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Combined therapy with clopidogrel and aspirin significantly increases the bleeding time through a synergistic antiplatelet action

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Objective: **Many thromboembolic events occur in patients taking aspirin. Dual therapy with aspirin and clopidogrel may prove effective at reduction of thromboembolic complications. However, the extent to which these two drugs interact may significantly increase the risk of bleeding in open surgery. Because of the increased use of combination antiplatelet therapy in populations with significant atherosclerotic disease, this risk needs to be evaluated by the assessment of the combined effect in vivo of clopidogrel and aspirin on bleeding time and platelet function.**

Outcomes: **In seven healthy subjects, addition of low dose clopidogrel (2 × 75 mg) to aspirin (150 mg) therapy significantly increased bleeding time (from 7.6 ± 3.4 minutes to 17.5 ± 8.6 minutes; $P < .05$), with concomitant falls in adenosine diphosphate (ADP)–induced platelet fibrinogen binding and aggregation ($P < .05$). Increasing the dose of clopidogrel to 300 mg increased bleeding time (to 24.9 ± 8.5 minutes; $P < .05$) without significant additional platelet inhibition. There was considerable variability in the individual subject platelet response to the lower dose of clopidogrel. Those patients with the highest ADP response at baseline had the least response, and subjects with a weak response to ADP at baseline achieved maximal platelet inhibition with the low dose of clopidogrel.**

Conclusion: **The increases in bleeding times should be considered in combination antiplatelet therapy in patients who undergo open vascular surgery. (J Vasc Surg 2002;35:1204-9.)**

Despite advances in the management of symptomatic atherosclerotic disease, thromboembolic complications still occur at sites of plaque instability. Endothelial disruption and platelet recruitment, activation, and aggregation are fundamental to the pathogenesis of arterial thrombosis.[1] Aspirin has been the mainstay of antiplatelet therapy, preventing platelet aggregation with irreversibly inhibiting the formation of thromboxane A_2, which reinforces the effects of other physiologic platelet agonists, such as adenosine diphosphate (ADP) and collagen.[2] Although a standard dose of aspirin has been shown to reduce the risk of vascular occlusion by as much as 25%,[3] most arterial thrombotic events still occur in patients who are currently taking aspirin.

Recent interest has focused on the novel group of antiplatelet drugs, the theinopyridine derivatives, which act as specific ADP receptor antagonists. Two drugs have been licensed: ticlopidine hydrochloride and the related compound, clopidogrel bisulfate, which gives rise to fewer adverse side effects.[4] The CAPRIE trial[5] showed that long-term therapy (mean, 1.91 years) with 75 mg per day clopidogrel gave an overall reduction in thrombotic events compared with 325 mg per day aspirin in patients at high risk of vascular occlusion. This was particularly significant in patients with peripheral arterial disease (relative risk reduction, 23.9%; 95% confidence interval, 8.9 to 36.2; $P = .0028$). The same study also indicated that clopidogrel had a safer drug profile than that of aspirin, with fewer hemorrhagic incidences.[5-7] However, theinopyridines still gave only a modest improvement over aspirin. The important issue is whether theinopyridines add to the benefit seen with aspirin without increasing the risk of bleeding. It is likely that increasing numbers of patients attending for vascular surgical procedures will be undergoing combination antiplatelet therapy. This may be of particular relevance in the acute setting, including intravascular interventions.

Studies in patients undergoing carotid endarterectomy have shown that, despite the use of aspirin and heparin therapy, thromboembolic events still occur after surgery[8] and increasing rates of postoperative embolization are associated with a significantly higher risk of thromboembolic stroke.[9,10] The ASA and Carotid Endarterectomy Trial showed that there may be a beneficial effect of low dose (81 mg or 325 mg) aspirin relative to higher doses (650 mg or 1300 mg).[11] Thus, in this setting, aspirin therapy alone is not sufficient to prevent platelet-mediated events. A recent study from this center,[12] which measured platelet reactivity in patients before carotid surgery, found that those patients with the highest numbers of embolic signals after surgery (determined with transcranial Doppler scan) had platelets that showed the greatest response to ADP before surgery. This was despite all patients taking 150 mg aspirin per day, and thromboembolic events did not correlate with the patient response to aspirin. These data strongly suggest that

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there may be a role for specific ADP receptor antagonists in carotid artery surgery, in addition to aspirin.

Studies in animals have indicated that aspirin enhances the antithrombotic efficacy of theinopyridines,[13-15] with only limited hemostatic impairment.[13-15] There is, however, little information on the effects of combining aspirin and clopidogrel therapy on bleeding time (BT) plus platelet function in humans. Most clinical experience with combination aspirin and ticlopidine or clopidogrel is in patients undergoing percutaneous transluminal angioplasty and stenting of coronary arteries.[16-18] In this setting, patients need small arterial puncture wounds to provide intraarterial access, and reports have indicated that the incidence rate of bleeding in patients with combined aspirin and ticlopidine or clopidogrel therapy was less than that with conventional anticoagulant regimes (generally heparin and warfarin). However, in considering the use of ADP receptor antagonists in combination with aspirin in open vascular surgery, a balance must be sought between prevention of thromboembolic events and achievement of adequate hemostasis. To address this issue, we conducted a study in healthy male subjects for the evaluation of the effects of aspirin alone or in combination with clopidogrel bisulfate on BT, platelet aggregation, and activation.

METHOD

Study design. Seven healthy, nonsmoking male subjects were studied, with ages ranging from 24 to 36 years (median, 30 years) and an average weight of 79.4 kg (range, 74 to 85 kg). Before the study, each volunteer underwent a full blood count and standard clotting screen to exclude underlying hematologic disorders. Subjects were also questioned concerning aspirin intolerance and to confirm that no cyclooxygenase inhibitor had been taken in the month before the study. The study was approved by the Ethics Committee of the Leicestershire University Hospitals' Trust.

Blood samples were collected at four timepoints for measurement of platelet activity. Baseline blood samples were collected on day 0 at 9 AM. The subjects then were given 150 mg aspirin (The Wallis Laboratory Ltd, Luton, England), and a second set of blood samples was taken 4 hours later. At 24 and 48 hours, the subjects received 75 mg of oral clopidogrel (Plavix; Bristol-Myers Squibb, Middlesex, United Kingdom) together with 150 mg aspirin per day. The third blood sample was collected on day 3 at 9 AM. The subjects then were given 300 mg of clopidogrel, and a final set of blood samples was collected 5 hours later. The doses of clopidogrel were chosen to give suboptimal and maximal ADP receptor inhibition. The 300 mg dose of clopidogrel induces a similar level of platelet inhibition to that seen in patients with 75 mg once a day on a long-term basis.

Blood was collected from the antecubital fossa with a standardized phlebotomy technique with minimal stasis, designed to minimize platelet activation.[19] Subjects were rested for 20 minutes before blood was collected via a 21-gauge butterfly needle into vacutainer tubes (Becton Dickinson, Oxford, United Kingdom). The first 3 mL of blood were taken into ethylenediamine tetraacetic acid (4.5 mg) and used to obtain a full blood count (A^CT Diff Analyser, Coulter Electronics Ltd, Luton, United Kingdom). Subsequent samples were taken into trisodium citrate (3.8% weight/volume) and processed for platelet aggregometry and flow cytometry.

Bleeding time measurement. BT measurements were performed on each of the four occasions in the contralateral arm immediately after the collection of blood samples times described previously. Measurements were performed by a single individual (P.B.) with extensive experience with this technique. All tests were performed on the volar surface of the forearm with a sphygmomanometer inflated to 40 mm Hg. Standardized incisions (5 mm long, 1 mm deep) were made parallel to the axis of the forearm with a dual blade spring-loaded device (Simplate IIR, Organon Teknika, Cambridge, UK). Excess blood was removed from the cut at 30-second intervals with blotting paper and with care to avoid contact with the wound. The BT was measured as the time of the incision to the time bleeding stopped. If bleeding exceeded 30 minutes (a time period agreed on by the Ethical Committee), compression was applied to the wound until bleeding stopped and the BT was recorded as 30 minutes.

Platelet aggregometry. Platelet rich plasma was prepared from citrated blood with centrifugation for 20 minutes at 160*g*. Platelet aggregation in response to 4×10^{-6} mol/L ADP (Sigma, Poole, UK), 6×10^{-6} mol/L thrombin receptor agonist peptide (TRAP) and SFLLRN peptide were synthesized in house by University of Leicester Protein & Nucleic Acid Laboratory, and 1.0×10^{-3} g/L collagen (Horm collagen, Nycomed, Cambridge, UK) was monitored with a PAP-4 aggregometer (Bio/Data Corporation, Alpha Laboratories Ltd, Hants, United Kingdom) as previously described.[20] Aggregation was recorded as the percentage of maximal aggregation, compared with light transmission through autologous platelet poor plasma.

Whole blood flow cytometric analysis. Whole blood flow cytometry was performed as described previously.[19,20] Samples were processed within 10 minutes of collection, with addition of 5 μL citrated blood with 50 μL Hepes-buffered saline solution containing appropriate fluorescein isothiocyanate (FITC)–conjugated antibodies and agonists and incubating for 20 minutes at room temperature (20° C to 22° C). Levels of glycoprotein (GP) IIb/IIIa on unstimulated platelets (expressed as mean fluorescence) were measured with a CD41/61 Mab, RFGP56, raised in house, and purified and conjugated to FITC by Cymbus Biotechnology Ltd (Chandlersford, Hants, United Kingdom). Platelet-bound fibrinogen was measured with a FITC-conjugated rabbit antibody to human fibrinogen (Rαfgn-FITC; Dako Ltd, High Wycombe, United Kingdom), and P-selectin expression was measured with a FITC-conjugated CD62P Mab, AK-6 (Serotec, Oxford, United Kingdom). Samples were analyzed without stimulation and in response to ADP (10^{-7}, 10^{-6}, and 10^{-5} mol/L) and TRAP (5×10^{-6} mol/L). At the end of the 20-minute incubation, the samples were diluted 100 times in formyl saline solution (0.2% [volume/volume] formaldehyde in 0.9%

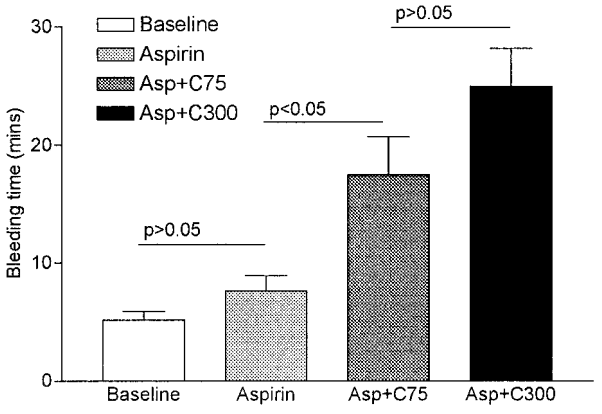

Fig 1. Template bleeding time measurements in healthy subjects (n = 7) given aspirin (150 mg), aspirin (150 mg/day) and clopidogrel (75 mg/day; *Asp+C75*) for 2 days, and aspirin (150 mg) and clopidogrel (300 mg; *Asp+C300*). Results are expressed as mean ± standard error of mean.

[weight/volume] NaCl) and analyzed, within 2 hours, in a Beckman Coulter MCL-XL flow cytometer (Beckman Coulter Ltd, Milton Keynes, UK). Fibrinogen binding and P-selectin expression are expressed as the percentage of platelets found positive for binding of fluorescent antibody. Negative controls for GPIIb/IIIa and P-selectin expression were set at 2% with FITC-conjugated IgG Mab, MOPC21C (Sigma). The negative control for fibrinogen binding was an unstimulated sample incubated with Rαfgn-FITC in the presence of 6 μmol/L ethylenediamine tetraacetic acid.

Statistical analysis. All data were analysed as the mean ± the standard deviation and presented graphically as the mean ± the standard error of the mean. Comparison of the different treatment groups was with analysis of variance, and 95% confidence intervals (CIs) are stated. Comparison of the variability of platelet fibrinogen binding in the high and low ADP responder groups (Fig 1) was by two-way analysis of variance, with the assumption of unequal variance. A *P* value of .05 or less was considered as statistically significant.

RESULTS

Effect on bleeding time of aspirin alone or aspirin combined with clopidogrel. A standard hematologic screen in the seven subjects showed no underlying abnormalities. The baseline BT was 5.1 ± 1.9 minutes (mean ± standard deviation), and all subjects had BTs within the healthy laboratory range of 4 to 10 minutes (Fig 2). Ingestion of 150 mg aspirin increased BT in six of the seven subjects, resulting in a nonsignificant increase in the BT to 7.6 ± 3.4 minutes ($P > .05$; 95% CI, –7.4 to 12.2). After 2 days of treatment with 75 mg clopidogrel and 150 mg aspirin per day, there was a significant 3.4-fold increase in BT relative to baseline to 17.5 ± 8.6 minutes ($P < .01$; 95% CI, 2.9 to 22.6). The BT was increased above the healthy range in six of the seven subjects. When the clopidogrel dose was increased to 300 mg, there was a significant five-fold increase in BT relative to baseline to 24.9 ± 8.5 minutes ($P < .001$; 95% CI, 10.0 to 29.6). After 300 mg of clopidogrel, there was a 47% increase in the BT relative to low dose clopidogrel ($P > .05$; 95% CI, –2.8 to 16.9). All subjects who received aspirin plus 2 days of 75 mg clopidogrel achieved hemostasis within the 30-minute time limit set for the BT, but with high-dose clopidogrel, three of the seven subjects needed compression at 30 minutes to stop bleeding from the forearm incision and two of these three had oozing from the BT wound within the subsequent 4 hours. It is likely that if the bleeding had been allowed to continue for an increased period of time, then there would have been a significant difference between the 75 mg and 300 mg of clopidogrel bisulfate in terms of BT.

Ex vivo platelet aggregation. ADP-induced platelet aggregation was significantly reduced from a baseline level of 71.5% ± 26.2% to 47.9% ± 21.0% after aspirin (Fig 2). This was further reduced to 34.7% ± 21.1% aggregation after 2 days of aspirin with 75 mg clopidogrel. Clopidogrel at 300 mg with aspirin gave a further reduction in ADP-induced platelet aggregation to 24.9% ± 27.4%. These reductions in aggregatory response were all significantly different from the baseline values and significantly different from each other ($P < .05$ for all).

Collagen-induced aggregation showed a similar significant trend to that seen with ADP (Fig 2), dropping from 85.9% ± 6.4% to 48.8% ± 10.5% with aspirin to 31.5% ± 10.7% with aspirin plus low dose clopidogrel and to 16.6% ± 26.7% with aspirin and high dose clopidogrel bisulfate ($P < .01$ for all). Aggregation in response to TRAP was significantly reduced with aspirin alone ($P < .05$), but addition of either 2 × 75 mg or 300 mg clopidogrel did not produce a further significant reduction in TRAP-induced platelet aggregation (Fig 2).

Whole blood flow cytometric analysis. The effects of the antiplatelet drugs on the platelet response to agonist stimulation, measured with flow cytometry, were similar to the results seen with aggregometry (Fig 3). Fibrinogen binding in response to ADP was reduced by 21% after aspirin ingestion from 63.5% ± 24.4% to 50.3% ± 18.9% ($P < .05$). Addition of 2 × 75 mg clopidogrel bisulfate and 300 mg clopidogrel gave further decreases in ADP-induced fibrinogen binding to 40.8 ± 29.1 (36% reduction from baseline) and 24.1 ± 19.1 (62% reduction from baseline), respectively. These reductions were both statistically significantly different from baseline ($P < .05$). However, although high dose clopidogrel gave a more than 40% reduction in ADP-induced fibrinogen binding to platelets when compared with low dose clopidogrel, this did not differ statistically, reflecting the high degree of intersubject variability in the response to the drug.

Aspirin produced a small (9%) but nonsignificant reduction in TRAP-induced fibrinogen binding. Addition of 2 × 75 mg clopidogrel gave a larger (48%; $P = .05$) decrease over and above that seen with aspirin alone, but increasing the dose of clopidogrel to 300 mg did not cause any further decrease in TRAP-induced fibrinogen binding.

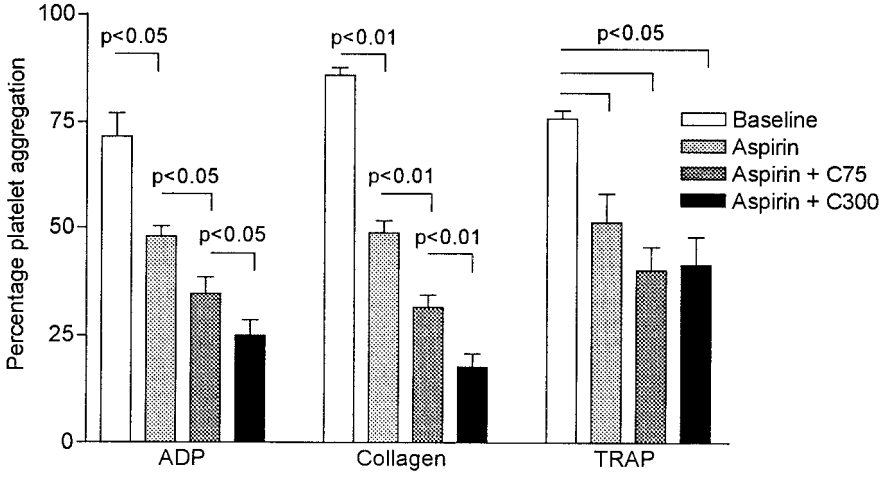

Fig 2. Effects of aspirin alone or in combination with low dose (2 × 75 mg; *C75*) or high dose (300 mg; *C300*) clopidogrel on platelet aggregation in response to adenosine diphosphate (*ADP*; 10^{-6} mol/L), collagen (1.0×10^{-3} g/mL), and thrombin receptor agonist peptide (*TRAP*; 6×10^{-6} mol/L). Results are expressed as mean ± standard error of mean.

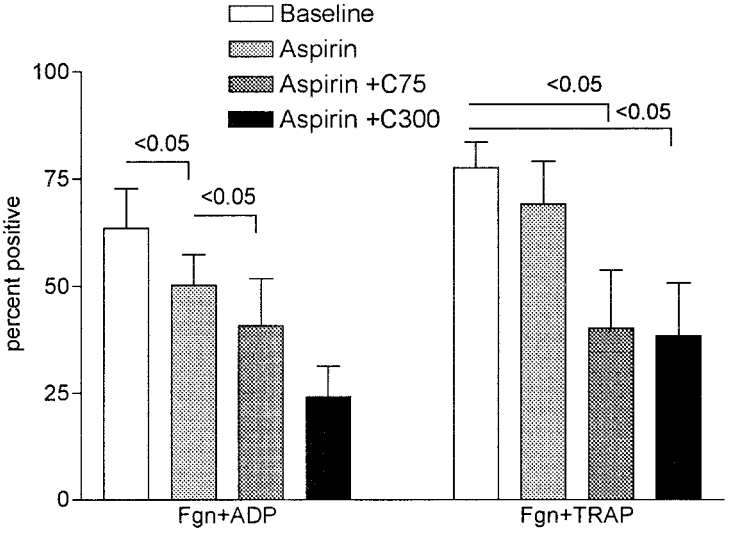

Fig 3. Effects of aspirin alone or in combination with low dose (2 × 75 mg; *C75*) or high dose (300 mg; *C300*) clopidogrel on platelet activation markers in response to adenosine diphosphate (*ADP*; 10^{-6} mol/L) and thrombin receptor agonist peptide (*TRAP*; 6×10^{-6} mol/L), measured with whole blood flow cytometry. Results are expressed as mean ± standard error of mean.

P-selectin expression in response to ADP and TRAP was not significantly reduced with any of the treatments (data not shown). Neither aspirin alone nor aspirin in combination with either dose of clopidogrel significantly affected the levels of bound fibrinogen or P selectin on unstimulated platelets, which were less than 3% in all cases, and expression of GPIIb/IIIa remained unchanged ($P > .3$ for all; data not shown).

Intersubject variability in response to clopidogrel bisulfate. There was considerable variability between individuals in response to ADP at baseline and subsequent response to low dose of clopidogrel. This is illustrated with the subject platelet fibrinogen binding in response to ADP (Fig 4). Subjects were divided into those who, at baseline, had higher than average levels of bound fibrinogen in response to ADP (n = 3) and those with lower than average responses (n = 4). With aspirin alone, both low ADP and high ADP responders showed a similar significant reduction in platelet fibrinogen binding at all levels of ADP used (Fig 4). The response to 2 × 75 mg clopidogrel was, however, different between the two groups. Low baseline ADP responders showed a significant fall (45% from base-

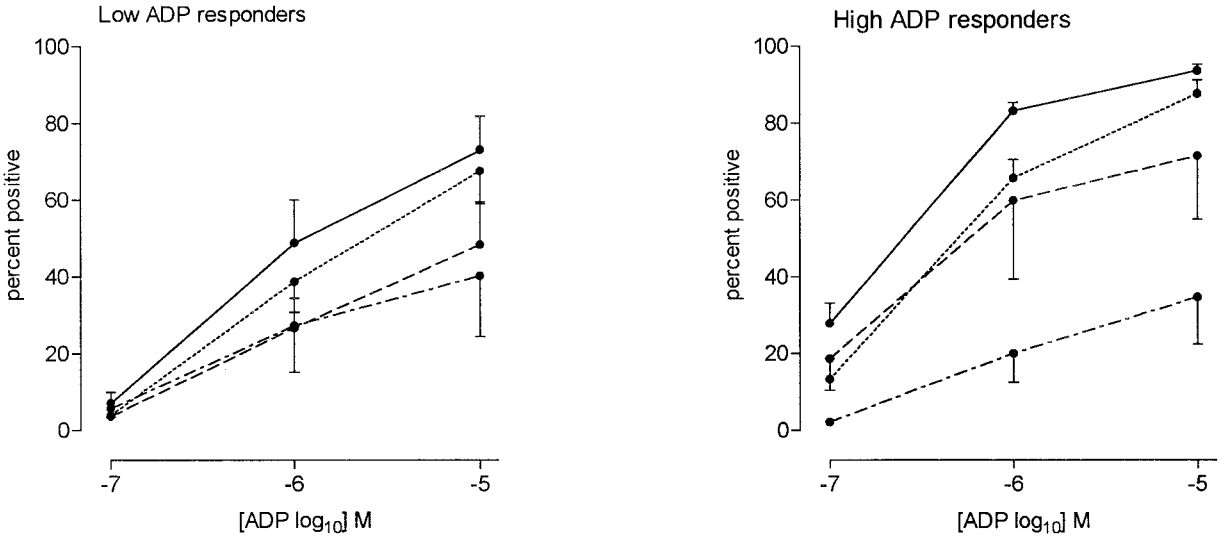

Fig 4. Adenosine diphosphate *(ADP)*–induced platelet fibrinogen binding, measured with whole blood flow cytometry. Measurements were made at baseline *(continuous line)*, after aspirin therapy alone *(dotted line)*, or with aspirin therapy in combination with low dose (2 × 75 mg; *dashed line*) or high dose (300 mg; *dashed/dotted line*) clopidogrel bisulfate. Subjects are divided into those with lower than average response to ADP (*low ADP responders*; n = 3) and those with higher than average response to ADP (*high ADP responders*; n = 4). Data are shown as mean ± standard error of mean.

line) in ADP-mediated platelet activation with the lower dose of clopidogrel and no subsequent fall in ADP response when the clopidogrel dose was increased to 300 mg. In contrast, the high ADP responders showed relatively little reduction in response to ADP with low dose clopidogrel (17.5% fall from baseline) but a large drop in ADP response (75.7% from baseline) after the higher clopidogrel dose. Despite these relative differences in the response to the lower dose of clopidogrel, the level of fibrinogen binding in response to ADP with the 300 mg dose of clopidogrel was similar in all subjects. There were similar findings with the aggregatory response to ADP (data not shown).

DISCUSSION

Although standard doses of aspirin can reduce the incidence rate of thromboembolic events, the effect is only partial[2] and does not inhibit the effect of physiologic agonists, such as ADP, directly. Long-term prophylactic treatment with the ADP receptor antagonists ticlopidine hydrochloride or clopidogrel bisulfate[5-7] has shown a small but significant benefit over aspirin alone, with no increase in bleeding. In the more acute setting of intracoronary intervention, a combination of aspirin and theinopyridine reduced thrombotic events without an increased incidence of bleeding, either after surgery or systemically, compared with conventional antithrombotic regimes.[16-18] This begs the question of whether combined therapy with these drugs would be beneficial in other vascular interventions, including open arterial surgery.

Therapeutically active doses of both ticlopidine and clopidogrel have been shown to prolong the BT in humans,[21,22] but there have been relatively few studies of the combined effects of clopidogrel with aspirin on bleeding risk in humans.[23] The combination of aspirin and ticlopidine or clopidogrel has been shown to have additive effects on ex vivo platelet function and in animal models of acute and subacute thrombosis with only a modest increase in BT.[12-15] There has been only one reported study of combination antiplatelet therapy in open surgery, with no systematic assessment of bleeding.[24]

The doses of clopidogrel used in this study were chosen to represent a submaximal (2 days at 75 mg per day) and maximum (300 mg for 5 hours) level of platelet inhibition.[25,26] Prolongation of the BT was seen with low-dose clopidogrel and aspirin in all but one subject, and prolonged bleeding was seen in all subjects after high dose clopidogrel with aspirin. This was reflected in concomitant reductions in platelet aggregation and fibrinogen binding in response to ADP and collagen. The reduction in platelet function with high dose clopidogrel, measured with flow cytometry, was not statistically different from low dose clopidogrel. This was because subjects with a low baseline response to ADP achieved maximal antiplatelet effect with the low dose of clopidogrel. Increasing the dose to 300 mg had little or no effect on ex vivo platelet function in these individuals, whereas the BT increased significantly.

Variability in response to clopidogrel has been previously ascribed to differences in drug metabolism or body weight between individuals, although this was not born out by the CAPRIE study.[5] The subjects in this study showed relatively little variation in body weight (74 to 85 kg), and there was no correlation between this variation and re-

sponse to clopidogrel. It would appear from this study that variability in response to clopidogrel is more closely linked to the ADP responsiveness of the subject platelets; those subjects with a high level of response to ADP needed a higher dose of clopidogrel to achieve maximum inhibition of ADP response.

There was no direct correlation between BT before or after treatment and the individual platelet response to agonists or to clopidogrel. This reflects the acknowledged variability and imprecision of standard BT measurements.[27] Although BT measurement also shows a relatively poor correlation with surgical bleeding,[28] it is a measure of the overall platelet hemostatic function and can be used to show the hemorrhagic effect of antiplatelet drugs. Newer laboratory-based methods, such as the PFA-100 analyzer,[29] may give a more relevant measure of the individual patient bleeding risk, but such a correlation has yet to be established.

TRAP-induced platelet aggregation and activation were reduced with the low dose of clopidogrel and aspirin, but increasing the dose of clopidogrel to 300 mg gave no further reduction in the thrombin response. Thus, platelet response to thrombin can be maintained, allowing a degree of hemostasis in the face of selective inhibition of thromboxane and ADP-mediated platelet activation, confirming earlier studies in a baboon model.[15]

Taken together, these data suggest that combining clopidogrel with aspirin for major cardiovascular or general surgical procedures may carry a significantly increased risk of bleeding. The recent CURE study has shown a significant benefit for the simultaneous administration of both drugs in the prevention of acute coronary syndromes.[30] It seems likely that patients undergoing chronic dual therapy will increasingly need consideration of vascular surgical intervention, and the significant potential for increased bleeding should be carefully considered.

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