Membrane Glycoproteins and Platelet Cytoskeleton in Immune Complex-Induced Platelet Activation

By Joonghee Kang, Claudia Cabral, Leslie Kushner, and Edwin W. Salzman

To clarify the mechanism of platelet activation by immune complexes and the possible involvement of surface glycoproteins (GP), we studied platelet activation induced by heat-aggregated IgG (HAG). We examined the effects of monoclonal antibodies (MoAbs) against GPIb, GPIb/IIIa, and the Fc receptor on resting platelets and on platelets stimulated by HAG. HAG increased the cytosolic ionized calcium concentration ([Ca\(^{2+}\)]\(_i\)) and stimulated protein (P47 and P20) phosphorylation, phosphatidic acid (PA) synthesis, serotonin secretion, and platelet aggregation. IV.3, an anti-Fc\(\gamma\)RII receptor MoAb, inhibited HAG binding to platelets and all subsequent platelet responses. Like IV.3, MoAbs against GPIb/IIIa (Tab, 10E5, AP-3) or GPIb (AP-1, 6D1) strongly inhibited platelet activation by HAG. However, while anti-GPIb/IIIa MoAbs inhibited binding of IV.3 and HAG to platelets, anti-GPIb MoAbs had little effect on platelet binding of IV.3 or HAG. These observations suggest a close topographical and functional association of GPIb/IIIa with Fc\(\gamma\)RII in the platelet response to HAG. Cytochalasin B, an inhibitor of actin polymerization, also inhibited platelet activation but not HAG or IV.3 binding. Measurement of the fluorescence of 7-nitrobenz-2-oxa-1,3-(NBD)-phallacidin, a specific marker for filamentous actin (F-actin), showed that both cytochalasin B and AP-1 blocked the increase of F-actin induced by HAG. The common effects of anti-GPIb MoAbs and of cytochalasin B suggest that unlike the activity of GPIb/IIIa, the ability of anti-GPIb to inhibit the activation of platelets by immune complexes is associated with perturbation of the cytoskeleton.

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Submitted February 10, 1992; accepted November 6, 1992.

Supported by National Heart, Lung, and Blood Institute Grant Nos. HL-37610 and HL-33014.

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Blood, Vol 81, No 6 (March 15), 1993: pp 1505-1512

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Flow Cytometric Studies

Measurement of HAG binding to platelets. HAG binding to platelets was determined as described by Rosenfeld et al., with some modifications. Platelets were suspended in calcium-free HEPES-Tyrode buffer containing 2 U/mL aprotinin and 0.5% bovine serum albumin, and incubated with 500 μg/mL HAG for 25 minutes at 37°C. After washing, fluorescein isothiocyanate (FITC)-conjugated (Fab′) rabbit anti-human-IgG was added (50 μg/mL final concentration). Platelets were examined by FacStar-plus flow cytometry (Becton Dickinson, Braintree, MA). FITC was excited with a 5 W argon laser at 200 mW power at a wavelength of 488 nm, and fluorescence was detected using a 530/30 nm band pass filter. In experiments using cytochalasin B, it was pre-incubated for 15 minutes. MoAbs, non-specific mouse IgG, and the synthetic peptide Arg-Gly-Asp-Ser (RGDS) were added 5 minutes before the addition of HAG.

Measurement of NBD-phallacidin binding to platelets. 7-Nitrobenz-2-oxa-1,3 (NBD)-phallacidin was purchased from Molecular Probes (Eugene, OR). Two minutes before HAG addition, the peptide RGDS (200 μmol/L final concentration) was added to washed platelet samples to prevent aggregation. After 2 minutes of incubation with HAG, the reaction was terminated by addition of 2.5% glutaraldehyde in 100 mmol/L phosphate-buffered saline (pH 7.4), containing 10 mmol/L EGTA. Fixed platelets were incubated with NBD-phallacidin 3.3 μmol/L. Fluorescence of NBD-phallacidin was detected in the flow cytometer as with FITC. Fluorescence intensity of NBD was calibrated with fluorescence-standard beads. Tab or AP-1 was added 5 minutes before and cytochalasin B was added 15 minutes before the addition of RGDS.

Protein (P47 and P20) Phosphorylation and Accumulation of PA

Platelets were resuspended in phosphate and calcium-free HEPES-Tyrode buffer and incubated with [32P] orthophosphate (50 μCi/mL) for 45 minutes at 22°C. Free [32P] orthophosphate was removed by gel filtration. After addition of HAG, a half volume of concentrated Laemmli buffer (187.5 mmol/L Tris-HCl, 9% sodium dodecyl sulfate (SDS), 21% Glycerol, pH 6.8) was added to terminate the reaction at each time point, and the samples were run in 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The phosphorylated 47-Kd protein (P47) and 20-Kd protein (myosin light chain) were de-detected by autoradiography on Kodak X-Omat AR film (Eastman Kodak, Rochester, NY). Radioactivity of each band was measured by scintillation spectrometry. Accumulation of phosphatidic acid (PA) was measured by thin-layer chromatography (TLC). After addition of HAG to [32P]-loaded platelets, 200-μL aliquots were removed and mixed with 750 μL of the following solution to stop the reactions: (chloroform/methanol/2N HCl = 2/1/0.5, vol/vol/vol). The lower chloroform fraction was applied to SILICA GEL 60 (Merck Co, Darmstadt, Germany) and developed using a chloroform/acetone/methanol/acetic acid/distilled water (30/15/13/12/7, vol/vol/vol/vol/vol) solvent system.

Materials

Carrier-free [35S] (Nal) and [32P] orthophosphate were obtained from New England Nuclear (Boston, MA). [3H]-5-HT was purchased from Amersham Co (Arlington Heights, IL). Control mouse IgG, cytochalasin B, and fluorescein isothiocyanate (FITC) were from Sigma. PGF2α was purchased from Biomol (Plymouth Meeting, PA). (Fab′) rabbit anti-human IgG was purchased from Accurate Chemical (Westbury, NY). The other materials were purchased from various sources and were highest reagent grades.

RESULTS

A typical experiment in which platelet activation is induced by HAG is shown in Fig 1. Addition of HAG (final concentration, 500 μg/mL) to aequorin-loaded platelets resulted in a detectable [Ca2+]i increase preceded by a short lag phase. Elevation of [Ca2+]i was accompanied by serotonin secretion and platelet aggregation. Stimulation of [32P]-loaded platelets with HAG (a. control: HAG 500 μg/mL). HAG was added at the time indicated by the small arrow. The numbers in left parentheses show the peak [Ca2+]i level, and the numbers in right parentheses show the [32P]-5-hydroxytryptamine ([3H]-5-HT) release. Secretion of [3H]-5-HT at 3 minutes is expressed as a percentage of total platelet [3H]-5-HT content. Other tracings show the inhibitory effect of aspirin (b. 1 mmol/L), cytochalasin B (c. 20 μmol/L), or IV.3 MoAb (d. 5 μg/mL). IV.3 MoAb was pre-incubated for 5 minutes, and aspirin and cytochalasin B were pre-incubated for 15 minutes before the addition of HAG.
Immune complex-induced platelet aggregation was studied in response to HAG (1), and the effect of IV.3 MoAb, aspirin, or cytochalasin B. Panel (A) shows the extent of phosphorylation of P47, a 47-Kd peptide, and panel (B) shows that of P20, a 20-Kd peptide, in response to HAG (500 μg/mL). The radioactivity of each band is expressed as a percentage of that of resting platelets. Panel (C) shows PA synthesis in response to HAG. The radioactivity of PA spots is presented as a percentage of that of resting platelets. The inhibitory effects of IV.3 MoAb (5 pg/mL), aspirin (1 mmol/L), and cytochalasin B (20 pmol/L) are also shown. The experiments shown are representative of three similar determinations.

It seemed possible that inhibition of HAG-induced platelet activation by anti-GPIIb/IIIa MoAbs might result from their blocking the interaction of the Fc receptor, FcγRII, with HAG in a fashion analogous to the action of IV.3. The effect of anti-GPIIb/IIIa MoAbs on platelet binding of IV.3 MoAb was examined (Fig 6) (control 1,146 ± 215 molecules/platelet at 10 minutes). Tab, 10E5, and AP-3 (but not nonspecific IgG) significantly reduced 125I-labeled IV.3 binding to platelets.

The effect of anti-GPIIb/IIIa MoAbs on binding of HAG to platelets was also examined (Table 1). Tab and 10E5 in the form of whole IgG molecules strongly inhibited HAG binding to platelets in a fashion similar to their effect on platelet binding of IV.3. However, Fab2 fragments of these MoAbs did not inhibit HAG binding to platelets (Table 1). This might suggest that the effect of anti-GPIIb/IIIa antibodies on HAG binding was caused by nonspecific occupation of the Fc receptor, but evidence to be detailed below argues against this relation.

The role of GPIb in immune complex-induced platelet activation was also examined. We studied two anti-GPIb MoAbs, AP-1 and 6D1, both of which inhibit ristocetin-induced binding of von Willebrand factor to platelets.6,7 We...
Fig 3. HAG binding to platelets studied by flow cytometry; inhibitory effect of IV.3, Tab or 6D1. (A) A typical binding pattern of HAG to platelets is shown. Log fluorescein isothiocyanate (FITC)-fluorescence was converted to linear channel number (0 through 255) (FL 1). Gel-filtered platelets were pre-incubated with HAG (500 pg/mL) for 25 minutes at 37°C (a., positive control). The negative control sample was not exposed to HAG (b.). It is composed of nonspecific IgG binding and platelet auto-fluorescence. In (c.) IV.3 (5 pg/mL) was added 5 minutes before HAG. (B) Typical binding pattern of HAG to platelets in the presence of IV.3 (a.), Tab (b.), or 6D1 (c.) MoAb. Each MoAb (10 pg/mL) was added 5 minutes before HAG.

found that they also inhibited certain aspects of platelet activation induced by HAG. Figure 4 illustrates the inhibitory effects of AP-1 on HAG-induced [Ca²⁺]i increase, serotonin release, [¹²⁵]I-IV.3 binding, or HAG binding to platelets, although it blocked platelet aggregation. These results suggest that neither access to the fibrinogen binding site of GPIIb/IIIa nor fibrinogen binding to GPIIb/IIIa is required for the initiation of HAG-induced platelet activation.

To inquire further into the mechanism by which anti-GPIb MoAbs inhibit HAG-induced platelet activation, we studied the effect of AP-1 on the platelet cytoskeleton, as represented by transformation of globular (G)- to filamentous (F)-actin, a regular feature of platelet activation by HAG that is inhibited by cytochalasin B (as discussed above). F-actin production was measured by flow cytometry using a fluorescent probe, NBD-phallacidin (Table 2). Increased F-actin formation by platelets stimulated by HAG was completely inhibited by cytochalasin B 20 μmol/L as Fox and Phillips previously observed using a DNAse I inhibition assay. Tab inhibited both F-actin formation and HAG binding to platelets, while AP-1 inhibited F-actin formation but not HAG binding. These observations suggested that inhibition of HAG-induced platelet activation by an antibody-recognizing GPIb was more likely to be the result of an association of GPIb with the cytoskeleton than of an effect on binding of HAG to platelet receptors.

**DISCUSSION**

Horsewood et al recently suggested that activation of platelets by certain MoAbs can result from cross-linking of mobile Fc receptors in clusters on the same or contiguous platelets. Platelet activation by many anti-platelet antibodies has been attributed to interaction of their Fc portion with the Fc receptor (FcγRII). However, it is very difficult to exclude an effect of the variable (Fab) portion of the IgG molecule. We investigated HAG-induced platelet activation as a model for FcγRII-mediated platelet activation, because HAG has been thought to bind to platelets exclusively via FcγRII. Cross-linking of FcγRII by HAG induces a [Ca²⁺]i increase, serotonin release, [¹²⁵]I-IV.3 binding, or HAG binding to platelets. These results suggest that neither access to the fibrinogen binding site of GPIIb/IIIa nor fibrinogen binding to GPIIb/IIIa is required for the initiation of HAG-induced platelet activation.

Table 1. HAG Binding to Platelets (flow cytometry)

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of Experiments</th>
<th>FITC-Fluorescence Intensity (% control) Mean (mode)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td></td>
<td>100 (100)</td>
</tr>
<tr>
<td>Pretreated with nonspecific IgG (10 μg/mL)</td>
<td>2</td>
<td>97 (103)</td>
</tr>
<tr>
<td>IV.3 (IgG) (5 μg/mL)</td>
<td>3</td>
<td>74 (70)</td>
</tr>
<tr>
<td>Tab (IgG) (10 μg/mL)</td>
<td>3</td>
<td>71 (77)</td>
</tr>
<tr>
<td>Tab (Fab) (10 μg/mL)</td>
<td>2</td>
<td>99 (105)</td>
</tr>
<tr>
<td>10E5 (IgG) (10 μg/mL)</td>
<td>2</td>
<td>81 (80)</td>
</tr>
<tr>
<td>10E5 (Fab) (10 μg/mL)</td>
<td>2</td>
<td>108 (115)</td>
</tr>
<tr>
<td>AP-1 (IgG) (10 μg/mL)</td>
<td>3</td>
<td>101 (107)</td>
</tr>
<tr>
<td>6D1 (IgG) (10 μg/mL)</td>
<td>3</td>
<td>100 (101)</td>
</tr>
<tr>
<td>RGDS (200 μmol/L)</td>
<td>3</td>
<td>95 (94)</td>
</tr>
<tr>
<td>Cytochalasin B (20 μmol/L)</td>
<td>3</td>
<td>95 (94)</td>
</tr>
</tbody>
</table>

* Control platelets were pre-incubated with 500 μg/mL HAG for 25 minutes at 37°C before addition of FITC-conjugated (Fab) rabbit anti-human IgG. Other platelets were pre-incubated with each material before addition of HAG. FITC-intensity of each sample is expressed as a percentage of mean or modal fluorescence of control platelets which are 100% by definition. The RGDS peptide, which inhibits binding of fibrinogen to the activated form of GPIIb/IIIa, had no effect on the
IMMUNE COMPLEX-INDUCED PLATELET AGGREGATION

Fig 4. Aggregation (top tracings) and aequorin-indicated \([\text{Ca}^{2+}]_i\) increase (bottom tracings) in response to HAG in the presence of anti-GPllb/llla MoAb (Tab and 10E5), anti-GPib MoAb (AP-1) or RGDS peptide. Tracing (a.) shows the aggregation pattern (top) and aequorin-indicated \([\text{Ca}^{2+}]_i\) increase (bottom) in response to HAG (500 \(\mu\text{g/mL}\), control). Other tracings show HAG-induced \([\text{Ca}^{2+}]_i\) increase and aggregation in the presence of nonspecific mouse IgG (b.), anti-GPllb/llla MoAb (Tab [d.] and 10E5 [e.]), anti-GPib MoAb (AP-1 [f.]), or RGDS peptide (c.). Peak \([\text{Ca}^{2+}]_i\) levels are presented in left parentheses. Percentage secretion of 5-HT is in right parentheses. Each MoAb (10 \(\mu\text{g/mL}\)), nonspecific mouse IgG (10 \(\mu\text{g/mL}\)), and RGDS peptide (200 \(\mu\text{mol/L}\)) was pre-incubated for 5 minutes at 37°C before the addition of HAG. These experimental results are representative of five similar determinations.

[\text{Ca}^{2+}]_i increase, enzymatic activation (eg, phospholipase C, protein kinase C, and myosin light chain kinase) and granule secretion, indicating that FcyRII is linked to a complex intracellular signaling mechanism. Aspirin, an inhibitor of cyclooxygenase, strongly inhibits HAG-induced \([\text{Ca}^{2+}]_i\) increase, protein phosphorylation (P47 and P20), serotonin release, and platelet aggregation. Thus, FcyRII-mediated platelet activation is largely caused by endogenous generation of thromboxane A2, but it is noteworthy that inhibition of thromboxane A2 formation by aspirin did not completely block all reactions of HAG, sparing \([\text{Ca}^{2+}]_i\) increase and PA synthesis. These data are consistent with Anderson and Anderson, who cross-linked FcyRII with IV.3 MoAb and (Fab') goat anti-mouse IgG, and showed that cross-linking

Fig 5. Effects of HAG (○) on protein phosphorylation (P47 and P20) in the presence of Tab (□), AP-1 (■), or RGDS peptide (○). Panel (A) shows P47 phosphorylation in response to HAG (500 \(\mu\text{g/mL}\)) and panel (B) shows phosphorylation of P20. HAG was added to \([^{32}\text{P}]\)-loaded platelets at time zero. Phosphorylation is expressed as a percentage of labeled \([^{32}\text{P}]\) incorporated into P47 or P20 bands in activated platelets, compared with radioactivity of equivalent bands in resting platelets. Each MoAb (10 \(\mu\text{g/mL}\)) or RGDS (200 \(\mu\text{mol/L}\)) was pre-incubated for 5 minutes at 37°C before the addition of HAG. The experiments shown are representative of three similar determinations.
of platelet FcγRII results in activation of phospholipase C. Our data are similar and indicate that cross-linking of immune-complexes (HAG) with FcγRII also initiates activation of phospholipase C (production of PA) and, thus, that there exists at least one activation mechanism of phospholipase C in addition to endogenous thromboxane generation.

The possible association of this process with the cytoskeleton is compatible with our observation that cytochalasin B, an inhibitor of actin polymerization, strongly inhibited accumulation of PA and the [Ca2+]i increase without impairing HAG binding to platelets. Cytochalasin B does not reduce enzymatic activity (phospholipase C, phospholipase A2, or cyclooxygenase) induced during platelet activation by conventional soluble agonists, such as ADP, U46619 (a PGG2/PGH2 analog), or arachidonic acid. Thus, mere FcγRII occupancy is not sufficient to initiate activation of platelets, including activity of phospholipase C. A concomitant process blocked by cytochalasin B, such as reorganization of the cytoskeleton (eg, polymerization of actin) is also required. Monomeric IgG does not trigger platelet activation.

We investigated the relation of the major membrane glycoproteins, GPIIb/IIIa and GPIb, with FcγRII. Three anti-GPIIb/IIIa MoAbs (Tab, 10E5, and AP-3) strongly inhibited binding of IV.3 MoAb and of HAG to platelets and reduced the subsequent platelet responses. If this was a steric effect, as seems likely, it suggests that the GPIIb/IIIa and FcγRII may be located in close proximity on the platelet surface. These data are consistent with previous suggestions of the propinquity and/or functional association of GPIIb/IIIa with FcγRII. The great excess of GPIIb/IIIa complexes (40,000/platelet) over FcγRII sites (1 to 2,000/platelet) would not be incompatible with such a scheme. In contrast, the synthetic peptide RGDS did not inhibit binding of IV.3 or HAG to platelets, or other HAG-induced platelet responses, except platelet aggregation. The latter effect presumably resulted from interference with fibrinogen binding to GPIb/IIIa, suggesting that FcγRII binding of HAG occurs independent of GPIIb/IIIa-fibrinogen interaction.

Rubinstein et al. recently described the likelihood of the propinquity of GPIIb/IIIa and FcγRII. They hypothesized that GPIIb/IIIa and FcγRII formed a multimolecular complex with GPIb, on the basis of observations that anti-GPIb MoAb inhibited anti-p24/CD9 MoAb-induced platelet activation. However, our data suggest that the role of GPIb in immune complex-induced platelet activation is more complicated. Anti-GPIb MoAbs (AP-1 and 6D1) strongly inhibited HAG-induced platelet activation, but not binding of IV.3 or HAG to platelets. An association of GPIb with FcγRII-mediated platelet activation was previously suggested: ristocetin-dependent agglutination of human platelets was in-

| Table 2. F-Actin Content (NBD-phallacidin binding) of Platelets |
|-----------------|-----------------|-----------------|-----------------|
|                | Resting         | Post-HAG        |
| Control (HAG)  | 100 (%)         | 160 ± 12 (%)    |
| Pretreated with Tab (10 μg/mL) | 109 ± 13 | 110 ± 18 |
| Cytochalasin B (20 μmol/L) | 94 ± 2 | 85 ± 13 |
| AP-1 (10 μg/mL) | 109 ± 12 | 114 ± 8 |

After 2 minutes incubation with HAG, reaction was terminated by the addition of 2.5% glutaraldehyde in phosphate-buffered saline (25 mmol/L sodium phosphate, 130 mmol/L NaCl, pH 7.4) at 37°C. NBD-phallacidin binding to resting platelets (control) was designated as 100%. All values are expressed as a percentage of that of control resting platelets and show the mean ± SD (n = 3). Only the difference in the control sample after HAG is statistically significantly different from the resting value.

Fig 6. 125I-IV.3 binding to platelets; effect of anti-GPIIb/IIIa MoAb, anti-GPIb MoAb, or RGDS peptide. Panel (A) shows the 125I-IV.3 binding to platelets in the absence or presence of anti-GPIIb/IIIa MoAb (Tab, 10E5, or AP-3). Panel (B) shows the effect of anti-GPIb MoAb (AP-1), RGDS peptide, or nonspecific IgG. Each MoAb (10 μg/mL), nonspecific IgG (10 μg/mL), and RGDS (200 μmol/L) was pre-incubated for 5 minutes before the addition of 125I-IV.3 (2 μg/mL). The radioactivity of a control sample at 10 minutes was designated as 100%, and other values were expressed as a percentage of the control 10-minute value. Each point represents mean ± SEM of at least three separate experiments. Statistical analysis was by Student's unpaired t-test, comparing each value to that of a sample pre-incubated with nonspecific IgG (*not significant; **p < .05, ***p < .01).
inhibited by aggregated IgG,\(^4\) and anti-GPIb MoAb inhibited anti-CD9 MoAb-induced platelet activation.\(^6\) We confirmed that anti-GPIb MoAbs inhibited platelet activation induced by anti-CD9 MoAb (ALB6 or PMMA2)\(^{39,44}\) without impairing binding of anti-CD9 MoAb (data not shown). That GPIb may function as an Fc receptor on platelets is a plausible explanation, but recent study showed this to be unlikely.\(^{38,40}\)

We recently reported that cleavage of GPIb by a metalloprotease from \textit{Serratia marcescens} did not diminish platelet activation induced by HAG,\(^5\) an observation that further de-emphasized a role for GPIb as Fc receptor. Anti-GPIb MoAb appeared to interfere with early events in HAG-induced platelet activation, including the \([\text{Ca}^{2+}]_i\) increase, PA accumulation, phosphorylation of P47, and the release reaction. The involved pathway may be related to the cytoskeleton. We found that platelet stimulation by HAG increased F-actin formation by as much as 60%. Both AP-1 and cytochalasin B strongly inhibited F-actin formation. These observations support the suggestion that GPIb participates in reorganization of the cytoskeleton during HAG-induced platelet activation. Recently, Fox and Berndt\(^31\) pointed out that phosphorylation of GPIb, a subunit of GPIb, by cyclic AMP-dependent A-kinase might control actin polymerization. However, because they studied platelets from a patient with Bernard-Soulier syndrome, it is not certain to what extent their data are applicable to normal platelets. The cytoskeleton is linked to a cytoplasmic domain of GPIbX complex via actin-binding protein,\(^23,55\) which is a possible participant in the maintenance of platelet morphology and in transmembrane signal transduction. If cross-linking of FcRII and subsequent cytoskeletal reorganization initiate platelet activation, anti-GPIb MoAbs and cytochalasin B may affect F-actin formation and alter the activity of phospholipases through changes in their microenvironment, rather than by competing with complicated agonists such as HAG for platelet binding sites, including the Fc receptors. Failure of antibodies against GPIb (AP-1, 6D1) or of nonimmune IgG to interfere with HAG or IV.3 binding to platelets would be compatible with such a scheme. Nakano et al\(^38\) reported that cytochalasin B strongly inhibited collagen-induced platelet activation; they interpreted this effect as evidence for the importance of the cytoskeleton in these reactions. The anti-GPIb antibodies AP-1 and 6D1 also inhibited collagen-induced platelet activation without blocking platelet binding of collagen.\(^{50,58}\) Both collagen and HAG apparently interact with platelets through multiple sites of attachment; the simultaneous association of these large molecules with multiple platelet receptors, including Fc and GPIb, linked to the cytoskeleton and coupled with activation of platelet phospholipases, may provide a general explanation for these observations.

ACKNOWLEDGMENT

We thank Drs Barry S. Coller, Thomas J. Kunicki, Rodger P. McEver, Peter J. Newman, Claude Boucheix, and Takaaki Hato for their kind donation of antibodies.

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