

Platelet Aggregation Is Not Initiated by Platelet Shape Change

Elisabeth Maurer-Spurej and Dana V. Devine

Canadian Blood Services, Vancouver, and Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY: Because the initial decrease in light transmission in platelet aggregometry is attributed to platelet shape change, it is widely held that platelet shape change is a prerequisite for platelet aggregation. We conducted this study to determine the basis of this initial optical effect in aggregometry. Platelets were activated with ADP, thrombin, or the thrombin receptor agonist peptide SFLLRN (TRAP₁₋₆). In every case the initial decrease in light transmission occurred with the concomitant formation of microaggregates. This was also seen when preactivated platelets, which cannot undergo further morphological changes, were used, and when platelets were activated in the presence of shape-change inhibitors such as cytochalasin D and vincristine. Microscopy analysis of samples fixed at minimum light transmission in the aggregometer, which is generally assumed to signal shape change, always showed the presence of microaggregates. Microaggregation appeared to be distinct from full aggregation, as it was not inhibited by the addition of CD61, an antibody to the β_3 integrin. To model these findings, fibrinogen-coated latex spheres, which cannot change shape, were aggregated with thrombin; the initial decrease in light transmission was still seen, and microaggregates formed at this time. These results indicate that platelet shape change is not a prerequisite for aggregation and that the signal widely believed to represent shape change reflects platelet microaggregation instead. We conclude that platelet aggregation occurs independently of shape change and that shape change is not necessarily followed by aggregation. These observations suggest an alternative role for platelet shape change of single platelets. (*Lab Invest* 2001, 81:1517–1525).

The assessment of platelet function is clinically important, because changes in platelet function can have a direct effect on both hemostasis and thrombosis (Barnhart, 1978; Zucker, 1980). A platelet biochemical defect (Caen and Michel, 1972), poor platelet quality (Kunicki et al, 1975), or the presence of inhibitors (Derrick et al, 1998) can reduce or abolish platelet function. Agonist-induced platelet activation is associated with optical effects that can be monitored in a platelet aggregometer (Born, 1962); this method is widely used to assess the functional status of platelets. Platelet aggregometry is a standard clinical technique that measures the *in vitro* aggregability of platelets in response to agonists by using the amount of light transmitted through platelet-rich plasma (PRP) (Holmsen, 1987). Aggregation leads to a substantial increase in light transmission because of the formation of large particles and the concomitant clearing of the sample. Severe abnormalities in platelet function, such as Bernard-Soulier syndrome, Glanzmann's thrombasthenia, or platelet storage-pool deficiency, are characterized by the inability of platelets to aggregate in response to certain agonists. In such cases the

expected increase in light transmission does not occur.

In contrast to the results obtained with general photometers (O'Brien, 1962), an initial decrease in transmission was observed with the devices specifically designed for platelet aggregometry by Born (Born et al, 1978; Latimer et al, 1977). It was suggested that this optical effect was caused by the morphological change of platelets from discs to spherical cells with pseudopodia (Latimer et al, 1977). Transmission electron microscopy was used to support the interpretation that the initial decrease in light transmission was caused by platelet shape change (Born et al, 1978). Since then, the initial decrease in light transmission detected in aggregometry is used as a measure for the extent of platelet shape change. Although a number of *in vivo* observations of the initial stages of platelet aggregation supported the possibility that platelet shape change did not precede aggregation (Bizzozero, 1882; Marcus, 1972; Stehbens and Biscoe, 1967), platelet shape change was, and still is, generally regarded to be the initial stage of platelet activation. Some researchers used alternative techniques to aggregometry, such as impedance measurements (Kitek and Breddin, 1980) or integrative light scattering (Hubell et al, 1991; Ozaki et al, 1994), to follow platelet activation and hypothesized that the initial changes in light transmission in an aggregometer could be due to microaggregation of the platelets. Nevertheless, measurement of the extent of shape change in an aggregometer is widely used to assess

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Address reprint requests to: Dr. Elisabeth Maurer-Spurej, University of British Columbia, Department of Pathology and Laboratory Medicine, 2211 Wesbrook Mall, Vancouver, BC, V6T 2B5, Canada. E-mail: emaurer@interchange.ubc.ca

low levels of platelet activation (Holme et al, 1998; Manual for the Chrono-Log Aggregometers, 1996).

The initial stages of platelet activation are very important for the initiation of hemostasis and thrombosis. Considering the discrepancies described above, it is desirable to better understand the optical effects associated with these initial stages. In this study, we examined the effect of platelet morphology on transmitted light in an aggregometer. We performed platelet aggregometry under experimental conditions that were reported to inhibit shape change and viewed the samples using phase contrast or scanning electron microscopy. We also modeled platelet aggregation using fibrinogen-coated latex spheres, which are particles that cannot change shape.

Results

Aggregation of Platelets with Thrombin, ADP, or TRAP₁₋₆ at 37° C

Thrombin, ADP, and TRAP₁₋₆ are three different platelet agonists that can cause platelet aggregation with or without fibrinogen polymerization. We analyzed the samples at the point of minimum light transmission to find the common cause for this optical effect. When platelets were kept at 37° C (Figs. 1A, 2A, and 3A), they were in a resting state and appeared as smooth discs. When the stirrer was turned on to 1000 rpm, the

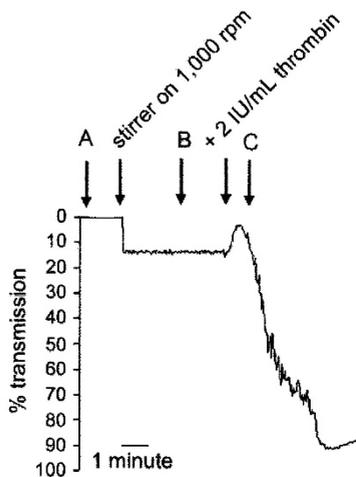
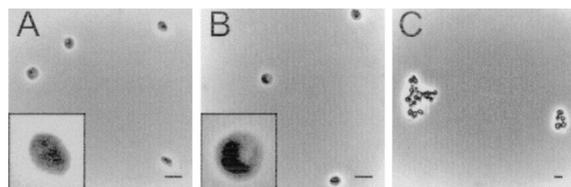


Figure 1.

Aggregation of platelets with thrombin at 37° C. Phase contrast micrographs show platelet morphology at three different stages during platelet aggregation, compared with the aggregometer trace: (A) resting platelets, (B) platelets stirred at 1000 rpm, and (C) microaggregates formed after the addition of thrombin at a final concentration of 2 IU/mL, which gave rise to the initial decrease in transmission. For the insets, images of platelets were enlarged four times. The size bars represent 5 μ m.

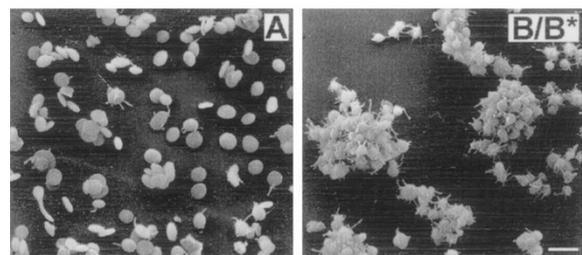
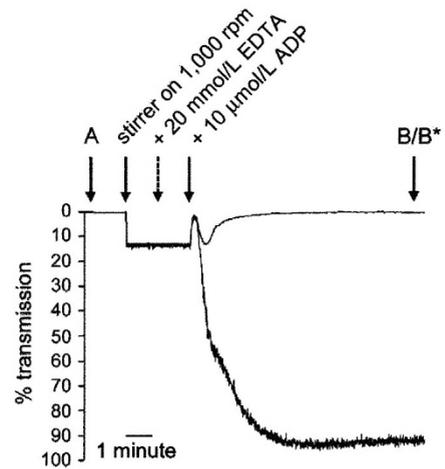
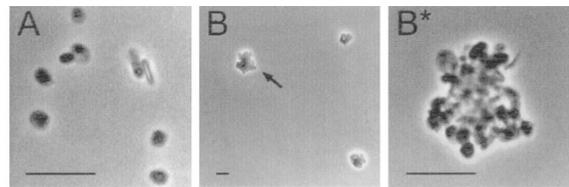


Figure 2.

Aggregation of platelets with ADP in the absence or presence of EDTA at 37° C. Phase contrast (top) and scanning electron micrographs (bottom) show the platelet morphology at two different stages during platelet aggregation, compared with the aggregometer trace. Discoid platelets (A) were stirred and ADP was added. In the absence of EDTA, 10 μ M ADP completely aggregated platelets, whereas, in the presence of 20 mM EDTA, full aggregation was inhibited and only microaggregates formed (B). The microaggregate marked with an arrow was recaptured with 10 times higher magnification (B*). The size bars represent 10 μ m for phase contrast and 5 μ m for the electron micrographs.

transmission increased abruptly, indicating that the concerted movement of the particles in solution allowed more light to pass through the sample. However, stirring did not have an effect on the morphology, as seen in Figure 1B. A stir speed of 1000 rpm in a 5-mm-diameter aggregometer cuvette is equivalent to an approximate shear stress of 0.03 Pa. This is low compared with the average shear stress that platelets experience in circulation, which is in the range of 0.1 to 2 Pa (Milnor, 1982). Addition of thrombin led to an initial decrease in transmission, and samples fixed at the peak of this deflection showed a high number of microaggregates when viewed under phase contrast microscopy (Fig. 1C).

Stimulation with 10 μ M ADP gave essentially the same results as 2 IU/mL thrombin. Figure 2A shows discoid platelets at the beginning of the experiment. The 10 μ M of ADP caused complete aggregation of

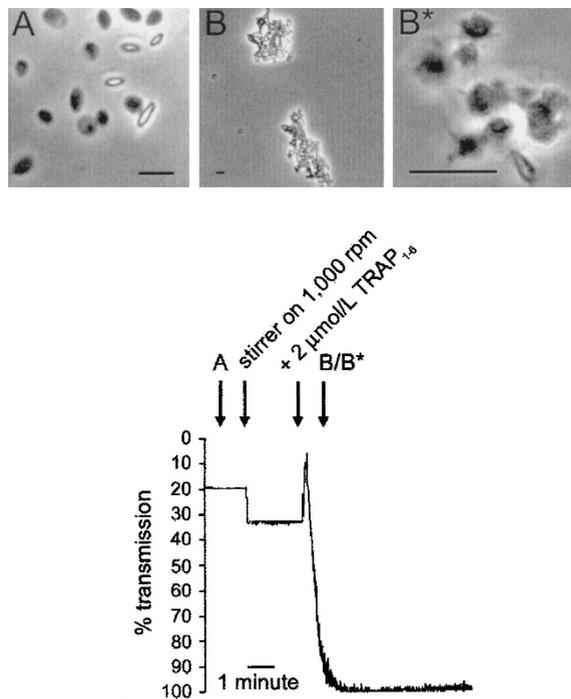


Figure 3.

Aggregation of platelets induced by TRAP₁₋₆ at 37°C. Phase contrast micrographs show the platelet morphology at two different stages during platelet aggregation, compared with the aggregometer trace. Discoid platelets (A) were activated with TRAP₁₋₆ (2 μM final concentration). Microaggregates formed concomitantly with the decrease in transmission (B and B*). The size bars represent 5 μm.

platelets, whereas only microaggregation occurred in the presence of 20 mM EDTA. The *dashed arrow* in Figure 2 indicates the time when EDTA was added. Microaggregates of different sizes were seen under both phase contrast and electron microscopy (Fig. 2, B and B*). Because phase contrast microscopy is faster and less likely to cause artifacts, we used this technique in most experiments.

TRAP₁₋₆ is a short peptide that activates the thrombin receptor on the platelet surface but does not polymerize fibrinogen. The addition of TRAP₁₋₆ to a final concentration of 2 μM caused discoid platelets to fully aggregate (Fig. 3). Analysis with phase contrast microscopy of a sample taken from the aggregometer cuvette at the point of minimum light transmission showed the presence of microaggregates (Fig. 3, B and B*). Microaggregates formed with thrombin, ADP, and TRAP₁₋₆. They are large particles that block the light and presumably cause the initial decrease in light transmission.

The addition of CD61, an antibody to the β₃ integrin also known as the glycoprotein (GP) IIIa, inhibited aggregation but not microaggregation (Fig. 4). Figure 4A shows the resting platelets in differential interference contrast (DIC) microscopy. The binding of CD61 was visualized by secondary staining with a fluorescently labeled secondary antibody (Fig. 4A*). Subsequent stimulation with 2 IU/mL thrombin, 10 μM ADP, or 2 μM TRAP₁₋₆ caused only microaggregation, which led to the initial decrease in light transmission.

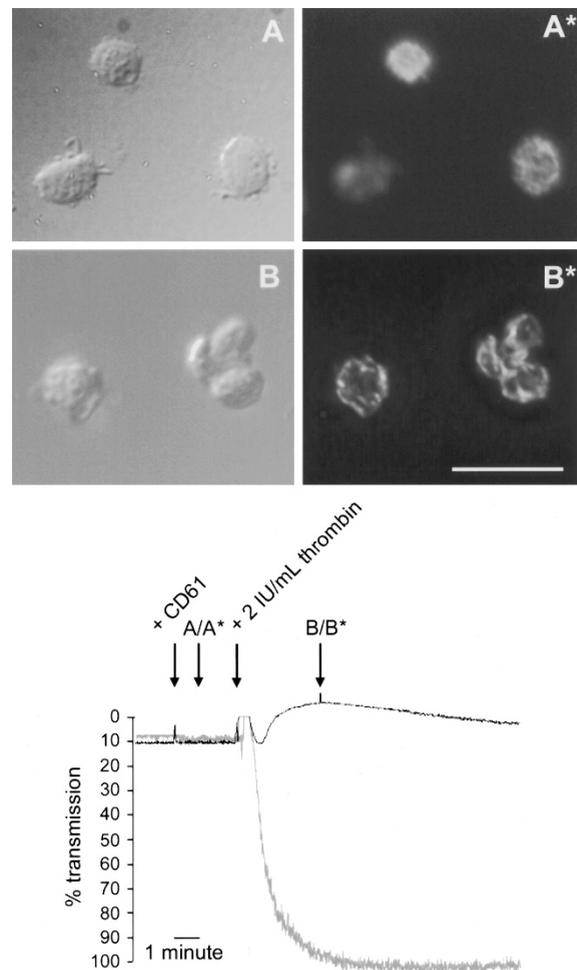


Figure 4.

Aggregation of platelets in the presence of CD61 with thrombin, ADP, or TRAP₁₋₆. PRP was stirred at 1000 rpm and 37°C in the aggregometer. CD61 inhibited aggregation, but not microaggregation, of platelets stimulated with 2 IU/mL thrombin, 10 μM ADP, or 2 μM TRAP₁₋₆ (*black curve*) compared with control samples, to which plasma was added instead of CD61 (*gray curve*). At the times indicated in the aggregometer trace, 50 μL aliquots were drawn from the CD61-containing samples, fluorescently labeled, fixed, and viewed with differential interference contrast (A and B) and fluorescence microscopy (A* and B*). The size bar represents 10 μm.

Control platelets fully aggregated in the presence of additional plasma instead of CD61. The aggregometer tracings in Figure 4 show curves in the presence (*black line*) or absence (*gray line*) of CD61, which are representative for stimulation with thrombin, ADP, or TRAP₁₋₆. These results suggest that, in contrast to aggregation, GPIIb-IIIa was not involved in microaggregation.

Aggregation of Preactivated Platelets with Thrombin at Room Temperature

The same samples that were prepared and tested at 37°C were brought to room temperature and examined again. Figure 5A shows the morphology of platelets after incubation at room temperature for 10 minutes, under phase contrast (top) and scanning electron microscopy (bottom). The lower temperature

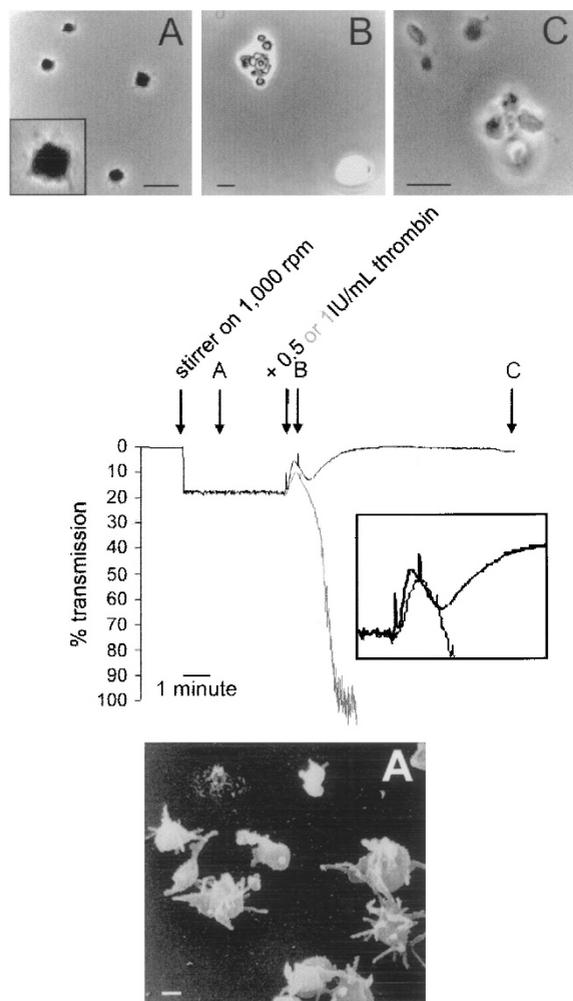


Figure 5.

Aggregation of preactivated platelets with thrombin at room temperature. Phase contrast and scanning electron microscopy show platelets that have been morphologically changed by exposure to low temperature at a stir rate of 1000 rpm (A); the inset shows a four-fold enlargement. Microaggregates formed after the addition of 0.5 or 1 IU/mL thrombin simultaneously to the initial decrease in transmission (B). At a final concentration of 0.5 IU/mL thrombin, small microaggregates formed (C), whereas 1 IU/mL thrombin caused full aggregation (*gray curve*). The size bars represent 5 μm and 1 μm for phase contrast and electron micrographs, respectively. The inset below the aggregometer trace shows an enlargement of the deflection of light transmission at room temperature (*black curve*) compared with that at 37°C (*gray curve*).

induced a change in platelet shape from a discoid morphology to spherical bodies with pseudopodia (Maurer-Spurej et al, 2001). These platelets were not expected to undergo additional morphological changes. For the aggregometry at room temperature, the thermostat in the aggregometer was turned off (see "Materials and Methods"). Turning on the stirrer caused a sudden increase in transmission. Although the platelets had already undergone a change in shape, the addition of 0.5 IU/mL thrombin resulted in a decrease in transmission. Phase contrast microscopy showed that microaggregates formed during the initial decrease in transmission (Fig. 5B). The small wave in the optical trace at this low dose of agonist might indicate the intermediate formation of unstable

larger aggregates. When the trace stabilized at a very low level of light transmission, microaggregates were still present in the sample (Fig. 5C). The inset below the aggregometer trace shows that the decrease in light transmission is comparable to the initial deflection with 2 IU/mL thrombin at 37°C (Fig. 1). Because the platelets had already undergone a change in shape before the agonist was added, the formation of microaggregates appears to be the only possible reason for the decrease in light transmission (Fig. 5, B and C). It is interesting to note that the concentration of agonist needed to induce full aggregation at room temperature was lower than at 37°C because platelets were preactivated. A final concentration of 1 IU/mL thrombin was sufficient to fully aggregate the preactivated platelets. The sensitivity of preactivated platelets to ADP stimulation was also increased (data not shown).

Aggregation of Platelets in the Presence of Cytochalasin D or Vincristine

To confirm that platelet shape change does not cause the initial decrease in light transmission in aggregometry, we conducted experiments similar to those described above in the presence of shape-change inhibitors. PRP was placed in the aggregometer at 37°C, the stirrer was turned on to 1000 rpm, and 60 μM cytochalasin D was added followed by 2 IU/mL thrombin. Addition of cytochalasin D preserved the discoid morphology of platelets (Fig. 6A) even after addition of thrombin. A thrombin concentration of 2 IU/mL caused full aggregation in the absence of cytochalasin D, as shown in Figure 1. Although the discoid shape of the platelets was preserved, thrombin induced an interaction between the platelets (Fig. 6B) and polymerized fibrin (Fig. 6C). Even without platelet shape change, thrombin induced the formation of microaggregates, which again blocked the light passing through the PRP in the aggregometer and caused a deflection in light transmission. The inset below the aggregometer trace compares the optical effect of microaggregate formation in the presence and absence of cytochalasin D. In the absence of the inhibitor, the deflection is higher, which is most likely due to the formation of larger microaggregates when cytochalasin D is not added.

Figure 7 shows the experimental results from platelet aggregometry in the presence of 100 μM vincristine and stimulation with 2 IU/mL thrombin at 37°C and 1000 rpm (Fig. 7A). Although vincristine interferes with a normal shape change reaction, light transmission decreased after the addition of thrombin to PRP. Phase contrast microscopy revealed once again the formation of microaggregates (Fig. 7B). Vincristine permitted the formation of larger microaggregates, leading to a high initial deflection in light transmission. The inset below the aggregometer trace shows the overlay of the deflection with (*black curve*) and without (*gray curve*) vincristine.

The platelet shape change inhibitor, prostaglandin E₁ (PGE₁), was used in the same way as cytochalasin

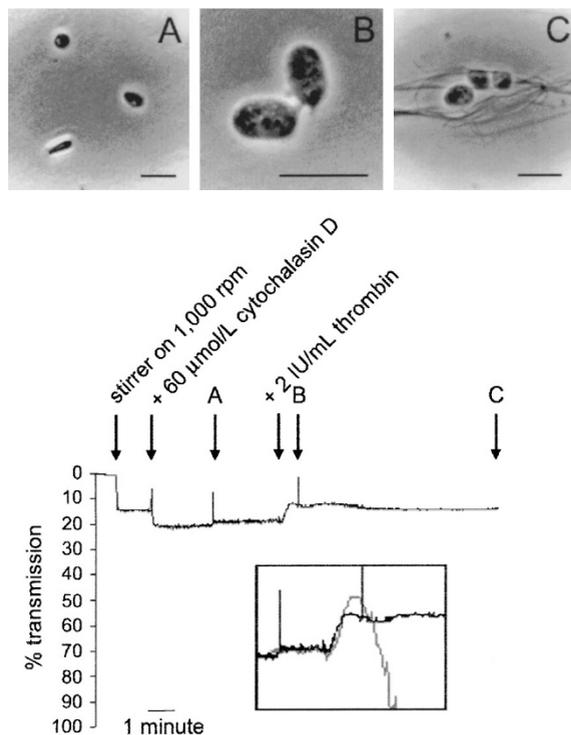


Figure 6.

Aggregation of platelets with thrombin in the presence of cytochalasin D at 37° C. Phase contrast micrographs show the platelet morphology at three different stages during platelet aggregation, compared with the aggregometer trace. The addition of 60 $\mu\text{mol/L}$ cytochalasin D had no effect on resting platelets (A). Small aggregates composed of discoid platelets formed after the addition of 2 IU/mL thrombin, coinciding with the peak of the initial decrease in light transmission (B), and were trapped by fibrin fibers (C). The size bars represent 5 μm . The inset below the aggregometer trace shows the decrease in light transmission in the absence (gray curve) and the presence of cytochalasin D (black curve).

D and vincristine. In our hands the addition of PGE₁ caused some formation of microaggregates, but morphological changes were largely inhibited. Stimulation with 2 IU/mL thrombin resulted in a further decrease in light transmission because of the formation of more and larger microaggregates (data not shown). In the presence of PGE₁, the formation of one tight aggregate does not occur, and therefore the sample does not completely clear. It seems that PGE₁ inhibits clot retraction.

Aggregation of Fibrinogen-Coated Latex Spheres with Thrombin

We showed that preactivated platelets and platelets inhibited from undergoing morphological changes were able to cause changes in light transmission and suggested that this optical effect stemmed from microaggregate formation. We designed the following experiment using latex spheres to exclude any cellular changes that might affect light transmission. Latex spheres with positive and negative surface charges were kept in distilled water at a low stir speed of 200 rpm and at room temperature to inhibit the spontaneous formation of microaggregates. Polyethylene glycol, molecular weight 3000-3700 (PEG₃₀₀₀) was

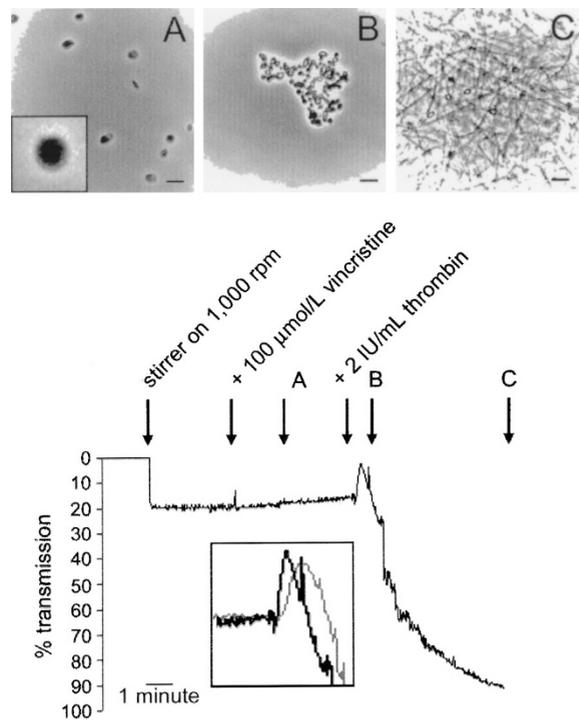


Figure 7.

Aggregation of platelets with thrombin in the presence of vincristine sulfate at 37° C. Phase contrast micrographs show the platelet morphology at three different stages during platelet aggregation, compared with the aggregometer trace: (A) platelets after the addition of 100 $\mu\text{mol/L}$ vincristine, (B) after the addition of 2 IU/mL thrombin at the peak of the initial decrease in light transmission, and (C) after complete aggregation and the formation of fibrin polymers. In the inset in Panel A, one representative platelet is shown, magnified four times. Although vincristine inhibits platelet shape change, thrombin induces a decrease in light transmission that correlates with the formation of microaggregates. The size bars represent 5 μm . The inset below the aggregometer trace shows the decrease in light transmission in the absence (gray curve) and the presence of vincristine (black curve).

added to increase the viscosity and reduce the probability of particle collisions. Addition of fibrinogen resulted in the coating of the latex spheres but no aggregation (Fig. 8A). We verified this with the use of Oregon Green-labeled fibrinogen and fluorescence microscopy (data not shown). After the addition of PBS and 15 IU/mL thrombin, the stir speed was increased and full aggregation of latex spheres occurred. Light transmission initially decreased, corresponding to the formation of microaggregates as seen in phase contrast microscopy of the fixed sample (Fig. 8B). Full aggregation of latex spheres (Fig. 8C) resulted in close to 100% light transmission. At higher stir speed, microaggregation and aggregation occur more rapidly, leading to a shorter existence of microaggregates and subsequently a reduced signal from microaggregation, which is indicated by a lower decrease in light transmission. These experiments were repeated three times. In the absence of latex particles, the light transmission stayed at 100%, indicating that fibrin strands have no effect on light transmission and their formation does not decrease the amount of transmitted light.

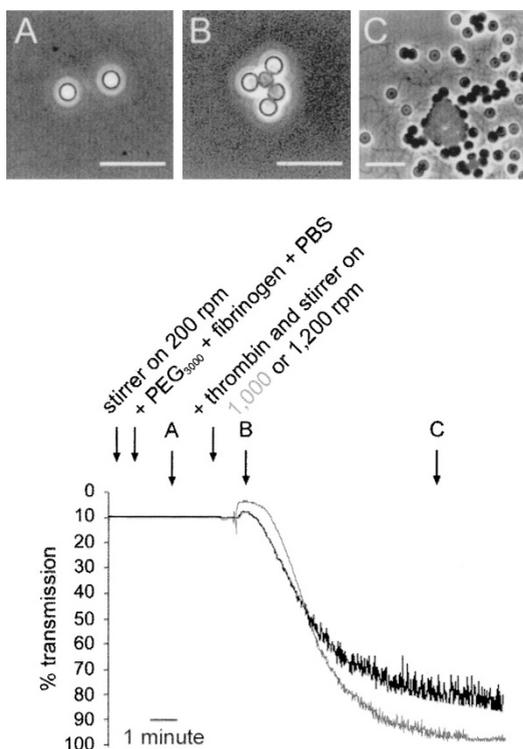


Figure 8.

Aggregation of fibrinogen-coated latex spheres with thrombin at room temperature. The effect of aggregation of fibrinogen-coated latex spheres on light transmission in the aggregometer is compared with phase contrast microscopy of the fixed samples at the stages indicated. The addition of thrombin to single latex spheres (A) caused the formation of microaggregates (B) corresponding to the decrease in light transmission. Kinetic differences were seen at a stir speed of 1000 rpm (gray curve) compared with 1200 rpm (black curve). Full aggregation of latex spheres resulted in close to 100% light transmission (C). Aggregation did not change the appearance of the single spheres. The size bars represent 5 μ m.

Discussion

In this study we challenged the general opinion that platelet shape change could be detected as the initial decrease in light transmission in an aggregometer. We investigated the morphology of platelets with phase contrast and scanning electron microscopy at the point of minimum light transmission in aggregometry after stimulation with thrombin, ADP, or TRAP₁₋₆, and found that microaggregation caused the optical effect.

After platelets have undergone a change in morphology, their shape is not expected to change any further. Exposure to room temperature has been shown to cause platelet shape change (Maurer-Spurej et al, 2001), but without additional stimulation these morphologically changed single platelets do not aggregate. However, although platelets exposed to room temperature had already changed shape, the initial decrease in light transmission that is generally attributed to platelet shape change was not abolished. In this study we have found that the optical signal is caused by microaggregates, which because of their size are much more likely to cause a change in light transmission than morphologically changed single platelets. Platelets that have undergone morphologi-

cal changes *in vitro* are hyperreactive, and the kinetics of their response to a certain dose of agonist is increased. A comparison of preactivated platelets with resting platelets exposed to the same dose of agonist can therefore show a reduction in, or even the loss of, the initial deflection in light transmission for the preactivated cells. In his early work O'Brien had already pointed out that kinetic parameters are very important for the process of aggregation (O'Brien, 1965).

In addition to kinetic considerations that affect the initial decrease in light transmission, the optical design of the instrument used to measure light transmission has been found to be critical. The initial decrease in light transmission was first observed with devices specifically designed for platelet aggregometry by Born (Born et al, 1978; Latimer et al, 1977), in which the angle of light collection is small. Based on theoretical calculations, Latimer (1983) predicted that in a Born-type aggregometer microaggregation leads to a decrease in light transmission. This is because, at a small angle of light collection, the blockage of light by large particles such as microaggregates is more important than an increase in forward scattering from these large particles. However, these findings were published several years after the decrease in light transmission was introduced as a measure for the extent of platelet shape change (Born et al, 1978; Latimer et al, 1977), and that method had already gained wide acceptance. Transmission electron microscopy was used to support the interpretation that shape change is the cause for the initial decrease in light transmission (Born et al, 1978). The sensitivity of platelets to environmental stresses such as exposure to room temperature can easily lead to the observation of morphologically changed platelets. In addition, it is difficult to differentiate between microaggregates and platelet clumps with electron microscopy because of the pelleting and resuspension steps required for sample preparation.

Cytochalasins, which freely cross the plasma membrane of cells, are reported to interfere with the reorganization of actin filaments, which is required for both cell motility and platelet shape change (Fox and Phillips, 1981; White and Rao, 1998). In this study, we observed that, although shape change was inhibited by the addition of cytochalasin D, platelets could still form microaggregates that gave rise to the initial decrease in light transmission. The addition of vincristine to platelets leads to disintegration of the microtubules located beneath the plasma membrane, and the cells round up but do not form pseudopodia (White and Rao, 1998). Again, this inhibition of normal shape change did not prevent microaggregation and the corresponding optical effect in this study. Unlike cytochalasin D, vincristine does not appear to interfere with clot retraction, and its addition resulted in a 100% increase in transmitted light.

It is well documented that for complete platelet aggregation the plasma membrane integrin $\alpha_{IIb}\beta_3$, also known as the GPIIb-IIIa complex, has to be activated and bind fibrinogen. This integrin is missing in the platelets of patients with Glanzmann's

thrombasthenia, an inherited disease. Although these platelets do not aggregate with normal doses of agonists in the aggregometer, they show the initial decrease in light transmission that is thought to be the shape change signal (Caen and Michel, 1972). We interpret this to mean that the platelets of these patients form microaggregates but do not completely aggregate. Our results using CD61 to mimic Glanzmann's thrombasthenia suggest that microaggregation is distinct from full aggregation and does not involve the integrin $\alpha_{IIb}\beta_3$. We hypothesize that microaggregation is induced by signaling processes different from full aggregation. It was recently reported that the shear-induced binding of von Willebrand factor (vWf) to the platelet GPIb/IX/V complex plays a key role in initiating platelet adhesion and aggregation at sites of vascular injury (Mistry et al, 2000). The finding that platelet adhesion and aggregation is enhanced in the presence of shape change inhibitors such as cytochalasin D, without involvement of the integrin $\alpha_{IIb}\beta_3$ (Mistry et al, 2000), supports our hypothesis that microaggregation is distinct from complete aggregation.

Several reports describing observations of the initial stages of platelet aggregation *in vivo* support the possibility that platelet shape change does not precede aggregation. When platelets were first discovered, more than half a century before the invention of platelet aggregometers, Bizzozero described the *in vivo* aggregation of platelets in the small artery of a rabbit or guinea pig (Bizzozero, 1882). In 1882, he published his microscopy observations illustrated with drawings showing that platelets aggregate without changing their shape and that shape change occurs only some time after isolation of the thrombus. Stehens and Biscoe (1967) used a cat model and reported that platelets aggregate *in vivo* in their discoid shape and subsequently change their morphology, which facilitates granule release and tightens the clot. Marcus (1972) also reported that in small aggregates human platelets appear in their discoid shape. He summarized the initial steps of the hemostatic reaction in response to an injury as follows: first, circulating platelets adhere to the site of injury within milliseconds and come in contact with collagen; subsequently, the aggregated platelets lose their discoid shape and form pseudopodia. Our results agree with the findings from these morphological studies.

We conclude that platelet shape change is not a prerequisite for aggregation *in vitro*. The data presented here support a model in which platelet shape change occurs after, rather than before, aggregation. In this context, aggregation means the gathering of discoid platelets (Marcus, 1972). The role of shape change would then be to condense the aggregate, provide necessary membrane receptors, and release serotonin for vasoconstriction as well as the α -granule contents. However, single platelets, which are not engaged in aggregation, can be stimulated to undergo shape change without subsequent aggregation (Maurer-Spurej et al, 2001). We suggest that these dramatic changes in platelet

shape have a function independent of platelet aggregation.

Materials and Methods

This study was approved by the Institutional Review Committee of the University of British Columbia and was conducted in accordance with the Declaration of Helsinki (Eighteenth World Medical Assembly, Helsinki, Finland, 1964).

Preparation of Platelet-Rich Plasma

Blood was collected from human volunteers who had provided informed consent. Whole blood was anticoagulated with either sodium citrate or acid citrate dextrose (ACD, pH 6.5). The anticoagulant had no effect on the results. The data presented were obtained from samples anticoagulated with sodium citrate. Platelet-rich plasma (PRP) was prepared at 37° C, because room temperature has been found to activate platelets (Maurer-Spurej et al, 2001). Briefly, 9 volumes of blood were drawn into a prewarmed tube containing 1 volume of sodium citrate (3.8% final concentration). The blood was kept warm and was centrifuged at 150 $\times g$ for 12 minutes at 37° C. The isolated PRP was kept at 37° C for 30 minutes before use. Platelet-poor plasma (PPP) was obtained by centrifuging the blood fraction for 15 minutes at 1500 $\times g$.

Phase Contrast, DIC, and Fluorescence Microscopy

Live-cell microscopy was performed using a Nikon Labophot 2 phase contrast microscope (Nikon, Tokyo, Japan) with a 100 \times /1.3 NA oil immersion objective. A 40 \times /0.65 NA or a 10 \times /0.25 NA air objective was used to capture more cells or aggregates in the field of view. The microscope was fitted with a laboratory-built temperature stage and a digital camera (Pixera, Los Gatos, California) for real-time imaging. All glass surfaces were siliconized with dichloro-dimethylsilane (Sigma, St. Louis, Missouri) to prevent adherence and spreading of live platelets on the glass slides. For the analysis of platelets at certain time points during platelet aggregometry, PRP was removed from the aggregometer cuvette and directly fixed 1:1 (v/v) in 4% paraformaldehyde (PFA; 2% final concentration). PFA was dissolved in phosphate-buffered saline (PBS; 50 mM NaH₂PO₄·H₂O, 5 mM KCl, 1.5 mM MgCl₂·6 H₂O, 80.1 mM NaCl, pH 7.3). DIC and fluorescence microscopy was carried out on a Zeiss Axiophot 2 microscope (Zeiss, Jena, Germany) with a 100 \times /1.3 NA Plan Neofluar oil objective.

Scanning Electron Microscopy

Scanning electron microscopy was performed using a Zeiss DSM 950 scanning electron microscope. Platelets were fixed in 3% glutaraldehyde in phosphate-buffered saline (PBS; 50 mM NaH₂PO₄·H₂O, 5 mM KCl, 1.5 mM MgCl₂·6 H₂O, 80.1 mM NaCl, pH 7.3) by incubating 400 μ L of PRP with 1 mL of glutaralde-

hyde/buffer solution for 30 minutes at 37° C or 20° C. Platelets were then washed with PBS and postfixed for 30 minutes in 2% OsO₄ in PBS at room temperature. The platelets were centrifuged at 1500 ×g for 5 minutes and resuspended in PBS, mixed with 2% agarose solution, and then dehydrated in a series of ascending ethanol concentrations. After critical-point drying, the samples were sputter-coated with gold and zirconium and examined.

Aggregometry

A Chrono-Log dual-channel lumi-aggregometer (Chrono-Log, Havertown, Pennsylvania) was used to measure the aggregation of platelets or latex particles. This instrument had custom-added temperature switches to turn off the temperature control. This allowed us to perform experiments at room temperature (24 ± 1° C). PRP was prepared as described earlier. The platelet count, determined with a Coulter counter, was 2 to 3 × 10¹¹/L and was therefore not adjusted, because a platelet count up to 3 × 10¹¹/L does not cause multiple scattering (Born et al, 1978; Latimer, 1983). PRP was incubated in small glass cuvettes appropriate for 0.5 mL of sample for at least 5 minutes at the measuring temperature. The baseline was set to the light transmission of nonstirred PRP against PPP; after stabilization of the baseline, the stirrer was turned on to a speed of 1000 rpm. For some experiments cytochalasin D, prostaglandin E₁ (PGE₁) (Sigma) or vincristine sulfate (Pharmacia, Mississauga, Ontario, Canada) was added to the PRP during stirring. Cytochalasin D and PGE₁ were dissolved in dimethylsulfoxide (DMSO). DMSO alone had no effect on platelet aggregation when added to PRP by itself. The final concentrations of cytochalasin D, PGE₁, and vincristine were 60 μM, 2 μM, and 100 μM, respectively.

Platelets were activated with ADP (10 μM final concentration), in the absence or presence of 20 mM EDTA, thrombin (2 IU/mL final concentration), or TRAP₁₋₆ (2 μM final concentration). In the case of platelets preactivated by incubation at 20° C for 30 minutes, the final concentration of thrombin was 0.5 or 1 IU/mL. Human thrombin, ADP, and EDTA, which was used to inhibit platelet aggregation with ADP, were purchased from Sigma, and TRAP₁₋₆ was purchased from Peninsula Laboratories Europe, Ltd. (Merseyside, England). Anti-CD61, a monoclonal mouse anti-human β₃ antibody (Beckman Coulter, Mississauga, Ontario, Canada), was used at a final concentration of 4 μg/mL in PRP, and will in the following be referred to as CD61. CD61 was added to PRP before stimulation with 2 IU/mL thrombin, 10 μM ADP, or 2 μM TRAP₁₋₆. Control samples were stimulated in the same way, but the volume of CD61 was replaced by PPP. To visualize the binding of CD61 to single platelets and platelet microaggregates, an Alexa488-labeled goat anti-mouse secondary antibody (Molecular Probes, Hornby, Ontario, Canada) was added at a final concentration of 40 μg/mL before fixation of the samples. Addition of only the secondary

antibody had no effect on platelet aggregation. All results shown are representative of at least three experiments with blood from different donors.

Surfactant-free aldehyde/amidine white polystyrene latex spheres of 1.2 μm mean diameter were purchased from Interfacial Dynamics Corporation (Portland, Oregon). The latex suspension was diluted with distilled water to a final particle concentration of 5.5 × 10¹⁰/L. Initially the particle suspension was stirred at 200 rpm at room temperature. Twenty microliters of PEG₃₀₀₀ (polyethylene glycol, molecular weight 3000–3700), fibrinogen (40 μg/mL final concentration), 50 μL of 10 × PBS for a physiological salt concentration, and thrombin (15 IU/mL final concentration) were added, and the stir rate was increased to 1000 or 1200 rpm. The low initial stir speed, the low temperature, and the addition of PEG₃₀₀₀ were necessary to slow down the aggregation process so that we could detect the initial decrease in light transmission followed by the increase in transmission with total aggregation. Fibrinogen was added under salt-free conditions to coat the latex spheres. Coating was confirmed by fluorescence microscopy (Nikon Labophot 2) of latex spheres in the presence of Oregon Green-labeled fibrinogen (Molecular Probes, Eugene, Oregon). All other reagents were purchased from Sigma.

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