Early Potent Antithrombotic Effect With Combined Aspirin and a Loading Dose of Clopidogrel on Experimental Arterial Thrombogenesis in Humans

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Background—We conducted a double-blind, randomized, crossover study to assess the antithrombotic effects of the combination of aspirin (acetylsalicylic acid, ASA) and clopidogrel, with or without a loading dose, versus ASA alone in a model of arterial thrombosis in humans.

Methods and Results—Eighteen male volunteers received the following 3 regimens for 10 days separated by a 1-month period: (1) 325 mg ASA daily, (2) 325 mg ASA+75 mg clopidogrel daily, (3) 325 mg ASA daily+300-mg clopidogrel loading dose on day 1 and +75 mg clopidogrel per day on days 2 to 10. The antithrombotic effect was measured 1.5, 6, and 24 hours after drug intake on day 1 and 6 hours after drug intake on day 10. Arterial thrombus formation was induced ex vivo by exposing a collagen-coated coverslip in a parallel-plate perfusion chamber to native blood for 3 minutes at an arterial wall shear rate. Without a loading dose, clopidogrel+ASA developed an antithrombotic effect within 6 hours after the first intake. It was superior to that produced by ASA, but it was moderate (P≤0.03). However, with the loading dose, the antithrombotic effect of clopidogrel+ASA appeared within 90 minutes, and after 6 hours it was comparable to that on day 10. On day 10, clopidogrel+ASA decreased platelet thrombus formation by ≈70%, and the effect was significantly more potent than that produced by ASA alone (P<0.001).

Conclusions—This study confirms the synergistic antithrombotic effects of a combined ASA and clopidogrel therapy and shows the early benefit obtained with a loading dose of clopidogrel. (Circulation. 2000;101:2823-2828.)

Key Words: aspirin ■ blood flow ■ collagen ■ platelets ■ thrombosis

A
spirin (acetylsalicylic acid, ASA) and ticlopidine interfere with different and complementary important pathways of platelet activation, that is, arachidonic acid and ADP pathways, respectively.1 Recent clinical studies have demonstrated that a combination of ASA and ticlopidine markedly reduced thrombotic events occurring during coronary artery stenting.2–9 However, ticlopidine therapy has adverse effects. Clopidogrel is a thienopyridine that is more potent than ticlopidine and produces fewer adverse effects.10,11 In addition, clopidogrel also has been shown to increase the antithrombotic effect of ASA in recent experimental studies performed in animals.12,13 However, no published clinical or experimental study has investigated the antithrombotic effect of the combination of ASA and clopidogrel in humans.

The antithrombotic effect of drugs can be experimentally investigated in humans with the use of an ex vivo model of thrombogenesis that closely mimics relevant clinical situations.14,15 In this model, native blood is drawn from healthy volunteers through a parallel-plate chamber device, where it interacts, in well-established flow conditions, with collagen, a relevant thrombogenic surface. Blood flow conditions mimic wall shear rates encountered in moderately stenosed arteries (2600 s⁻¹). The efficacy of antithrombotic drugs is determined by quantifying the respective thrombus content in platelets and fibrin by immunoenzymatic methods.16 This model has been used to investigate a number of different antithrombotic strategies.17–21 Thus, we previously showed that combined ASA and ticlopidine therapy potentiates in a synergistic manner the antithrombotic effect of each drug alone.21 By using this ex vivo model of acute initial thrombus formation, we designed a randomized, non–placebo-controlled, double-blind study to determine the antithrombotic effects of combined clopidogrel+ASA therapy versus ASA.

A major clinical goal of antithrombotic therapy, notably in patients undergoing coronary stent implantation, is to be effective as soon as possible to prevent early stent thrombosis. Clopidogrel must undergo hepatic modification to cause
selective inhibition of ADP-induced platelet aggregation.\textsuperscript{10} Thus, inhibition of platelet aggregation is noted 2 hours after the administration of 75 mg, but it reaches a steady state within 3 to 7 days. The onset of action of the combination of clopidogrel+ASA is not known. Therefore, we also studied the pharmacodynamics of the antithrombotic effect of the combined clopidogrel+ASA therapy. In this regard, whereas clopidogrel is indicated for the reduction of atherosclerosis events at a dose of 75 mg daily, we tested whether an initial loading dose of 300 mg given on the first day of treatment accelerates its onset of action.

**Methods**

**Subjects**

The study population consisted of 18 healthy white male volunteers 20 to 30 years of age. They had no history or clinical signs of any disease and were not taking any medication known to affect blood coagulation or platelets during the study period. The volunteers each smoked <10 cigarettes per day, and they did not smoke on the day of the perfusion experiments. Clinical chemistry and hematological and hemostatic laboratory values were within the normal ranges. All subjects gave written informed consent to the protocol, which was approved by the local Human Subjects Committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Toulouse).

**Study Design**

This monocentric, randomized, non–placebo-controlled, double-blind study was carried out in the Center for Clinical Investigation at Hôpital Purpan, Toulouse, France. After selection for the trial, an initial perfusion experiment was performed on each volunteer who satisfied the inclusion and exclusion criteria: This perfusion experiment was considered as the baseline experiment. Each volunteer then randomly received 1 of the 3 tested regimens in a 3-period crossover design: either (1) 325 mg ASA daily for 10 days, (2) 325 mg ASA plus 75 mg clopidogrel daily for 10 days, or (3) 325 mg ASA plus 300 mg clopidogrel on day 1, followed by 325 mg ASA plus 75 mg clopidogrel daily for the last 9 days. The 3 regimens were given separately with a washout period of 3 to 6 weeks between each of them. All drugs were supplied by Sanofi Recherche in a blister-pack that indicated the day and time to be taken. The subjects were requested to come to the study center for blood sampling at different times after the drug was given. A total of 4 perfusion experiments were performed: 1.5, 6, and 24 hours after drug intake on day 1 and 6 hours after drug intake at the end of the treatment period on day 10. Blood was also collected just before each perfusion experiment for platelet aggregation tests. All adverse effects were recorded, and appropriate follow-ups were obtained.

**Preparation of Thrombogenic Surface**

Equine collagen (Collagen Reagent Horm, Nycomed) was spray-coated onto Thermanox plastic coverslips (Miles Laboratories) to a final density of 0.5 μg/cm². They were stored at room temperature for 15 to 20 hours before use.\textsuperscript{21}

**Perfusion Experiments**

Perfusion experiments were performed with a parallel-plate perfusion chamber device at 37°C.\textsuperscript{14,15} After blood sample collection, native blood was drawn directly from an antecubital vein of the volunteers through a 19-gauge infusion set (Ohmeda) over the collagen-coated coverslip positioned in the parallel-plate perfusion chamber. The blood flow rate was maintained at 10 mL/min by a peristaltic roller pump (MultiPerfex LKB, Pharmacia) placed distal to the chamber. The wall shear rate was 2600 s\(^{-1}\), which corresponds to that encountered in moderately stenosed arteries. The blood perfusion experiment lasted for 3 minutes and was followed by a 30-second perfusion of PBS at the same flow rate to wash out blood from the flow channel. The coverslip covered by thrombotic deposits was placed in a plasmin solution and processed as described below.

**Immunological Determination of Fibrin Deposition**

Fibrin deposition was quantified by immunological determination of fibrin degradation products of plasmin-digested thrombi.\textsuperscript{16} After perfusion, the thrombus was immediately incubated in 2 mL of a plasmin solution (Chromogenix, 0.7 U/mL, in Tris-buffered saline, pH 7.4) for 30 minutes at 37°C. Plasmin digestion was stopped by aprotinin (2000 kIU/mL, Bayer Pharma). The solution was centrifuged (4°C, 4300 g, 15 minutes) and the supernatant frozen at −80°C for measurement of fibrin degradation products and P-selectin levels (see below). Fibrin degradation products were measured with the use of an immunoenzymatic assay (Asserachrom D-Di, Stago). The amount of deposited fibrin is directly determined from the levels of fibrin degradation products expressed in fibrin equivalent units as indicated by the manufacturer.

**Other Laboratory Procedures**

Red cell, leukocyte, and platelet counts and hemoglobin and hematocrit were measured by an electronic counting device (Model S plus, Coulter Electronics). For platelet aggregation tests, blood was collected into a citrated Vacutainer (Becton Dickinson, ref 367704), containing 0.5 mL of 0.129 mol/L trisodium citrate for 4.5 mL of blood. Platelet-rich plasma was obtained after a centrifugation at 150g for 15 minutes at room temperature and, platelet-poor plasma was obtained after a second centrifugation at 1500g for 15 minutes. Platelet aggregation was performed with a platelet aggregometer (Helena Laboratories). The aggregating agents were ADP (5 μmol/L final concentration, Stago), equine collagen (10 μg/mL final concentration, Nycomed), and arachidonic acid (1 mmol/L final concentration, BioData Corp). The maximum amplitude of platelet aggregation was measured and expressed as a percentage of the difference between platelet-rich plasma and platelet-poor plasma.

**Statistical Analysis**

Results are expressed as mean±1 SEM. Analysis of fixed effects was performed on log-transformed data. The model included fixed effect terms for treatment, period, and subject within sequence as the random term. The carry-over effect was checked in the form of treatment by period interaction, and because it was not significant, it was dropped from the model. Pairwise comparisons of treatments were performed, and the estimates (with standard error and 95% confidence intervals) of the differences between treatments were obtained. All statistical tests of hypothesis were 2-tailed and were performed at the 0.05 level of significance.
Results

Study Population
Eighteen healthy male, white subjects, 20 to 30 years of age (mean age 24 years), were enrolled in the study from April 1998 through February 1999 and randomly assigned to the different treatments. None prematurely stopped the trial. All 18 volunteers completed the study according to the protocol.

Antithrombotic Effect of ASA and Clopidogrel + ASA on Day 10
The effects of the 3 antithrombotic treatments on collagen-induced thrombus formation 10 days after their first administration are shown in Figure 1. Compared with baseline, ASA had a modest but significant antithrombotic effect: Platelet and fibrin deposition were inhibited by 24% and 35%, respectively ($P=0.002$ and $P=0.03$, respectively). However, in volunteers treated with clopidogrel + ASA, the antithrombotic effect was much greater, since clopidogrel + ASA decreased platelet thrombus formation and fibrin deposition by 71% and 74%, respectively. This effect was significantly more potent than that produced by ASA alone ($P<0.01$), and it was comparable whether or not a 300-mg clopidogrel loading dose had been given ($P>0.10$).

Pharmacodynamics of Antithrombotic Effect of ASA and Clopidogrel + ASA
The pharmacodynamics of the antithrombotic effect of the 3 antithrombotic treatments on collagen-induced thrombus formation are shown in Figures 2, 3, and 4. Interestingly, ASA did not fully express its antithrombotic effect on the first day of administration. Its effect on platelet deposition was delayed: On day 1, ASA decreased platelet deposition by <10% ($P=NS$), whereas on day 10 it was decreased by 24% ($P=0.03$). However, the effect of ASA on fibrin deposition was not time-dependent: The reduction of fibrin deposition was comparable on the first and tenth days of treatment (35%, $P=0.010$).

Without a loading dose of clopidogrel, clopidogrel + ASA developed an antithrombotic effect within 6 hours after the first dose. This effect was moderate, since at this time period platelet and fibrin deposition were reduced by 34% and 60%, respectively ($P=0.042$ and $P<0.001$, respectively) as compared with 71% and 74%, respectively, on day 10. However, it was significantly superior to that produced by ASA alone ($P=0.030$).

With the 300-mg clopidogrel loading dose, the antithrombotic effect of clopidogrel + ASA appeared within 90 minutes after the first dose. As early as 6 hours after the first administration, it was very potent (61% and 75% of platelet and fibrin reduction, respectively, $P<0.001$) and comparable to that obtained after 10 days of treatment. As compared with ASA alone, this treatment was significantly more potent for preventing platelet deposition on collagen-coated surfaces at each time point ($P=0.040$). It also prevented fibrin deposition significantly more than ASA at 6 and 24 hours and at 10 days after the first dose ($P=0.004$). It was also superior to clopidogrel + ASA given without the 300-mg loading dose at each time point on the first day of treatment ($P=0.030$).

Figure 1. Effect of antiplatelet treatment on deposition of platelets and fibrin on collagen-coated coverslips. Surface was exposed for 3 minutes to nonanticoagulated blood at shear rate of 2600 s$^{-1}$. Volunteers were given for 10 days either 325 mg ASA daily, 325 mg ASA + 75 mg clopidogrel daily, or 325 mg ASA + 300 mg clopidogrel per day on days 2 to 10. Baseline perfusion was performed with blood from volunteers before administration of ASA or ASA + clopidogrel. Values are mean±SEM (n=18). *$P<0.01$ vs ASA.

Figure 2. Effect of ASA on deposition of platelets and fibrin on collagen-coated coverslips. Surface was exposed for 3 minutes to nonanticoagulated blood at shear rate of 2600 s$^{-1}$. Volunteers were given 325 mg ASA daily for 10 days, and perfusion experiments were performed 1.5, 6, and 24 hours after drug intake on day 1 and 6 hours after drug intake on day 10. Baseline perfusion was performed in volunteers before ASA administration. Values are mean±SEM (n=18). *$P<0.05$, **$P<0.01$ vs baseline.
Effect of Treatment on Platelet Aggregation
Platelet aggregation was evaluated on blood samples drawn from volunteers before each perfusion experiment. Results are shown in the Table. ASA inhibited collagen-induced Platelet Aggregation was evaluated on blood samples drawn from volunteers before each perfusion experiment. Results are shown in the Table. ASA inhibited collagen-induced (P<0.010) but not ADP-induced platelet aggregation. This inhibition was comparable on days 1 and 10. On day 1, the inhibition was time dependent: ASA inhibited collagen-induced platelet aggregation at 6 hours but not at 1.5 and 24 hours after drug administration.

ADP- and collagen-induced platelet aggregation were significantly more inhibited by the combination of clopidogrel+ASA than by ASA alone; this inhibition was maximum 10 days after the drug was given, and, at this time period, it was comparable with or without the 300-mg loading dose of clopidogrel administered. However, on day 1, the inhibition of platelet aggregation was time dependent. ADP-induced platelet aggregation was inhibited more quickly when a 300-mg loading dose of clopidogrel was given. Interestingly, with or without a loading dose of clopidogrel, collagen-induced platelet aggregation was less inhibited at 24 hours than at 6 hours after the drug was administered.

Finally, arachidonic acid–induced platelet aggregation was fully inhibited in all 18 volunteers, with all 3 tested regimens, and at all time points.

Discussion
By using a model of human thrombogenesis, we show in the present study that the antithrombotic effect of the combination of clopidogrel and ASA is significantly more potent than the antithrombotic effect of ASA alone. Second, we show that when clopidogrel is given with a 300-mg loading dose on the first day of treatment, the optimal antithrombotic effect of clopidogrel+ASA is obtained within 6 hours after the first dose.

With or without a 300-mg loading dose, clopidogrel increased the antithrombotic effect of ASA (Figure 1). Comparable findings have been shown in recent experimental studies performed in animals.2,3 Previously, we also showed that combined ASA and ticlopidine therapy dramatically potentiated the antithrombotic effect of each drug alone. Taken together, these results indicate that both ADP and thromboxane A2 play a major and synergistic role in mediating platelet thrombus formation and that both pathways must be simultaneously blocked to achieve a maximum antithrombotic effect.

Thienopyridine derivatives have a delayed onset of action.10 Thus, pharmacological studies have indicated that maximum inhibition of ADP-induced platelet aggregation occurs only after 3 to 5 days of oral administration of 250 mg BID of ticlopidine.22 In the present study, the antithrombotic effect of clopidogrel+ASA was time dependent. However, it is interesting to note that when a 300-mg loading dose of clopidogrel was given on day 1, the antithrombotic effect of the combined clopidogrel+ASA therapy appeared within 90 minutes after oral intake and that at 6 hours it was comparable to that seen on day 10. This finding is particularly important in patients undergoing coronary stent implantation or having acute coronary syndrome, in which effective antithrombotic effects are needed as early as possible to prevent thrombosis.
The antiplatelet drug regimens affected coagulation as well, since fibrin deposition was significantly reduced. The apparent anticoagulant effect provided by antiplatelet regimens may be the direct consequence of a reduction in platelet deposition, since fibrin deposition on collagen substrate generally occurs subsequent to platelet thrombus formation. 1 It is also possible that this finding is a result of reduced platelet activation, since activated platelets amplify the coagulation cascade by binding activated coagulation factors to form the tenase and prothrombinase complexes. 1

Criticisms with respect to the significance and clinical relevance of the ex vivo model of human thrombogenesis used may be raised. In our study, thrombus formation was only determined on a collagen-coated surface. Whereas collagen is an important determinant of the thrombogenicity of ruptured human atherosclerotic lesions, 25 there are other components at least as important, notably tissue factor. 26 We did not study the antithrombotic effect of clopidogrel+ASA on tissue factor, since we previously showed that ticlopidine+ASA did not inhibit the thrombotic process on tissue factor. 21 In addition, we examined the effect of antithrombotic drugs on early acute platelet thrombus formation. Perfusion times were only 3 minutes because thrombus formation in this model is maximum at 3 minutes. 16 Also, the study of antithrombotic drugs on the very early events of thrombus formation are important, since they have profound impact on later events. However, longer perfusion times would give additional information on thrombus growth and thrombus stabilization. But, as discussed above, our thrombosis model has previously been shown to be useful in evaluating different antithrombotic agents. 17–21 One can note that results obtained with largely used antithrombotic agents appear consistent with clinical data. 27

In conclusion, this study confirms that combined ASA and clopidogrel therapy exerts a potent synergistic antithrombotic effect significantly superior to that given by ASA alone. Since clopidogrel is well tolerated, this result warrants clinical trials in which clopidogrel is associated with ASA. In this regard, the present demonstration that a 300-mg loading dose of clopidogrel given on day 1 accelerates the rate with which combined clopidogrel+ASA exerts its antithrombotic effect is important, since a major clinical goal of antithrombotic therapy is to be rapidly effective.

### References


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