Regulation of Autoimmune Anti-Platelet Antibody-Mediated Adhesion of Monocytes to Platelet GPIIb/GPIIa: Effect of Armed Monocytes and the Mac-1 Receptor

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Platelet autoantigen-autoantibody-monocyte interaction was studied by utilization of a specific monoclonal antibody (MoAb) 10E5 to trap and immobilize the GPIIb-GPIIa complex on microtiter plates. Peripheral blood mononuclear cells (PBMC) or purified monocytes formed distinct morphologic clusters after incubation with immobilized antigen for 18 hours at 37°C. PBMC of 18 of 19 patients with autoimmune thrombocytopenic purpura (ATP) formed 48 ± 6.8 (SEM) clusters/well compared with 7.4 ± 1.0 for control subjects, P < .001. The number of clusters per well correlated inversely and exponentially with platelet count, r = −.8, n = 21, indicating that the GPIIb-GPIIa autoantigen is pathophysiologically relevant. Binding of ATP PBMC to immobilized GPIIb-GPIIa could be inhibited by F(ab)2 fragments of immunoglobulin (Ig) G of ATP patients, indicating that monocyte IgG bound to autoantigen by its F(ab)2 domain. Optimal cluster formation could be obtained with normal monocytes if preincubated with ATP IgG but not with F(ab)' fragments of ATP IgG, indicating that ATP IgG binds to monocytes by its Fc domain. Armed monocytes (ie, normal monocytes preincubated with ATP IgG) bound to immobilized autoantigen 5.8-fold greater than normal monocytes incubated with immobilized autoantigen opsonized with ATP IgG. Armed monocyte adhesion could be inhibited 81% from 18.9 ± 1.6 to 3.6 ± 0.5 clusters/well by prior fixation with 0.1% formalin, whereas fixation of IgG before arming of monocytes was not inhibitory. MoAb MM41, directed against the αIIb chain of the Mac-1 adhesive protein receptor of monocytes, inhibited cluster formation by 79%. Thus, (1) armed monocyte interaction with autoantigen is considerably more effective than monocyte interaction with opsonized autoantigen; (2) armed monocyte interaction requires specific F(ab)2-antigen recognition; and (3) monocyte-autoantigen interaction requires a secondary nonimmunologic adhesive event.

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MATERIALS AND METHODS

Patient population. Sixteen classic autoimmune thrombocytopenic purpura (ATP) patients, consisting of 11 women and 5 men, were studied. All 16 had a history of thrombocytopenia, increased megakaryocytes in the bone marrow, negative anti-nuclear antibodies, and absent secondary clinical disorders associated with thrombocytopenia. All responded to treatment with either prednisone, danazol, intravenous gamma globulin, or splenectomy. Platelet counts at the time of study ranged between 22,000 and 309,000/μL. Healthy control subjects with normal platelet counts consisted of 4 women and 4 men, who were age- and sex-matched for the patients studied. A second control group consisted of 8 patients with nonimmune thrombocytopenia (2 with chemotherapy-induced marrow aplasia, 3 with disseminated intravascular coagulation, and 3 with sepsis) and platelet counts of 21,000 to 111,000/μL. A third control group consisted of six patients with inflammatory and infectious disorders with normal platelet counts (pneumonia, rheumatoid arthritis, cellulitis with diabetes mellitus, malignant melanoma, subacute bacterial endocarditis, and trauma).

Preparation of mononuclear adherent cells. Peripheral blood mononuclear cells (PBMC) were prepared from 40 mL of blood anticoagulated with heparin, by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). PBMC were washed three times in RPMI/10% fetal calf serum (RPMI/FCS) (Gibco, Grand Island, NY) and incubated for 4 to 6 hours at 25°C tissue culture flasks (Becton-Dickinson, Lincoln Park, NJ) at 37°C. Nonadherent cells were decanted and adherent cells removed with 0.02% EDTA/RPMI/FCS. These cells were 80% to 85% esterase positive as determined by flow cytometry (see below).

Highly purified monocytes (greater than 95% esterase positive) were prepared by separation of contaminating B cells from the adherent PBMC preparation with rabbit anti-human immunoglobulin (Ig) M and complement. Adherent PBMC were incubated with a 1:100 dilution of rabbit anti-human IgM (Hyclone, Logan, UT) for 1 hour at 4°C, washed three times in RPMI/FCS, and resuspended in the same media containing a 1:4 dilution of baby rabbit complement (Pel Freeze, Rogers, AK) for 1 hour at 37°C. Cells were rewashed twice before use.

Adhesion assay. Initial PBMC adhesion experiments were performed with intact washed platelets made adherent to microtiter plates, as described previously. Further experiments were per-
formed with MoAbs used to trap platelet antigens. F(ab′)2, fragments of MoAbs 10E5, 6D1 (gifts of Dr Barry Coller, SUNY, Stonybrook, NY), or 3B2* (directed against GPIIb-GPIIIa, GPIb, or GPIIb, respectively), or buffer controls, were applied to 96-well flat-bottomed microtiter plates (Becton-Dickinson) in a volume of 50 µL at a concentration of 5 µg/mL in 0.3 M sodium carbonate buffer, pH 9.0, for 1 hour at 37°C, followed by blocking nonspecific binding sites with RPMI/10% FCS. Fifty microliters of a whole platelet preparation, prepared by solubilization of 10⁷ washed platelets in 1.0 mL 1% Triton X-100 (Sigma, St Louis, MO) followed by centrifugation at 100,000 × g to remove insoluble sediment, was then added for 1 hour at 37°C. The microtiter wells were washed with RPMI/10% FCS, and 50 µL of 10⁷ monocytes added to each well for 18 hours at 37°C in 5% CO₂. Similar results were noted after 3 hours of incubation, with optimum effect at 18 hours. The supernatant medium was decanted, and 100 µL of 0.02% EDTA/RPMI/10% FCS added for an additional hour at 37°C, 5% CO₂. The supernatant medium and nonadherent cells were then removed by gentle aspiration and the adherent cells stained with Diff Quick (American Hospital Products, McGaw Park, IL). Adherent clusters (greater than 5 cells) were enumerated for the entire microtiter well. Each experiment was performed in triplicate, with the mean applied for each data point.

Preparation of IgG and F(ab′)2, fragments. IgG was prepared from serum by 50% saturated ammonium sulfate precipitation, followed by DEAE ion exchange chromatography. It was centrifuged at 100,000 × g for 1 hour before use in the adhesion assay. F(ab′)2 fragments were prepared by pepsin digestion, followed by passage through a sepharose-protein A column as described previously. F(ab′)2, fragment purity and molecular weight were verified by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Preparation of PBMC supernatants. PBMC of control subjects or ATP patients were suspended in RPMI/10% FCS at a concentration of 10⁷ cells/mL and incubated for 72 hours at 37°C in 5% CO₂. Supernatants were collected by aspiration and stored at −70°C until use.

Indirect adhesion assay. Control or patient monocytes (10⁷/mL) were incubated with 200 µL of control or ATP IgG or F(ab′)2 fragments, or adherent PBMC supernatants at varying concentrations for 1 hour at 4°C, followed by washing twice in RPMI/10% FCS before use in the adhesion assay.

Armed monocyte assay. Control monocytes, 1 × 10⁶, were incubated with control IgG or ATP patient IgG at 200 µg/mL for 1 hour at 37°C, washed twice in RPMI/10% FCS, and then added to microtiter wells containing immobilized IIb-IIIa. This adhesion was compared with adhesion of control monocytes, preincubated, and washed as above with RPMI/10% FCS, and then incubated with immobilized GPIIb-IIIa that had been sensitized with the same concentration of ATP IgG as above, or incubated with control IgG for 1 hour at 37°C, and similarly washed.

Effect of MM41 (anti–Mac-1 MoAb). Washed armed monocytes were incubated in 50 µL of a 1:400 dilution of MM41 (murine IgG2a directed at the Mac-1 aβ chain [CD11b], gift of Dr Robert Winchester, New York University Medical School), 50 µL of MoAb 6D1 (murine IgG2a directed against platelet glycoprotein GPIb, gift of Dr Barry Coller), or 50 µL of buffer, before incubation with immobilized GPIIb-GPIIIa.

Flow cytometry. A Technicon H1 automated peripheral blood cell analyzer (Tarrytown, NY) was used to monitor esterase positive cells. The monocyte population was defined as the larger esterase positive cells. IgG coating of monocytes was determined by incubation of 10⁶ purified monocytes with a 1:20 dilution of 20 µL of fluorescence isothiocyanate (FITC)-labeled anti-human IgG (Hy Clone). Nonspecific Fc binding was blocked with nonimmune murine IgG, 100 µg/mL. Cells were then washed in phosphate-buffered saline/bovine serum albumin (PBS/BSA), fixed in 0.1% formalin/PBS/BSA and analyzed by cytofluorograph (FACSCAN, Model FC, Becton-Dickinson, Mountainvale, CA) at 488 nm.

RESULTS

Adherence of mononuclear cells to specific platelet antigens. Preliminary experiments showed that adherent PBMC were capable of adhering to an immobilized platelet monolayer on microtiter plates that were resistant to washing with RPMI/10% FCS-0.02% EDTA (data not shown). Further experiments were performed with MoAb immobilized platelet antigens. Figure 1A shows the adhesive cluster formation noted when F(ab′)2, fragments of 10E5 were used to immobilize the platelet IIb-IIIa antigen. This should be compared with control wells in which MoAb alone was used (Fig 1B), control wells in which platelet extract was added to microtiter plates in the absence of 10E5 (Fig 1C), or control wells in which neither 10E5 or platelet extract was used (Fig 1D). Note that control wells had minimal cluster formation, although individual monocytes did show some adherence.

Antibody 6D1, which immobilizes GPIb, increased adherent PBMC cluster formation twofold in 3 of 6 experiments (data not shown). Antibody 3B2, which binds to IIb, increased cluster formation twofold in 3 of 6 experiments (data not shown). Antibody 10E5, which binds to the IIb-IIIa complex, enhanced cluster formation in 18 of 19 experiments (Fig 2). Note the absence of enhanced cluster formation with adherent PBMC of eight thrombocytopenic control patients, as well as six patients with inflammatory disorders. Thus, 19 ATP patients formed 48 ± 6.8 (SEM) clusters compared with 7.4 ± 1.0 clusters for control subjects (P < .001).

These results are in agreement with other investigators who observed evidence for anti–GPIIb-IIIa activity in 75% to 95% of patients with ATP, and anti-GPIb activity in 28% of such patients.

The number of clusters per well correlated inversely (r = −.8, n = 21, P < .001) and exponentially with the patient’s platelet count according to the equation y = 637x⁻⁰·⁶, where y is the number of clusters per well and x is the platelet count per microliter × 10⁻³ (Fig 3).

Effect of incubation of immobilized GPIIb-IIIa with F(ab′)2, fragments of control and ATP IgG before incubation with control or patient’s adherent PBMC. F(ab′)2 fragments of control or patient IgG were incubated with immobilized GPIIb-IIIa and were capable of adhering to control PBMC. F(ab′)2, fragments of control or patient IgG were incubated with immobilized GPIIb-IIIa before the addition of adherent PBMC of control subjects and ATP patients. F(ab′)2, fragments of ATP patients reduced the number of clusters/well in a concentration-dependent manner by 42% to 58% at 100 and 200 µg/mL of F(ab′)2, respectively (P < .03, n = 4), whereas control F(ab′)2, fragments had no effect (Fig 4).

Effect of capping on adhesion of adherent PBMC to immobilized GPIIb-IIIa. Inhibition of cluster formation of ATP adherent PBMC by F(ab′)2, fragments of ATP IgG suggested that adherent PBMC of ATP patients required antigen-specific IgG on their surface. To test this hypothesis, adherent PBMC were subjected to conditions known to result in capping with internalization of surface IgG. This was accomplished by incubating adherent PBMC for 24 hours at 37°C in 5% CO₂ before their utilization in the adhesion
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Fig 1. Photomicrograph of adherence of PBMC to platelet GPIIb-GPIIIa. GPIIb-GPIIIa of a Triton-solubilized platelet extract was immobilized on wells of plastic microtiter plates with the F(ab')2 fragment of MoAb 10E5, as described in Materials and Methods. Unoccupied sites were blocked with RPMI/10% FCS. The wells were then incubated with PBMC, washed free of nonadherent cells, and stained with Diff Quick for adherent clusters (greater than 5 cells). (A) Complete incubation system; (B) complete system minus platelet extract; (C) complete system minus MoAb 10E5; (D) complete system minus MoAb 10E5 as well as platelet extract.

Fig 2. Comparison of scattergram of adherence of PBMC of ATP patients with PBMC of thrombocytopenic nonimmunologic patients and PBMC of inflammatory-disorder patients.

assay. Twenty-four hours of incubation reduced antigen-specific adherence by 88%, compared with freshly prepared adherent PBMC from $38.6 \pm 6.2$ clusters/well to $4.8 \pm 1.8$ ($P = .005$, $n = 4$, Fig 5A). This was associated with an 88% decrease in surface IgG content, as determined by flow cytometry (Fig 5B). Similar results were obtained with the control subject’s adherent PBMC as well as surface IgG (data not shown).

To rule out the possibility that 24-hour incubation may have damaged the monocytes, the following experiment was performed: Monocytes (prepared as described in Materials and Methods) were incubated for 24 hours to induce capping, treated with ATP IgG for 1 hour at 37°C, and then tested for adhesion to GPIIb-IIIa. These treated monocytes demonstrated $31.8 \pm 4.9$ clusters/well compared with $5.9 \pm 0.5$ for similarly capped monocytes treated with control IgG.

Effect of removal of contaminating B cells on adherence of monocytes to immobilized GPIIb-IIIa antigen. Flow cytometric analysis of adherent PBMC showed 80% to 85% esterase positive cells. The remainder of the cells were a smaller sized population bearing IgM and IgG on their surface, and presumed to be B cells. Because these cells could be secreting anti-platelet antibody in vitro, it was necessary to remove them to clarify the role of monocytes coated by antibody. Therefore, monocytes were purified, as described in Materials and Methods, and then used in the adhesion assay. Removal of B cells decreased the adhesion of monocytes by 76%, from $24.1 \pm 2.3$ clusters/well to $5.5 \pm 0.8$ ($P = .0001$, $n = 8$, Fig 6). Control monocytes, similarly
purified, also decreased their adhesion by 65%, from 7.0 ± 1.2 clusters/well to 1.8 ± 0.4 (P = .005). However, despite the decreased adhesion noted after removal of B cells, purified ATP monocytes developed significantly more clusters per well than purified control monocytes (5.5 ± 0.8 vs 1.8 ± 0.4, P = .001, n = 8).

Effect of repletion of autologous B cells to purified monocytes. When purified monocytes were supplemented with autologous B cells, monocyte cluster formation was restored in a concentration-dependent manner. Addition of 20% B cells to ATP monocytes increased adhesion from 4.3 ± 1.0 clusters/well to 16.4 ± 1.5 (P = .001, n = 4), whereas addition of control B cells to control monocytes increased adhesion from 2.7 ± 0.4 clusters/well to 5.1 ± 1.1 (P > .05, n = 4, Fig 7).

Effect of purified IgG on adherence of monocytes to immobilized GPIIb-IIIa antigen. Control monocytes were incubated with control or ATP patient IgG at varying concentrations before performing the adhesion assay. Figure
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Fig 6. Effect of removal of B cells from PBMC on adhesion of remaining monocytes (greater than 95% esterase positive) to immobilized GPIIb-GPIIIa.

8 shows the concentration-dependent enhancement of cluster formation by ATP patient IgG of 4.0- to 5.8-fold over baseline values at 110 to 250 μg/mL of IgG preincubation. No effect was noted with control IgG at similar preincubation concentrations (n = 4), or with ATP patient F(ab′)2, at 200 μg/mL (n = 2, data not shown).

Effect of supernatants of ATP and control PBMC on the adhesion of control monocytes to immobilized GPIIb-IIIa. The observations that removal of B cells from adherent PBMC decreased cluster formation, and that addition of ATP IgG to purified control monocytes increased cluster formation, suggested that B cells were secreting IgG during the 18-hour incubation of adherent PBMC with immobilized GPIIb-IIIa. Therefore, experiments were designed to determine whether supernatants of PBMC of ATP patients would enhance cluster formation of purified control monocytes. This proved to be the case. Thus, ATP supernatants preincubated with control monocytes stimulated cluster formation by 8.6-fold compared with control supernatants preincubated with control monocytes: 1.0 ± 0.7 to 8.6 ± 0.9 clusters/well (P = .003, n = 5). The IgG concentration of ATP and control supernatants was 3.8 ± 1.0 and 1.6 ± 0.8 μg/mL, respectively.

Identification of the role of armed monocytes in antigen-specific adhesion. The effect of preincubation of monocytes with ATP IgG (200 μg/mL) was compared with preincubation of immobilized GPIIb-IIIa with ATP IgG. Arming of control monocytes with ATP IgG led to the formation of 26.5 ± 5.5 clusters/well, whereas preincubation of immobilized IIIa with the same concentration of ATP IgG resulted in 4.6 ± 1.6 clusters/well (P = .002, n = 5, Fig 9). An enzyme linked immunosorbent assay (ELISA) of the opsonized GPIIb-GPIIIa showed the presence of 45 ng IgG per well, compared with 7 ng IgG/5 x 10⁴ armed monocytes added per well (mean of two experiments). These data suggested that monocytes per se could be contributing to the adhesive process, rather than simply IgG bound to the Fc receptor of monocytes. This hypothesis was tested by fixing armed monocytes with 0.1% formalin for 1 hour before its utilization in the adhesion assay. When compared with unfixed armed monocytes, fixed cells decreased cluster formation 81%, 18.9 ± 1.6 versus 3.6 ± 0.5 (P = .001, n = 3). To determine whether fixation impaired monocyte function versus IgG conformation, IgG alone was incubated with 0.1% formalin for the same time interval, centrifuged at 100,000 x g for 1 hour at 4°C to remove aggregates, protein concentration adjusted to 200 μg/mL, and then used to arm control monocytes. This manipulation had no effect on enhanced armed monocyte adhesion, 16.8 ± 1.9 versus 20.5 ± 3.3 for fixed and unfixed IgG, respectively, compared with 4.0 ± 1.5 for monocytes armed with control IgG.

Effect of MoAb MM41 (anti-Mac-1) on armed monocyte adhesion. Because GPIIb-IIIa may be a unique antigen in that it is a member of the β3 integrin family, and because monocytes have integrin receptors for the β2 integrin family, we elected to determine whether the monocyte per se contribution to cluster formation could be related to integrin
raise the possibility that some of the autoantibody on platelets may be due to nonspecific Fc binding to GPIIIa.

The enhanced adhesion was specific and not due to the adhesion of ATP monocytes to MoAb 10E5, since enhanced adhesion did not take place with MoAb alone (ie, absence of platelet extract). In addition, the F(ab′)2 fragment of the MoAb was used to avoid adhesion of unoccupied monocyte Fc receptors to the Fc domain of murine IgG. It is unlikely that the enhanced adhesion was due to nonspecific activation of monocytes, since enhanced adhesion was not noted with PBMC of patients with inflammatory disorders. The enhanced adhesion was specific for the F(ab′)2 domain of IgG autoantibody in that F(ab′)2 fragments of serum IgG of ATP patients, when incubated with immobilized GPIIb-GPIIIa, were capable of inhibiting the enhanced adhesion of PBMC of ATP patients; F(ab′)2 fragments of control subjects were not. The enhanced adhesion was noted as monocyte clustering. This phenomenon, for which we have no explanation, requires further study.

The adherence assay is clinically relevant in that adhesion of PBMC of ATP patients correlated inversely with platelet count. The inverse correlation was exponential rather than linear, suggesting the presence of a threshold for the occupancy of monocyte receptors before the activation of the adhesion reaction. An inverse relationship between platelet count and adhesion would be expected if the major pathophysiologic mechanism for the thrombocytopenia is due to the opsonization of platelets by autoantibody, followed by ingestion and destruction by phagocytic cells. This concept is supported by the classic experiments of Harrington et al, who noted a precipitous and rapid fall of the platelet count after the infusion of plasma IgG from an ATP patient into volunteer recipients, as well as the rapid clinical response to splenectomy and/or prednisone.

Our studies on the mechanism of adhesion of ATP PBMC...
show the following: Monocytes bind the Fc domain of autoantibody to their Fc receptors, since enhanced adhesion does not occur with the F(ab')₂ fragment of ATP IgG which, as an intact molecule, is capable of enhancing adhesion. ATP patient IgG is required because ATP PBMC depleted of lymphocytes diminish their adherence, which can be reconstituted by repletion with lymphocytes, addition of ATP IgG, or addition of conditioned media of B cells of ATP patients. The Fc receptors on ATP monocytes are not completely saturated with autoimmune anti–GPIIb-GPIIIa or have undergone capping during in vitro incubation since (1) ATP monocytes depleted of B cells lose 76% of their adherence capacity, although they are still capable of adhering to immobilized GPIIb-GPIIIa threefold greater than control monocytes; (2) IgG or conditioned media of PMBC of ATP patients is capable of restoring the enhanced adhesion noted with either B-cell repletion or ATP IgG addition; and (3) ATP PBMC incubated for 24 hours lose their surface IgG as well as their ability to adhere to immobilized GPIIb-GPIIIa.

A particularly relevant finding was the observation that armed monocytes were capable of dramatically enhancing adhesion to immobilized GPIIb-GPIIIa when compared with unarmed monocyte adhesion to opsonized GPIIb-GPIIIa. This was not due to the presence of more IgG on armed monocytes since sixfold greater IgG was present on opsonized GPIIb-GPIIIa compared with armed monocytes. We postulate that this effect could be due to the orientation or clustering of autoimmune IgG on the monocyte surface, thus increasing its effective concentration and/or affinity, or to a secondary adhesive event between the monocyte and autoantigen.

The latter suggestion is supported by two lines of evidence: (1) formalin-fixed ATP monocytes lose their ability to elicit enhanced adhesion to immobilized GPIIb-GPIIIa, whereas formalin-fixed ATP IgG does not; and (2) armed monocytes lose their ability to elicit enhanced adhesion when preincubated with an anti–Mac-1 MoAb directed against an integrin receptor on monocytes and neutrophils, which has been shown to be required for optimum monocyte IgG-Fc receptor-mediated phagocytosis, as well as adhesion to C3bi and factor X.

We conclude that armed monocyte binding to antigen is considerably more effective for binding than opsonized antigen. We propose that armed monocyte–platelet interaction rather than monocyte-opsonized platelet interaction may be more pathophysiologically relevant in the destruction of platelets, since opsonized platelets would have to compete with plasma IgG for monocyte Fc receptors.

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 Regulation of autoimmune anti-platelet antibody-mediated adhesion of monocytes to platelet GPIIb/GPIIa: effect of armed monocytes and the Mac-1 receptor

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