Journal of the American College of Cardiology Vol. 46, No. 4, 2005

Published by Elsevier Inc.
ISSN 0735-1097/05/$30.00
doi:10.1016/j.jacc.2005.02.092



# Matching the Evaluation of the Clinical Efficacy of Clopidogrel to Platelet Function Tests Relevant to the Biological Properties of the Drug

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**OBJECTIVES** This study aimed to explore platelet function tests relevant to the biological effects of clopidogrel that could help the clinical monitoring of drug efficacy.

**BACKGROUND** Clopidogrel selectively inhibits the $P2Y_{12}$ receptor, the major role of which is stabilization of aggregation, whereas initiation of aggregation depends on activity of both $P2Y_1$ and $P2Y_{12}$ receptors.

**METHODS** Tests used were peak aggregation ($Agg_{max}$) and late aggregation ($Agg_{6min}$), and disaggregation, relating to $P2Y_1$ and $P2Y_{12}$ activity, respectively; and monoclonal antibody binding activated glycoprotein (GP) IIb/IIIa receptors (PAC-1) and P-selectin, measuring activation and secretion. A first study compared hirudin/PPACK (r-hirudin and D-phenylalanyl-prolyl-arginine chloromethyl ketone) with citrate as blood anticoagulant (16 patients), and a second control study compared the effects of clopidogrel, aspirin, or both (20 normal controls).

**RESULTS** Clopidogrel similarly inhibited adenosine 5′-diphosphate (ADP)-induced $Agg_{max}$ with either anticoagulant, but significantly more $Agg_{6min}$ (75% vs. 31%), P-selectin (72% vs. 53%), and PAC-1 (62% vs. 24%) in hirudin/PPACK. In the control study, it inhibited $Agg_{max}$ by 22%, and $Agg_{6min}$, P-selectin, and PAC-1, by 69%, 66%, and 55%, respectively (all $p < 0.05$). Disaggregation at six min reached 62% with clopidogrel, but was virtually absent with placebo and aspirin. Non-responsiveness as evaluated by inhibition of $Agg_{max}$ in citrate was diagnosed in 35% of patients; in half this rate by $Agg_{6min}$, P-selectin, and PAC-1; and in 6% to 12% with the latter tests performed in hirudin/PPACK.

**CONCLUSIONS** The evaluation of clopidogrel responsiveness by platelet function tests is largely influenced by the choice of blood preservative and functional tests. Measures of aggregation stabilization, and of consequent secretion and activation, identified most patients as responders, contrasting with measures of peak aggregation, by likely reflecting better the interactions clopidogrel and the $P2Y_{12}$ receptor. (J Am Coll Cardiol 2005;46:638–45) © 2005 by the American College of Cardiology Foundation

Clopidogrel is a thienopyridine that specifically inhibits the purinergic $P2Y_{12}$ receptors. The drug is recommended as alternative therapy to aspirin in the secondary prevention of cardiovascular events and in combination with aspirin for patients with an acute coronary syndrome or undergoing stent implantation. It is currently investigated as adjunct therapy to fibrinolysis in acute myocardial infarction and in the prevention of thromboembolic events in high-risk patients. Despite its growing use, a high degree of inter-individual variability is described in response to clopidogrel, with non-response in a substantial number of patients (1–3). Considering the potentially serious consequences of such poor response, many have expressed a need for a platelet function tests that could permit reliable evaluation of clinical efficacy (4). This need is reinforced by the results of a few small studies that described a higher risk of events in patients whose condition responded poorly (5–7). These results, however, and the risk gradient associated with a poor response, remain to be validated in larger datasets and prospective studies. Most studies to date have defined clopidogrel resistance as an inadequate inhibition of peak adenosine 5′-diphosphate (ADP)-induced ex vivo platelet aggregation in blood sampled in sodium citrate (2,5,6,8,9). These methods, although measuring the activity of platelet purinergic receptors, may have drawbacks because clopidogrel, and other thienopyridines as well, blocks the purinergic $P2Y_{12}$ receptor but not the $P2Y_1$ receptor; the former is mainly involved in stabilization of aggregates (10), and the latter in initiation of platelet shape changes and of platelet aggregation. On the other hand, although ADP is considered a weak platelet agonist, a number of direct or indirect pharmacodynamic effects have been described with clopidogrel that could be useful to the clinical monitoring of drug responsiveness (11,12).

See page 646

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Manuscript received November 15, 2004; revised manuscript received February 3, 2005, accepted February 8, 2005.

**Abbreviations and Acronyms**

| | |
|---|---|
| ADP | = adenosine 5′-diphosphate |
| $Agg_{6min}$ | = late aggregation |
| $Agg_{max}$ | = peak aggregation |
| ASA | = acetylsalicylic acid |
| GP | = glycoprotein |
| hirudin/PPACK | = r-hirudin and D-phenylalanyl-prolyl-arginine chloromethyl ketone |
| PAC-1 | = monoclonal antibody binding activated glycoprotein IIb/IIIa receptors |
| TRAP | = thrombin receptor agonist peptide |

This study explored issues related to the ex vivo evaluation of clopidogrel efficacy and various platelet function tests with the goal of identifying a test that could eventually be clinically useful. Tests related to platelet aggregation, activation, secretion, and disaggregation after initial aggregation, the latter reflecting better the activity of the $P2Y_{12}$ receptor than that of the $P2Y_1$ receptor, were compared in blood preserved with either citrate or antithrombins.

## METHODS

**Study populations and designs.** Two separate studies were performed after being approved by the Montreal Heart Institute Internal Ethics Committee and were carried out in compliance with the Declaration of Helsinki's recommendations. All participants signed an informed consent form before inclusion. Individuals with a contraindication to antiplatelet therapy or using a drug other than aspirin that interferes with platelet function or coagulation were excluded.

The first study was designed to characterize the influence on platelet function tests of using antithrombins as an anticoagulant in place of sodium citrate. This study was open labeled and included 16 patients with stable angina already using aspirin 80 mg daily and scheduled for an angioplasty on an outpatient basis. Clopidogrel at a daily maintenance dose of 75 mg was initiated seven days before the planned procedure. The first blood sample was obtained immediately before the initiation of clopidogrel, and the second before the angioplasty procedure.

The goal of the second study was to describe the effects of clopidogrel on different platelet function tests; a randomized, double-blind, placebo-controlled design was used. The study included 20 patients partitioned in one of three parallel groups (Fig. 1). Blood samplings were obtained 24 h apart in a fasting state on three consecutive days. The research nurse administered the study drugs immediately after the baseline sampling on day 1, and after the second sampling on day 2. The loading dose of clopidogrel was 300 mg and the maintenance dose was 75 mg, and those of aspirin were 300 mg and 80 mg, respectively. Subjects in the first group received the loading dose of clopidogrel and placebo-aspirin on the first day, and a maintenance dose of clopidogrel and the loading dose of aspirin on the second day. In the second group, they received on day 1 placebo-clopidogrel and the loading dose of aspirin, and on day 2 the loading dose of clopidogrel and the maintenance dose of aspirin. In the third group, placebo-clopidogrel and placebo-aspirin were used on both the day 1 and day 2 (Fig. 1).

**Platelet aggregation studies.** For the first study, blood was collected from a forearm vein and withdrawn into two tubes, one containing sodium citrate 3.8% (Becton Dickinson, Mississauga, Ontario), and the second containing hirudin 2 U/ml and D-phenylalanyl-prolyl-arginine chloromethyl ketone (PPACK) 40 μmol/l (Calbiochem, La Jolla, California). Based on results from the first study, only the hirudin/PPACK tube was used in the second randomized study.

The same instrumentation and methodology was otherwise used for the two studies, except for different concentrations of the two agonists used. In the first study, ADP and a thrombin receptor activating peptide-6 (TRAP-6, Sigma Chemical Co., Oakville, Ontario, Canada) were used each at a higher concentration of 10 μmol/l to better differentiate the effects of citrate and of the antithrombins; in the second study, the agonists were used at lower concentrations of 2.5 μmol/l to more selectively study the effects of ADP on dependent receptors and pathways.

Platelet aggregation was measured by light transmission (model 570VS with AggroLink software package, Chronolog Corp., Havertown, Pennsylvania) in stirred (1,000 rpm) platelet-rich plasma (PRP). The platelet-rich plasma was prepared by the centrifugation from whole blood at 130 *g* for 10 min and platelet-poor plasma by the centrifugation of the remaining blood at 1,800 *g* for 15 min. The platelet count was adjusted to 250,000 platelets/μl with platelet-poor plasma; platelet-rich plasma and platelet-poor plasma were used to set the light transmission to 0% and 100%, respectively. Aggregation was measured at peak ($Agg_{max}$), which is seen approximately 1 min after the addition of the agonists, and at the end of the test after 6 min ($Agg_{6min}$). Inhibition of aggregation was calculated as the percent decrease in aggregation values obtained at baseline and on treatment. A percentage of disaggregation (D) between $Agg_{max}$ values and $Agg_{6min}$ was calculated as: $D\ (\%) = 100 \cdot (1 - Agg_{6min}/Agg_{max})$. Figure 2 illustrates a series of

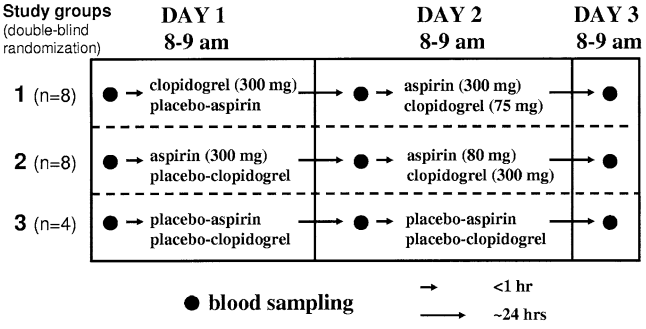


**Figure 1.** Design of the double-blind placebo-controlled randomized study performed in normal volunteers (Study 2).

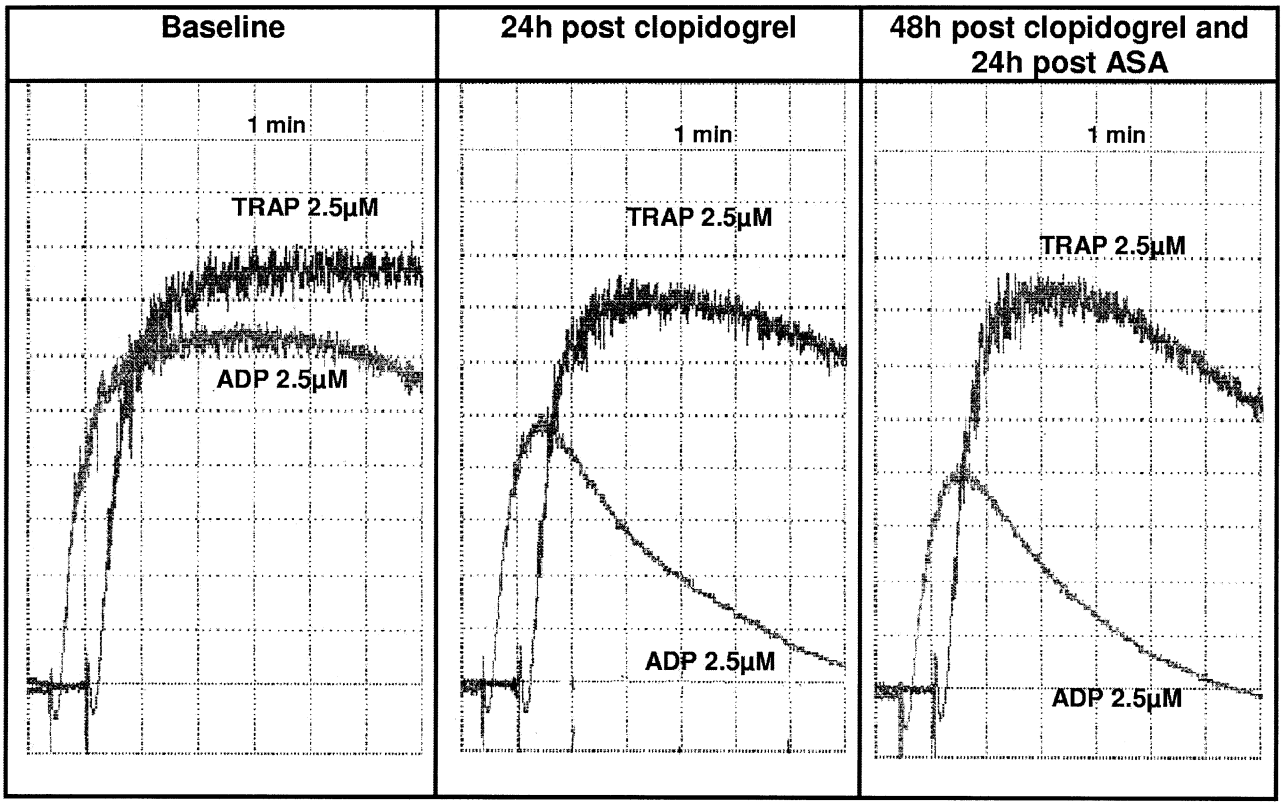


**Figure 2.** Aggregation curves from a normal individual, obtained after the addition of adenosine 5′-diphosphate (ADP) 2.5 $\mu$M and thrombin receptor agonist peptide (TRAP) 2.5 $\mu$M in platelet-rich plasma at baseline **(left)**; 24 h after a loading dose of clopidogrel **(middle)**; and 24 h later, after a dose of clopidogrel 75 mg and aspirin (ASA) 300 mg **(right)**. Disintegration is minimal at baseline and nearly complete after clopidogrel in the presence of ADP and significant in the presence of TRAP. Aspirin added no additional disintegration.

aggregation curves obtained on three different days in one normal individual from Group 1 of the randomized study.

**Platelet secretion and activation.** Platelet membrane expression of P-selectin, secreted from the $\alpha$-granules, and of monoclonal antibody binding activated glycoprotein (GP) IIb/IIIa receptors (PAC-1), a monoclonal antibody that selectively binds the GP IIb/IIIa receptor when activated, was quantified in whole blood by flow cytometry. They serve as measures of platelet secretion and activation, respectively. The hirudin/PPACK blood was diluted in a 1:5 ratio within 15 min of being drawn with a modified Tyrode buffer solution (TBS) (containing NaCl, 137 mM; KCl, 2.8 mM; MgCl2, $6H_2O$, 1 mM; $NaHCO_3$, 12 mM; $Na_2HPO_4$, 0.4 mM; bovine serum albumin, 0.35%; HEPES, 10 mM; and glucose, 5.5 mM; pH, 7.4); 13 $\mu$l diluted blood was divided and poured into Eppendorf tubes containing saturating concentrations of antibodies and 12 $\mu$l ADP 100 $\mu$M (Sigma Chemical Co.) dissolved in Tyrode buffer solution to a final concentration of 10 $\mu$M. The samples were incubated with the antibodies without stirring for 30 min at room temperature, and adding 500 $\mu$l of formaldehyde 1% stopped further reaction. The antibodies used were PAC-1, a fluorescein isothiocyanate-conjugated immunoglobulin M polyclonal antibody that specifically binds the activated GP IIb/IIIa receptors, and anti-CD 62P, a phycoerythrine-conjugated murine monoclonal antibody immunoglobulin $G_1$ directed against platelet membrane P-selectin (Becton Dickinson, San Jose, California). Flow cytometer readings were done within one h of blood sampling on a total of 5,000 platelets per sample. Forward scatter identified the platelet population for cell size and side scatter for cell granularity. The percentage of fluorescence-positive platelets was obtained in duplicate from the instrument computer system (Beckman Coulter Epics XL, Miami, Florida).

**Statistical analysis.** Because most data were non-normally distributed, non-parametric methods were used and data are expressed as median and interquartile range; 5th and 95th percentiles are also shown in the figures. Statistical analyses were performed with the SPSS 10.0 for Windows statistical software (SPSS Inc., Chicago, Illinois). Paired comparisons of aggregation, and P-selectin and PAC-1 expression values were done with the Wilcoxon paired test, and unpaired comparisons were done with the Mann-Whitney and Kruskal-Wallis tests. Correlations were evaluated using Spearman non-parametric rho coefficients. The response to clopidogrel was assessed using chi-square tests by referring to the criteria published by Lau et al. (8), i.e., $<$10% relative inhibition being non-response; 10% to 30%, poor response, and $>$30%, good response. These criteria were also applied to quantify P-selectin and PAC-1 expression. The level of significance for two-sided hypothesis tests was set at a p

value >0.05; no correction factors were introduced for multiple analyses.

## RESULTS

**Study populations and platelet parameters.** Sixteen patients (1 woman, 15 men; median age, 70 years; range, 59 to 75 years) were enrolled in the first study, and 20 normal volunteers (9 women, 11 men; median age, 42 years; range, 36 to 46 years) in the randomized study. No significant differences in clinical characteristics emerged between the three groups in the latter study. Basal whole blood platelet counts were similar in patients and controls, as were counts in the serial samples obtained throughout the course of the two studies. Results of platelet function tests were in general little influenced by the concentration of 10 $\mu$M used in the first study and of 2.5 $\mu$M in the second study: peak aggregations were 65% and 70%; percent inhibitions by clopidogrel were 31% and 22%; PAC-1 binding, 62% and 65%; P-selectin expression, 42% and 42%; and disaggregation rates, 4% and 1.4%. No controls and no patients showed a second aggregation wave.

The responses to ADP and TRAP at baseline were also the same in the three parallel groups of the randomized study, and were highly reproducible on the three consecutive days' sample among individuals who were only administered the placebo.

**Influence of hirudin/PPACK and of citrate.** In the basal state, the usage of either hirudin/PPACK or citrate as a blood preservative did not significantly influence measures of the agonist effects of ADP and of TRAP on aggregation and disaggregation rates and on P-selectin expression. However, PAC-1 binding was reduced in response to ADP ($p$ = NS) and to TRAP ($p < 0.01$) in hirudin/PPACK.

With clopidogrel, the inhibition of peak aggregation was similar in antithrombins and in citrate, but the inhibition of late aggregation, P-selectin expression, and PAC-1 binding in response to ADP was significantly higher in antithrombins. Figure 3 illustrates the distribution of the individual data points. Thus, a modest 20% inhibition (−6% to 38%) in peak ADP-induced aggregation was observed in citrate, whereas an important 75% (54% to 94%) inhibition of late aggregation occurred in hirudin/PPACK plasma. Similarly, the inhibition of TRAP- induced late aggregation and PAC-1 binding was slightly although non-significantly greater in hirudin/PPACK than in citrate (Fig. 3). Moreover, although no difference was measured at baseline (1% vs. 4%; $p = 0.6$), disaggregation of ADP-aggregated platelets by clopidogrel was greatly enhanced in analyses performed in hirudin/PPACK compared with citrate (66% vs. 29%; $p = 0.001$).

**Effects of aspirin in healthy donors (randomized study).** Aspirin administered alone or with clopidogrel had no detectable effects on peak and late aggregation to ADP and TRAP-6. Aspirin increased ADP- and TRAP-induced P-selectin expression by 3% ($p = 0.03$) and 5% ($p = 0.04$), respectively, and TRAP-induced PAC-1 binding by 8% ($p = 0.01$). These activations, however, were prevented with the pre-administration of clopidogrel.

**Effects of clopidogrel.** Clopidogrel inhibited ADP-induced peak aggregation by a median of 22% ($p = 0.01$) and TRAP-induced aggregation by 7% (NS). By contrast, as seen in Table 1, late aggregations to ADP and TRAP were markedly more reduced with clopidogrel by 69% ($p <$

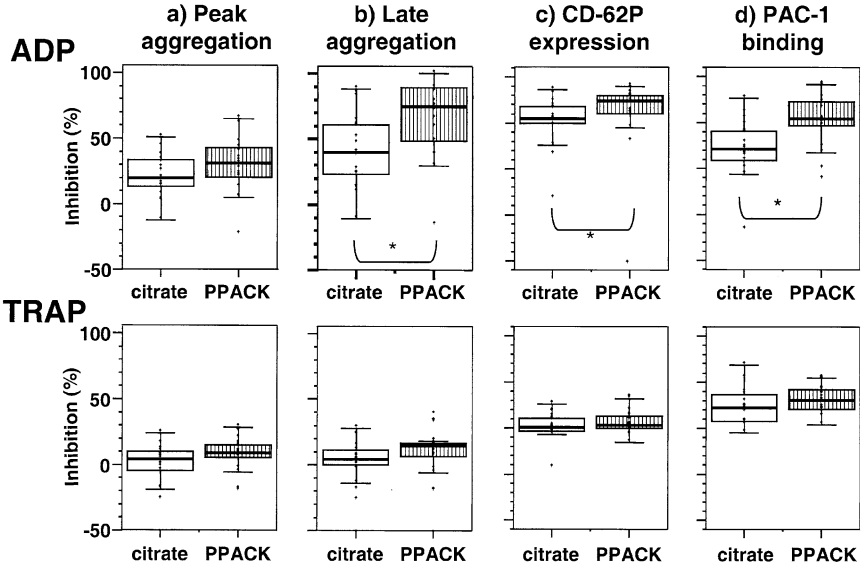


**Figure 3.** Inhibition of platelet functions in platelet-rich plasma prepared in sodium citrate **(open boxes)** versus r-hirudin and D-phenylalanyl-prolyl-arginine chloromethyl ketone (hirudin/PPACK) **(boxes with vertical lines)** (*$p < 0.05$). **(a)** Peak aggregation, **(b)** late aggregation, **(c)** P-selectin expression, **(d)** glycoprotein (GP) IIb/IIIa activation by monoclonal antibody binding activated GP IIb/IIIa receptors (PAC-1) measure after agonist stimulation with either a thrombin receptor activating peptide-6 **(left panels)** or adenosine 5′diphosphate (ADP) **(right panels)**. **Horizontal lines** are medians, **boxes** are the interquartile range, **whiskers** are the 5th and 95th percentiles, and p values were obtained using the Wilcoxon signed-rank test; *$p < 0.05$.

**Table 1.** Platelet Function Tests in PRP Before and After Clopidogrel (CLOP) and/or Acetylsalicylic Acid (ASA)

| | Baseline | | | | 24 h After First Dosing | | | | 48 h After Second Dosing | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Agonist Treatment | $Agg_{max}$ | $Agg_{6min}$ | PAC | Psel | $Agg_{max}$ | $Agg_{6min}$ | PAC | PSel | $Agg_{max}$ | $Agg_{6min}$ | PAC | Psel |
| ADP | | | | | | | | | | | | |
| CLOP-ASA (n = 8) | 68 (64–75) | 66 (60–75) | 56 (49–68) | 36 (25–55) | 54† (50–60) | 22*† (1–34) | 23† (13–31) | 7† (3–24) | 51 (35–61) | 16*† (0–33) | 20† (12–32) | 9† (3–24) |
| ASA-CLOP (n = 8) | 70 (64–80) | 69* (57–78) | 73 (46–82) | 45 (37–49) | 70 (66–72) | 70* (62–70) | 68 (59–80) | 48† (45–61) | 54‡ (43–68) | 21*† (1–60) | 33‡ (14–58) | 22‡ (6–35) |
| Placebo (n = 4) | 76 (70–77) | 74 (66–77) | 75 (69–83) | 46 (28–72) | 74 (68–80) | 71 (65–78) | 72 (67–81) | 48 (33–70) | 74 (69–79) | 72 (65–79) | 78 (69–81) | 45 (31–73) |
| TRAP | | | | | | | | | | | | |
| CLOP-ASA (n = 8) | 76 (70–81) | 76 (67–81) | 50 (37–79) | 63 (44–88) | 69 (61–73) | 61*† (36–67) | 28† (5–37) | 53 (20–74) | 69 (59–74) | 56*† (31–68) | 26 (7–41) | 57 (26–76) |
| ASA-CLOP (n = 8) | 84 (70–81) | 80* (72–87) | 55 (42–59) | 71 (56–76) | 74 (70–80) | 73 (64–80) | 63† (55–68) | 76† (66–86) | 71 (61–78) | 56*‡ (35–73) | 35‡ (8–45) | 52‡ (40–70) |
| Placebo (n = 4) | 81 (75–87) | 81 (75–87) | 77 (74–81) | 86 (78–89) | 81 (76–86) | 81 (76–85) | 76 (69–79) | 87 (82–91) | 86 (80–90) | 86 (78–90) | 76 (73–80) | 87 (82–91) |

Randomized study in normal volunteers with design is summarized in Figure 1. Values are median and 25th and 75th percentiles; p values were assessed by Wilcoxon test. *$p < 0.05$ versus $Agg_{max}$; †$p < 0.05$ versus baseline; ‡$p < 0.05$ versus 24 h.
ADP = adenosine 5′-diphosphate; $Agg_{max}$ = peak aggregation; $Agg_{6min}$ = late aggregation; PAC-1 = monoclonal antibody labeling the activate glycoprotein IIb/IIIa receptor; Psel = membrane P-selectin expression measured with an anti-CD62-P monoclonal antibody; TRAP = thrombin receptor agonist peptide.

0.001) and 29% (p = 0.001), respectively, because of rates of disaggregation from peak to 6 min of 62% (36% to 98%; p = 0.01) for ADP and of 11% (10% to 41%; p = 0.01) for TRAP. Disaggregation was virtually absent with the placebo, 1% (0% to 9%; p = 0.7) and 0% (0% to 1%; p = 0.3) respectively, and with aspirin, 2% (0% to 9%; p = 0.5) and 0 % (0% to 8%; p = 0.6). Figure 2 illustrates the aggregation curves of a representative case, and Figure 4 shows the data in each normal individual.

The P-selectin expression with ADP and TRAP was reduced by 66% (p = 0.01) and 22% (p = 0.04), respectively, and PAC-1 binding by 55% (p < 0.001) and 54% (p < 0.001). The magnitudes of all of these inhibition rates were similar regardless of whether aspirin was administered after (Group 1) or before (Group 2) clopidogrel (Table 1).

While on clopidogrel, strong correlations existed between inhibition of aggregation at 6 min and both P-selectin expression (r = 0.7, p = 0.001) and PAC binding (r = 0.6, p = 0.02), and also between the latter two (r = 0.8, p < 0.001).

**Clopidogrel resistance.** The application of the conventional criteria of responsiveness to clopidogrel based on inhibition of peak aggregation measured in platelet-rich plasma prepared in citrate divided our population of stable patients into approximately one-third responders, one-third poor responders, and one-third non-responders (Table 2). When peak aggregation was measured in blood with the antithrombins, twice as many patients were responders and three times fewer patients were non-responders. Similar shifts in distribution were observed, on the one hand, with late aggregation, P-selectin, and PAC-1 measured in hirudin/PPACK compared with citrate, and on the other hand, in late aggregation compared with peak aggregation, both being measured in hirudin/PPACK. Thus, measurements of late aggregation, P-selectin, and PAC-1 in hirudin/PPACK identified 94%, 82%, and 77% of patients respectively as responders, and 6%, 6%, and 12% as non-responders. Furthermore, the demarcation between responders and non-responders was also more clearly delineated, with fewer patients being borderline poor responders (Table 2).

Similar response rates were observed in the randomized study of normal volunteers in which only hirudin/PPACK was used as an anticoagulant. With the loading dose of 300 mg clopidogrel, measures of peak aggregation, late aggregation, P-selectin, and PAC-1 resulted in non-response rates of 25%, 6%, 0% and 6%, respectively.

## DISCUSSION

This study shows high degrees of inter-individual variability in the response to clopidogrel and high rates of non-response, comparable with those reported in the literature, when using the conventional approach of measuring inhibition of ADP-induced peak aggregation in citrated

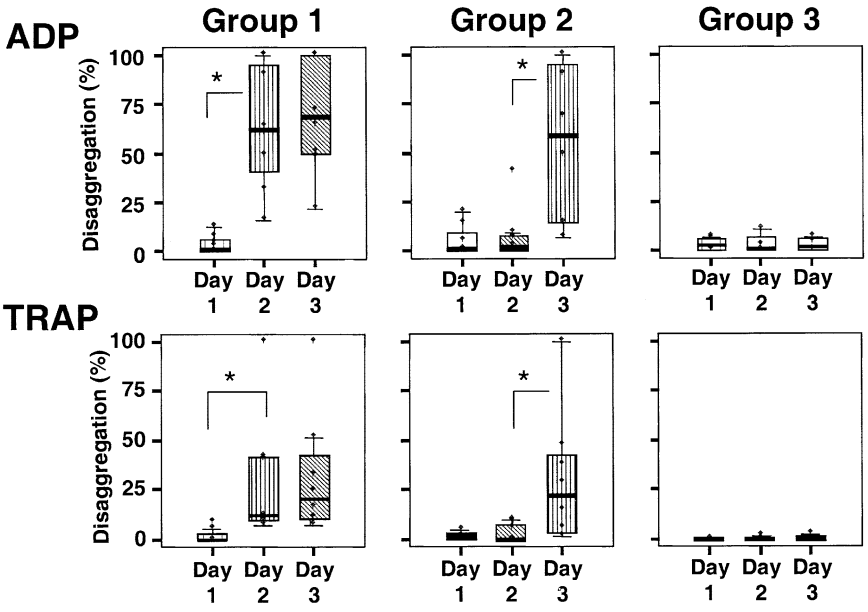


**Figure 4.** Percent disaggregation from peak aggregation to six min after the addition of adenosine 5′-diphosphate (ADP) **(top)** and thrombin receptor agonist peptide (TRAP) **(bottom)** in healthy volunteers, at baseline on Day 1, 24 h after the first drug administration on Day 2, and 24 h after the last drug administration on Day 3. **Open boxes** are either at baseline or after placebo, **boxes with diagonal lines** are after aspirin, **boxes with vertical lines** are after clopidogrel (doses shown in Fig. 1, and an actual tracing in Fig. 2). The **horizontal lines** are medians, the **boxes** are the interquartile range, **whiskers** are the 5th and 95th percentiles, and p values were obtained by the Wilcoxon signed-rank test; *$p < 0.05$.

platelet-rich plasma. Based on such results, a concept of clopidogrel resistance was elaborated (2,5,13,14). Our results, however, challenge the significance of such a concept based on a simple test of aggregation that is influenced by activity of both the $P2Y_1$ and $P2Y_{12}$ purinergic receptors, whereas clopidogrel blocks only the latter. Indeed, a much lower rate of variability was found by looking at stabilization of aggregation, measured by disaggregation and late aggregation, which is mainly driven by the activity of $P2Y_{12}$ receptor, and at markers of platelet secretion and activation. The variability was further reduced when these tests were performed in blood preserved with antithrombins rather than citrate. Combining these approaches, the non-response rate was reduced from 35% to 6%.

**Hirudin/PPACK versus sodium citrate.** Contrasting with other antiplatelet drugs, no studies to our knowledge have directly compared the use of an antithrombin versus citrate in measuring the effects of clopidogrel (15). Hirudin/PPACK in this study provided better detection of the inhibitory effects of clopidogrel on disaggregation, late aggregation, P-selectin expression, and GP IIb/IIIa activation. Interestingly peak aggregation measures were not influenced by the selection of the preservative. Although widely used as an anticoagulant when studying ex vivo platelet functions, sodium citrate possesses shortcomings because it promotes P-selectin expression (16) and microaggregate formation likely in relation with platelet activation signaled by the binding of fibrinogen to GP IIb/IIIa receptors (17,18).

**Effects of ASA.** Aspirin had no detectable effect on the tests used in this study regardless of the presence of clopidogrel, consistent with published data that described no effects of aspirin on ADP-induced aggregation (19–21), CD62P, CD63, and PAC-1 expression, and platelet-leukocytes conjugate formation (11). On the other hand, acetylsalicylic acid (ASA) can potentiate the effects of clopidogrel on less specific function tests such as bleeding time (22) and aggregation induced by type 1 collagen or TRAP 15 $\mu$M (19). The ASA also inhibits the second phase of ADP-induced aggregation by preventing thromboxane $A_2$ production. No second wave of aggregation was seen in this study because the 10-$\mu$M concentration was used in patients pretreated with ASA, and the subthreshold concentration of 2.5 $\mu$M was used in controls. In the two instances, however, clopidogrel clearly prevented sustained aggregation because it consistently promoted dis-

**Table 2.** Responsiveness to Clopidogrel by Blood Preservative and Tests of Platelet Function Used

| | Sodium Citrate | | | | Hirudin/PPACK | | | |
|---|---|---|---|---|---|---|---|---|
| | $Agg_{max}$ | $Agg_{6min}$ | PAC | Psel | $Agg_{max}$ | $Agg_{6min}$ | PAC | Psel |
| Good response (%) | 29 | 59 | 41 | 75 | 53 | 94 | 77 | 82 |
| Low response (%) | 35 | 24 | 30 | 12.5 | 35 | 0 | 11 | 12 |
| Non-response (%) | 35 | 18 | 29 | 12.5 | 12 | 6 | 12 | 6 |

Study performed in patients with stable coronary artery disease. See text for definition of good, low, and non-response.
Hirudin/PPACK = r-hirudin and D-phenylalanyl-prolyl-arginine chloromethyl ketone; other abbreviations as in Table 1.

aggregation. The TRAP effects were not influenced by ASA, as previously documented (20,23).

**Effects of clopidogrel.** Clopidogrel produced a profound and predictable inhibition of late aggregation in relation to rapid disaggregation of emerging aggregates, contrasting with the modest and variable inhibition of peak aggregation observed in this and other studies (1,2,6,14). Only one previous study reported on late aggregation, and did not refer to variability in response (21).

The TRAP data extend to disaggregation our previous observation on the inhibitory effects of clopidogrel on peak aggregation, P-selectin, PAC-1, and platelet-monocyte conjugates formation (12). Although less pronounced than for ADP, the disaggregation after TRAP was of greater magnitude than the inhibition of $Agg_{max}$. These data are consistent with the known influence of purinergic receptors on the activity of protease activated receptor-1 (24). The physiologic importance of the observations may be debatable because TRAP may release calcium through mechanisms different than that of thrombin that involve protease activated receptor-4 delayed signaling (24,25). The inhibitions of PAC-1 and on P-selectin by clopidogrel were predictable because they relate to ADP agonists' effects on platelet activation.

**Resistance to clopidogrel.** Previously reported prevalence rates of clopidogrel resistance, measured as absolute difference to baseline aggregation, were within the range of 24% to 40% (1,2,9,26). Using more restrictive definitions of <10% relative inhibition, Muller et al. (6) and Lau et al. (8) could reduce these rates to 5% and 22% of patients, respectively. The non-response rates of 35% observed through using peak aggregation in this study could be reduced to 12% when the analyses were performed in hirudin/PPACK blood rather than in citrate, and further down to 6% when late aggregation was studied instead of peak aggregation. Disaggregation was always present with clopidogrel and absent without clopidogrel. Similar low non-response rates of 6% were seen with both P-selectin expression and PAC-1 binding when assessed in hirudin/PPACK, compared with rates of 12.5% and 29%, respectively, in sodium citrate.

**Study limitations and clinical implications.** The low non-response rates observed herein are unlikely related to the concentrations of agonists (4) because they were observed at both low and high concentrations of ADP and of TRAP, and at concentrations similar to that used in most studies that showed these high rates. Although other factors related to individuals studied or to methodologic aspects could have influenced the results, they were unlikely of importance because results were consistent in two different populations, with two different agonists, two different concentrations of ADP, and different tests of platelet function examining platelet secretion, activation, and aggregation and disaggregation.

Although reproducible, the findings of this study are not a direct documentation of clinical relevance. For this purpose, large clinical trials matching clinical efficacy to biological effects will be required.

Nonetheless, the results of the study, along with the conclusive efficacy data derived from clinical trials and the known selective blocking effect of clopidogrel on the $P2Y_{12}$, strongly support a measure of late aggregation or disaggregation as a more appropriate assay to measure clopidogrel efficacy than aggregation. These mechanistic and conceptual issues are relevant to the evaluation of clopidogrel efficacy and carry implications for patients' treatment for and development of new generations of ADP receptor blockers.

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