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Relationships Between Optical Aggregometry (Type Born) and Flow Cytometry in Evaluating ADP-Induced Platelet Activation

Silverio Sbrana,[1]* Francesca Della Pina,[1] Antonio Rizza,[2] Manuela Buffa,[1] Rossella De Filippis,[1] Jacopo Gianetti,[1] and Aldo Clerico[3]

[1]Laboratory of Hematology and Flow Cytometry, CNR Institute of Clinical Physiology, Massa, Italy

[2]Cardiology and Hemodynamics Department, CNR Institute of Clinical Physiology, Massa, Italy

[3]Scuola Superiore S. Anna, Pisa, Italy

Background: Platelet response to activating agents is used to monitor the efficacy of anti-aggregation therapies. The aim of our study has been to demonstrate the existence of relationships between early events of ADP-induced platelet activation, measured by flow cytometry and platelet-rich plasma aggregation, quantified by optical aggregometry.

Methods: We evaluated peripheral blood of 12 donors. The following parameters were quantified by cytometry after stimulation with adenosine diphosphate (ADP) (0.5, 1, 2, 5, 10, 20 μM): CD62P (P-selectin) and PAC-1 expression, and cytosolic Ca^{2+} mobilization. Aggregation was measured by optical aggregometry. We also studied 13 patients, undergoing coronary stenting, treated with aspirin (before procedure) or with aspirin plus clopidogrel (after procedure). We evaluated CD62P and PAC-1 expression, aggregation, and vasodilator-stimulated phopshoprotein phosphorylation (platelet reactivity index, PRI).

Results: Flow procedures were more sensitive than aggregometry, with a lowest interindividual variability. Linear relationships existed in donors between CD62P expression and Ca^{2+} mobilization ($P < 0.0001$), and between aggregation and Ca^{2+} mobilization ($P < 0.0001$). Linear relationships existed between aggregation and CD62P expression, as percentage ($P < 0.0001$), or relative fluorescence intensity (RFI) ($P < 0.0001$). Exponential equations related aggregation and PAC-1 expression, as percentage ($P < 0.0001$), or RFI ($P < 0.0001$). Linear relationships between aggregation and CD62P expression (as percentage) existed in the patients before ($P = 0.0022$) and after procedure ($P = 0.0020$). Exponential relationships between aggregation and PAC-1 expression (as percentage) existed before ($P = 0.0012$) and after procedure ($P = 0.0024$). Linear correlations related aggregation response predicted on CD62P expression, and measured aggregation inhibition after clopidogrel ($P = 0.0013$) as well as predicted aggregation and PRI inhibition ($P = 0.0031$).

Conclusions: Tight relationships between aggregation and cytometric quantification of platelet markers in whole blood, in particular CD62P, allow to predict aggregation response to ADP from flow data in patients treated with aspirin alone or with aspirin plus clopidogrel.

Key terms: platelet; ADP; optical aggregometry; flow cytometry; anti-aggregation therapies

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Platelet aggregation represents the final step of a complex activating sequence in which the contact with a damaged endothelium and/or with soluble factors of the coagulation pathway triggers dramatic intracellular changes such as cytoskeleton rearrangement and shape change, release reaction of stored

*Correspondence to: Silverio Sbrana MD, PhD, CNR Institute of Clinical Physiology, "G.Pasquinucci" Hospital, 54100, Massa, Italy.

E-mail: sbrana@ifc.cnr.it

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granules, and membrane glycoproteins modifications (1–3).

Exogenous added adenosine diphosphate nucleotide (ADP) and ADP released from platelet dense bodies following activation bind two types of platelet G-protein coupled purinergic receptors ($P2Y_1$ and $P2Y_{12}$) (4,5). It appears that $P2Y_1$ has a role in the initiation of platelet activation, while $P2Y_{12}$ is essential for a sustained, full aggregation response to ADP (6). A reciprocal cross-talk between $P2Y_1$ and $P2Y_{12}$ has been also demonstrated at the level of calcium signaling in human platelets (7). The $P2Y_{12}$ receptor stimulation by ADP is inhibited by clopidogrel, a thienopyridine prodrug, which has to be metabolized in the liver to acquire its anti-platelet aggregation properties (8). The normal $P2Y_{12}$ stimulation by ADP determines the dephosphorylation of the vasodilator-stimulated phosphoprotein (VASP), an intracellular actin regulatory protein involved in platelet cytoskeletal rearrangement, and GPIIb-IIIa conformational changes. Conversely, inhibition of $P2Y_{12}$ receptor by clopidogrel induces phosphorylation of VASP (9). Flow cytometric quantification of intraplatelet VASP phosphorylation is now widely used to monitor the efficacy of clopidogrel therapy in ischemic cardiovascular diseases (10,11).

The aim of our study has been to demonstrate in healthy subjects the presence of relationships between the early events of ADP-induced platelet activation measured by flow cytometry, such as Ca^{2+} mobilization, α-granules release (CD62P expression), GPIIb-IIIa receptor conformational changes (PAC-1 expression), and the quantification of maximal platelet-rich plasma (PRP) aggregation by optical aggregometry (type Born). Moreover, we investigated the role of platelet activation markers (CD62P and PAC-1) in predicting the aggregation response to ADP in ischemic patients undergoing elective coronary stenting, and treated with aspirin alone (before procedure) or with a combination of aspirin plus clopidogrel (after procedure).

MATERIALS AND METHODS

Donors, Patients, and Specimens

Whole blood was obtained from 12 healthy adult volunteers (range 26–45 years, with a male/female ratio of 6/6) who had not taken medications or anti-platelet drugs for the preceding 2 weeks. To reduce artefactual platelet activation, blood samples were drawn from an antecubital vein using a 19-gauge needle, and the first 2.5 ml were discarded. All samples for flow cytometric and aggregometric evaluations were collected in Vacutainer tubes (Becton Dickinson, San Jose, CA) containing 3.8% trisodium-citrate solution as anticoagulant.

We also studied 13 patients who had ischemic disease and were undergoing elective coronary stenting. Patients' mean age was 71 years (range 58–85 years, with a male/female ratio of 8/5). Over the daily therapy with aspirin (100 mg/day), all patients started treatment with clopidogrel. In particular, they received a loading dose of clopidogrel (300 mg) immediately after stenting, followed by a regimen therapy of 75 mg/day. Patients' blood was drawn under therapy with aspirin alone (before procedure), and 48 h after stenting, under a combination therapy of aspirin and clopidogrel (after procedure).

The study protocol was approved by the local ethical committee, and informed consent was obtained from each subject before enrollment.

Data Acquisition and Analysis

Platelet count in PRP was carried out with the automated cell analyzer SF-3000 (SYSMEX, Kobe, Japan). Flow cytometry was performed with a FACScan instrument and CellQuest software (Becton Dickinson). Overtime monitoring of instrumental performance was carried out with CaliBRITE beads (Becton Dickinson). Flow data analysis of platelet surface molecules and intracellular calcium mobilization was based on measurement of positive events (percentage), or on their relative fluorescence intensity (RFI) quantification, using the statistic function of overlaid and subtracted histograms (12). The presented net values of data (percentages or RFI) concerning CD62P and PAC-1 surface staining were calculated after subtracting the values measured in resting samples from that obtained following ADP activation. This calculation excludes that the level of platelet activation observed after addition of exogenous ADP depends also by ADP released from erythrocytes.

No spontaneous Ca^{2+} mobilization or PRP aggregation was observed in resting condition (addition of PBS alone).

Aggregometry was performed with a dual channel optical aggregation system (CHRONO-LOG series 490, Havertown, PA), equipped with an infra red light beam and the AGGRO/LINK software (CHRONO-LOG).

Sample Preparation for Flow Cytometric Analysis

Platelet activation markers

CD62P. Five microliter samples of 3.8% trisodium-citrate anticoagulated blood were gently suspended in 30 μl of N2-hydroxy-ethyl-piperazine-N′-2-ethanesulfonic acid (HEPES)-buffered phosphate saline (PBS) solution, containing appropriate volumes of fluorescein isothiocyanate (FITC) CD41a (Becton Dickinson) and phycoerythrin (PE) conjugated CD62P (Pharmingen, San Diego, CA) monoclonal antibodies, together with different final concentrations of ADP (0.5, 1, 2, 5, 10, 20 μM) (code number A-2754) (Sigma Chemical, St. Louis, MO). A resting sample was also performed to determine the spontaneous level of CD62P expression. All samples were incubated for 8 min at room temperature (RT) in the dark. For each activation point, a mouse isotype control was carried out in parallel. At the end, the samples were diluted and fixed with 500 μl of ice-cold 1.0% formaldehyde solution in PBS, and maintained on ice until analyzed.

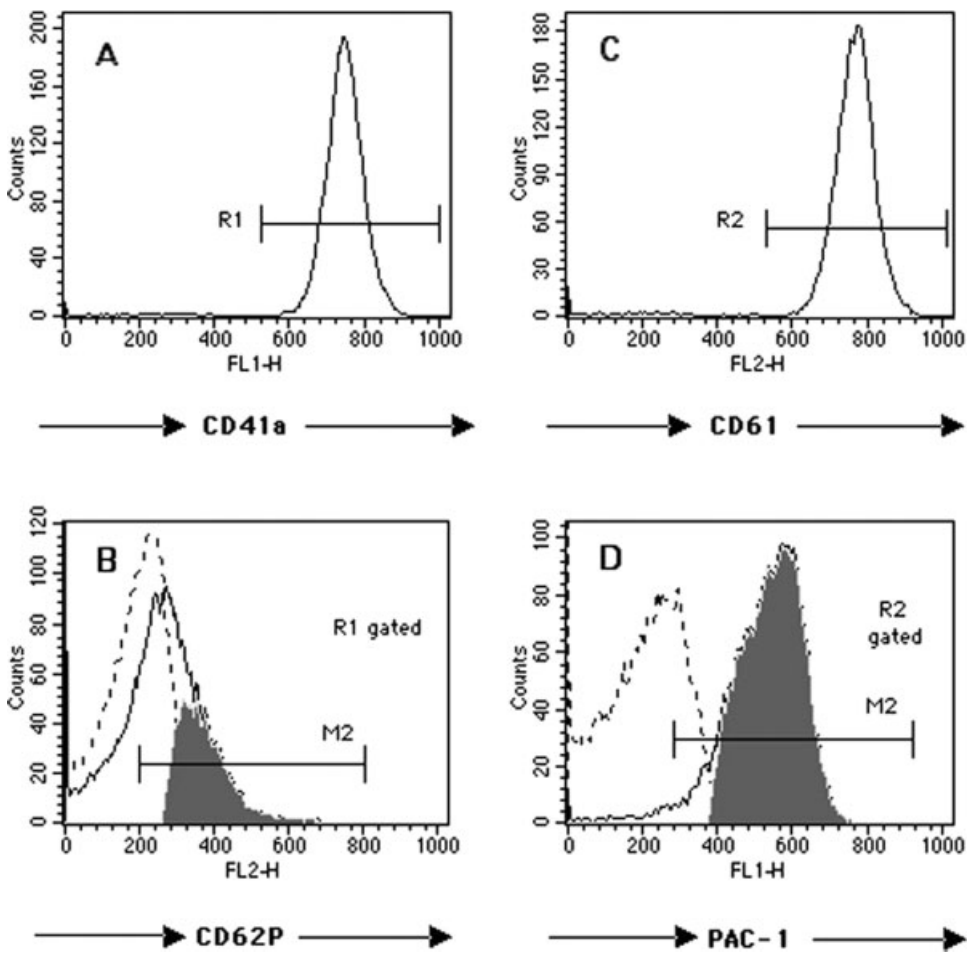

FIG. 1. Representative example of ADP-induced platelet activation (0.5 μM) in whole blood sample. The platelets, previously clustered by a dual-parameter dot plots of side versus forward scatter, both collected in four-decade log scales, are subsequently identified on the basis of their bright CD41a (FL1; **A**) and CD61 (FL2; **C**) fluorescence intensities. The overlaid histograms subtraction (test histogram–control histogram) (**B**, **D**) is used to quantify the percentages of CD62P and PAC-1 positive platelets (events in M2, filled histograms) and their RFI (linear scale) with respect to the corresponding isotype controls (dotted histograms).

PAC-1. Whole blood samples were treated according to the manufacturer's instructions, as already described (13). In brief, 5 μl of trisodium-citrate anticoagulated blood were gently suspended in a mixture containing appropriate volume of CD61 (PE; Immunotech, Beckman Coulter, Fullerton, CA) and PAC-1 (FITC; Becton Dickinson) monoclonal antibodies, together with different final concentrations of ADP (0.5, 1, 2, 5, 10, 20 μM) (Sigma Chemical). A resting sample was also performed to determine the spontaneous level of PAC-1 expression. All samples were incubated for 8 min at RT in the dark. For each activation point, a mouse isotype control was carried out in parallel. Then, the samples were diluted and fixed with 500 μl of ice-cold 1.0% formaldehyde solution in PBS, and maintained on ice until flow analysis.

For both staining procedures, the acquisition process was stopped after 20,000 platelets gated morphologically by a dual-parameter plot of four-decade log scales of side versus forward scatter, or on the basis of their CD41a (FL1) and CD61 (FL2) bright fluorescence intensities were collected for each sample (Fig. 1).

Kinetic measurement of intracellular platelet Ca^{2+} mobilization. Blood samples were treated according to the flow cytometric procedures already described (14), with some modifications. In brief, 100 μl of trisodium-citrate anticoagulated whole blood, diluted 1:10 in Krebs–Ringer bicarbonate buffer (K 4002) (Sigma Chemical) modified by addition of sodium bicarbonate ($NaHCO_3$) 1.26 g/l, bovine serum albumin 0.35%, and HEPES 10 mM were loaded with the fluorescent calcium indicator Fluo-4, AM (Molecular Probes, Eugene, OR) (5 μM final) by incubating for 30 min at 37°C in the dark. To assist the dispersion in aqueous media of the nonpolar tracer Fluo-4 and reduce the leakage from platelets of its de-esterified form, we supplemented the staining buffer with the nonionic surfactant Pluronic®F-127 (Molecular Probes) (0.02% final) and with the anion-transport inhibitor probenecid (2.5 mM final), as already described (15–17). At the end, the loaded suspension was gently resuspended and 25 μl of diluted blood were incubated with 5 μl of CD41a (PE) (Pharmingen) monoclonal antibody, for 15 min at RT in the dark, to identify platelet population. Then, stained samples were further diluted with 1 ml of modified buffer, and aliquots of 500 μl, maintained at RT in the dark, were used to determine kinetic variations of intracellular Ca^{2+}. After an initial acquisition of 25 s of baseline at low speed, in which the instruments aspirates 5 μl of suspension, we added to

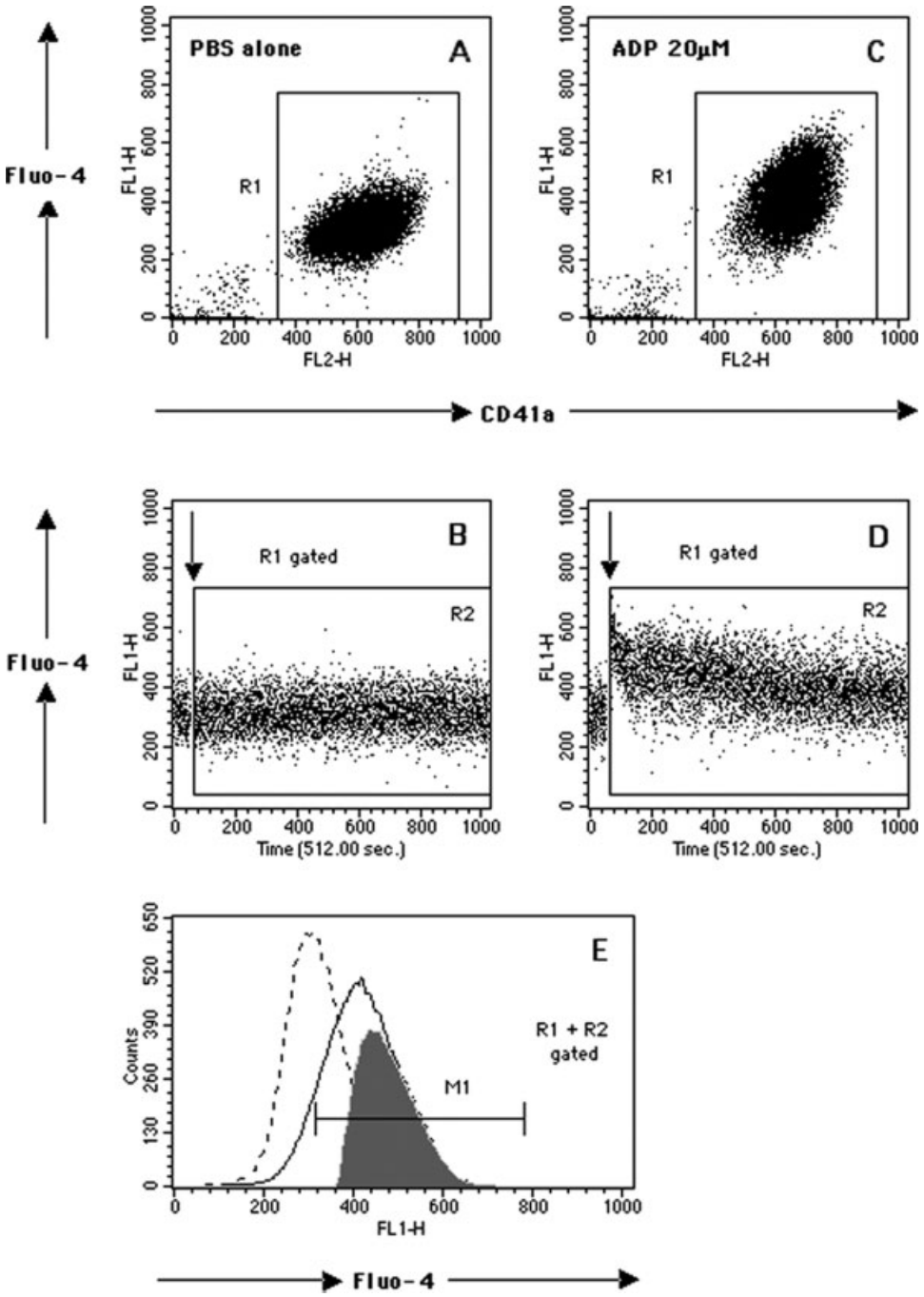

FIG. 2. Representative example of ADP (20 µM)-induced intracellular platelet Ca^{2+} mobilization in whole blood sample. The Fluo-4 loaded platelets, previously clustered by a dual-parameter dot plots of side versus forward scatter, both collected in four-decade log scales, are subsequently identified on the basis of their bright CD41a (FL2; **A** and **C**) fluorescence intensity. The dual-parameter dot-plots of FL1 versus time show the changes over-time in Fluo-4 fluorescence intensity after stimulation (arrow) with ADP (20 µM final) (**D**) or with an irrelevant stimulus (PBS alone) (**B**). In the overlaid histograms subtraction of test histogram–control histogram (*R*1 + *R*2 gated) (**E**), the percentage of ADP-responding platelets and their RFI (events in M1, filled histogram) are compared with the PBS-stimulation (dotted histogram), over 8 min.

each sample 5 µl of appropriate ADP working solutions, to obtain final agonist concentrations of 0.5, 1, 2, 5, 10, 20 µM. The entire acquisition process was stopped after about 8 min starting from ADP addition (Fig. 2).

Platelet VASP phosphorylation. Intracellular platelet VASP phosporylation level was measured in the patients using the dedicated *PLT* VASP/P2Y12 kit (Biocytex, Marseille, France). The staining procedure and the calculation of the platelet reactivity index (PRI) were performed according to the manufacturer's protocol.

Plasma Samples and Aggregometry

PRP was prepared by slow centrifugation at 100*g* for 15 min, at RT. The remaining blood was recentrifuged at 2400*g* for 20 min, at RT, to obtain Platelet-Poor Plasma. Appropriate ADP working solutions in PBS were prepared to reach the scheduled final concentrations of agonist (0.5, 1, 2, 5, 10, 20 µM). The aggregation process was monitored over 8 min starting from agonist addition (50 µl of working solution) to stirred PRP ali-

Table 1
Coefficients of Variation (C.V.) of Flow Cytometric Procedures

	CD62P	PAC-1	Ca^{2+}
% Positive events	1.9%	0.3%	2.9%
RFI	2.4%	1.7%	2.4%

RFI, relative fluorescence intensity.

quots (450 μl) into glass tubes preheated to 37°C. Before each measurement, the aggregometer was calibrated at 100% of transmittance, according to the manufacturer's instructions.

Statistical Analysis

Mean values of measurements and their standard errors of the mean, as well as the relationships among the different groups of data, were determined with the StatView 5.0 software (SAS Institute, Cary, NC). The choice of relationships between platelet functional parameters or between platelet functional parameter and PRP aggregation was always based on the evaluation of best statistical significance characterizing the fitting curve. $P < 0.05$ was considered statistically significant.

RESULTS

The accuracy of flow cytometric procedures (coefficients of variation, C.V.) was determined on blood from one healthy donor by 10 repeated measurements following platelet activation with ADP 5 μM (Table 1).

Also optical aggregometry exhibited a good reproducibility at the same activatory conditions, with a C.V. of 6.7%.

As shown in Figure 3, flow cytometric procedures performed on blood from healthy donors, in particular PAC-1 expression, are more sensitive (higher percentage of response) than optical aggregometry in evaluating platelet activation induced by low concentrations of ADP (0.5 and 1 μM). Moreover, flow procedures reach a plateau of response more rapidly than aggregometry. Finally, optical aggregometric response exhibits a larger interindividual variability, starting from ADP 1 μM, when compared with flow cytometric protocols.

The relationship observed in healthy subjects between CD62P surface expression (percentage of positivity) and platelet Ca^{2+} mobilization (percentage of responding platelets) (Fig. 4A), following activation with increasing ADP concentrations, is described by the equation:

$$y = 2.568x - 47.423 \quad (1)$$

with $R^2 = 0.9899$ and $P < 0.0001$.

The linear regression existing between PRP maximal aggregation and platelet Ca^{2+} mobilization (Fig. 4B) is described by the equation:

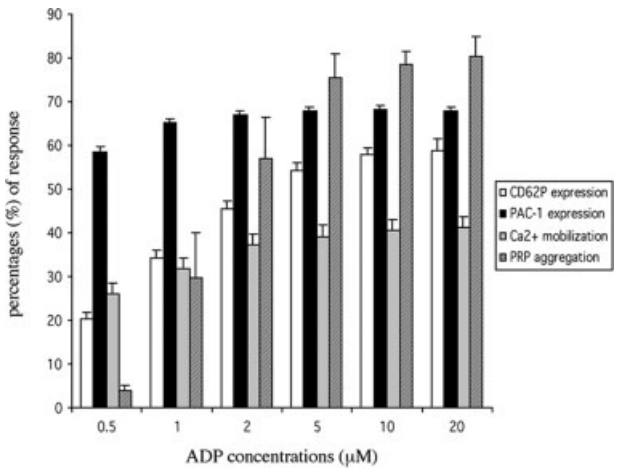

FIG. 3. Graph showing the differences observed between flow cytometry and optical aggregometry in quantifying the percentages of responding platelets following ADP-induced activation. The reported percentages (net values) of CD62P and PAC-1 positive events are calculated after subtracting the values measured in resting samples. Moreover, no Ca^{2+} mobilization or PRP aggregation was observed using an unrelated stimulus (PBS alone).

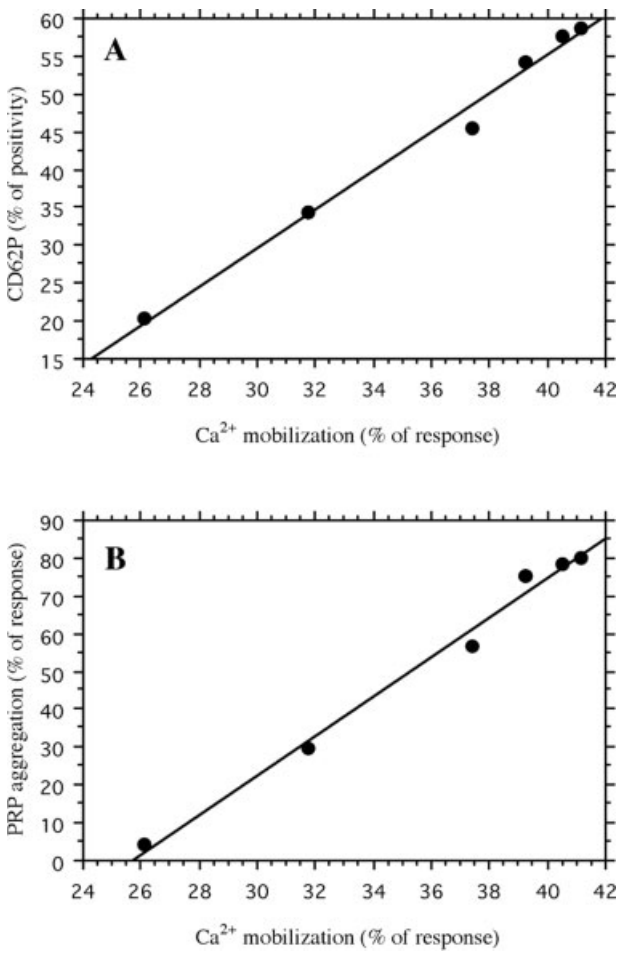

FIG. 4. Relationships between platelet Ca^{2+} mobilization and CD62P expression (**A**) as well as between platelet Ca^{2+} mobilization and PRP aggregation (**B**), after stimulation with different concentrations of ADP (0.5, 1, 2, 5, 10, 20 μM). Each point represents the mean values of 12 independent experiments. See the text for the corresponding equations and statistical significance.

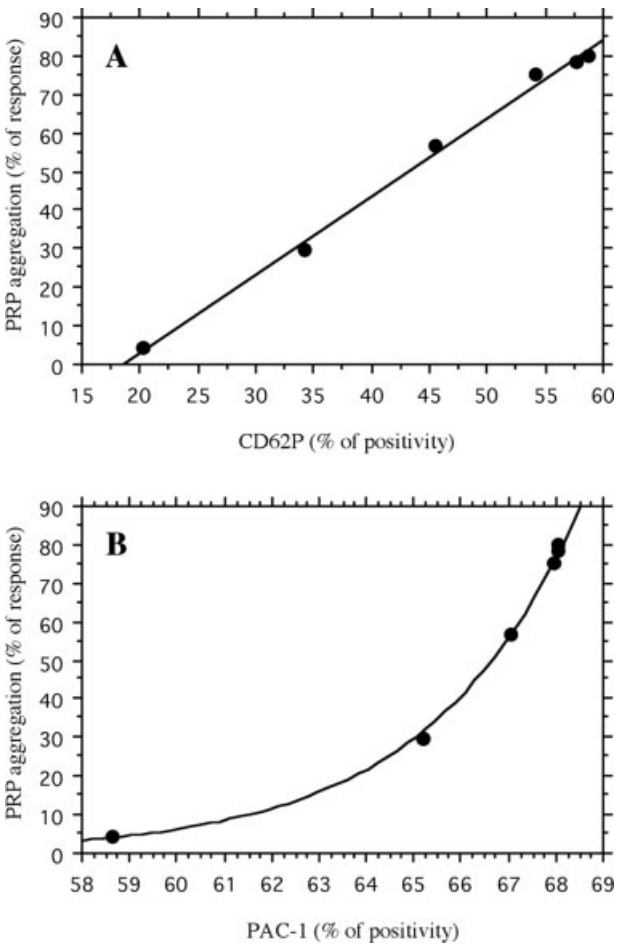

FIG. 5. Relationships between CD62P expression and PRP aggregation (**A**) as well as between PAC-1 expression and PRP aggregation (**B**), after stimulation with different concentrations of ADP (0.5, 1, 2, 5, 10, 20 μM). Each point represents the mean values of twelve independent experiments. See the text for the corresponding equations and statistical significance.

$$y = 5.229x - 134.372 \quad (2)$$

with $R^2 = 0.9903$ and $P < 0.0001$.

A linear regression characterizes also the relationship between PRP maximal aggregation and CD62P surface expression (percentage of positivity) as follows (Fig. 5A):

$$y = 2.031x - 37.556 \quad (3)$$

with $R^2 = 0.9953$ and $P < 0.0001$, while PRP maximal aggregation and PAC-1 surface expression (percentage of positivity) are related by the following exponential function (Fig. 5B):

$$y = e^{0.315x} \cdot 3.891 \cdot 10^{-8} \quad (4)$$

with $P < 0.0001$.

Similarly, when comparing PRP maximal aggregation and CD62P RFI (Fig. 6A), we obtain a linear regression:

$$y = 1.196x - 44.672 \quad (5)$$

with $R^2 = 0.9893$ and $P < 0.0001$, while an exponential function relates PRP maximal aggregation and PAC-1 RFI (Fig. 6B):

$$y = e^{0.05x} \cdot 2.0 \cdot 10^{-3} \quad (6)$$

with $P < 0.0001$.

The relationships found in healthy donors between the mean expressions of platelet activation markers (CD62P and PAC-1) and the mean values of PRP maximal aggregation, in response to different ADP concentrations, seem to be maintained also in patients treated with aspirin alone or a combination of aspirin plus clopidogrel.

In fact, linear regressions relate PRP maximal aggregation and CD62P surface expression (percentage of posi-

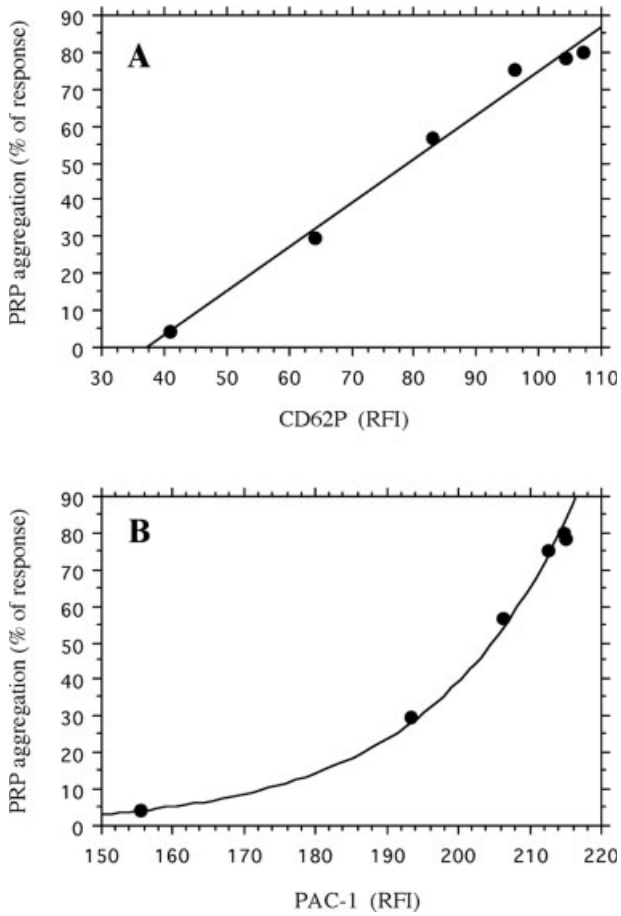

FIG. 6. Relationships between CD62P RFI and PRP aggregation (**A**) as well as between PAC-1 RFI and PRP aggregation (**B**), after stimulation with different concentrations of ADP (0.5, 1, 2, 5, 10, 20 μM). Each point represents the mean values of twelve independent experiments. See the text for the corresponding equations and statistical significance.

tivity), under treatment with aspirine alone, or with aspirin plus clopidogrel respectively, as follows:

$$y = 2.787x - 105.199 \quad (7)$$

with $R^2 = 0.996$ and $P = 0.0022$, and

$$y = 2.012x - 39.231 \quad (8)$$

with $R^2 = 0.996$ and $P = 0.0020$.

Similarly, significant linear relationships are observed when comparing PRP maximal aggregation and CD62P RFI (data not shown).

On the other hand, exponential relationships relate PRP maximal aggregation and PAC-1 surface expression (percentage of positivity), under treatment with aspirine alone, or with aspirin plus clopidogrel respectively, as follows:

$$y = e^{0.535x} \cdot 3.554 \cdot 10^{-13} \quad (9)$$

with $P = 0.0012$, and

$$y = e^{0.285x} \cdot 3.408 \cdot 10^{-6} \quad (10)$$

with $P = 0.0024$.

Similarly, significant exponential relationships are observed when comparing PRP maximal aggregation and PAC-1 RFI (data not shown).

The before- and after-procedure mean levels of fibrinogen were 285.1 ± 24.1 and 314.14 ± 18.17 mg/dl, respectively, while the corresponding mean platelet counts in the PRP (number of cells per microliter) were 316,419 ± 25,865 and 327,019 ± 22,166, respectively.

The before- and after-procedure mean values of the VASP-P PRI (as percentage) were 79.243 ± 1.531 and 60.554 ± 4.342, respectively.

DISCUSSION

In the present study we applied flow cytometric protocols and optical aggregometry (type Born) to evaluation of blood from healthy subjects, to establish eventual mathematical relationships between early events of platelet activation and PRP aggregation, after stimulation with different concentrations of ADP.

Moreover, we extended the evaluation to a group of ischemic patients under two different settings of antiplatelet aggregation, with aspirin alone or with a combination of aspirin plus clopidogrel, respectively.

Our preliminary results from donors showed the existence of a linear relationship between the earliest event of platelet activation, represented by Ca^{2+} mobilization from internal stores, and the release reaction of α-granules, indirectly quantified as surface expression (percentage) of the P-selectin molecule (CD62P) [Eq. (1)]. Taking into account that the granules release reaction is directly dependent on cytoskeleton rearrangement induced by Ca^{2+} mobilization, the Eqs. (2), (3), and (5) seem to confirm that platelet aggregation relates linearly with modifications of actin filaments and cytoplasmic Ca^{2+} variations upon activation with ADP. The simultaneous evaluation of PRP aggregation and flow cytometric measurement of platelet filamentous actin (F-actin) content (18,19) could confirm this hypothesis.

Moreover, platelet PAC-1 surface expression related with cytoplasmic Ca^{2+} variations by statistically significant polynomial relationships (data not shown), as percentage of positivity ($P = 0.0008$) or RFI ($P = 0.0076$), and reached its maximum at concentration of ADP 20 μM, with a saturation-like kinetics. Probably, this aspect represents the resulting phenomenon of epitopes conformational changes (2), receptor clustering (20), as well as ADP dose-dependent receptor internalization (21).

Exponential relationships [Eqs. (4) and (6)] were observed between PRP aggregation and platelet PAC-1 expression, as percentage or RFI.

At present, a combined anti-platelet aggregation therapy with aspirin plus clopidogrel is widely used in coronary ischemic patients to prevent acute thrombosis and stent restenosis (22–24).

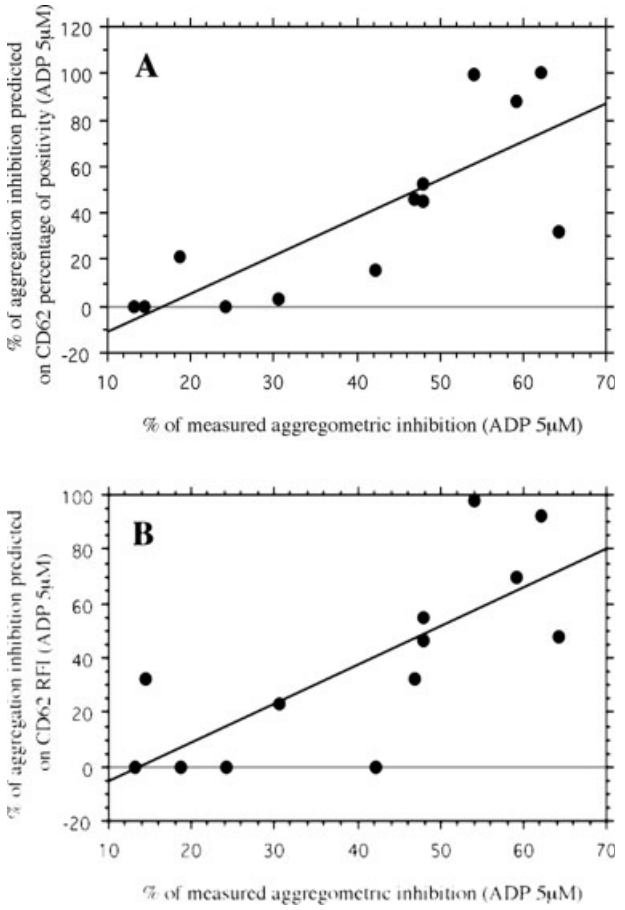

FIG. 7. Linear correlations between the actually measured percentages of aggregation inhibition (ADP 5 μM) and that predicted using the relationships based on CD62P percentage of positivity (**A**, $R^2 = 0.627$, $P = 0.0013$) or RFI (**B**, $R^2 = 0.577$, $P = 0.0026$).

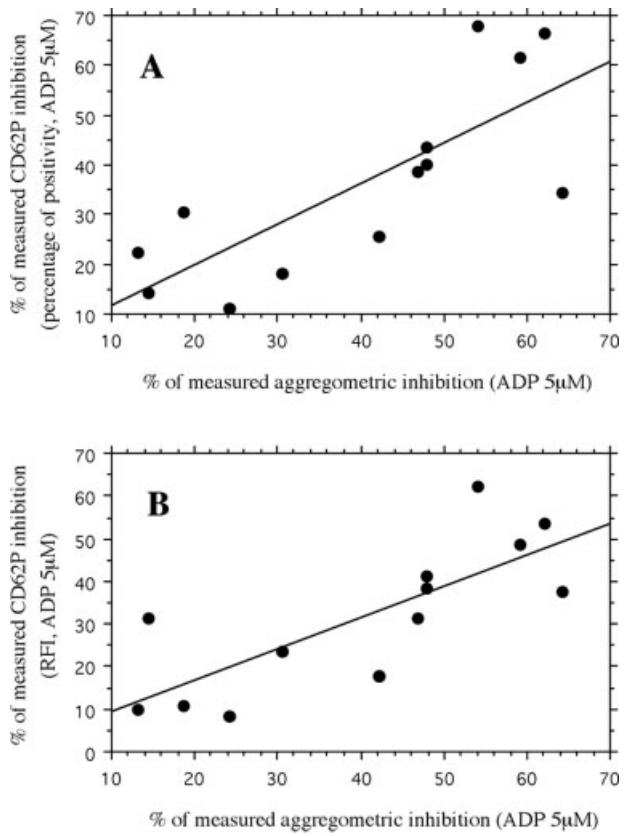

FIG. 8. Linear correlations between the actually measured percentages of aggregation inhibition (ADP 5 μM) and the individually measured variations (%) of CD62P percentage of positivity (**A**, $R^2 = 0.602$, $P = 0.0018$) or RFI (**B**, $R^2 = 0.614$, $P = 0.0015$).

In both the clinical settings of our patients, we observed that the relationships between the expression of platelet activation markers and PRP aggregation in response to ADP were similar to that observed in healthy subjects. This demonstrates the persistence of conserved patterns of platelet response, and suggests the possibility to use flow data to predict aggregometric response under therapies with platelet ADP receptors inhibitory drugs.

We found that the highest percentage of PRP aggregation inhibition (mean value = 40.193%), after clopidogrel administration, was obtained with ADP 5 μM.

Therefore, on the basis of the above indicated relationships obtained before and after procedure, we used flow cytometric data, actually obtained from each patients at this ADP concentration (5 μM), to verify their capacity to predict aggregation inhibition.

Significant correlations existed between the percentages of aggregation inhibition, predicted on the basis of the CD62P expression relationships (as percentage of positivity or RFI) obtained before and after procedure, and the actually measured aggregation inhibitions (Fig. 7).

These correlations were similar to that obtained using the percentages of CD62P expression inhibition (as percentage of positivity or RFI) actually measured for each patient before and after procedure (Fig. 8).

Linear correlations existed also between the percentages of aggregation inhibition, predicted on the basis of the CD62P percentage of positivity ($P = 0.0031$, $R^2 = 0.563$) or RFI ($P = 0.0018$, $R^2 = 0.602$), and the down-modulation of the VASP-P PRI.

Moreover, linear correlations were observed between the percentages of CD62P expression inhibition (as percentage of positivity) ($P = 0.0025$, $R^2 = 0.579$) or RFI ($P = 0.005$, $R^2 = 0.527$), and the VASP-P PRI actually measured for each patient before and after procedure.

On the contrary, the exponential relationships, since lowering and expanding the values corresponding respectively to the lowest and to the highest measurements of the PAC-1 expression range (as percentage, or RFI), did not show a significant correspondence between the predicted and the actually measured aggregometric values (data not shown). Also linear and semi-logarithmic transformations were unable to establish a significant correspondence (data not shown).

Moreover, the individual flow cytometric PAC-1 expression down modulation (as percentage of positivity or RFI) was not significantly correlated with the actually observed aggregation inhibition (data not shown).

On the other hand, the individual flow cytometric PAC-1 expression down modulation correlated signifi-

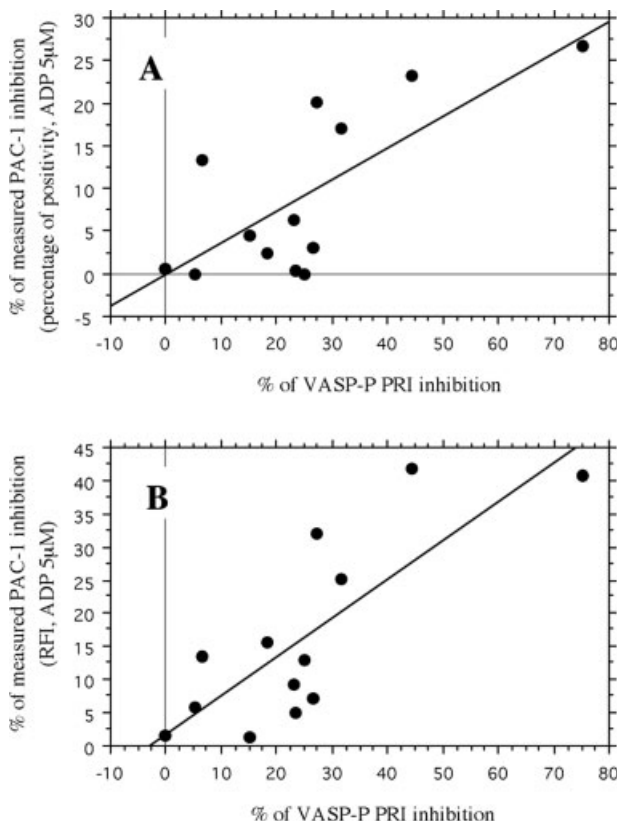

FIG. 9. Linear correlations between the actually measured VASP-P PRI inhibition (%) and the individually measured variations (%) of PAC-1 percentage of positivity (**A**, $R^2 = 0.539$, $P = 0.0043$) or RFI (**B**, $R^2 = 0.635$, $P = 0.0011$).

cantly with the individual VASP-P PRI inhibition, as percentage of positivity or RFI (Fig. 9).

Interestingly, only a weak correspondence ($P = 0.0699$) existed for each patient between the actually measured aggregation inhibition (ADP 5 μM) and the before and after procedure down-modulation of the VASP-P PRI. This observation brings new insights to the conclusions reported in a recent paper (25), were a clear correlation between VASP-P assay and light-transmission aggregometry has been found in in vitro experiments performed on blood from young healthy subjects.

If the association of aspirin and clopidogrel is supposed to ameliorate the efficacy of the antiplatelet aggregation regimens in ischemic patients, our data suggest that the evaluation of platelet cytoskeleton modifications in response to ADP represents an important functional aspect to predict the efficacy of treatment. In particular, the changes of the CD62P molecule expression, linearly correlated with intra-platelet Ca^{2+} levels, seem to be predictive of the aggregometric response to ADP of patients undergoing therapy with $P2Y_{12}$ inhibitory drugs.

In fact, the significant relationships observed between the CD62P-based predicted aggregometric variations and the down modulation of VASP-P PRI indicates that the expression of this molecule is able to reflect simultaneously the global platelet cytoskeletal modifications in response to ADP, or the individual clopidogrel-induced $P2Y_{12}$ receptor signaling inhibition.

These preliminary data relating CD62P expression and PRP aggregation should be extended on a more wide number of patients, accordingly with our settings of therapy. Moreover, the used ADP concentrations should be further subdivided in more closed intervals, to determine accurate and always valid relationships during therapy with aspirin alone or with aspirin plus clopidogrel. Finally, it should be interesting to evaluate if the above described relationships can be improved using impedance-based whole blood platelet aggregation methods that, as reported in the literature, offer a higher sensitivity for anti-platelet drug effects than optical aggregometry (26,27).

On the contrary, the complex relationships between PAC-1 expression and intra-platelet Ca^{2+} modifications as well as between PAC-1 expression and platelet aggregation make the modulation of this marker ineffective in predicting the aggregation response to ADP. The significant correspondence found between the individual PAC-1 expression inhibition and VASP-P PRI down-modulation suggest that this activation marker can be used only to monitor the level of inhibition of the $P2Y_{12}$ receptor intraplatelet signaling induced by clopidogrel.

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