Functional Significance of Mobile Receptors on Human Platelets

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Mobile receptors on surface- and suspension-activated platelets bind a variety of specific and nonspecific antigens and particulates and clear them from the plasma membrane to channels of the open canalicular system (OCS). The present study examined the interaction of platelets with latex spherules of increasing size to identify the role of mobile receptors and binding sites in hemostatic physiology. Small latex spherules 0.09 to 3.13 μm in average diameter were cleared from peripheral margins to central zones and the OCS on surface-activated platelets and from the surface membrane to the OCS of platelets in suspension. Larger spheres, 6.4 μm in diameter, could not be moved across the membranes of solid phase- and fluid phase-activated platelets. Instead, platelets moved their surface membranes through the contact sites fixed on the surface of immobile spheres, bringing interior membranes of the OCS channels to the latex. This change, in which mobile membranes move through fixed contact sites rather than mobile receptor complexes moving across plasma membranes, results in evagination of almost all OCS channels on large latex spheres. A similar mechanism may be involved in the interaction of platelets with relatively flat surfaces, such as denuded subendothelium. (Arteriosclerosis and Thrombosis 1993;13:1236-1243)

Key Words • glycoprotein IIb/IIIa • surface activation • suspension activation • open canalicular system • receptor/ligand complex translocation

Receptors on the platelet surface are mobile. The fibrinogen receptor, glycoprotein (GP) GPIIb/IIIa, is nonfunctional in resting platelets but is rapidly activated after stimulation of platelets on surfaces or in suspension.1-3 Initially, the receptor complexes are randomly dispersed over the plasma membrane of stimulated platelets. Shortly thereafter, the receptors and bound ligands translocate across the cell membrane toward cell centers and channels of the open canalicular system (OCS).4 GPIb receptors bind von Willebrand factor (vWF), and after platelets are activated, these receptor/ligand complexes are also cleared to the OCS.5 The purpose served by mobile receptor reorganization in platelet physiology is unknown.

Much of the information regarding mobility of GPIIb/IIIa and GPIb comes from studies using electron-dense tracers or immunofluorescent tags.4-13 Binding of antibodies or ligands coupled to visible tracers selective for GPIIb/IIIa or GPIb causes and/or demonstrates clustering, patching, capping, and endocytosis similar to receptor responses on immunoreacted lymphocytes.5-9 However, platelets lack the polarity of nucleated lymphocytes and monocytes14 and possess an extensive OCS that is absent in other blood cells.15 As a result, it is difficult to understand the functional basis for such events on surface- or suspension-activated platelets.

The present study has examined the interaction of platelets with latex particles ranging from 0.09 to 6.4 μm in diameter.16,17 Small latex particles were transported across the platelet surface into channels of the OCS and, in some cases, to platelet α-granules.18,19 Larger latex particles were also taken up into platelets by channels of the OCS.20 If ingested particles were very large, the separate channels combined with the surface membrane to enclose the spherule in a vacuole. Extremely large latex particles were too big to ingest. Platelet membrane binding sites were attached to the latex surface, but the spherule could not be moved to the interior. Instead, the platelet evaginated its plasma membrane and linings of OCS channels through the fixed binding sites to the latex spherule. Without mobile binding sites and receptors, the spreading that is essential for the hemostatic reaction of platelets to surfaces and each other could not take place.

Methods

Preparation of Platelets

Blood for the present study was obtained after informed consent from well-characterized normal donors who had not taken aspirin for at least 10 days. After venipuncture, the samples were mixed immediately with citrate-citric acid, pH 6.5, (93.0 mmol/L sodium citrate, 7.0 mmol/L citric acid, and 140 mmol/L dextrose) in a ratio of 9 parts blood to 1 part anticoagulant.21 Platelet-rich plasma (PRP) was separated by centrifugation at room temperature for 20 minutes at 100g. Some samples were combined with an equal volume of the citrate-citric acid anticoagulant, centrifuged to pellets at 900g for 15 minutes, and resuspended in Hanks’ balanced salt solution.
solution (HBSS) containing calcium, magnesium, and 0.1% albumin.22

Preparation of Latex Spherules

Latex particles with average diameters of 0.09±0.024, 0.3±0.02, 0.77±0.015, 3.13±0.004, and 6.40±1.7 μm were obtained from Sigma Chemical Co (St Louis, Mo). The stock suspensions were centrifuged to pellets, washed, and resuspended in HBSS.23,24 Washing and resuspension were repeated three times. The final suspension was diluted to 1/10th the concentration of latex in the original stock suspension.

Latex Interaction With Surface-Activated Platelets

Drops of washed platelets were placed on carbon-stabilized, Formvar-coated grids and allowed to interact with the surface for 20 minutes at 37°C to produce maximum surface activation.24-27 The grids were placed in a moist chamber and put into a 37°C oven to maintain constant temperature.28 Nonadherent platelets were gently rinsed off the grid with HBSS. The grid was then covered with a drop containing latex particles of a particular size and again placed in the oven at 37°C for 1, 2, 3, 4, or 5 minutes, then fixed in 2% glutaraldehyde in cacodylate buffer, air dried, and studied in the electron microscope.23,24

Interaction of Suspended Platelets With Latex

Latex particles of a particular size were added in 0.1-mL volumes to 0.9 mL of PRP or washed platelets.18,19 The tubes were inverted once and placed in a 37°C water bath for 5, 10, 15, 30, or 60 minutes. After incubation, the samples were fixed by adding an equal volume of 0.1% glutaraldehyde in White’s saline, a 10% solution of a 1:1 mixture of (a) 2.4 mmol/L NaCl, 0.1 mmol/L KCl, 46 mmol/L MgSO4, 64 mmol/L Ca(NO3)2·4H2O and (b) 0.13 mol/L NaHCO3, 8.4 mmol/L NaH2PO4, and 0.1 g/L of phenol red, pH 7.4.29 After 15 minutes, the samples were centrifuged to pellets, and the supernatant fixative was removed and replaced with 2% glutaraldehyde in the same buffer. The samples resuspended in the second aldehyde fixative were maintained at 4°C for 30 minutes and then sedimented to pellets. The supernatant was removed and replaced with either 1% OsO4 in Zetterquist’s buffer or 1% OsO4 in distilled water containing 1.5% K4Fe(CN)6 for 1 hour at 4°C.

Tannic acid was used on some samples of platelets combined with latex to identify the OCS in surface-activated cells.20,21 As in previous studies, the procedure modified from Somlyo21 was used. Samples were fixed initially with 0.2% glutaraldehyde in White’s saline followed by sedimentation to a pellet, removal of the supernatant, and replacement with 3% glutaraldehyde in the same buffer for 2 hours at 4°C. After completion of the initial fixation steps, pellets were washed three times and stored overnight in 0.1 mol/L cacodylate buffer, pH 7.2. The buffer was decanted the following day and replaced with 2% tannic acid in 0.1 mol/L cacodylate buffer, pH 7.2, for 4 hours at room temperature. The supernatant was decanted, the pellets were washed in cacodylate buffer, and the samples were postfixed in 2% OsO4 alone or combined with 1.5% K4Fe(CN)6 for 2 hours at 4°C.

Effects of Inhibitors

The nature of the membrane binding sites interacting with latex spherules is unknown. To determine whether specific receptors were involved, antibodies against GPIIb/IIIa and GPIb/IX complexes and F(ab')2 fragments from goat anti-human immunoglobulin G (IgG) were obtained and tested for their influence on latex binding and translocation on suspended and surface-activated platelets. Monoclonal antibodies 6D1 against GPIb and 10E5 and 7E3 against GPIIb/IIIa were kindly supplied by Dr Barry Coller. Monoclonal antibody API against GPIb and APII against GPIIb/IIIa came from Dr Tom Kunicki. The F(ab')2 fragment of goat anti-human IgG was purchased from Sigma. Platelets were also obtained from a patient with thrombasthenia lacking GPIIb/IIIa and another patient with Bernard-Soulier syndrome lacking GPIb/IX complex. The defects in both patients have been characterized previously.30

Results

Latex Interaction With Surface-Activated Platelets

Platelets allowed to interact with the surface of Formvar grids for 20 minutes at 37°C are largely converted to spread forms.25-27,33 About 1% remain discoid, and 5% to 15% develop a dendritic appearance. Small latex particles, 0.09 μm in diameter, incubated with the surface-activated cells for 5 minutes do not react with discoid platelets but cover the bodies and pseudopods of dendritic forms.23,24 On spread platelets, the latex spherules move from the peripheral margins toward platelet centers (Fig 1). When grids of spread platelets reacted with latex for 5 minutes are placed on drops of HBSS, the spherules are concentrated into caps over cell centers (Fig 2). Slightly larger latex particles (average diameter, 0.3 μm) were processed in the same manner as small spherules (Fig 3). Latex spherules 0.7 μm in diameter also attached to the peripheral margin of spread platelets after 1 minute of incubation and were translocated to cell centers by 5 minutes (Fig 4).

Latex spherules with an average diameter of 3.13 μm were also bound to the peripheral margins of spread platelets initially and then transported to cell centers (Figs 5 and 6). The relation of even larger latex spherules (6.4 μm) with spread platelets could not be determined because they obscured the platelets. No obvious association between them could be defined.

Latex Interactions With Platelets in Suspension

The smallest latex particles used in this study (0.09 μm) bound to discoid platelets in suspension. During 15 to 60 minutes of incubation, the particles were cleared from the cell surface to channels of the OCS (Fig 7). Most cells retained a discoid form despite particle uptake, but a few became irregular in form. A few latex spherules (6.4 μm) with spread platelets could be seen but obscured the platelets. No obvious association between them could be defined.
but remained discoid for 15 to 30 minutes during the process of ingestion.

By 30 and 60 minutes after the start of incubation, latex spherules frequently appeared together within a membrane-enclosed vacuole (results not shown). Previous studies using tannic acid staining and other electron-dense tracers, however, revealed that the vacuoles were dilated sacs formed by fusion of latex-containing OCS channels. They retained their direct connection to OCS channels inside the platelet and through those conduits to the cell exterior.

Larger latex particles (average diameter, 3.13 μm) were also cleared from the platelet surface to channels of the OCS during incubation (Fig 9). Interiorization of
Facing page, Figs 1 through 6. Photomicrographs.

Fig 1. Platelet from a sample of washed cells incubated on a Formvar grid for 20 minutes and then combined with small latex particles averaging 0.09 μm in diameter for 5 minutes. The surface of the cell is arbitrarily divided into three areas: the peripheral zone (PZ), the intermediate zone (IZ), and the central zone (CZ). The latex (Lx) particles have moved from the peripheral zone toward the central zone of the spread platelet. Magnification ×6000.

Fig 2. Platelet prepared in the same manner as the cell in Fig 1, except for one additional step. After incubation with latex for 5 minutes, the grid was placed on a drop of Hanks’ balanced salt solution for another 10 minutes. Small latex (Lx) particles have moved from the peripheral zone (PZ) into a cap in the central zone (CZ) of the spread platelet. IZ, intermediate zone. Magnification ×8000.

Fig 3. Platelet prepared in the same manner as the cells in Figs 1 and 2. However, in this example, the cell was spread for 20 minutes and then incubated with latex particles averaging 0.3 μm in diameter. The latex (Lx) spherules have migrated from the peripheral margin to the platelet center. Magnification ×8000.

Fig 4. Fully spread platelet interacted with latex particles 0.7 μm in average diameter for 5 minutes. The latex (Lx) particles have moved from the periphery to the spread cell center. Magnification ×16 000.

Fig 5. Sample of spread platelets reacted with latex spherules 3 μm in average diameter. After the 5-minute incubation, the large latex (Lx) particles adhered selectively to the platelets and were transported to cell centers. Magnification ×2500.

Fig 6. Another example of a fully spread platelet that interacted with latex (Lx) spherules with an average diameter of 3 μm. Two spherules have attached to a single spread cell and migrated to the central region. Magnification ×8000.

Spherules of this size did not immediately trigger activation, although shape change did develop in most platelets after incubation with the large latex particles for 30 to 60 minutes.

Interaction of platelets with the largest latex particles (average diameter, 6.4 μm) used in this study produced a variety of appearances. In some examples, platelets surrounded the spheres, suggesting that they had successfully ingested the large particles (Figs 10 and 11). More often, the platelets tried to internalize the latex but were unable to do so (Figs 12 and 13). As a result, the platelet response resembled interaction with flat surfaces (Figs 14 through 18). Platelets spread into thin films on the large spheres. Tannic acid staining revealed a virtual absence of OCS channels in the cytoplasm of cells spread on latex (Figs 17 and 18). Organelles were also absent from most latex-activated platelets, but some retained α-granules concentrated in cell centers. The appearance was identical to spread platelets examined in thin section after surface activation on glass slides or Formvar grids.

Role of Known Platelet Receptors in Interaction of Latex Particles With Platelet Membrane

Attempts to determine whether the specific receptor complex was responsible for binding latex particles to platelets were unsuccessful. Monoclonal antibodies 6D1 and AP2 against GPIb/IX and 10E5, 7E3, and AP2 against GPIIIa were used at concentrations sufficient to inhibit binding of fibrinogen-coated colloidal gold or immunogold staining of GPIb/IX on surface-activated platelets. Except for API, the antibodies did not prevent binding and translocation of latex beads. Antibody API appeared to depress latex binding, and because this antibody interacts with the GPIb/IX complex, platelets from a patient with Bernard-Soulier syndrome who was deficient in this complex were examined. Patient platelets bound and translocated latex spherules in the same manner as normal cells. Thrombasthenic platelets deficient in GPIb/IIIa complex also bound and translocated latex beads. These results indicate that neither the GPIb/IX nor the GPIIIa/IIa complex is responsible for latex binding to platelets. To block the potential interaction of IgG absorbed on latex particles with the Fc receptor, the F(ab')2 fragments of goat anti-human IgG were used. These antibody fragments also failed to inhibit binding of latex by human platelets. Thus, the nature of the binding site for latex on human platelets remains unresolved.

Discussion

The present study and earlier investigations have demonstrated that discoid platelets in suspension can bind small latex spheres, move the particles to channels of the surface-connected OCS, and on occasion, transfer latex from outside the cell into apparently intact α-granules without changing shape. Plasma membranes of the discoid platelets and linings of the OCS must be specially adapted to serve a role in the binding, translocation, and interiorization, since these phenomena occur in the absence of the actin filament assembly associated with pseudopod extension and internal transformation.

The binding of latex spherules by suspended platelets appears to be random, but translocation of the particles has a directional component. Investigation of suspended platelet interaction with thorium dioxide has shown that all of the ligands are cleared to OCS channels. If the translocation lacked direction, then the various particulates would remain dispersed, rather than being cleared from plasma membranes to the OCS.

Additional support for this concept has come from studies of bovine platelets. Hemostatic cells from the cow lack the OCS found abundantly in platelets from human subjects. Bovine platelets bind Fgn/Au after surface activation in the same manner as human cells but do not translocate or interiorize the receptor/ligand complexes.

Emphasis in earlier studies was focused on movement of latex and other particulates across exposed plasma membranes and into OCS channels of resting discoid platelets in suspension. Recently, we have evaluated the interaction of latex with surface-activated platelets. Even the fully spread, terminally activated cells bound small latex particles and transported them toward channels of the OCS. The mobility of the receptors binding latex particles and the direction in which receptor/ligand complexes move suggested similarities to GPIb/IIIa after complexing Fgn/Au. However, the binding of latex did not require calcium, and platelets from a patient with thrombasthenia lacking GPIb/IIIa receptors bound
and translocated latex in a normal manner. Platelets from a patient with Bernard-Soulier syndrome also bound and translocated latex spherules in the same manner as normal cells. Attempts to prevent latex binding to surface-activated platelets by prior exposure of the cells to monoclonal antibodies revealed that blockade of GPIb/IX and GPIIb/IIIa did not interfere with particle attachment or translocation. Thus, some mobile binding system other than GPIIb/IIIa or GPIb/IX appears to be responsible for translocating latex and probably other nonspecific ligands.
The possibility that the Fc receptor was involved was eliminated when the F(ab')\textsubscript{2} fragment of goat anti-human IgG failed to affect latex binding or translocation.

The present investigation has shown that plasma membranes of surface-activated, fully spread platelets can bind and transport latex spheres from random positions to cell centers, even when the particles are nearly as large as resting platelets. Albrecht et al\textsuperscript{9} have previously shown that fully spread cells bind and move discoid or dendritic platelets to their centers in the same manner as large latex spheres. This feat is accomplished by thin films of fully spread cells fixed in position by receptors coupled across the plasma membrane to the cytoskeleton and foreign surface and unable to move.\textsuperscript{42} Under these conditions, actin polymers are generating isometric tension to hold the spread cell to the surface and do not shorten.\textsuperscript{43} The findings suggest existence of a force-generating mechanism within the plasma membrane and membrane cytoskeleton capable of driving mobile receptor/ligand complexes across the surface membrane separate from and largely independent of cytoplasmic actin.\textsuperscript{43}
prevents assembly of new cytoplasmic actin filaments without affecting the short, already established actin filaments making up the cytoskeleton of the surface membrane, does not block adherence to grids but prevents pseudopod formation and spreading. Fgn/Au added to CB-treated platelets after their placement on grids is rapidly cleared to channels of the OCS in 95% of the cells. Cytoplasmic actin cannot assemble in the CB-treated platelets. Therefore, the membrane-associated actin filaments resistant to the action of CB provide the driving force for receptor/ligand complex clearance to the OCS. The same mechanism is most likely involved in the platelet interaction with both small and large latex spherules.
In summary, the present study has shown that as the size of latex particles is increased, the response of platelets to them is altered. When the latex is small, platelets move the particles to the OCS. If the particles are large, platelets move the OCS to the latex. Short actin filaments associated with the plasma membrane cytoskeleton appear to provide the driving force for movement of small particles from the surface to the OCS. On the other hand, assembly of cytoplasmic actin into a peripheral weave provides the power for movement of the membrane through the fixed receptors attached to large spheres.

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