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A Colorimetric Method for the Measurement of Platelet Adhesion in Microtiter Plates

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A procedure for the determination of the adhesion of human platelets to protein-coated culture microwells was developed. The number of platelets was quantitated by measuring the activity of acid phosphatase, a platelet enzyme whose activity is stable independently of platelet stimulation and is not released. Isolated and washed platelets were incubated in 96-well microtiter plates with flat-bottom wells that had been precoated with various compounds, including collagen, fibrinogen, human plasma, and human albumin. At the end of incubation (optimal time: 40–60 min), nonadherent platelets were washed out, adherent platelets were solubilized with Triton X-100, and the acid phosphatase activity was measured by using the substrate *p*-nitrophenyl phosphate. The *p*-nitrophenol produced was measured with a microplate reader at 405 nm and the percentage of adhesion was calculated with reference to known platelet standards. ADP and thrombin stimulated platelet adhesion in a dose-dependent manner to fibrinogen and human plasma, but not to human albumin. Platelets adhered to collagen even in the absence of stimulants. Simultaneous evaluation of adhesion and aggregation demonstrated that with ADP as stimulus, but not with thrombin, the two platelet responses were dissociated. Microscopic examination of culture wells showed that most of platelets adhered as single cells and not as aggregates. The sensitivity of this method allowed the assay of platelet adhesion by using only 2.5×10^5 platelets/well.

One important mechanism by which blood platelets perform their functions is adhesion to the injured vessel wall, which may be regarded as the first and crucial step of the haemostatic process. Acquired or genetic defects of platelet adhesion may seriously compromise the hemostatic process, while unnecessary increase of adhesiveness may result in enhanced risk of vascular disorders. Therefore, analysis of the adhesion function of platelets is of great importance in the differential diagnosis and follow-up of bleeding and thrombotic syndromes.

Adhesion is a complex event involving a series of plasma and subendothelial tissue components that specifically bind to several different membrane glycoproteins. Platelets possess receptors for various proteins such as collagen, fibrinogen, fibronectin, von Willebrand factor, laminin, thrombospondin, vitronectin, and glycoproteins carrying sialic acid residues (1–7). Physiological platelet agonists such as ADP, collagen, and thrombin activate stimulus–response coupling pathways that may increase either the number or the ligand affinity of specific adhesion receptors. Some membrane adhesion complexes, such as GPIa/IIa and probably also GP IIb/IIIa, function not only as adhesive protein receptors, but also as signal transducing elements (8–10). Study of adhesion to different surfaces and following the challenge of the cells with different agonists is therefore necessary in order to characterize the multiple molecular mechanisms by which platelet–substrate contact interactions occur.

The use of methods based on multiwell microplates is of obvious convenience when a number of different experimental conditions has to be evaluated and this is the case when dealing with complex functions such as platelet adhesion. In this report we describe a simple and reproducible cell adhesion assay based on the determination of acid phosphatase activity of platelets. We previously developed an acid phosphatase assay that has proven to be particularly useful for the measurement of neutrophil adhesion (11,12). Here we show that this enzymatic activity is contained also in platelets and, after proper modifications of assay conditions, is high enough to make its determination a sensitive marker of platelet number in microwells.

The kinetics of adhesion in response to two well-known platelet agonists (ADP and thrombin) and the specificity of platelet adhesion to various substrates have been investigated.

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MATERIALS AND METHODS

Materials

p-Nitrophenyl phosphate, human fibrinogen (type I), and Triton X-100 were purchased from Sigma Chemical Company (St. Louis, MO), collagen from Menarini (Florence, Italy), ADP from Boehringer (Mannheim, Germany), thrombin from Calbiochem (La Jolla, CA), and purified human albumin from Behring Institut (Marburg, Germany). Dulbecco's phosphate-buffered saline (PBS)[1] was from Gibco Ltd. (Paisley, Scotland); the composition of PBS was 0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 0.047 g/liter $MgCl_2$, 8 g/liter NaCl, 1.15 g/liter Na_2HPO_4 (pH 7.4). Human plasma (pool from five to eight healthy subjects) was prepared from heparin–anticoagulated blood, centrifuged at 1300*g* for 15 min. Sterile 96-well microtiter plates with flat-bottom wells (Linbro type) were from Flow Laboratories. Other materials and reagents were of the highest purity available.

Microplate Coating

Preliminary experiments showed that platelets incubated in microplates spontaneously adhered to the plastic bottom of the wells, thus preventing the possibility of studying the cell adhesion and activation under specific conditions. Nonspecific adhesion was abolished by coating the microplate wells with suitable substrates. Coating was performed overnight at +4°C by adding 100 μl/well of one of the following solutions: (a) human plasma, diluted 1/1 in PBS; (b) 2 mg/ml human albumin, in PBS; (c) 2 mg/ml human fibrinogen, in PBS; or (d) 20 μg/ml collagen, diluted in 0.9% NaCl. Immediately before use, the plates were washed two times with 0.9% NaCl using an automatic plate washer (Easy Washer 2, SLT Labs Instruments).

Isolation of Platelets

Platelets were harvested from human blood by differential centrifugation (13). A final volume of 10 ml of blood was drawn by venipuncture in 1.66 ml of anticoagulant solution (15 g/liter citric acid, 20 g/liter dextrose, 25 g/liter sodium citrate), and platelet-rich plasma was obtained by centrifugation at 300*g* for 10 min. The platelet-rich plasma was recentrifuged at 700*g* for 15 min and platelets were gently suspended (5×10^7/ml) in a buffer composed of 145 mmol/liter NaCl, 5 mmol/liter KCl, 10 mmol/liter Hepes, 0.5 mmol/liter Na_2HPO_4, 6 mmol/liter glucose, and 0.2% human serum albumin, pH 7.4 (buffer A). The platelet suspension was kept at room temperature and utilized within 1 h. Ten minutes before use, platelets were warmed up to 37°C.

[1] Abbreviation used: PBS, phosphate-buffered saline.

Assay of Adhesion

The 96-well microtiter plates were prepared according to various schemes and combinations, depending on the test assay to be carried out (e.g., various incubation times or various concentrations of test compounds, etc.) and on the number of compounds to be tested. Assays were performed in triplicate or quadruplicate. Operatively, immediately after coating and washing, the wells were supplemented with 25 μl of the test agonist (3× the final desired concentration) in buffer A supplemented with 3 mM $CaCl_2$ and 3 mM $MgSO_4$. This was done because agonists, $CaCl_2$ and $MgSO_4$, are diluted three times in the final incubation mixture. The plate was then brought to 37°C, and 50 μl of the platelet suspension (2.5×10^5 platelets), prewarmed at 37°C, was added to each well using a multichannel pipet. The plate was incubated for the desired time under static conditions in humidified thermostat at 37°C.

At the end of incubation, the plate was transferred to the automatic washer (Easy Washer 2, SLT Labs Instruments) and subjected to two washing cycles with PBS at room temperature. Washing was carefully calibrated in order to obtain an optimal sensitivity and reproducibility of adhesion measurements. Each cycle was carried out as follows: aspiration, filling by gentle jet for 1.5 s, and, after about 30 s, aspiration. In preliminary experiments, washing was also performed manually by plate inversion followed by gentle shaking to remove residual droplets, dipping of the plate (held vertically) into PBS, emptying of the plate by inversion, dipping in a new PBS solution, and finally inversion and shaking to remove residual droplets. The results obtained with these two different washing procedures did not differ appreciably and the automatic washing procedure was consequently adopted.

After washing, the wells were rapidly supplemented with 150 μl of 0.1 M citrate buffer, pH 5.4, containing 5 mM *p*-nitrophenyl phosphate and 0.1% Triton X-100. This concentration of Triton X-100 caused instantaneous lysis of platelets and did not affect acid phosphatase activity. After incubation at room temperature for the indicated time (routinely: 60 min) the reaction was stopped and the color was developed by the addition of 100 μl of 2 N NaOH. The *p*-nitrophenol produced by the reaction was measured with a microplate reader (Reader 400, SLT Labs Instruments) at 405 nm against a platelet-free blank. The percentage of adherent cells was calculated on the basis of a standard curve obtained with known number of platelets.

Assay of Aggregation

Common methods for evaluating aggregation are based on the decrease of turbidity of a platelet suspension (14,15). A modification of this method, consisting of the measurement of turbidity at 620 nm directly into

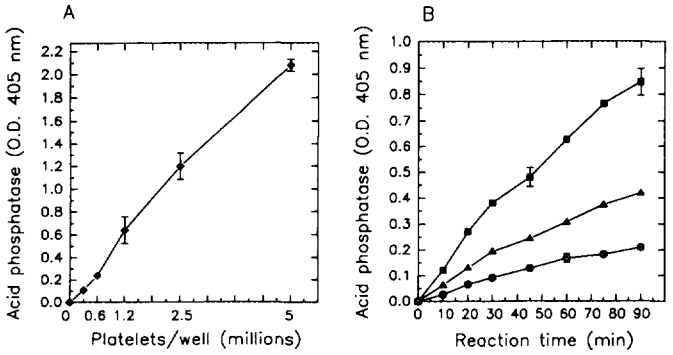

FIG. 1. Optimization of the platelet acid phosphatase assay. Twenty microliters of platelet suspensions containing the indicated cell number was dispensed in triplicate wells of uncoated plates and then the acid phosphatase activity was measured as O.D. increase at 405 nm as described under Materials and Methods. (A) Enzyme activity as a function of cell number (60 min of incubation). (B) Enzyme activity as a function of incubation time (■, 1.2×10^6 platelets/well; ▲, 6×10^5 platelets/well; ●, 3×10^6 platelets/well). Values are means ± SD of triplicates from a typical experiment.

microplates, allowed detection of platelet aggregation under conditions very similar to those used for the adhesion assay. The assays were carried out by using exactly the same incubation mixture used for the adhesion (see above), with the difference that a 4× final volume (300 μl instead of 75 μl) was used. This was done in order to reach a sensitivity high enough for the turbidimetric reading. The wells were supplemented with 100 μl of the test agonist (3× the final desired concentration) in buffer A supplemented with 3 mM $CaCl_2$ and 3 mM $MgSO_4$. After addition of 200 μl of the platelet suspension, the plate was incubated at 37°C and the decrease of absorbance at 620 nm was followed over time.

RESULTS

Optimization of Platelet Acid Phosphatase Assay

A set of experiments was preliminarly carried out to establish optimal conditions for the measurement of platelet number based on acid phosphatase activity. The absorbance of blanks was low (0.120–0.140 O.D. units), while in the presence of increasing numbers of cells the assay procedure gave net absorbance values in the range of 0.050 to 2.0 O.D. units, making the method highly sensitive (Fig. 1A). A linear relationship exists between optical density at 405 nm and cell number in the range between 3×10^5 and 5×10^6 platelets/well. Due to such sensitivity, the adhesion assays could be consequently performed by using 2.5×10^6 platelets/well, corresponding to 50 μl of a 5×10^7 platelets/ml suspension. Over 5 ml of this suspension, enough to perform assays utilizing all of the wells of a microtiter plate, may be harvested from a 8-ml blood sample with a normal platelet count.

The absorbance increase was linear up to at least 60 min (Fig. 1B), a time that was therefore adopted as optimal for the reaction. The time courses of enzyme reactions in citrate buffer were more linear than those in acetate buffer, previously used in the leukocyte adhesion assay (11). The optimum pH of the reaction was found to be 5.4 (data not shown).

The acid phosphatase activity of platelets isolated from a group of healthy subjects corresponded to 0.589 ± 0.120 (SD) O.D. units/60 min/10^6 platelets (n = 32, max 0.823, min 0.380).

Figure 2 shows that the enzyme activities of lysates from unstimulated platelets and ADP- and thrombin-activated platelets were similar. Moreover, when these lysates were subjected to centrifugation, most of the enzyme activity was recovered in the cell pellet, indicating that the enzyme is cell-bound and is not released following cell activation. Taken together, these experiments demonstrate that acid phosphatase activity of platelet lysates may be considered as a reproducible and reliable marker of platelet number.

Adhesion Kinetics and Dose Responses

Platelets incubated in stationary cultures progressively adhered to the surfaces of microwells and the adhesion was higher in the presence of ADP (Fig. 3). The time-course of adhesion was markedly influenced by

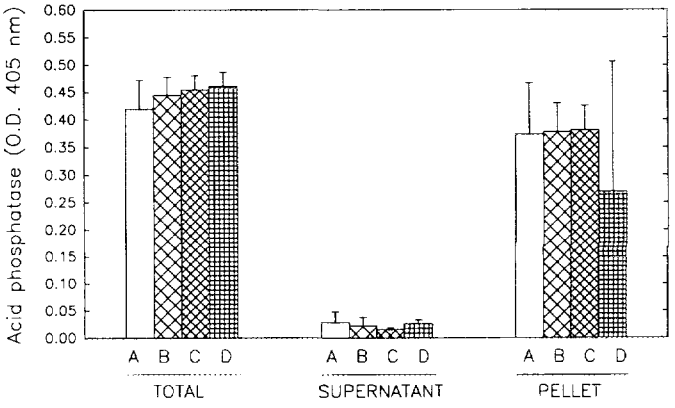

FIG. 2. Acid phosphatase activity in platelet lysates and distribution after centrifugation of resting and stimulated platelets. Twenty microliters of platelet suspensions (3.75×10^7/ml in buffer A containing 1 mM $CaCl_2$ and 1 mM $MgSO_4$), either unstimulated (A) or treated for 10 min with 1 μM ADP (B), 10 μM ADP (C), or 0.1 U/ml thrombin (D), was assayed for acid phosphatase activity as described under Materials and Methods (Total). Aliquots of the same suspensions were centrifuged at 3000g for 10 min and the cell pellets were resuspended in buffer A containing 1 mM $CaCl_2$ and 1 mM $MgSO_4$ at the same initial volume. Twenty microliters of supernatants and resuspended pellets were assayed for acid phosphatase activity as described under Materials and Methods. Values are means ± SD of quadruplicates from a typical experiment. Resuspended pellets from thrombin-stimulated platelets contained macroscopic aggregates and this explains the high variation of the data.

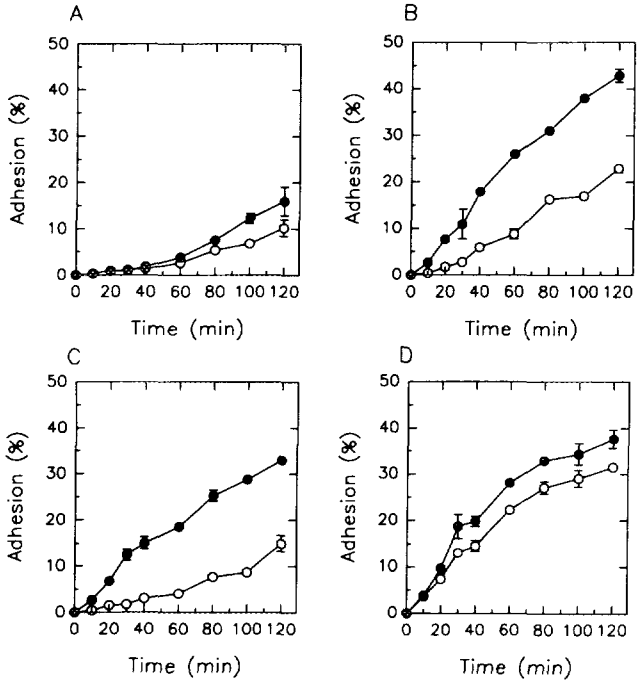

FIG. 3. Time course of adhesion of unstimulated and ADP-stimulated platelets to microplate wells coated with various compounds. Platelets were incubated for the indicated time in the absence (○) and in the presence (●) of 10 μM ADP in microwells coated with: (A) human albumin, (B) human fibrinogen, (C) human plasma, (D) collagen. Coating, plate washing, and acid phosphatase assay were performed as described under Materials and Methods. Values are means ± SD of triplicates from a typical experiment.

the type of coating used and by the cell activation, indicating that the adhesion response is specific for molecular membrane–substrate interactions. In particular, the data of Fig. 3 show that: (a) the adhesion to albumin-coated surfaces was almost undetectable, at least until 60 min of incubation; from 60 min onward a slight increase of adhesion, particularly in the presence of ADP, was observed; (b) the adhesion to fibrinogen was much greater and faster, exhibiting an almost linear trend, reaching a 40% adhesion after 120 min of incubation; the adhesion was markedly stimulated by ADP, the difference being mostly evident in the first 60 min of incubation; (c) the adhesion to plasma-coated surfaces was slightly lower than the adhesion to fibrinogen, the main difference regarding the adhesion of unstimulated cells, which was minimal over plasma until up to 60 min; therefore, under these conditions the effect of cell stimulation was particularly evident; (d) when the coating was performed with collagen, rapid and consistent platelet adhesion was observed even in the absence of agonists, and ADP increased adhesion by only a minor fraction. Similar time–courses of adhesion were obtained after stimulation of platelets with thrombin (0.1 U/ml) (not shown). On the basis of these results, incubation times between 40 and 60 min were chosen as optimal for sensitivity and detection of significant differences related to type of coating and cell activation.

Figure 4A shows the extent of adhesion to fibrinogen-coated wells of platelets incubated in the presence of increasing doses of ADP and thrombin. The adhesion response was clearly agonist dose-dependent.

It is well known that platelet activation under appropriate conditions is followed by aggregation and, as a matter of fact, aggregation stimulated by specific agonists represents the most widespread *in vitro* test for evaluating platelet function. It was therefore of interest to verify whether adhesion could be dissociated from aggregation in the assay system described here. Figure 4b shows that under these experimental conditions ADP induced platelet adhesion without any concomitant aggregation, while thrombin induced aggregation at doses comparable with the doses inducing adhesion. Microscopic examination clearly showed agonist-stimulated adhesion and spreading of platelets to the bottom of microplate wells (Fig. 5). In the absence of stimulants (Fig. 5A) and in the presence of ADP (Fig. 5B), platelets adhered as single cells and not as aggregates, only small

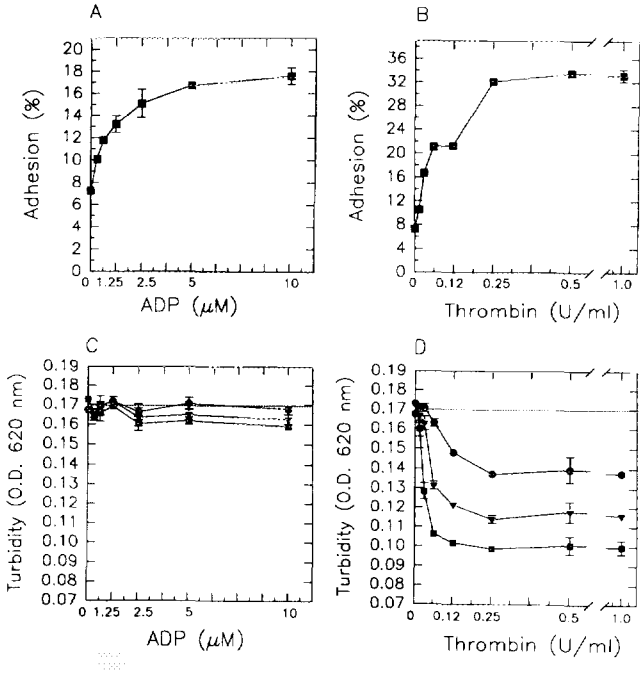

FIG. 4. Platelet adhesion and aggregation in dependence of increasing doses of ADP (A and C) and of thrombin (B and D). The incubation of platelets for adhesion assay (A and B) was carried out for 60 min on a fibrinogen-coated plate as described under Materials and Methods, and in parallel a plate containing a fourfold volume of platelet suspensions incubated in the corresponding experimental conditions was assayed for aggregation by turbidimetry at interval times after the addition of platelets (C and D). ●, 3 min; ▼, 30 min; ■, 60 min. Values are means ± SD of quadruplicates from a typical experiment.

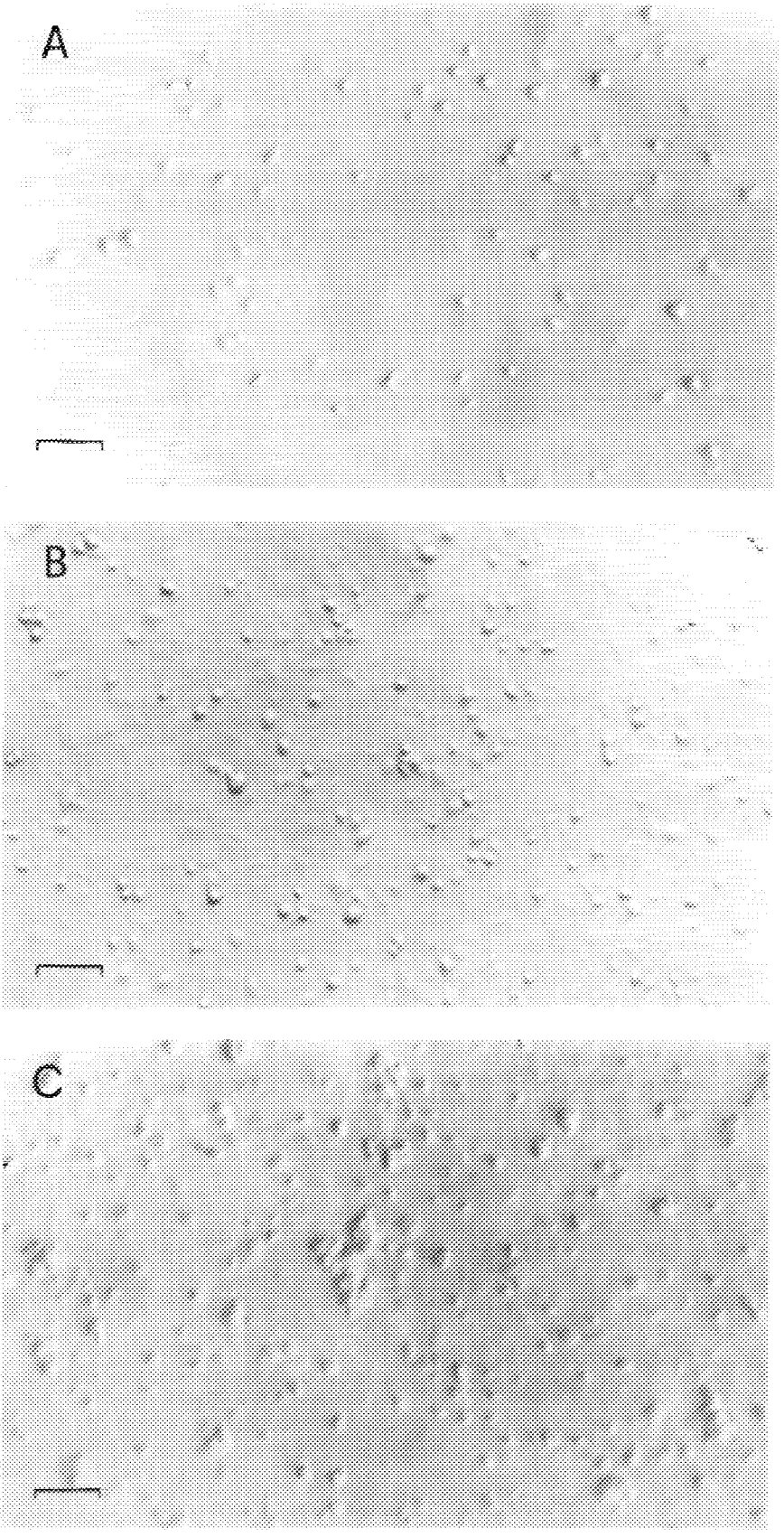

FIG. 5. Microscopic examination of adherent platelets. Platelets were incubated in human plasma-coated wells for 60 min in the absence of simulants (A), in the presence of 10 μM ADP (B) and 1 U/ml of thrombin (C), as described under Materials and Methods. After washing out nonadherent platelets, the wells were supplemented with 0.2 ml of PBS and examined with phase-contrast microscopy. Bar = 20 μm.

TABLE 1

Adhesion of Resting and ADP-Stimulated Human Platelets to Microplate Wells Coated with Various Compounds

	Stimulant		
Coating	None	1 μM ADP	10 μM ADP
Human plasma	2.7 ± 1.8	8.6 ± 3.8	16.9 ± 4.1
Fibrinogen	4.9 ± 2.6	13.3 ± 3.2	20.7 ± 2.6
Collagen	27.4 ± 3.4	28.5 ± 3.6	29.6 ± 3.8

Note. The values are from 25 healthy subjects assayed in separate experiments and are expressed as % adhesion/60 min of incubation ±SD.

and sporadic aggregates being detectable. With thrombin (Fig. 5C), platelet adhesion was also observed, but a higher contamination by small platelet aggregates was present.

Normal values of the adhesion of unstimulated and ADP-stimulated platelets to various substrates were established in a group of healthy human volunteers (Table 1). The adhesion response measured by this method was very consistent in different subjects and was substrate-sensitive and dose-dependent, allowing detection of intermediate states of platelet activation *in vitro*.

DISCUSSION

The aim of this study was to apply the basic methodology developed for studying leukocyte adhesion (11,12) to the quantitative evaluation of adhesion of platelets to various substrates in culture wells. Following suitable modifications, the acid phosphatase assay has proven to be a simple and sensitive method for the measurement of platelet mass and number. The activity of this enzyme is distributed in a narrow range in a sample of normal human subjects and, more important, is not affected by the functional state of the cell, thus allowing the evaluation of platelet number irrespective of whether the cells are resting or agonist-stimulated. Moreover, since acid phosphatase is not significantly released, the number of both resting and activated adherent platelets may be calculated with reference to a curve made with a known number of unstimulated platelets in suspension.

Cell adhesion was specific, since little or no cell attachment was obtained when plates were coated with human albumin, while time- and dose-dependent adhesion could be easily and consistently demonstrated by using fibrinogen and human plasma as coating agents. The fact that the spontaneous adhesion of unstimulated normal platelets to plasma-coated surfaces was very low might be important for the detection of small increases in adhesion, such as those induced by stimuli at low doses or possibly for the *ex vivo* detection of a partial platelet activation such as that described in vasculopathic and dislipidemic subjects (16–18). On collagen-coated surfaces, a marked time-dependent adhesion was observed even in the absence of agonists, in agreement with the fact that collagen receptors are constitutively expressed on the platelet membrane (19). These results suggest that the method is suitable for the study of the various complex phenomena involved in the functional expression and/or activation of platelet adhesion molecules.

Several different methods have been developed in order to study platelet–surface interactions. Some of them, such as those employing perfusion chambers and systems that mimic the conditions of laminar flowing blood (20–22), are undoubtedly the most informative, allowing investigation of platelet functions at shear rates throughout the physiological range. However, these methods require special equipment that is only available in specialized laboratories and present limitations when many subsequent assays and changes in experimental conditions have to be performed. Other approaches utilize adhesion to culture wells containing endothelial monolayers or coated with plasma proteins. These methods are based on principles similar to those of the method here described, but they measure the number of adherent platelets using different systems, such as direct counting, radioactive labeling, and protein assays (19,23–28). With respect to these approaches, our method presents the following advantages: (a) the use of radiolabeled (^{51}Cr, ^{111}In) platelets is more time-consuming and presents the problems of radioactivity handling; (b) others have measured adhesion as platelet-associated proteins after solubilization of adherent platelets (27,28), but these methods require the use of platelet suspensions which are about 10 times more concentrated than those used here, due to the lower sensitivity of protein assay with respect to the enzymatic assay; (c) counting of adherent platelets by light microscopy or fluorescent videomicroscopy (reviewed in Ref. 24) is very reliable and helps in distinguishing adhesion from aggregation (see below), but becomes highly time-consuming when applied to the evaluation of multiple samples such as in multiwell plate-based assays.

One of the main problems limiting the information obtained with methods of platelet adhesion is the fact that they do not differentiate between platelet–surface and platelet–platelet interactions associated with receptor-mediated activation. This is particularly true for tests using filtration through glass beads or glass wool filters, because retention of platelets in a column is clearly a product of both adhesion and aggregation (24). In our test and similar procedures based on adhesion to culture dishes, this problem is circumvented, because aggregates that may be present in suspension are removed by aspiration and washing. However, if any platelet aggregates bind to coated surfaces with strength suf-

ficient to resist the washing stress, they are counted as adherent platelets. For this reason, it has been suggested to use the term "platelet deposition" to indicate the number of platelets deposited, including all types of platelet–surface and platelet–platelet interactions, and the term "platelet adhesion" to indicate platelet–surface interaction under conditions which do not trigger platelet–platelet interaction, i.e., when nonthrombogenic surfaces are used or platelet aggregation is inhibited (21).

The assay system described in this work has been designed to study adhesion under conditions where the aggregation is highly discouraged: in fact, (a) the incubation is performed in stationary culture, while aggregation is facilitated by stirring which facilitates membrane contacts; (b) the platelets in the final incubation are present in a concentration of 3.75×10^7 platelets/ml, about one order of magnitude lower than the platelet number in blood, and it has been reported that *in vitro* aggregation is reduced when the count is below 10^8/ml (15); (c) the incubation buffer does not contain fibrinogen, which is known to act as a molecular "glue" for aggregation, bridging the gap between platelets. With stimuli such as thrombin and collagen the fibrinogen can be secreted from the platelet α-granules, but with stimuli such as ADP and epinephrine, which require aggregation before secretion occurs, fluid-phase fibrinogen must be present for aggregation (15). Our data demonstrate that, by using unstimulated and ADP-stimulated platelets on surfaces coated with fibrinogen and human plasma, the assay described here detects adhesion in the absence of aggregation. This conclusion is supported also by microscopic examination of resting and ADP-stimulated adherent platelets. On the other hand, in the presence of thrombin, a condition where a significant aggregation in the platelet suspension occurs, also small platelet aggregates were found to adhere to the bottom of microplate wells. These microaggregates do not affect overall binding of platelets to the surfaces, but they make the method less specific for adhesion when thrombin is used as the stimulatory agent.

The method is sensitive and versatile and could be useful for the study of the complex mechanisms involved in the activation and regulation of platelet functions. The assay may also have a number of applications in studies related to the diagnosis of clinically relevant platelet defects, in the identification and functional characterization of surface molecules that mediate adhesion, and in the investigation of the new anti-adhesion therapies.

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