Adenosine Diphosphate–Induced Platelet Aggregation Is Associated With P2Y<sub>12</sub> Gene Sequence Variations in Healthy Subjects

Pierre Fontana, MD; Annabelle Dupont, MSc; Sophie Gandrille, PhD; Christilla Bachelot-Loza, PhD; Jean-Luc Reny, MD, PhD; Martine Aiach, PhD; Pascale Gaussem, PhD

Background—The adenosine diphosphate (ADP) receptor P2Y<sub>12</sub> plays a pivotal role in platelet aggregation, as demonstrated by the benefit conferred by its blockade in patients with cardiovascular disease. Some studies have shown interindividual differences in ADP-induced platelet aggregation responses ex vivo, but the mechanisms underlying this variability are unknown.

Methods and Results—We examined ADP-induced platelet aggregation responses in 98 healthy volunteers, and we identified 2 phenotypic groups of subjects with high and low responsiveness to 2 μmol/L ADP. This prompted us to screen the recently identified G<sub>i</sub>-coupled ADP receptor gene P2Y<sub>12</sub> for sequence variations. Among the 5 frequent polymorphisms thus identified, 4 were in total linkage disequilibrium, determining haplotypes H1 and H2, with respective allelic frequencies of 0.86 and 0.14. The number of H2 alleles was associated with the maximal aggregation response to ADP in the overall study population (P<0.007). Downregulation of the platelet cAMP concentration by ADP was more marked in 10 selected H2 carriers than in 10 noncarriers.

Conclusions—In healthy subjects, ADP-induced platelet aggregation is associated with a haplotype of the P2Y<sub>12</sub> receptor gene. Given the crucial role of the P2Y<sub>12</sub> receptor in platelet functions, carriers of the H2 haplotype may have an increased risk of atherothrombosis and/or a lesser clinical response to drugs inhibiting platelet function. (Circulation. 2003;108:989-995.)

Key Words: platelets | genetics | receptors

One of the most important mediators of hemostasis and thrombosis is adenosine diphosphate (ADP).<sup>1</sup> The effect of ADP on platelets is mediated by two P2Y receptors, designated P2Y<sub>1</sub> and P2Y<sub>12</sub>. Transduction of the ADP signal involves both a transient rise in Ca<sup>2+</sup> mediated by the G<sub>q</sub>-coupled P2Y<sub>1</sub> receptor<sup>2</sup> and inhibition of adenylate cyclase mediated by the G<sub>i</sub>-coupled P2Y<sub>12</sub> receptor.<sup>3,4</sup> Simultaneous activation of the G<sub>q</sub> and G<sub>i</sub> pathways by ADP is necessary for normal aggregation.<sup>5,6</sup> Activation of the G<sub>q</sub> pathway through P2Y<sub>1</sub> leads to platelet shape changes and a rapidly reversible wave of platelet aggregation,<sup>2,6</sup> whereas activation of the G<sub>i</sub> pathway through P2Y<sub>12</sub> amplifies G<sub>i</sub>-mediated responses and alone may elicit slowly progressive and sustained platelet aggregation.<sup>7</sup> The effect of P2Y<sub>12</sub> is not limited to inhibition of adenylate cyclase and a subsequent reduction in intracellular cAMP content. Indeed, it has been shown that P2Y<sub>12</sub> receptor activates glycoprotein (GP) IIb/IIIa integrin via a phosphoinositide (PI) 3–kinase pathway<sup>8</sup> and/or another unidentified G protein.<sup>9</sup> P2Y<sub>12</sub> thus appears to have a pivotal role in the irreversible wave of platelet aggregation that occurs on exposure to ADP.

The importance of P2Y<sub>12</sub> is emphasized by the fact that it is the target of the thienopyridine drugs ticlopidine and clopidogrel.<sup>3</sup> The P2Y<sub>12</sub> gene, which encodes the 342–amino acid receptor, was recently identified.<sup>3,4,10</sup> Four patients have been described whose platelets, when exposed to ADP, undergo little, if any, aggregation and show a decrease in the normal adenylate cyclase inhibition of prostaglandin E<sub>1</sub> (PGE<sub>1</sub>)–stimulated platelets.<sup>11,12</sup> In 1 patient, this phenotype was associated with a P2Y<sub>12</sub> gene abnormality.<sup>3</sup>

Considerable interindividual variations in the concentration of ADP required to produce irreversible aggregation have been reported.<sup>13</sup> Because inherited factors have a major role in platelet aggregation,<sup>14</sup> we looked for sequence variations in the P2Y<sub>12</sub> gene that might explain the interindividual variability of platelet aggregation responses to ADP.

Methods

Subjects

Ninety-eight unrelated healthy white male volunteers age 18 to 35 years were recruited and investigated in the Clinical Investigation
Center of Hôpital Européen Georges Pompidou. The volunteers were nonsmokers and denied taking any medication for at least 10 days before blood collection. Exclusion criteria were personal or family history of excessive bleeding or thrombosis. A physical examination and routine laboratory tests (including white blood cell, red blood cell, and platelet counts; mean platelet volume; prothrombin time; activated partial thromboplastin time; plasma fibrinogen; and C-reactive protein assays) were performed before inclusion. Blood obtained from all the volunteers on day 0 (visit 1) and day 7 (visit 2) was subjected to aggregation tests. A third blood sample was obtained from a subset of 20 volunteers for platelet cAMP assay and comparison of maximal aggregation in citrated and in hirudin-anticoagulated platelet-rich plasma (PRP). All the subjects gave their written informed consent, and the local ethics committee approved the study protocol.

Sample Preparation
Venous blood was collected between 8:00 and 10:00 AM, after an overnight fast, in tubes containing either 0.105 mol/L sodium citrate (1:9 v/v; BD Vacutainer, Becton Dickinson) or 50 μg/mL lepirudin (Refudan, Hoechst) by using a 19-gauge needle and no tourniquet. The first 2 mL of blood was discarded. PRP was obtained by centrifugation at 150 g for 10 minutes at room temperature. Autologous platelet-poor plasma, obtained by further centrifugation at 2300g for 15 minutes, was used to adjust the platelet count of PRP to 250 x 10^9/L.

Platelet Aggregation Studies
Aggregation studies were performed within 2 hours after blood collection. Aggregation was measured at 37°C by using a photometric method on a 4-channel aggregometer (Reguest). A 280-μL aliquot of PRP was incubated for 3 minutes at 37°C and was then stirred at 1100 rpm for 2 minutes before adding 20 μL of saline or ADP (Sigma-Aldrich) at 1, 2, or 5 μmol/L (final concentration). The platelet aggregation response was recorded for 5 minutes.

cAMP Measurement
Platelet cAMP content was measured by incubating 270 μL of PRP with 10 μL of a solution of the stable prostaacyclin analog iloprost (20 μg/L, final concentration; Iломédine, Schering-Plough) for 1 minute with stirring, then adding 20 μL of saline or ADP (1, 2, or 5 μmol/L). The reaction was stopped 3 minutes later by adding 20 μL 12% trichloroacetic acid. cAMP was then extracted and measured with an enzyme immunoassay (Amersham, Pharmacia Biotech) according to the manufacturer’s instructions, without knowledge of the subject’s P2Y12 genotype.

Genotyping Studies
Genomic DNA was isolated from peripheral blood mononuclear cells by using the Qiamp Maxi Kit (Qiagen) according to the manufacturer’s instructions.

P2Y12 Receptor
We took advantage of the cDNA sequence reported by Hollopeter et al., which is available under GenBank accession number AF313449. Assuming that P2Y12 gene organization was the same as that of other seven-transmembrane-domain receptor genes, most of which comprise 2 exons separated by a single intron, we performed a preliminary amplification experiment by using 2 oligonucleotides, designated E and J, as sense and antisense primers; they were located at the 5’ and 3’ ends of the reported cDNA (Figure 1). The amplification product was then purified with the Pre-Sequencing Kit (Amer- sham Pharmacia Biotech). Purification was followed by a cycle sequencing reaction by using primers E, B, and J. Subsequent sequencing using several other primers (G, H, K, and L) (Figure 1) allowed us to determine the nucleotide (nt) sequence of the amplification product. Sequence analysis was performed with an ABI Prism 3700 (Applied Biosystems). Screening of 40 subjects in a population is sufficient to identify polymorphisms with a frequency of ≥5%, with a CI of 95%. Thus, a series of the first 48 consecutive healthy volunteers were screened for P2Y12 gene polymorphisms by sequencing the 58 nt of exon 1 with primer K; the 947 nt of its 3’ flanking region with primers E and G; the 1274 nt of exon 2 with primers L, C, and M; and the 570 nt of its 5’ flanking region with primer H. The primers are described in Figure 1. The 3’untranslated regions of the exons, in Figure 2. The P2Y12 gene was then sequenced in the other 50 subjects by using primers targeting the identified polymorphisms, i.e., primer E for the i-C139T, primer G for the i-T744C and i-ins801A, and primer L for the C34T and G52T.

GP IIb/IIIa
We used restriction fragment-length polymorphism analysis to detect the substitution of cytosine for thymidine at position 1565 in exon 2 of the GP IIb/IIIa gene, which is responsible for the P1α2β1 polymorphism. 15 PCR and sequencing results were analyzed without knowledge of the platelet aggregation results. Genotyping was repeated in ambiguous cases. All 98 subjects were successfully genotyped.

Platelet RNA Extraction and Reverse Transcription
Four milliliters of PRP (10^9 platelets) was pelleted by centrifugation for 15 minutes at 2300g. RNA was extracted with the RNeasy kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed by using 10 μL RNasin ribonuclease inhibitor (Promega), 100 μL Superscript II Raushe reverse transcriptase (Invitrogen), and 1.5 mmol/L random hexanucleotides (Amerham Pharmacia biotech). The cDNA was stored at −20°C.

Statistical Analysis
Data are shown as mean±SEM, except for skewed variables, which are expressed as medians. Individual subjects’ values obtained at visits 1 and 2 were compared by using a concordance test.16 The χ2 test was used to compare the observed allele and genotype frequencies with the Hardy-Weinberg equilibrium prediction. Trends across genotype groups were tested by using the nonparametric Kruskall-Wallis test. Two-way ANOVA was used to compare cAMP values and maximal aggregation between genotype groups according to ADP concentrations. The association between the genotype and the maximal platelet aggregation response to ADP was tested, after adjustment for other variables, by using multivariable logistic regression models in which maximal aggregation <50% was coded as 0 and maximal aggregation ≥50% was coded as 1. Considering the small number of subjects homozygous for the mutated allele (<7), whatever the polymorphism, these subjects were pooled together with heterozygotes for regression analysis.
Statistical tests were performed by using the STATA 7.0 software package (Stata Corp), and differences with probability values $P < 0.05$ were considered statistically significant.

**Results**

To assess the variability of ADP-induced platelet aggregation, we tested 2 samples, obtained 1 week apart, from each of 98 healthy volunteers. Figure 3 compares the maximal aggregation responses to $20 \mu M$ ADP in citrated PRP at the 2 visits. At least 2 phenotypic groups were identified. Maximal aggregation was $<50\%$ at both visits in 47 subjects (48.0%). Moreover, 43 (91.5%) of the subjects in this subgroup had aggregation profiles consistent with a reversible primary phase. Conversely, maximal aggregation was $<50\%$ at both visits in 29 subjects (29.6%), and 28 (96.5%) of these subjects had an irreversible second phase of ADP-induced aggregation. As maximal aggregation was stable between the 2 visits ($r^2 = 77\%$, $P < 0.001$), we used the mean value for each subject (referred to below as maximal aggregation) for subsequent analyses.

The existence of these 2 distinct phenotypic groups of platelet aggregation, together with the stability of ADP responses over time, pointed to possible genetic control of the ADP effect on platelet aggregation. Thus, we analyzed the P2Y$_{12}$ gene in the study population and found that it contained at least 2 exons separated by 1 intron $\approx 1700$ bp in length, located upstream of the ATG codon (Figure 1). Exon 2 encodes the entire 342-amino-acid protein. Because of a 6-nt repeat sequence (Table 1), the exact 3' end of exon 1 and 5' start site of exon 2 could not be determined. Table 1 describes 3 possible sequence patterns. We arbitrarily chose pattern 1 to number the polymorphisms described below.

We found 4 single-nt polymorphisms (SNPs) and a single-nt insertion polymorphism (Table 2). Two variations were located 139 nt and 744 nt after the 5' intron start site, consisting of a C-to-T (i-C139T) and a T-to-C (i-T744C) transition, respectively. Another polymorphism consisted of a single-nt insertion (A) at position 801 of the intron (i-ins801A). The remaining 2 polymorphisms were found in exon 2 and consisted of a C-to-T transition (C34T) and a G-to-T transversion (G52T), respectively, 34 nt and 52 nt after the 5' start site of exon 2 (Figure 2); neither modified the encoded amino acid (Asn 6 and Gly, 12 respectively).

As the i-C139T, i-T744C, i-ins801A, and G52T polymorphisms were in complete linkage disequilibrium in our population, we designated H1 as the major haplotype (a C in position 139, a T in position 140, a C in position 141, and absence of the i-ins801A in the intron, as well as a G in position 52 of exon 2) and H2 as the minor haplotype (a T in position 139, a C in position 140, a T in position 141, and presence of the i-ins801A in the intron).
position 744, presence of the i-ins801A in the intron, and a T in position 52 of exon 2). The respective frequencies of haplotypes H1 and H2 were 86% and 14%. The allele frequencies of all the polymorphisms were in Hardy-Weinberg equilibrium.

The H2 haplotype was associated with higher maximal aggregation in response to ADP, with median values of 34.7% in subjects carrying none of the H2 alleles (H1/H1, n = 21), 67.9% in subjects carrying 1 H2 allele (H1/H2, n = 21), and 82.4% in the 3 subjects carrying 2 H2 alleles (H2/H2; P = 0.0071) (Figure 4). Two of the subjects who were homozygous for the H2 haplotype had values of 82% and 86%, whereas the third subject had a value of only 27%.

Multivariate logistic regression analysis indicated that the OR of this association (OR, 3.3; 95% CI, 1.1 to 10.4) did not change substantially after adjustment for age, body mass index, platelet count, mean platelet volume, white blood cell count, fibrinogen, prothrombin time, activated partial thromboplastin time, and the PlA1/P2A2 genotype. The C34T polymorphism was not associated with maximal aggregation in response to ADP.

To investigate the association of the H2 haplotype and a possible splice variant of the intron located between exon 1 and 2, we amplified and sequenced P2Y12 cDNA obtained by reverse transcription of platelet mRNA from volunteers with the H1/H1 (n = 3), H1/H2 (n = 3), and H2/H2 (n = 3) genotypes. Nucleotide sequencing revealed no variations, whatever the haplotype. Moreover, the sequence profile was consistent with amplification of both alleles in H1/H2 subjects, suggesting that both mRNA variants are transcribed (data not shown).

To further study the association between the H2 haplotype and ADP-stimulated platelet aggregation, we randomly selected 10 carriers and 10 noncarriers of the H2 haplotype. As ADP-induced platelet aggregation is considered to be dependent on the extracellular Ca\(^{2+}\) concentration,\(^{17}\) we measured maximal aggregation to 1, 2, and 5 \(\mu\text{mol/L}\) ADP in medium containing a low Ca\(^{2+}\) concentration (citrate-anticoagulated PRP) and in medium containing a physiological Ca\(^{2+}\) concentration (hirudin-anticoagulated PRP). Carriers of the H2 allele had an increased maximal platelet aggregation to ADP compared with that of noncarriers in citrated PRP (\(P = 0.027\)) (Figure 5A). However, in hirudin-anticoagulated PRP, maximal aggregation did not reach significance, although following the same trend (\(P = 0.061\)) (Figure 5B). This is probably owing either to the small number of subjects in each group, and/or to the lack of sensitiveness to reveal a difference in this medium in terms of aggregation.

To identify a possible link between the H2 haplotype and P2Y\(_{12}\) regulation of adenylate cyclase, we determined the capacity of ADP to inhibit iloprost-stimulated cAMP accumulation.

### Table 1. Possible Nucleotide Sequence Patterns Describing the Exact 3’ End of Exon 1 and the 5’ Start Site of Exon 2

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Exon 1 and 3’-Flanking Region</th>
<th>Exon 2 and 5’-Flanking Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>...ACAGTTATCGgtatgttatattttttt...</td>
<td>...tgtctctAGTAACCAAAG...</td>
</tr>
<tr>
<td>2</td>
<td>...ACAGTTATCGGTAAggttatattttttt...</td>
<td>...tgtctctagtgtaaCAAACAG...</td>
</tr>
<tr>
<td>3</td>
<td>...ACAGTTATCGgtatgttatattttttt...</td>
<td>...tgtctctagtgtaaCAAACAG...</td>
</tr>
</tbody>
</table>

The repeat sequence is underlined. We arbitrarily chose pattern 1 for nucleotide numbering.

### Table 2. Description of the Polymorphisms Detected in the Study Population

<table>
<thead>
<tr>
<th>Name of SNP</th>
<th>Amino Acid</th>
<th>Region</th>
<th>Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>i-C139T</td>
<td>...</td>
<td>Intron</td>
<td>C:86.2% T:13.8%</td>
</tr>
<tr>
<td>i-T744C</td>
<td>...</td>
<td>Intron</td>
<td>T:86.2% C:13.8%</td>
</tr>
<tr>
<td>i-ins801A</td>
<td>...</td>
<td>Intron</td>
<td>Insertion of an A: 13.8%</td>
</tr>
<tr>
<td>G52T</td>
<td>G/G</td>
<td>Exon 2</td>
<td>G:86.2% T:13.8%</td>
</tr>
<tr>
<td>C34T</td>
<td>N/N</td>
<td>Exon 2</td>
<td>C:72.5% T:27.5%</td>
</tr>
</tbody>
</table>

The H1/H2 haplotype is defined by the i-C139T, i-T744C, i-ins801A and G52T polymorphisms.
mulation in platelets from the same 20 subjects. In citrated blood, ADP inhibited cAMP formation in platelets from noncarriers in a concentration-dependent manner, with 13%, 31%, and 37% inhibition at 1, 2, and 5 μmol/L ADP, respectively, compared with 31%, 39%, and 50%, respectively, in carriers of the H2 haplotype (P = 0.028) (Figure 6A). Similar results were found when platelets were obtained from hirudin-anticoagulated PRP (P = 0.01) (Figure 6B).

Discussion
We identified 3 SNPs and 1 nt insertion in the P2Y12 receptor gene, which determined 2 haplotypes designated H1 and H2. The H2 haplotype was associated with increased maximal platelet aggregation in response to ADP. This was related to differences in the mechanism regulating cAMP inhibition by ADP.

It has been shown in a large population study that ADP-induced platelet responses exhibit considerable variability as regards the ADP concentration required to produce a biphasic response with >50% aggregation.13 In the present study, ADP-induced aggregation in the 98 healthy volunteers was stable between 2 measurements 1-week apart, ie, after renewal of most platelets. These results pointed to genetic control of ADP-induced platelet aggregation.

To further study the difference in the aggregation response to ADP between carriers and noncarriers of the H2 haplotype, we examined adenylate cyclase inhibition of iloprost-stimulated platelets and found a correlation between the H2 haplotype and the reduction in the intracellular cAMP concentration by ADP.

Data obtained with specific P2Y12 receptor inhibitors such as the thienopyridines ticlopidine and clopidogrel confirm that P2Y12 is the only known platelet ADP receptor responsible for adenylate cyclase inhibition and the subsequent reduction in intracellular cAMP.18 Moreover, selective P2Y1 receptor antagonists have no effect on ADP-induced adenylate cyclase inhibition.2 Thus, the difference in the platelet cAMP concentration between carriers and noncarriers of the H2 haplotype after ADP stimulation may be involved in maximal ADP-induced aggregation observed in these 2 groups of subjects.

One of the 3 H2/H2 subjects had a maximal aggregation value of only 27% and a reversible aggregation profile. P2Y12...
gene sequence and platelet function of this subject were studied a third time, 11 months after visit 2. Similarly to visits 1 and 2, platelet aggregation to 2 μmol/L ADP was 37%, with a reversible aggregation profile. However, bleeding time was normal (7 minutes), as was platelet response to arachidonic acid and collagen. These data argue for a selective impairment of the ADP pathway. However, the capacity of ADP to inhibit iloprost-stimulated cAMP accumulation in platelets was found in the same range of the one showed by carriers of the H2 allele. Moreover, sequencing of exon 1 and exon 2 of P2Y12 gene in this subject ruled out a loss of function mutation. Possible explanations of the poor ADP response in this H2/H2 subject include a defect in the P2Y1 receptor, which is necessary for full ADP responsiveness, and a defect in the GP Ib/IIIa activation cascade mediated by ADP. The molecular mechanism by which the H2 haplotype increases platelet aggregation in response to ADP remains to be determined. As the complete coding sequence of the P2Y12 gene was screened in our study population, an amino acid substitution affecting the protein structure can be ruled out. Moreover, cDNA analysis of both haplotypes showed normal sequences at the exon 1–exon 2 junction, ruling out a splice variant. Thus, an increase in the number of receptors on the platelet surface is most likely to explain the association between the H2 haplotype and platelet responsiveness to ADP. Indeed, the H2 haplotype could be linked to a sequence variation in the promoter region that could increase transcription efficiency.

Considerable evidence suggests that the P2Y12 receptor plays a central role in the formation of hemostatic plugs and in the occurrence of arterial thrombosis. Our identification of a P2Y12 receptor haplotype that is strongly associated with increased ADP-induced platelet aggregation may have important clinical implications, particularly in screening for subjects at risk of atherothrombosis. Because thienopyridines only provide partial P2Y12 blockade, and aspirin does not inhibit P2Y12-mediated amplification of platelet responses, carriers of the H2 allele may have less protection of these platelet inhibitors.

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