paraformaldehyde was neutralized
with NH4Cl, the platelets were pelleted by centrifugation, and the
supernatants assayed for 3-thromboglobulin.

Analytical procedures. Polyacrylamide gel electrophoresis in
sodium dodecyl sulfate (SDS-PAGE) was performed in the buffer
system of Laemmli.27 When required for sample reduction, 1%
mercaptoethanol was used. Molecular weights were estimated
from the electrophoretic mobilities of the test samples relative to
those of protein standards. For quantitation of the intensity of
autoradiographic bands, a Zeineh soft laser scanning densitometer
equipped with an integrator was used. For molecular exclusion
chromatography by high performance liquid chromatography
(HPLC), a TSK 4000 column (0.75-X60 cm) was used. The column
was equilibrated in PBS at pH 6.75 and run at 0.9 mL/min. Elution
profiles were monitored at 280 nm or by collecting 1-minute
fractions and testing them for the platelet aggregating activity. For
OKM5, 45 µg of the antibody was applied to the column and the
aggregating activity of the fractions was detected by added 10-µL
samples to PRP.

RESULTS

Binding of OKM5 to platelets. To assess the direct
interaction of OKM5 with platelets, purified OKM5 was radiiodinated to a specific activity of approximately 1.5
µCi/µg. The radiolabeled antibody was characterized by
Okm5 and Platelet Function

Figure 1. Binding of Okm5 to platelets. Thrombin-stimulated (0.25 U/mL) or resting (PGE, + theophylline at 1 µg/mL and 1 mmol/L, respectively) washed human platelets were incubated with varying concentrations of Okm5. After 30 minutes at 22°C, the number of antibody molecules bound per cell was determined. The data are not corrected for nonspecific binding, which was estimated to be less than 0.1% by the Ligand Computer Program. The number of Okm5 binding sites per platelet under all experimental conditions was approximately 10,000. Platelet stimulation and/or divalent ion conditions had only a minimal influence on the number of binding sites. From the apparent kd values listed in Table 1, it is clear that Okm5 bound to both stimulated and resting platelets with high affinity, and the interaction was not affected by divalent ion availability. Thrombin stimulation increased the apparent kd of the interaction slightly. The binding of 125I-Okm5 to platelets was not inhibited by large excesses of Tspl-3 or PMI-1, two MoAbs of the same subclass as Okm5, whereas it was inhibited by nonlabeled Okm5 derived from three different lots of the antibody.

**Okm5 as a platelet stimulus.** Okm5, at 20 nmol/L, was added to platelets at 1 x 10^8/mL in the presence of 300 nmol/L Fg and 1 mmol/L Ca^2+ and Mg^2+. After a short lag phase of approximately 2 minutes, an increase in light transmittance was observed in a platelet aggregometer, consistent with an aggregation or agglutination response. A classical platelet stimulus, adenosine diphosphate (ADP), induced aggregation of the same preparation of platelets without a detectable lag phase (Fig 2). The response induced by 20 nmol/L Okm5 was completely blocked by addition of EDTA (5 mmol/L), PGE, (1 µg/mL) and theophylline (1 mmol/L), the arginyl-glycyl-aspartyl-serine tetrapeptide (0.5 mmol/L), and the ADP scavenger system of creatine phosphate (12.7 mg/mL)/creatine phosphokinase (50 µg/mL). All of these agents inhibited Fg-dependent platelet aggregation induced by ADP and low concentrations of other platelet agonists such as epinephrine and thrombin, but are known not to effect platelet agglutination such as in response to ristocetin. Okm5 also aggregated platelets in plasma in the same dose range as it was active with washed platelets. However, in plasma the inhibition by ADP scavengers could be overcome by a further increase (fivefold) in Okm5 concentration. The existence of ADP-dependent and independent pathways for platelet aggregation is also noted for many platelet agonists.

The capacity of Okm5 to induce a platelet secretory response was also examined. Serotonin and β-thromboglobulin were used to monitor dense and α granule secretion, respectively. As shown in Table 2, Okm5 induced both serotonin and β-thromboglobulin release. The extent of the secretion of the α and dense granule markers induced by

<table>
<thead>
<tr>
<th>Table 1. Okm5 Binding Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelet Stimulation</strong></td>
</tr>
<tr>
<td><strong>Divalent Ions</strong></td>
</tr>
<tr>
<td><strong>kd (nmol/L)</strong></td>
</tr>
<tr>
<td><strong>No. of Sites</strong></td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Ca^2+ + Mg^2+</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
<tr>
<td>Thrombin</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
<tr>
<td>Thrombin</td>
</tr>
<tr>
<td>EDTA</td>
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<td>Thrombin</td>
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<tr>
<td>EDTA</td>
</tr>
<tr>
<td>Thrombin</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
</tbody>
</table>

Varying quantities of 125I-Okm5 were added to platelets at 2 x 10^8/mL in the presence of Ca^2+ + Mg^2+ (2 mmol/L each) or EDTA (6 mmol/L). The cells were either stimulated with 0.25 U/mL of thrombin or maintained in PGE, and theophylline at 1 µg/mL and 1 mmol/L, respectively, to prevent stimulation. Binding was measured after 30 minutes at 22°C. The data were subjected to Scatchard plot analyses, using the Ligand Computer Program, and the results are summarized above.

SDS-PAGE. Under both reducing and nonreducing conditions, 125I-Okm5 and nonlabeled Okm5 exhibited the same electrophoretic properties. In preliminary experiments, the radiolabeled antibody was found to bind rapidly to platelets; apparent equilibrium binding was observed within 5 minutes, and the extent of binding did not change for at least 60 minutes. When varying quantities of 125I-Okm5 were added to washed human platelets, saturable binding of Okm5 to both resting and stimulated platelets was observed at approximately 10 nmol/L (Fig 1). At a saturating concentration of 125I-Okm5, a fivefold excess of nonlabeled Okm5 inhibited binding of the radiolabeled ligand by greater than 80%. Data such as those shown in Fig 1 were analyzed with the Ligand Computer Program, and binding parameters derived for nonstimulated and thrombin-stimulated platelets in the presence or absence of divalent ions are summarized in Table 1. The nonsaturable binding, N1, was extremely low; and, as it did not exceed 0.1% of total added 125I-Okm5 radioactive under all conditions, subtraction of nonspecific binding was not necessary to see clear evidence of saturation of binding (see Fig 1). For example, at a 10-nmol/L input concentration, 8,500 molecules of 125I-Okm5 were bound per platelet of which 320 molecules were nonspecifically associated. The mean number of Okm5 binding sites per platelet under all experimental conditions was 11,900 ± 1,800. Platelet stimulation and/or divalent ion conditions had only a minimal influence on the number of binding sites. From the apparent kd values listed in Table 1, it is clear that Okm5 bound to both stimulated and resting platelets with high affinity, and the interaction was not affected by divalent ion availability. Thrombin stimulation increased the apparent kd of the interaction slightly. The binding of 125I-Okm5 to platelets was not inhibited by large excesses of Tspl-3 or PMI-1, two MoAbs of the same subclass as Okm5, whereas it was inhibited by nonlabeled Okm5 derived from three different lots of the antibody.

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OKM5 was similar to that obtained with 0.25 U/mL thrombin. ADP caused only modest α and dense granule secretion; but, when added in combination with OKM5, extensive secretion was observed.

To further assess the ability of OKM5 to stimulate platelets, its capacity to induce Fg receptors was examined. Addition of 20 nmol/L OKM5 to resting platelets resulted in an increase in 125I-Fg binding to the cells (Table 3). The extent of Fg binding induced by OKM5 was similar to that supported by ADP and thrombin. PGE₁ and theophylline inhibited Fg binding induced by OKM5.

To investigate the basis for the stimulatory activity of OKM5, F(ab')₂ fragments of the antibody were prepared. Densitometric scanning of SDS-PAGE established that greater than 75% of the intact antibody was digested to a 105-Kd F(ab')₂ fragment. While 20 nmol/L intact OKM5 induced aggregation of washed platelets, a fivefold higher concentration of the F(ab')₂ fragments did not (Fig 3). Moreover, the antibody fragments blocked the capacity of the intact antibody to induce platelet aggregation. This effect was specific as the platelets retained their capacity to respond to ADP. BSA in buffer, exposed to the same protocol used to prepare the OKM5 F(ab')₂ fragments, did not block OKM5-induced platelet aggregation. When OKM5 was subjected to the protocol, except that the pepsin was omitted, the aggregating activity of the antibody was retained. The requirement for intact antibody was also shown for induction of Fg binding. In an experiment in which 5 nmol/L intact OKM5 induced the binding of 4,600 Fg molecules per platelet, 100 nmol/L F(ab')₂ fragments supported the binding of only 900 Fg molecules, very similar to the level of 700 Fg molecules bound per platelet without any added stimulus.

To determine if the stimulatory activity of intact OKM5 was a function of the monomeric antibody, OKM5 was subjected to HPLC analysis on a TSK 4000 molecular exclusion column and 1.0-minute fractions were collected.

### Table 2. Effect of OKM5 on Platelet Secretion

<table>
<thead>
<tr>
<th>Platelet Stimulation</th>
<th>Serotonin Secretion</th>
<th>β-Thromboglobulin Secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (PGE₁ + theophylline)</td>
<td>0</td>
<td>&lt;9</td>
</tr>
<tr>
<td>OKM5</td>
<td>51</td>
<td>53</td>
</tr>
<tr>
<td>Thrombin</td>
<td>57</td>
<td>50</td>
</tr>
<tr>
<td>Thrombin + OKM5</td>
<td>89</td>
<td>ND</td>
</tr>
<tr>
<td>ADP</td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td>ADP + OKM5</td>
<td>75</td>
<td>46</td>
</tr>
</tbody>
</table>

Table 2. Effect of OKM5 on Platelet Secretion

PRP was incubated with 3H-serotonin for 1 hour at 37°C and the platelets were then isolated by gel filtration. The washed cells were incubated with prostaglandin E₁ (PGE₁) and theophylline, at 1 μg/mL and 1 mmol/L, respectively, 20 nmol/L OKM5, 0.25 U/mL thrombin, 10 μmol/L ADP, or a combination of these reagents in the presence of 2 mmol/L Ca²⁺ and 2 μmol/L imipramine. After 10 minutes, the cells were centrifuged through 20% sucrose, and the cell pellet was solubilized and counted to determine the percent serotonin release. β-thromboglobulin secretion was measured by radioimmunoassay. Washed human platelets at 2 × 10⁶/mL were incubated with the same reagents indicated above. After 10 minutes, the cells were placed on ice and fixed with 2% paraformaldehyde for 30 minutes (see Materials and Methods). The cells were then pelleted by centrifugation, and the supernatants were assayed for β-thromboglobulin by radioimmunoassay. Percent secretion is calculated relative to a sample of lysed platelets, which contained 26.4 μg β-thromboglobulin/10⁶ cells.

Abbreviation: ND, not determined in this experiment. (In a separate experiment, the combination of OKM5 + thrombin produced 8% greater β-thromboglobulin release than OKM5 alone, and 27% greater release than thrombin alone).

### Table 3. Induction of Fg Receptors by OKM5 and Other Platelet Stimuli

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Fibrinogen Bound (molecules/platelet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>560</td>
</tr>
<tr>
<td>OKM5</td>
<td>17,230</td>
</tr>
<tr>
<td>ADP</td>
<td>14,230</td>
</tr>
<tr>
<td>Thrombin</td>
<td>25,800</td>
</tr>
<tr>
<td>PGE₁ / theo + OKM5</td>
<td>480</td>
</tr>
</tbody>
</table>

Table 3. Induction of Fg Receptors by OKM5 and Other Platelet Stimuli

Washed human platelets were incubated in Tyrode's-BSA buffer containing 1 mmol/L Ca²⁺ with OKM5 (20 nmol/L), ADP (10 μmol/L), thrombin (0.25 U/mL), or 20 nmol/L OKM5 + PGE₁, and theophylline (1 μg/mL and 1 mmol/L, respectively). After 5 minutes, 125I-Fg was added to a final concentration of 300 nmol/L. Following a 30-minute incubation at 22°C, the molecules of Fg bound per platelet were determined. The data are the means of triplicate determinations within a single experiment and are representative of eight similar experiments.
Platelet aggregatory activity eluted in the fractions from 19.0 to 23.0 minutes, coinciding with the elution position of a control MoAb, TSPI-1 (protein peak at 20.2 minutes). No platelet aggregation activity was detected in fractions eluting before 18.0 minutes. Fg, with a molecular weight of 340 Kd, eluted with a peak at 14.7 minutes, establishing that the 19.0- to 23.0-minute range was not the void volume of the column. Thus, the platelet stimulatory activity of OKM5 was not dependent on aggregates of the antibody.

Effects of OKM5 on the interaction of adhesive proteins with the platelet surface. In the course of experiments to assess the effects of OKM5 in combination with other platelet stimuli on 125I-Fg binding to platelets, unanticipated results were obtained. Namely, Fg binding did not occur when both OKM5 and thrombin were added to platelets. In these studies, OKM5 was preincubated with washed platelets before addition of thrombin. As shown in Fig 4, Fg binding to thrombin-stimulated platelets was suppressed by OKM5; in a dose-dependent manner; and, at concentrations as low as 5 nmol/L, the inhibition exceeded 90%. Indeed, it should be noted that the inhibitory effect of OKM5 occurred in a dose range consistent with the estimated affinity of the antibody for platelets. The estimated kd of OKM5 for thrombin-stimulated platelets was 1.7 nmol/L (Table 1) and 125I-Fg binding to thrombin-stimulated platelets was 50% inhibited by approximately 1 nmol/L OKM5 (Fig 4), showing a concordance between the binding of OKM5 to platelets and its inhibitory activity. Thus, the combination of OKM5 and thrombin abrogated the component of Fg binding induced by thrombin alone or by the antibody alone. In contrast, OKM5 concentrations as high as 22 nmol/L had no effect on ADP-induced Fg binding. With phorbol myristate acetate (PMA) as the platelet stimulus, intermediate inhibition was observed, which reached 48% at 22 nmol/L OKM5.

Under the same experimental conditions that resulted in effective inhibition of Fg binding to thrombin-stimulated platelets, OKM5 (4.5 nmol/L) also suppressed the binding of Fn by 77% and vWF by 93%. As with Fg, a partial inhibition of binding was noted for PMA-stimulated platelets; Fn and vWF binding were inhibited by 50% and 51%, respectively, under conditions where Fg binding was inhibited by 48%. In contrast, TSP binding to thrombin-stimulated platelets was not inhibited by OKM5. With 125I-TSP added at 90 nmol/L, no inhibition was observed either in the presence of 1 mmol/L Ca2+ + Mg2+ or in the presence of 1 mmol/L EDTA. At this TSP input concentration, 72% of the binding was divalent ion dependent. Thus, neither the divalent ion dependent nor independent component of TSP binding was affected by OKM5.

As inhibition of TSP binding by OKM5 is predicted from the data of Asch et al,9 the capacity of the MoAb to influence the surface expression of endogenous TSP was also evaluated. In this analysis, the surface expression of TSP on thrombin-stimulated platelets was monitored with 125I-TSPI-1,22 and 125I-Fg binding was measured in parallel. Divalent ion conditions were used to attain maximal surface expression for both ligands. Under these conditions, the stimulated cells bound 13,900 molecules of TSP-1 (the divalent ion-dependent component of the measured TSP surface expression accounted for 68% of the total TSP-1 binding22) and 29,000 molecules of Fg. As shown in Fig 5, OKM5 did not inhibit the surface expression of TSP at a concentration of 9
Fig 5. Effect of OKM5 on TSP surface expression. Conditions were used to obtain maximal TSP surface expression or Fg binding. For TSP surface expression, washed human platelets at 2 x 10^8/mL in the presence of 2 mmol/L Ca\(^{2+}\) and 2 mmol/L Mg\(^{2+}\), were incubated with varying concentrations of OKM5 in the presence of PGE\(_1\) and theophylline (1 µg/mL and 1 mmol/L, respectively) to prevent OKM5 induced secretion. After the 5-minute preincubation, thrombin was added at 0.75 U/mL followed by a saturating concentration of ^{125}I-TSPI-1 (1.1 nM). After 60 minutes, the molecules of TSPI-1 bound per cell was determined. For Fg binding, washed human cells at 1 x 10^8/mL were incubated with varying concentrations of OKM5 in the presence of 2 mmol/L Ca. After 5 minutes, the cells were stimulated with 0.25 U/mL thrombin. The molecules of Fg bound per cell were determined after 30 minutes at 22°C.

Table 4. Relationship Between the Effect of OKM5 on Fg Binding and the Extent of Platelet Aggregation

<table>
<thead>
<tr>
<th>Platelet Stimulation</th>
<th>Platelet Aggregation (single platelets/grid)</th>
<th>Fibrinogen Bound (molecules/platelet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>113</td>
<td>600</td>
</tr>
<tr>
<td>OKM5</td>
<td>44</td>
<td>7,000</td>
</tr>
<tr>
<td>Thrombin</td>
<td>61</td>
<td>16,200</td>
</tr>
<tr>
<td>OKM5 + thrombin</td>
<td>12</td>
<td>2,100</td>
</tr>
<tr>
<td>PMA</td>
<td>52</td>
<td>20,100</td>
</tr>
<tr>
<td>OKM5 + PMA</td>
<td>33</td>
<td>8,900</td>
</tr>
<tr>
<td>ADP</td>
<td>45</td>
<td>5,200</td>
</tr>
<tr>
<td>OKM5 + ADP</td>
<td>40</td>
<td>6,500</td>
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Washed human platelets were incubated for 5 minutes with 20 nmol/L OKM5 before the addition of a second stimulus (thrombin at 0.25 U/mL, PMA at 0.2 µmol/L, or ADP at 10 µmol/L) or buffer. Aliquots of the fresh cells were used to determine the extent of ^{125}I-Fg binding. The rest of the cells were fixed with paraformaldehyde and resuspended in Tyrode’s buffer. After diluting the cells 1/10 with isotonic saline, the single platelets were counted on a hemacytometer. These results were representative of at least four experiments.

Stimulated with thrombin in the presence of OKM5 exhibited a significant decrease in single platelets as compared with cells stimulated with thrombin alone. In addition to this marked decrease in free platelets, the aggregates themselves were massive (Fig 6), containing large numbers of platelets. These changes were not observed with ADP-stimulated cells, and an intermediate response was observed with PMA. In parallel with nonfixed cells, OKM5 inhibited the binding of Fg by 87% to thrombin-stimulated cells, 0% to ADP-stimulated cells, and 55% to PMA-stimulated cells. Thus, in all samples exhibiting an inhibition of ^{125}I-Fg binding by OKM5, there was also a marked increase in aggregate formation as reflected by a decrease in the number of single platelets. Other combinations of agonists did not have the

![Fig 6. Formation of massive aggregates in the presence of OKM5 + thrombin. Platelets in the left tube were unstimulated. Platelets in the middle tube were identical except for the addition of thrombin (0.25 U/mL) followed by PPACK (3 µmol/L) before the addition of Fg. In the tube on the right the cells were exposed to 20 nmol/L OKM5 before the addition of thrombin. The concentrations of cells (4 x 10^8/mL), divalent ions (Ca\(^{2+}\) 2 mmol/L), and Fg (300 nmol/L final) was the same in all tubes. The arrow indicates the massive aggregates formed when platelets were exposed to both OKM5 and thrombin.](image-url)
same effect as OKM5 and thrombin. Stimulating the cells with thrombin and PMA resulted in 44 free cells per grid and 18,500 molecules of Fg bound per cell. If OKM5 was also added with the thrombin and PMA, the number of free cells per grid was reduced to 7 and the molecules of Fg bound per cell was reduced to 1,700.

Because platelet aggregation should be less extensive at low cell concentrations, the ability of OKM5 to inhibit thrombin-induced Fg binding was assessed as a function of cell concentration. The results of these analyses are summarized in Table 5. The molecules of Fg bound per thrombin-stimulated platelet in the absence of OKM5 remained relatively constant, varying from 27,400 to 32,600 over the range of cell concentrations tested in one experiment, and from 14,200 to 15,200 in the second experiment. However, the inhibition of Fg binding induced by OKM5 varied considerably with cell concentration. The inhibition was only 14% at the lowest platelet concentration tested in contrast to 83% at the highest concentration tested.

Additional evidence for the role of platelet aggregation in the inhibitory effects of OKM5 was obtained in experiments altering the timed additions of thrombin, OKM5, and 125I-Fg to the platelets. Maximal inhibition of Fg binding by OKM5 required that the cells be preincubated with the antibody before the addition of the adhesive protein. A saturating concentration of OKM5 (20 nmol/L) inhibited Fg binding by 92% (19,500 molecules/platelet to 1,500 molecules/platelet) when the antibody was preincubated with the cells for 5 minutes before thrombin stimulation. Similar inhibition was observed when OKM5 and thrombin were added simultaneously to the cells but still before Fg. In contrast, when the same concentration of OKM5 was added after thrombin and simultaneously with the 125I-Fg, only 42% inhibition of Fg binding was observed. These changes in inhibition were paralleled by changes in platelet aggregation in the samples. When incubated for 30 minutes in the absence of either thrombin or OKM5, most of the platelets (85%) remained as single, nonaggregated cells. OKM5 alone or thrombin alone caused some platelet clumping during the 30-minute incubation, but single cells still predominated in the suspension and the size of the platelet aggregates was small, usually containing approximately two to five platelets. When the platelets were treated first with OKM5 and then with thrombin, the condition that resulted in greater than 90% inhibition of Fg binding, most of the platelets were aggregated, and the aggregates were massive. In contrast, when the platelets were treated first with thrombin followed by the simultaneous addition of OKM5 and Fg, the condition at which the inhibition of Fg binding was less extensive, platelet aggregation was also considerably less extensive.

The effect on Fg binding of F(ab')2 fragments of OKM5, which, as shown above, do not induce platelet aggregation, was tested. With thrombin-stimulated (0.1 U/mL) platelets, 32,900 ± 1,000 Fg molecules were bound per platelet at an input concentration of 300 nmol/L Fg. OKM5, at a 20 nmol/L concentration reduced Fg binding to 4,700 ± 100, an 86% inhibition. In contrast, the F(ab')2 fragments, at a concentration as high as 100 nmol/L, did not inhibit Fg binding (36,700 ± 1,050 Fg molecules/platelet).

### DISCUSSION

The OKM5 antigen is expressed by a variety of cells16-18, and, on platelets, the antibody reacts with the membrane protein designated GPIV. Our analysis of the interaction of OKM5 with platelets provides a quantitative estimate of the density of GPIV. The number of OKM5 epitopes per platelet was estimated to be approximately 12,000. Assuming one epitope per GPIV, this membrane protein is present at 1/4 the abundance of GPIIb-IIIa and GPIb.

OKM5 can act as platelet stimulus. The antibody induced platelet aggregation, secretion, and expression of Fg binding sites, causing a response pattern similar to that induced by thrombin. That these effects were due directly to the antibody, and not a contaminating component within the OKM5 preparations, is shown by: (1) the low concentrations of OKM5 required to induce these effects; (2) the failure of other MoAbs, isolated by an identical protocol, to induce these effects; (3) the failure of protease inhibitors, including PPACK and p-amidinophenylmethyl-sulfonyl fluoride, to abrogate these effects; and (4) the capacity of F(ab')2 fragments of OKM5 to selectively block the stimulatory activity of intact OKM5. The later results show that the Fc region of antibody is required for induction of platelet responses. As many antibodies to platelet antigens, including those of the same subclass as OKM5, such as PM-1, do not stimulate platelets, engagement of the target antigen by the antibody combining site, as well as secondary Fc-dependent interactions, are required for platelet stimulation.

When thrombin-stimulated platelets were preincubated with OKM5, the capacity of these cells to bind 125I-Fg was markedly decreased. Thus, the combination of these two platelet agonists suppressed Fg binding induced by either agonist individually. The effect was observed at OKM5 concentrations consistent with the measured affinity of the antibody for the platelet. This effect was not due to neutralization of the thrombin as shown by the observations that: (1) thrombin retained its capacity to induce platelet secretion in the presence of OKM5 (Table 2); (2) thrombin activity measured with the synthetic substrate S2238 (Ortho Diagnostics) was not affected by OKM5 (data not shown); and (3) Fg binding induced by PMA was also inhibited by OKM5,

### Table 5. Effect of Platelet Concentration on the Inhibition of Fg Binding by OKM5

<table>
<thead>
<tr>
<th>Platelet Concentration (platelets/mL)</th>
<th>Fibrinogen Bound (% inhibition)</th>
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<tbody>
<tr>
<td>1 x 10⁷/mL</td>
<td>14.5</td>
</tr>
<tr>
<td>5 x 10⁷/mL</td>
<td>62</td>
</tr>
<tr>
<td>1 x 10⁸/mL</td>
<td>78</td>
</tr>
<tr>
<td>2 x 10⁹/mL</td>
<td>83</td>
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Washed human platelets, at varying concentrations, were incubated with or without 20 nmol/L OKM5 for 5 minutes before thrombin stimulation (0.25 U/mL). After a 5-minute incubation, the thrombin was inactivated with PPACK and 125I-Fg added (300 nmol/L). The molecules of Fg bound per cell were determined after 30 minutes at 22°C. Percent inhibition was calculated by dividing the Fg molecules bound in the presence of OKM5 by the number bound in the absence of the MoAb and is the mean of two experiments.
although less extensively than with thrombin. OKM5 was not a general inhibitor of platelet stimulation as Fg binding to ADP-stimulated platelets was unaffected and the antibody itself could induce Fg binding in the absence of other stimuli. Moreover, these effects were not restricted to Fg binding, as vWF and Fn binding to thrombin or PMA-stimulated platelets were also affected. By direct examination, it was found that conditions under which OKM5 inhibited Fg binding to platelets also resulted in extensive platelet aggregate formation. Furthermore, when aggregation was less extensive, at lower platelet counts or by altering the order of thrombin and OKM5 addition, OKM5 produced minimal inhibition of Fg binding. Taken together, these observations suggest a close proximal relationship between the inhibition of Fg binding by OKM5 and its induction of aggregates within the platelet suspension. However, cell-cell contact cannot account entirely for the inhibition of Fg binding by OKM5. Platelet suspensions stimulated with thrombin also showed a reduced number of free platelets, but the extent of Fg binding to thrombin-stimulated platelets was independent of cell concentration. One difference in the platelets stimulated with thrombin alone or OKM5 alone and those treated with OKM5 + thrombin may reside in the massive size of the platelet aggregates formed. The massive aggregates may prevent access of Fg to its receptors or unoccupied Fg receptors may be downregulated on aggregated platelets.

The present study has shown that an MoAb reactive with a target antigen other than GPIIb-IIIa can inhibit the binding of adhesive proteins to platelets. We have previously demonstrated that MoAbs to GPIIb-IIIa can inhibit the binding of an adhesive protein to a receptor independent of GPIIb-IIIa; ie, binding of TSP to platelets does not appear to be mediated by GPIIb-IIIa. Nevertheless, certain MoAbs to GPIIb-IIIa inhibit TSP binding to platelets. These observations suggest a close proximal relationship between the TSP receptor and GPIIb-IIIa, as suggested by Leung, and further emphasize the need for considerable caution in interpreting antibodies inhibition of receptor-ligand interactions. Finally, the differences in the data presented in this study with those presented by Asch et al require comment. In contrast to our results, these investigators found that OKM5 inhibited surface expression of TSP and concluded that the OKM5 antigen was the TSP receptor on platelets. We have previously found that TSP binds to platelets through two distinct mechanisms, a divalent ion-dependent and a divalent ion-independent pathway. The OKM5 antibody did not appear to interfere with either of these two pathways, as measured by binding of added TSP or surface expression of endogenous TSP. Other pathways for TSP surface expression may exist. TSP interacts with Fg with high affinity. Based on our data, such a pathway could be inhibited by OKM5 if the antibody induced extensive platelet aggregation. Finally, while our data raise questions regarding the mechanism by which OKM5 might inhibit TSP surface expression on platelets, they do not exclude a role of GPIV as a TSP receptor. A potential role of GPIV in TSP binding to platelets has recently been supported, although TSP receptors unrelated to GPIV have now been identified on other cell types.

ACKNOWLEDGMENT

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REFERENCES

Effects of OKM5, a monoclonal antibody to glycoprotein IV, on platelet aggregation and thrombospondin surface expression [see comments]

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