Conclusions—Interindividual variability in the platelet inhibitory response from clopidogrel occurs in patients undergoing resistance. However, the uniformity of inhibition after clopidogrel therapy and the incidence of drug resistance has not been investigated extensively. Interindividual variability in drug response as measured by all markers that showed a normal distribution. Resistance, defined as baseline aggregation (%) minus posttreatment aggregation (%) ≤10% by 5 μmol/L ADP, was present in 31% and 15% of patients at 5 and 30 days, respectively. Patients with the highest pretreatment platelet reactivity remained the most reactive at 24 hours after treatment (P<0.0001).

Methods and Results—Platelet aggregation (5 and 20 μmol/L ADP), the activation of glycoprotein IIb/IIIa (PAC-1 antibody), and the expression of P-selectin were measured in patients undergoing elective coronary stenting (n=96) at baseline and at 2 hours, 24 hours, 5 days, and 30 days after stenting. All patients received aspirin (325 mg). Clopidogrel (300 mg) was administered in the catheterization laboratory and followed by 75 mg daily. There was marked interindividual variability in drug response as measured by all markers that showed a normal distribution. Resistance, defined as baseline aggregation (%) minus posttreatment aggregation (%) ≤10% by 5 μmol/L ADP, was present in 31% and 15% of patients at 5 and 30 days, respectively. Patients with the highest pretreatment platelet reactivity remained the most reactive at 24 hours after treatment (P<0.0001).

Conclusions—Interindividual variability in the platelet inhibitory response from clopidogrel occurs in patients undergoing elective coronary stenting. Patients with high pretreatment reactivity are least protected. Alternative pharmacological strategies and the association of adverse ischemic events should be investigated in these patients. (Circulation. 2003;107:2908-2913.)

Key Words: drugs ■ platelets ■ stents

Clopidogrel with aspirin is the regimen of choice to prevent stent thrombosis. The CURE study (Clopidogrel in Unstable angina to prevent Recurrent Events) showed that combination clopidogrel and aspirin antiplatelet therapy reduces ischemic events compared with aspirin therapy alone. These findings are consistent with those of the CAPRIE study (Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events), which showed superior reduction in ischemic events with clopidogrel therapy compared with aspirin, and which may be explained in part by aspirin resistance. However, the uniformity of inhibition after clopidogrel therapy and the incidence of drug resistance has not been investigated extensively. Interindividual variability in response to clopidogrel may affect clinical outcomes.

We studied the individual responses to clopidogrel therapy in patients undergoing elective coronary artery stenting by measuring platelet aggregation and other markers of platelet activation by flow cytometry for 30 days after the procedure. The frequency of drug resistance is reported. We also studied the influence of pretreatment platelet reactivity on drug response.

Methods
This study was approved by the Investigational Review Board. Consecutive patients undergoing elective coronary stenting were enrolled after giving informed consent. All ages were included. The exclusion criteria were a history of bleeding diathesis, acute myocardial infarction within 48 hours, cerebrovascular event within 3 months, illicit drug or alcohol abuse, prothrombin time >1.5 times control, platelet count <100 000/mm³, hematocrit <25%, creatinine >4.0 mg/dL, and thienopyridine or glycoprotein (GP) IIb/IIIa use before the procedure.

Per protocol, GP IIb/IIIa inhibitors were not given. Clopidogrel (300 mg) was given to all patients in the catheterization laboratory after successful coronary artery stent implantation followed by 75 mg daily for 30 days. In addition, all patients had received at least 81 mg of aspirin for 7 days before the procedure (>90% received 325 mg) and were administered 325 mg on the day of the procedure and daily thereafter. Heparin to achieve an activated clotting time >300 seconds was administered as a bolus to all patients in the catheterization laboratory immediately before stenting.

Blood Sampling
Blood was collected in evacuated container tubes containing 3.8% trisodium citrate that were filled to capacity and then inverted 3 to 5 times for gentle mixing. Samples were obtained before clopidogrel administration (baseline) and at 2 hours, 24 hours, 5 days, and 30 days after stenting.

Platelet Aggregation
The blood-citrate mixture was centrifuged at 1200g for 2.5 minutes. The resulting platelet-rich plasma was kept at room temperature for use within 1 hour. The platelet count was determined in the
platelet-rich plasma sample and adjusted to 3.5×10^9/mL with homologous platelet-poor plasma. Platelets were stimulated with ADP (5 and 20 μmol/L), and aggregation was assessed as described previously with a Chronolog Lumi-Aggregometer (model 560-Ca) with the AggroLink software package.6 Platelet aggregation was expressed as the maximal percent change in light transmittance from baseline, with platelet-poor plasma used as a reference. Curves were analyzed according to accepted standards.3

Flow Cytometry
The surface expression of platelet receptors was determined by flow cytometry with monoclonal antibodies. Briefly, the blood-citrate mixture (50 μL) was diluted with 450 μL of Tris buffered saline (10 mmol/L Tris, 0.15 mol/L sodium chloride) and mixed by inverting an Eppendorf tube gently 2 times. The corresponding antibody was then added (5 μL) and incubated at room temperature for 30 minutes. After incubation, 400 μL of 2% buffered paraformaldehyde was added for fixation. The samples were analyzed on a Becton Dickinson FACSScan flow cytometer set up to measure fluorescent light scatter as described previously.8 All parameters were collected with four-decade logarithmic amplification. The data were collected in list-mode files and then analyzed. The PAC-1 antibody (Becton Dickinson) binds only to the active αIIbβ3 receptor, and therefore the total amount of αIIbβ3 was not determined.6 PAC-1 was expressed as log mean fluorescence intensity. P-selectin (Pharmingen) was measured after stimulation with 200 μmol/L ADP and is expressed as percent positivity (ie, the percentage of platelets positive for the antibody) as described previously.10 The dose of agonist was chosen on the basis of data reporting maximum expression of P-selectin induced by 100 μmol/L ADP in the absence of an ADP blocker.6

Drug Resistance Definition
Drug resistance was defined as an absolute difference between baseline aggregation and posttreatment aggregation (Aggregation [%]) of 10% or less with 5 μmol/L ADP used as the agonist. Because Δ aggregation (%) = baseline aggregation (%) − posttreatment aggregation (%), a negative Δ aggregation would indicate posttreatment platelet reactivity greater than baseline, and a positive Δ aggregation would indicate platelet inhibition.

Statistical Analysis
The responders and nonresponders were compared with t tests. Standard regression analysis was used to correlate 5 and 20 μmol ADP/L-induced aggregation and 5- and 30-day aggregation and P-selectin expression (Statistica software). To assess the effect of pretreatment reactivity on drug response, patients were divided into high, moderate, and low baseline reactivity.11 Two separate analyses were performed on the basis of aggregation and P-selectin expression. For 5 μmol/L ADP-induced aggregation, high reactivity was defined as percent aggregation 50%; moderate, 40% to 50%; low, <40%. Comparisons were made between groups by 1-way ANOVA (Statistica software). The Wilks-Shapiro test was used to assess conformity with a normal distribution. Curves were plotted of the best fit to a normal distribution by Statistica software. Given the normal distribution of data, the mean±SD and mean±SE were used. P<0.05 was considered significant.

Results

Patient Data
Ninety-six patients had complete platelet studies performed at baseline, and of these patients, 92 had adequate poststent samples. The patient demographics on these 92 patients are shown with respect to the response to 5 μmol/L ADP-induced aggregation at day 5 in the Table. The patients were elderly, and most were males. Multiple cardiovascular risk factors were frequent. Concomitant drug use did not differ significantly between groups. A trend of higher calcium antagonist and ACE inhibitor use was observed in the nonresponders. Most patients were treated with 1 stent. There were no significant procedural differences between responders and nonresponders except for total stent length. Follow-up at 30 days revealed no cases of Q-wave myocardial infarction, stent thrombosis, target-vessel revascularization, cerebrovascular ischemic events, or death.

Platelet Aggregation
Histograms of the response to clopidogrel are shown in Figures 1 and 2. Baseline aggregation to 5 and 20 μmol/L ADP was 62±18% and 83±21%, respectively. The response to 5 and 20 μmol/L ADP showed a shift to the right between 2 and 24 hours after treatment, which indicates increased platelet inhibition. Aggregation by 5 μmol/L ADP was maximally inhibited by 24 hours (P<0.05 compared with baseline). Platelet aggregation was 58±22% at 2 hours, 37±22% at 24 hours, 32±18% at 5 days, and 31±15% at 30 days. At 2 hours after stenting, 63% of patients met the definition of resistance and platelet reactivity was greatest, with 42% of patients having greater aggregation than at baseline. At 24 hours, resistance fell to 31%, and 24% of patients still had greater aggregation than at baseline. No
further changes were seen at 5 days, when resistance was observed in 31%. However, at 30 days after stenting, the incidence of resistance fell to 15%, but 11% still had aggregation greater than baseline.

The response to 20 μmol/L ADP showed a similar pattern. Aggregation was 80±24% at 2 hours and fell to 60±25% at 24 hours (P<0.05 compared with baseline). At 5 days, aggregation remained stable (57±23%), with a nonsignificant decrease at 30 days (52±14%). A Δ aggregation of 10% or less was present in 53% of patients at 2 hours, 35% at 24 hours, 32% at 5 days, and 21% at 30 days. The correlation between the 5- and 20-μmol/L ADP aggregation response was strong (r=0.6).

**Correlation of Responses at 5 and 30 Days**
A strong correlation was observed between the 5- and 30-day responses to 5 μmol/L ADP (r=0.8). Moreover, strong correlations were also observed between 5- and 30-day responses for 20 μmol/L ADP (r=0.8) and P-selectin (r=0.7).
Platelet Receptor Expression

**P-Selectin**
Baseline stimulated P-selectin expression was $45 \pm 16\%$ and fell over 24 hours, as indicated by a shift in the curve to the right (Figure 3). Maximum inhibition of P-selectin expression occurred within 24 hours ($24 \pm 13\%; P<0.05$ compared with baseline) and was unchanged at 5 days ($22 \pm 13\%$) and 30 days ($23 \pm 10\%$), as observed in the aggregation studies. An absolute change in percent positivity of 10% or less was observed in 44% of patients at 2 hours, 25% at 24 hours, 12% at 5 days, and 29% at 30 days, which again suggests resistance to the standard clopidogrel regimen.

**PAC-1**
PAC-1 binding showed similar response variability (Figure 4). Baseline expression was $14.9 \pm 13.1$, and inhibition of the expression of active GP IIb/IIIa was maximal within 24 hours ($8.5 \pm 5.1; P<0.05$ compared with baseline). No significant changes as compared with 24 hours were observed at 5 days ($8.1 \pm 3.7$) or 30 days ($8.6 \pm 5.3$).

**Effect of Pretreatment Platelet Reactivity on Drug Response**
High pretreatment reactivity, defined by the response to 5 $\mu$mol/L ADP, was present in 31 patients, moderate reactivity in 25 patients, and low reactivity in 40 patients. High-reactivity patients had a greater incidence of diabetes (71%; $P<0.05$) than those with moderate (24%) and low (40%) reactivity. Patient weight, gender, age, statin use, smoking history, incidence of hyperlipidemia, history of prior infarc-
tion, total contrast load, procedure duration, and number of vessels treated were not significantly different between groups.

Platelet Aggregation
At baseline, by definition, the high-reactivity group had markedly greater reactivity (78 ± 6%) than the moderate (65 ± 3%; P < 0.00001) and low (48 ± 9%; P < 0.00001) groups (Figure 5A). At day 1 after stenting, the high-reactivity group continued to have the most reactive platelets (67 ± 11%; P = 0.004 versus moderate [56 ± 15%] and P < 0.0001 versus low [52 ± 15%]). By day 5, platelet reactivity by this marker was similar among groups.

P-Selectin Expression
At baseline, the high-reactivity group had greater expression (67 ± 7%) than the moderate (44 ± 3%; P < 0.00001) and low (29 ± 6%; P < 0.00001) groups (Figure 5B). The effect that pretreatment reactivity had on the inhibitory response to clopidogrel at day 1 was remarkably similar to findings with ADP-induced light-transmittance aggregometry. At day 1, patients with high pretreatment P-selectin expression remained the most reactive (P < 0.001 versus low reactivity). At day 5 of therapy, patients in the high-reactivity group had a trend to greater P-selectin expression than the moderate (31 ± 12% versus 19 ± 13%; P = 0.06) and low (20 ± 7%; P = 0.04) groups.

Discussion
The present study illustrates the variable platelet inhibitory response to the standard administered dose of clopidogrel. The platelet aggregation studies used 2 agonist concentrations that showed a strong correlation. In addition, platelet receptor expression showed similar findings. These uniform observations, irrespective of the methodology chosen to detect inhibition, strengthen our conclusions that the response to clopidogrel therapy is indeed heterogeneous and that drug resistance occurs. Our observations are in agreement with one other report of 18 patients with stable angina treated with the same clopidogrel regimen after coronary intervention. Those investigators demonstrated variable inhibition of ADP-induced fibrinogen binding on day 2 after stenting.

Our definition of drug resistance was empirical because there have been no extensive reports on this subject among patients treated with clopidogrel. Clopidogrel inhibits aggregation in response to ADP, and therefore, we studied the response to 2 different concentrations of this agonist with light-transmittance aggregometry. Moreover, we assessed the expression of an established marker of platelet activation (P-selectin) in response to a maximal agonist concentration and studied the response of a sensitive platelet activation–dependent marker (PAC-1 binding) in nonstimulated blood.5,9

The present study suggests that the maximum inhibitory response to a 300-mg loading dose followed by 75 mg/d occurs within 24 hours. These findings are consistent with
reports in healthy volunteers and in patients undergoing coronary stenting.13,14

The response to clopidogrel appears to be patient specific. The robust correlations demonstrated that in most patients, the 30-day inhibitory response from clopidogrel was predicted by the 5-day response. Of equal importance, the present investigation also suggests that resistance to clopi-
dogrel does not accrue over time.

The present study is the first to demonstrate that the level of platelet reactivity after the standard clopidogrel regimen for coronary stenting is critically dependent on the pretreat-
ment reactivity. The present in vitro tests suggest that patients with the greatest pretreatment platelet activity have the least antithrombotic protection, particularly within the first 24 hours of therapy. The level of platelet reactivity has been correlated with adverse events by others.4 Moreover, an examination of P-selectin expression suggests that this relation-
ship is true even after 5 days of therapy, when those patients with the greatest baseline expression of this activation-dependent receptor tended to be more reactive than those with baseline low or moderate expression. The present findings may help to explain why ticlopidine without a loading dose did not prevent stent thrombosis in the first 3 days after the procedure.15 The similar findings at 24 hours using 2 different established markers of platelet activity strengthen our conclusion that the response to clopidogrel therapy is indeed dependent on pretreatment reactivity. Pre-
vious investigations using aggregometry and P-selectin ex-
pression as markers of reactivity have shown that loading doses higher than 300 mg may enhance and accelerate platelet inhibition in patients undergoing coronary interven-
tions.16–19 Similar strategies may particularly benefit patients with high pretreatment reactivity.

Limitations
The present study included patients undergoing elective coronary stenting, which is known to increase platelet reac-
tivity.8 Because pretreatment reactivity affected the reactivity measured after antiplatelet therapy, postdrug platelet reactiv-
ity may be less in studies of healthy volunteers and in patients with stable coronary artery disease. Our definition of resis-
tance involves the amplitude of maximal platelet aggregation and can be influenced by various factors, including intrapa-
tient variability. The current rates of stent thrombosis ob-
served in elective stenting are much lower than the incidence of clopidogrel resistance in the present study, which suggests that our definition may be an overestimate or that resistance to clopidogrel is not a primary factor influencing stent thrombosis in these patients. However, the present data imply that nonresponders with high pretreatment reactivity may be at greatest risk.

In conclusion, the platelet inhibitory response to the standard dosing regimen of clopidogrel for coronary stenting is variable, follows a normal distribution, and appears stable over 30 days. Patients with high pretreatment reactivity are the least protected within the first 5 days of treatment. Further study is necessary to investigate the mechanisms of these findings and how they correlate with the occurrence of ischemic events. The present work would also support further investigations to determine whether higher clopidogrel doses may overcome interindividual differences in drug response.

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