Evaluation of Dose-Related Effects of Aspirin on Platelet Function
Results From the Aspirin-Induced Platelet Effect (ASPECT) Study

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Background—The antiplatelet effect of aspirin is attributed to platelet cyclooxygenase-1 inhibition. Controversy exists on the prevalence of platelet resistance to aspirin in patients with coronary artery disease and effects of aspirin dose on inhibition. Our primary aim was to determine the degree of platelet aspirin responsiveness in patients, as measured by commonly used methods, and to study the relation of aspirin dose to platelet inhibition.

Methods and Results—We prospectively studied the effect of aspirin dosing on platelet function in 125 stable outpatients with coronary artery disease randomized in a double-blind, double-crossover investigation (81, 162, and 325 mg/d for 4 weeks each over a 12-week period). At all doses of aspirin, platelet function was low as indicated by arachidonic acid (AA)-induced light transmittance aggregation, thrombelastography, and VerifyNow. At any 1 dose, resistance to aspirin was 0% to 6% in the overall group when AA was used as the agonist, whereas it was 1% to 27% by other methods [collagen and ADP-induced light transmittance aggregation, platelet function analyzer (PFA-100)]. Platelet response to aspirin as measured by collagen-induced light transmittance aggregation, ADP-induced light transmittance aggregation, PFA-100 (81 mg versus 162 mg, \( P \leq 0.05 \)) and urinary 11-dehydrothromboxane B₂ was dose-related (81 mg versus 325 mg, \( P = 0.003 \)). No carryover effects were observed.

Conclusions—The assessment of aspirin resistance is highly assay-dependent; aspirin is an effective blocker of AA-induced platelet function at all doses, whereas higher estimates of resistance were observed with methods that do not use AA as the stimulus. The observation of dose-dependent effects despite nearly complete inhibition of AA-induced aggregation suggests that aspirin may exert antiplatelet properties through non–cyclooxygenase-1 pathways and deserves further investigation.

(Circulation. 2007;115:3156-3164.)

Key Words: aggregation ■ aspirin ■ coronary disease ■ platelets ■ resistance

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Platelet responsiveness to aspirin during treatment with 325 mg/d aspirin was recently studied in patients who underwent elective coronary stenting and patients with a history of stent thrombosis by AA-induced light transmittance aggregometry (LTA) and thrombelastography. Approximately 3% of the patients were found to be noncompliant with aspirin therapy, all of whom were sensitive after...
in-hospital treatment. Finally, among the entire group only 1 patient (0.4%), who also had a history of stent thrombosis, was resistant to aspirin treatment. In another study of patients with a history of myocardial infarction, 9% of patients were found to be noncompliant with aspirin therapy and only 1 patient was resistant to 325 mg aspirin therapy as measured by AA-induced LTA. These studies suggest that platelet resistance to aspirin is infrequent in patients administered 325 mg/d aspirin when measured by a laboratory method that is directly dependent on platelet COX-1 activity. The high prevalence of aspirin resistance as reported in previous studies may in part be explained by the implementation of assays that do not use AA as the agonist.

Although many investigations have focused on quantifying the percentage of patients who display aspirin resistance, little information is available to compare simultaneous analyses of aspirin within the individual patient. In addition, limited information is available to compare simultaneous analyses of various commonly used assays. On the basis of our previous study, we hypothesized that the prevalence of platelet resistance to aspirin is rare at all doses when measured by methods that use AA as the stimulus. Our primary aim was to determine the degree of platelet responsiveness to aspirin as measured by commonly used methods and study the relation of dose to platelet inhibition.

Methods

Patients
The study was approved by the Western Institutional Review Board (Olympia, Wash.) and all patients were >18 years of age. A total of 125 outpatients with coronary artery disease (CAD) documented by coronary angiography or ultrafast computed tomography scan were enrolled in a single-center, double-blind, double-crossover study. Overencapsulated, non–enteric-coated aspirin (Bayer HealthCare, Morristown, NJ) was prepared on-site. In a 3-period 3-treatment crossover that followed a Williams design, subjects were assigned to 1 of 6 treatment sequences (81, 162, and 325 mg/d for 4 weeks each) with a washout period of 1 week between treatments. The study consisted of 5 18-week periods. Subjects were instructed to return their bottle of aspirin to count remaining capsules to calculate percent compliance.

Blood and Urine Sampling
Blood was collected from the antecubital vein into Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) between 8 AM and 12 PM on the day of the last aspirin dose. Tubes that contained 3.2% trisodium citrate were used for LTA and PFA-100; 17 USP/mL lithium heparin tubes were used for TEG. In addition, 1 tube that contained 3.2% sodium citrate (Greiner Bio-One Vacuette North America, Inc., Monroe, NC) was collected for VerifyNow measurements. After the first 2 to 3 mL of free-flowing blood was discarded, the tubes were filled to capacity and gently inverted 3 to 5 times to ensure complete mixture of the anticoagulant. Urine samples were collected for measurements of 11-dihydro-TxB2 at the end of each aspirin treatment period into tubes that contained indomethacin (Corgenix, Denver, Colo) and stored at −70°C until analysis.

Light Transmittance Aggregometry
The blood-citrated tubes were centrifuged at 120g for 5 minutes to recover platelet-rich plasma and further centrifuged at 850g for 10 minutes to recover platelet-poor plasma. The platelet-rich plasma and platelet-poor plasma were stored at room temperature to be used within 30 minutes. Platelet aggregation was assessed as described previously. Briefly, platelets were stimulated with 5 μmol/L ADP, 2 μg/mL collagen, and 1, 2, and 5 mmol/L AA. Aggregation was assessed with a Chronolog Lumi-Aggregometer (Model 490 to 4D) with the Aggrolink software package. Aggregation was expressed as the maximum percent change in light transmittance from baseline, with platelet-poor plasma used as a reference.

Thrombelastograph PlateletMapping Assay
The TEG Hemostasis Analyzer with PlateletMapping assay relies on the measurement of thrombin-induced clot strength to enable a quantitative analysis of platelet function. In the PlateletMapping assay, heparin is used to eliminate thrombin activity in the sample. Reptilase and factor XIIIa are used to generate a cross-linked fibrin clot to isolate the fibrin contribution to the clot strength. The contribution of the cyclooxygenase pathway to clot formation can be measured by the addition of 1 mmol/L AA. Blood samples were analyzed according to the manufacturer’s instructions as described previously.

VerifyNow Aspirin Assay
VerifyNow is a turbidimetric-based optical detection system that measures platelet aggregation in whole blood. The PFA-100 point-of-care assay utilizes a cartridge that contains a capillary, a sample reservoir, a collagen/epinephrine-coated membrane, and an aperture that exposes platelets to high shear conditions. When platelets in whole blood come in contact with epinephrine and collagen, they become activated and aggregate at the aperture, and thus gradually reduce and finally arrest blood flow. The PFA-100 records the time in seconds from the start of the test until the platelet plug occludes the aperture (closure time).

Flow Cytometry
The expression of platelet GPIIb/IIIa and P-selectin were determined by whole blood flow cytometry with a multicolor analysis method that used the following monoclonal antibodies: fluorescein isothiocyanate-conjugated PAC-1 (recognizes the active GPIIb/IIIa receptor), R-phycocerythrin-conjugated CD41a (recognizes the total GPIIb/IIIa receptor population), and phycoerythrin-cyanine5-
conjugated CD62p (recognizes P-selectin). All antibodies were obtained from BD Biosciences (San Diego, Calif). ADP- and AA-induced expression of GPIIb/IIIa receptors and P-selectin were measured as described previously.10

Urinary 11-dh-TxB$_2$

The AspirinWorks ELISA assay has been described elsewhere.15 Briefly, 100 $\mu$L of urine in assay buffer was incubated with a monoclonal antibody followed by the addition of 11-dh-Tx B$_2$-alkaline phosphate tracer. Urinary 11-dh-TxB$_2$ concentrations were determined by measurement of color development at 405 nm with an ELISA reader and expressed as pg/mg creatinine.

Aspirin Resistance Definitions

Aspirin resistance definitions included $\geq$20% AA-, $\geq$70% ADP-, and $\geq$70% collagen-induced aggregation by LTA;16 $\geq$50% AA-induced whole blood aggregation by TEG,8 $\geq$550 aspirin reaction units by VerifyNow,13 $\leq$193 seconds by PFA-100;14 and upper quartile pg 11-dh-TxB$_2$/mg creatinine during treatment with 81 mg.17

Statistical Analysis

Aspirin resistance definitions included $\geq$20% AA-, $\geq$70% ADP-, and $\geq$70% collagen-induced aggregation by LTA;16 $\geq$50% AA-induced whole blood aggregation by TEG,8 $\geq$550 aspirin reaction units by VerifyNow,13 $\leq$193 seconds by PFA-100;14 and upper quartile pg 11-dh-TxB$_2$/mg creatinine during treatment with 81 mg.17

The present study used a 3-period 3-treatment crossover design. Each subject was randomized to 1 of 6 treatment sequences and administered doses of aspirin (81, 162, and 325 mg) according to their treatment sequence. For a given subject, measurements of aspirin resistance were recorded over the 3 treatment periods for each dose level. As a result, the observations within a given subject became correlated, which caused the assumptions of the usual ANOVA to be violated. Therefore, the analysis performed here used a repeated measures logistic regression with a random subject effect to account for the dependencies from the crossover design. Carryover effects were included in the model because washout periods were not possible. Carryover effects were modeled with dummy variables (0 or 1) to specify the order in which patients received the different treatments (ie, if a patient received a dosage of 81 mg in period 1, an effect for carryover from 81 mg would be included for that patient in period 2). Wald $t$ tests were used to analyze differences between dosage levels (81 mg versus 162 mg, 81 mg versus 325 mg, and 162 mg versus 325 mg) and to correlate resistance measured by 2 mmol/L AA-induced LTA with resistance measured by all other methods.19 In addition, Wald $\chi^2$ tests were used to test the overall difference between dosage levels and overall carryover effect.

An unpaired $t$ test was used to compare measurements of platelet function between patients and healthy volunteers at all doses; $P<0.05$ was considered significant. Statistical analyses were performed with SAS version 9.1.3. (SAS Institute Inc, Cary, NC).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Patients

Patient demographics are shown in Table 1. Briefly, the majority of patients were elderly white men with a history of hyperlipidemia who received standard medical treatment for CAD, such as statins, $\beta$-blockers, and angiotensin-converting enzyme inhibitors. Ninety-six percent (n=120 patients) of the study population completed platelet testing at all 3 aspirin doses, and data on these patients are reported. Five patients were terminated from the study after completion of at least 1 dose for the following reasons: transient ischemic attack, gastritis, self-withdrawal (n=2 patients), and inability to obtain blood. Overall compliance with daily aspirin therapy was 98%.

| Age, y  | 65±10 |
| Male, n (%) | 80 (70) |
| Weight, lbs | 196±51 |
| Systolic BP, mm Hg | 130±18 |
| Diastolic BP, mm Hg | 71±6 |
| Ethnicity, n (%) | White 79 (69) | Black 32 (28) | Asian 3 (3) |
| Risk factors/past medical history, n (%) | Smoking (previous or current) 46 (40) | Family history of CAD 40 (35) | Hypertension 73 (64) | Hyperlipidemia 100 (87) | Diabetes mellitus 30 (26) | PVD 9 (8) | Prior MI 25 (22) | Prior CABG 36 (32) | Prior PCI 39 (34) | Prior CVA 6 (5) |
| Medications, n (%) | $\beta$-Blockers 69 (61) | ACE inhibitors 57 (50) | Calcium channel blockers 20 (18) |
| Lipid-lowering therapy 96 (84) |
| Laboratory data | WBC, $\times$1000/mm$^3$ 6.4±2.1 | Platelets, $\times$1000/mm$^3$ 237±62 | Hemoglobin, g/dL 13.7±2.2 | Hematocrit, % 40.5±5.0 | Creatinine, g/dL 1.0±0.2 |

Data are expressed as mean±SD unless otherwise indicated. BP indicates blood pressure; CAD, coronary artery disease; PVD, peripheral vascular disease; MI, myocardial infarction; CABG, coronary artery bypass grafting; PCI, percutaneous coronary intervention; CVA, cerebrovascular accident; ACE, angiotensin converting enzyme; and WBC, white blood cells.

| TABLE 1. Patient Demographics | Patients (n=120) |

Primary Outcome

A statistically significant difference was observed between the prevalence of aspirin resistance measured by the PFA-100 and 2 mmol/L AA-induced LTA at all doses of aspirin (at 81 mg, n=32 versus n=2, $P<0.001$; at 162 mg, n=14 versus n=0, $P<0.001$; and at 325 mg, n=21 versus n=0, $P<0.001$, respectively) (Table 2).
Analysis of Aspirin Treatment Effects and Carryover Effects

Table 3 presents the estimated difference in resistance between aspirin doses. For the logistic regression model, this estimated difference corresponds to an estimate of the log of the odds ratio between 2 doses. The odds ratio is the ratio of the probability that resistance is present to the probability that resistance is not present. For consistency, each odds ratio refers to the odds for the higher dose divided by the odds for the lower dose. For example, the first column of Table 3 corresponds to the log of the odds ratio for the patients who received 162 mg versus 81 mg. Similarly, the columns titled “325 mg versus 81 mg” and “325 mg versus 162 mg” provide the comparisons between 325 mg versus 81 mg and 325 mg versus 162 mg. A log of the odds ratio of 0 would characterize that the odds of aspirin resistance for 2 different doses are equal, whereas a negative value would indicate that a decrease in resistance occurs as the dose increases. Positive values are similarly interpreted. For example, the estimate for 5 μmol/L ADP in the 162 mg versus 81 mg column of Table 3, −1.2104, indicates that the odds of resistance measured by 5 μmol/L ADP LTA when given 162 mg of aspirin is less than the odds of resistance measured by the 5 μmol/L ADP when given 81 mg. The log of the odds ratios for “325 mg versus 81 mg” are also negative, which indicates a decrease in the odds of observing resistance for the higher dose. The columns of probability values indicate that the difference is not statistically significant for some of the methods, which points to possible differences among the methods to detect aspirin resistance. In addition, Table 3 presents the Wald t statistics (t), standard errors (SE), and the probability values for each test that correspond to these estimates. Table 4 presents the results of the tests for the carryover effects from each of the 3 dosage levels. For completeness, Tables 3 and 4 also include χ² tests and probability values that test the overall treatment and carryover effects. Results are further described under each method.

### TABLE 3. Analysis of Aspirin Treatment Effects

<table>
<thead>
<tr>
<th>Method</th>
<th>162 mg vs 81 mg</th>
<th></th>
<th></th>
<th>325 mg vs 81 mg</th>
<th></th>
<th></th>
<th>325 mg vs 162 mg</th>
<th></th>
<th></th>
<th>Overall Treatment</th>
</tr>
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<tr>
<td></td>
<td>Estimated</td>
<td>SE</td>
<td>t</td>
<td>P</td>
<td></td>
<td></td>
<td>Estimated</td>
<td></td>
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<tr>
<td>LTA</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μmol/L ADP</td>
<td>−1.2104</td>
<td>0.6107</td>
<td>−1.98</td>
<td>0.049</td>
<td></td>
<td></td>
<td>−1.1307</td>
<td>0.6078</td>
<td>−1.86</td>
<td>0.065</td>
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<tr>
<td>2 μg/mL Collagen</td>
<td>−2.9794</td>
<td>1.1674</td>
<td>−2.55</td>
<td>0.012</td>
<td></td>
<td></td>
<td>−3.7225</td>
<td>1.4375</td>
<td>−2.59</td>
<td>0.011</td>
</tr>
<tr>
<td>2 μmol/L AA</td>
<td>−2.9012</td>
<td>4.3951</td>
<td>−0.66</td>
<td>0.510</td>
<td></td>
<td></td>
<td>−6.5075</td>
<td>7.0303</td>
<td>−0.93</td>
<td>0.357</td>
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<td>TEG-1 mmol/L AA</td>
<td>−1.3392</td>
<td>1.0162</td>
<td>−1.32</td>
<td>0.190</td>
<td></td>
<td></td>
<td>−0.5896</td>
<td>1.073</td>
<td>−0.55</td>
<td>0.584</td>
</tr>
<tr>
<td>VerifyNow</td>
<td>−1.6059</td>
<td>0.4912</td>
<td>−3.27</td>
<td>0.001</td>
<td></td>
<td></td>
<td>−0.9091</td>
<td>0.4472</td>
<td>−2.03</td>
<td>0.044</td>
</tr>
<tr>
<td>Urinary thromboxane B2</td>
<td>−0.9034</td>
<td>0.7557</td>
<td>−1.06</td>
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<td></td>
<td></td>
<td>−0.6159</td>
<td>0.8032</td>
<td>−0.77</td>
<td>0.445</td>
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</table>

**AA-Induced Platelet Aggregation Measured by LTA**

Aspirin resistance was rare at all doses (Figure 1, Table 2). The treatment and carryover effects of aspirin dosing on AA-induced platelet aggregation are analyzed in Tables 3 and 4. AA-induced aggregation was low in patients, and no additional reduction in aggregation occurred at doses >81 mg (Table 5). One patient was resistant at 1 mmol/L AA-LTA while on 81 mg aspirin. At 2 mmol/L AA-LTA, the latter patient and another patient were resistant while on 81 mg aspirin. At 5 mmol/L AA-LTA, these 2 patients were resistant at 81 mg and 1 of these 2 patients was resistant at 162 mg. No patients were found to be aspirin resistant during treatment with 325 mg daily aspirin (Figure 1).

**AA-Induced Platelet Aggregation Measured by TEG**

In total, 11 patients were resistant at ≥1 dose of aspirin; 5, 3, and 5 patients were aspirin-resistant during treatment with 81 mg, 162 mg, and 325 mg, respectively (Table 2). One patient was resistant at both 81 mg and 162 mg, and another patient was resistant at both 162 mg and 325 mg; none were resistant at all 3 doses (Table 2). Similar to LTA, aggregation was low; treatment and carryover effects of aspirin doses on AA-induced platelet aggregation were not observed (Tables 3 to 5), and aspirin resistance was infrequent at all doses (Table 2).

**VerifyNow Aspirin Assay**

In total, 13 patients were resistant at ≥1 dose of aspirin; 7, 4, and 4 patients were aspirin-resistant during treatment with 81
mg, 162 mg, and 325 mg, respectively (Table 2). Two patients were resistant at both 81 mg and 162 mg doses; none were resistant at all 3 doses (Table 2). Significant treatment or carryover effects were not observed (Tables 3 and 4).

**Urinary 11-dh-TxB₂**

In total, 42 patients were resistant at ≥1 dose of aspirin; 31, 22, and 14 patients were aspirin-resistant during treatment with 81 mg, 162 mg, and 325 mg, respectively (Table 2). Sixteen patients were resistant at 2 doses, and 5 patients were resistant at all 3 doses (Table 2). An incremental treatment effect of aspirin dosing on urinary 11-dh-TxB₂ levels was observed in patients, with significant differences in levels ($P=0.003$) demonstrated between 81 mg and 325 mg (Tables 3 and 5), uninfluenced by carryover effects (Table 4).

**Collagen-Induced Platelet Aggregation Measured by LTA**

In total, 14 patients were resistant at ≥1 dose of aspirin; 12, 2, and 1 patients were aspirin-resistant during treatment with 81 mg, 162 mg, and 325 mg, respectively (Table 2). Only 1 patient was resistant at both 81 mg and 325 mg aspirin doses; none were resistant at all 3 doses (Table 2). A significant effect of aspirin treatment on collagen-induced platelet aggregation was observed between 81 mg and 162 mg ($P=0.012$) with no further inhibition demonstrated between 162 mg and 325 mg (Tables 3 and 5). No carryover effects between aspirin dosing were observed (Table 4).

**ADP-Induced Platelet Aggregation Measured by LTA**

Overall, 27 patients were resistant at ≥1 dose of aspirin; 19, 11, and 10 patients were aspirin-resistant during treatment with 81 mg, 162 mg, and 325 mg, respectively (Table 2). In total, 7 patients were resistant at 2 aspirin doses, and 3 patients were resistant at all 3 doses of aspirin (Table 2). A significant effect of aspirin treatment on ADP-induced platelet aggregation was observed between 81 mg and 162 mg ($P=0.049$), with no further inhibition demonstrated between 162 mg and 325 mg (Tables 3 and 5). No carryover effects between aspirin dosing were observed (Table 4).

**PFA-100 Assay**

With the PFA-100 method, 42 patients were found to be aspirin-resistant at ≥1 dose of aspirin; 32, 14, and 21 patients were aspirin-resistant during treatment with 81 mg, 162 mg, and 325 mg, respectively (Table 2). In total, 15 patients were resistant at any 2 aspirin doses, and 5 patients were resistant at all 3 doses of aspirin (Table 2). Interestingly, treatment with 325 mg aspirin was associated with shorter closure times and increased rates of

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**TABLE 4. Analysis of Carryover Effects**

<table>
<thead>
<tr>
<th>Method</th>
<th>Carryover From 81 mg</th>
<th>Carryover From 162 mg</th>
<th>Carryover From 325 mg</th>
<th>Overall Carryover</th>
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<tr>
<td></td>
<td>Estimated SE t P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μmol/L ADP</td>
<td>−0.9391 0.6908 −1.36 0.177</td>
<td>−0.9806 0.6504 −1.51 0.134</td>
<td>−0.6823 0.6233 −1.09 0.276</td>
<td>3.65743 0.301</td>
</tr>
<tr>
<td>2 μg/mL collagen</td>
<td>0.9348 1.2637 0.74 0.461</td>
<td>−1.6206 0.9107 −1.78 0.078</td>
<td>−1.11 0.7831 −1.42 0.159</td>
<td>4.89409 0.180</td>
</tr>
<tr>
<td>2 mmol/L AA</td>
<td>2.9798 4.3875 0.68 0.498</td>
<td>0.06461 1.4716 0.04 0.965</td>
<td>−3.5324 0.6881 −0.58 0.563</td>
<td>0.84129 0.840</td>
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<tr>
<td>TEG-1 mmol/L AA</td>
<td>2.2977 1.1178 2.06 0.060</td>
<td>−0.4254 1.2213 −0.35 0.728</td>
<td>0.871 1.0994 0.79 0.430</td>
<td>5.48605 0.139</td>
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<tr>
<td>VerifyNow</td>
<td>−0.7626 0.9175 −0.83 0.408</td>
<td>−1.1865 0.9233 −1.29 0.201</td>
<td>−0.263 0.7559 −0.35 0.729</td>
<td>2.07524 0.557</td>
</tr>
<tr>
<td>Urinary thromboxane</td>
<td>0.2431 0.4922 0.49 0.622</td>
<td>−0.2994 0.5086 −0.59 0.557</td>
<td>−0.2982 0.4752 −0.63 0.532</td>
<td>1.15715 0.763</td>
</tr>
</tbody>
</table>

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**Figure 1.** Individual platelet aggregation data measured after stimulation by 3 concentrations of AA by LTA at 3 different doses of aspirin. At 1 mmol/L AA-LTA, 1 patient ( construed) while on 81 mg aspirin was resistant. At 2 mmol/L AA-LTA, this patient and another patient (c) were resistant while on 81 mg aspirin. At 5 mm AA-LTA, these 2 patients at 81 mg were resistant and 1 of these 2 patients was resistant at 162 mg. No patients were found to be aspirin resistant during treatment with 325 mg aspirin daily. Dashed line indicates cut point for resistance. AA indicates arachidonic acid; LTA, light transmittance aggregometry.
resistance compared with 162 mg aspirin (Tables 2 and 5). Like collagen- and ADP-induced platelet aggregation, there was a statistically significant treatment effect, uninfluenced by carryover effects, between 81 mg and 162 mg as determined by PFA-100 ($P=0.001$), and no further treatment effect was observed between 162 mg and 325 mg (Tables 3 to 5).

Comparison of Platelet Function Measurements in Healthy Volunteers and Patients
A significant effect of aspirin treatment in patients compared with healthy volunteers who were not given aspirin at all doses was observed by use of ADP-, collagen-, and AA-induced LTA, AA-induced TEG, VerifyNow, PFA-100, and urinary thromboxane measurements (Table 5; $P=0.05$ for all measurements).

Correlation Between Methods
Table 6 compares the log of the odds ratios of resistance for the various assays with 2 mmol/L AA LTA. With the exception of 1 mmol/L and 5 mmol/L AA-induced LTA, no significant correlations existed between 2 mmol/L AA-induced LTA and any other methods of aspirin resistance assessment.

Flow Cytometry
A comparison of platelet GPIIb/IIIa and P-selectin expression in patients and healthy volunteers is shown in Figure 2. Nonstimulated total GPIIb/IIIa expression was nonsignificantly higher in patients versus healthy volunteers ($P=0.134$), whereas activated GPIIb/IIIa reached significance ($P=0.033$). AA-stimulated expression of total and activated GPIIb/IIIa was significantly lower in patients treated with aspirin as compared with healthy volunteers ($P=0.0001$) (Figure 2A). Despite potent inhibition of AA-induced aggregation by aspirin therapy, we observed prominent activation of GPIIb/IIIa and P-selectin expression after AA stimulation.

ADP-stimulated expression of total GPIIb/IIIa was nonsignificantly higher ($P=0.108$) in patients treated with aspirin as compared with healthy volunteers, whereas expression of activated GPIIb/IIIa reached significance ($P=0.02$) (Figure 2B). Nonstimulated P-selectin expression was nonsignificantly higher in patients versus healthy volunteers ($P=0.06$). AA-stimulated P-selectin expression was lower in patients than in healthy volunteers ($P<0.0001$). In contrast, ADP-stimulated P-selectin expression remained higher in patients than in healthy volunteers ($P=0.032$) (Figure 2C) despite lower aggregation (Table 5). A dose-related effect of aspirin therapy was not observed by any flow cytometric measurement (Figure 2).

Discussion
The present study assessed platelet responsiveness to 3 different, frequently used aspirin doses by various commonly used assays in patients with CAD. Our study was in part stimulated by the controversy that surrounds the relationship

<table>
<thead>
<tr>
<th>Difference from LTA (2 mmol/L AA)</th>
<th>Healthy Volunteers (n=10)</th>
<th>CAD Patients (n=120)</th>
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</thead>
<tbody>
<tr>
<td>LTA 5 μmol/L ADP</td>
<td>70±10</td>
<td>58±13*</td>
</tr>
<tr>
<td>2 μg/mL collagen</td>
<td>80±13</td>
<td>38±26*</td>
</tr>
<tr>
<td>1 mmol/L AA</td>
<td>77±10</td>
<td>3±9</td>
</tr>
<tr>
<td>2 mmol/L AA</td>
<td>80±11</td>
<td>6±13*</td>
</tr>
<tr>
<td>5 mmol/L AA</td>
<td>78±5</td>
<td>7±15*</td>
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<tr>
<td>VerifyNow, ARU</td>
<td>627±39</td>
<td>454±58*</td>
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<tr>
<td>PFA-100 closure time, secs</td>
<td>142±39</td>
<td>222±73*</td>
</tr>
<tr>
<td>Urinary thromboxane, pg</td>
<td>614±108</td>
<td>378±182*</td>
</tr>
<tr>
<td>11-dh-TxB2/mg Cr</td>
<td>614±108</td>
<td>378±182*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. ASA indicates aspirin.

*P<0.005 for healthy volunteers compared to CAD patients.

**TABLE 5.** Platelet Function in Healthy Volunteers and Patients

**TABLE 6.** Analysis of Method Effects
of aspirin dose to treatment effect.\textsuperscript{20,21} As a double-crossover study, each patient served as his or her own control. The results of the study have several significant implications in the interpretation of platelet responsiveness to aspirin and resistance. First, the estimation of aspirin resistance is highly assay-dependent. Assays that use AA to stimulate platelet aggregation (LTA, TEG, VerifyNow) result in resistance estimates that are lower than methods that use stimuli other than AA. Second, aspirin is a highly effective inhibitor of AA-induced aggregation. However, despite complete or near-complete inhibition of AA-induced aggregation at all agonist concentrations and at all doses of aspirin, platelet aggregation stimulated by other agonists can be robust. Third, the platelet response to aspirin as measured by collagen- and ADP-induced LTA, PFA-100, and urinary thromboxane was dose-related. Last, the overall variability in the measurement of aspirin resistance was most notable for assays that did not use AA as the stimulus.

High Aspirin Efficacy in the Inhibition of AA-Induced Aggregation

Earlier studies demonstrated that repeated 50 to 100 mg daily doses of aspirin were associated with highly effective inhibition of COX-1 as demonstrated by >95% inhibition of serum TxB\textsubscript{2}.\textsuperscript{1} Maximum inhibition of serum TxB\textsubscript{2} can also be attained within 4 hours by a single 325-mg aspirin dose.\textsuperscript{22} Our results agree with these observations. Maximum inhibition of COX-1 activity was present at the lowest aspirin dose as measured by AA-induced aggregation in platelet-rich plasma at 3 different agonist concentrations. When the cut points for resistance defined by the manufacturers are used, the prevalence of resistance was low by all 3 methods that measured platelet aggregation after stimulation by AA (LTA, TEG, VerifyNow). The results also agree with a previous study by our group that demonstrated the high efficacy of a 325-mg aspirin dose in the suppression of AA-induced aggregation in patients who underwent coronary stenting.\textsuperscript{8}

Previous investigations with VerifyNow have reported a higher prevalence of aspirin resistance than the present investigation.\textsuperscript{13,23} It is interesting that these earlier investigations used cationic propyl gallate as the agonist instead of AA. The use of AA as the agonist in the present investigation resulted in low estimates of resistance by VerifyNow measurements. The presence of uninhibited COX-2 in whole blood that contributed to thromboxane A\textsubscript{2}
production may explain a higher prevalence of resistance as compared with LTA. The measurement of urinary thromboxane also suggested a dose-related effect with superior reduction in levels observed with doses >81 mg. Because urinary thromboxane levels indicate total in vivo thromboxane production, an unblocked COX-2 source may partially explain our findings.

**Platelet Aggregation Despite COX-1 Inhibition**

Our results agree with prior studies that demonstrated the presence of residual platelet reactivity to agonists other than AA in patients treated with aspirin. We have referred to the latter methods as COX-1–nonspecific because they may not directly indicate residual COX-1 activity. The presence of high platelet aggregation in the latter methods has been interpreted as evidence for aspirin resistance. Our study, which used various concentrations of AA, suggests that platelet COX-1 activity is inhibited in the majority of patients. Our results therefore argue against residual COX-1 activity as the primary explanation for aspirin resistance as indicated by the presence of high platelet reactivity to either collagen or ADP, or rapid shear-induced aggregation as measured by PFA-100.

Because the currently accepted explanation for the antithrombotic effect of aspirin is the inhibition of COX-1, aspirin resistance has been defined as the presence of persistent COX-1 activity during aspirin therapy. However, our data suggest that aspirin may influence targets in addition to COX-1 in platelets. Consideration should be given to the observed dose-dependent effects of aspirin in the present study when platelet responsiveness to aspirin is evaluated in future studies.

**COX-1–Independent Effects of Aspirin**

The occurrence of lower collagen-, ADP-, and shear-induced aggregation in patients treated with aspirin as compared with healthy volunteers clearly demonstrates that aspirin plays an important role in the modification of the response to these stimuli. Our findings are consistent with numerous prior reports that confirm the same. However, the dose-related effect of aspirin observed after stimulation by collagen in the presence of complete or near-complete inhibition of AA-induced aggregation suggests that aspirin may exert antplatelet effects beyond acetylation of COX-1. Persistent thromboxane production after collagen stimulation cannot be ruled out on the basis of our study; however non–COX-1–mediated effects deserve further investigation. Interestingly, maximum inhibition of aggregation stimulated by collagen and shear occurred at a 162-mg dose of aspirin with no further reduction observed at the 325-mg dose.

**Flow Cytometry as a Method to Assess Aspirin Responsiveness**

Our study demonstrates that, despite aspirin therapy, patients exhibit higher platelet reactivity than healthy volunteers not on aspirin therapy. The latter conclusion is supported by higher levels of circulating active platelets as measured by both P-selectin and activated GPIIb/IIIa expression on nonstimulated platelets as well as greater expression of these receptors after ADP stimulation. Interestingly, we observed an uncoupling of the relationship between platelet function and receptor expression in patients on aspirin therapy. Despite greater expression of P-selectin and activated GPIIb/IIIa after ADP stimulation in patients, lower ADP-induced aggregation was observed than in healthy volunteers. Moreover, only partial inhibition of AA-induced expression of activated GPIIIa and P-selectin occurred in patients treated with aspirin, although platelet function was profoundly inhibited. The former findings suggest that aspirin therapy may inhibit the function of the activated GPIIb/IIIa receptor. The latter data also suggest that AA stimulation may directly increase P-selectin and activated GPIIb/IIIa expression through a mechanism independent of COX-1.

**Limitations**

Pretreatment studies could not be conducted because all patients had CAD and were therefore on aspirin therapy at the time of enrollment. The patients in the present investigation will be followed to track the development of clinical events that will be correlated with their laboratory findings. Finally, although in vitro thromboxane generation was not measured, the observation of very low platelet aggregation induced by various concentrations of AA lends support to the conclusion that aspirin is highly effective in the blockade of COX-1. The primary goal of our study was to assess commonly used platelet functional measurements. The present study represents an exhaustive analysis that addresses platelet response to multiple doses of aspirin with the use of common functional assays. In the present study we determined 11-dh-TxB2 levels, a method that has been linked to clinical events in the Heart Outcomes Prevention Evaluation (HOPE) Study.

In conclusion, aspirin is a highly effective blocker of AA-induced platelet function in stable outpatients with CAD. The prevalence of aspirin resistance was found to be highly dose-dependent, with significantly higher measurements of resistance found with methods that use agonists other than AA. The observation of dose-related effects despite near-complete inhibition of AA-induced aggregation suggests that aspirin may also exert antplatelet effects through non–COX-1 pathways. In addition, despite greater ADP-induced expression of activated GPIIIa in patients treated with aspirin than in healthy volunteers, platelet function in patients was lower, which suggests that aspirin therapy may inhibit the function of the activated GPIIIa receptor. Finally, our observations suggest that AA may also stimulate platelet activation through a COX-1–independent pathway. Further investigations are required to better define the underlying mechanisms for the antithrombotic effects of aspirin. A clearer understanding of the mechanistic effects of aspirin is necessary to establish a uniform definition of aspirin “resistance”.

**Sources of Funding**

The study was supported by Bayer HealthCare LLC, Morristown, NJ, and Sinai Hospital of Baltimore, Md.

**Disclosures**

Dr Gurbel has received research grants and honoraria from Hemoscope, AstraZeneica, Schering Plough, Medtronic, Lilly/Sankyo, Sanofi, Boston-Scientific, and Bayer. The remaining authors report no conflicts.

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Controversy surrounds the optimal dose of aspirin for the treatment of patients with coronary artery disease and the concept of resistance to aspirin therapy. Currently, aspirin is administered without assessment of its effect on platelet function. Low-dose daily aspirin is a uniformly accepted strategy for secondary prevention in patients with cardiovascular disease. However, prospective information is limited on potential dose-related platelet effects of aspirin and their relation to clinical outcomes. In the present study we assessed platelet responsiveness to 3 different frequently used aspirin doses by various assays in patients with stable coronary artery disease. We demonstrated that aspirin at all doses is a highly effective blocker of platelet function when measured by methods that use arachidonic acid as the agonist. A significantly higher prevalence of resistance was observed with methods that used agonists other than arachidonic acid to stimulate platelets. The observation of dose-dependent and non–dose-dependent effects of aspirin on platelet function suggests that aspirin also exerts antithrombotic effects through pathways other than cyclooxygenase-1. A clearer understanding of the mechanistic effects of aspirin is necessary to establish a definition for aspirin resistance and its clinical relevance. On the basis of the dose–dependent antiplatelet effects observed in the present study, we believe that higher aspirin doses may improve clinical outcomes in selected patients. Large-scale prospective clinical trials are needed to support the link between superior dose-related effects of aspirin on platelet function and clinical outcomes.

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Evaluation of Dose-Related Effects of Aspirin on Platelet Function: Results From the Aspirin-Induced Platelet Effect (ASPECT) Study
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_Circulation_. 2007;115:3156-3164; originally published online June 11, 2007;
doi: 10.1161/CIRCULATIONAHA.106.675587
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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