Antibodies to Platelet Factor 4–Heparin After Cardiopulmonary Bypass in Patients Anticoagulated With Unfractionated Heparin or a Low-Molecular-Weight Heparin

Clinical Implications for Heparin-Induced Thrombocytopenia

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Background—Cardiopulmonary bypass (CPB) induces platelet activation with release of platelet factor 4 (PF4), and patients are exposed to high doses of heparin (H). We investigated whether this contributes to the development of antibodies to H-PF4 and heparin-induced thrombocytopenia (HIT).

Methods and Results—CPB was performed with unfractionated heparin (UFH) in 328 patients. After surgery, patients received UFH (calcium heparin, 200 IU·kg⁻¹·d⁻¹) (group 1, n=157) or low-molecular-weight heparin (LMWH, Dalteparin, 5000 IU once daily) (group 2, n=171). Eight days after surgery, antibodies to H-PF4 were present in 83 patients (25.3%), 46 in group 1 and 37 in group 2 (P=0.12). Most patients (61%) had IgG1 to H-PF4, but only 8 samples with antibodies induced platelet activation with positive results on serotonin release assay. HIT occurred in 6 patients in group 1, but no thrombocytopenia was observed in subjects receiving LMWH, although 2 had high levels of antibodies with positive serotonin release assay results. When antibodies to H-PF4 were present, mean platelet counts were lower only in patients with FcγRIIA R/R131 platelets.

Conclusions—These results provide evidence that the development of antibodies to H-PF4 after CPB performed with UFH is not influenced by the postoperative heparin treatment. The antibodies associated with high risk of HIT are mainly IgG1, which is present at high titers in the plasma of patients continuously treated with UFH. (Circulation. 1999;99:2530-2536.)

Key Words: cardiopulmonary bypass • heparin • platelets • antibodies
During the 15 months from May 1996 to June 1997, and informed written consent was systematically obtained according to the principles of the declaration of Helsinki. Of these patients, 156 had CABBG and 137 had valve replacement or repair, with CABBG in 19. Sixteen other patients underwent CPB for another indication (Table 1).

All patients had previously been exposed to UFH during cardiac catheterization 1 to 3 months before surgery. Anticoagulation for CPB was achieved with sodium UFH (Léon) or with an intravenous (IV) bolus of UFH, corresponding to 250 IU/kg before aortic cannulation. Additional heparin was then administered with a continuous IV infusion to maintain the activated clotting time (Hemochron) over 1.5 to 2.5 times the control range. On completion of CPB, the anticoagulant effect of heparin was reversed by slow IV infusion of protamine sulfate until the activated clotting time returned to the preheparinization level.

Patients were then divided into 2 groups according to the type of surgery and the individual assessment of risk factors for thromboembolism. Most patients in group 1 had a valve replacement (n = 148), and a few exhibited cardiac failure, arrhythmia, myocardial infarction, and/or a previous history of thrombosis (n = 9). They received continuous IV infusion of sodium UFH at a dose of 120 IU/kg before aortic cannulation. Additional heparin was then administered with a continuous IV infusion to maintain the activated clotting time (Hemochron) over >600 seconds and plasma heparin levels between 2 and 4 IU/mL. On completion of CPB, the anticoagulant effect of heparin was reversed by slow IV infusion of protamine sulfate until the activated clotting time returned to the preheparinization level.

The patients in group 2 (n = 171) had an estimated lower risk of thromboembolism because most of them had isolated ischemic heart disease and underwent CABBG (n = 150). A few other patients had surgery for patent foramen ovale (n = 13) or mitral valve repair (n = 8). They all received LMWH (dalteparin or Fragmin, Pharmacia and Upjohn) with 1 subcutaneous injection of 5000 IU anti-Xa per day for 1 month.

The mean duration of CPB for patients in group 1 was significantly longer than for those in group 2 (Table 1, P < 0.0001).

### Blood Samples and Platelet Counts

Whole blood was collected on EDTA for platelet counts or on 0.129 mol/L sodium citrate (9:1) for the other biological assays, including DNA analysis. Platelet-poor plasma was isolated by centrifugation of blood samples at 2500 g for 15 minutes and stored at −80°C for no longer than 18 months until assay.

Platelet counts were obtained with an automatic counter (Coulter STKS, Coultronics) before surgery, during CPB, and at least 3 times in the postoperative period (on days 3 to 4, 5 to 7, and 8 to 10).

Blood samples for the detection of antibodies to H-PF4 complexes by ELISA were drawn before CPB and twice in the postoperative period (on days 3 to 5 and 7 to 10).

### ELISA for Detection of Antibodies to H-PF4 Complexes

H-PF4 ELISA was performed with the Asserachrom HPIA kit (Diagnostica Stago). For the overall assay measuring IgG, IgA, and IgM isotypes, the result was defined as negative if absorbance at 492 nm (A492) was < 0.5 or as positive if A492 was ≥ 0.5.

In 80 patients with antibodies to H-PF4, isotopic distribution was analyzed with monovalent anti-IgG-, anti-IgA-, and anti-IgM-peroxidase conjugates instead of polyclonal anti–human IgG, IgA, and IgM, and A492 values ≥ 0.20 were considered positive. The cutoff values were determined on the basis of the mean value ± 3 SD of 50 control samples taken from normal subjects and from patients with thrombocytopenia from causes other than HIT.

In 45 patients with IgG antibodies, the subclasses were analyzed with monoclonal mouse anti–human IgG subclass–specific antibodies diluted at 1:5000 for anti-IgG1 (clone MH161-1 ME) and 1:200 for anti-IgG2 (clone MH162-1 ME), anti-IgG3 (clone MH163-1 ME), and anti-IgG4 (clone MH 164-4 ME) (CLB). The cutoff value for each IgG subclass was determined on the basis of the mean of control absorbance values (+ 3 SD) measured with 37 samples from patients without antibodies to H-PF4. All ELISA values were confirmed with ≥ 2 separate experiments.

### Analysis of FcγRIIA Polymorphism

After isolation of genomic DNA, the FcγRIIA-H/H(31) polymorphism was analyzed by an allele-specific restriction enzyme digestion method. Amplification was carried out with a mutagenic sense primer (5'GGGAAATCCCGAAATTCCTGGC-3') that created a BstUI site on the PCR product only if the codon for amino acid 131 resulted in an arginine.

A second mutagenic antisense primer (5’-CAACAGCCTGACATCCATTACCGGGG-3’) introduced an internal BstUI site (in bold type) into every PCR product serving as a control for the restriction enzyme digestion. Reactions were performed in a final volume of 50 μL containing 0.1 to 1 μg of DNA, 1 μmol/L of each primer, 200 μmol/L of each dNTP, 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 0.01% (wt/vol) gelatin, 1.5 mmol/L MgCl2, 0.1% Triton X-100, and 0.5 μL of Super Taq DNA Polymerase (ATGC Biotechnologie). Amplification was performed in the GeneAmp PCR system 2400 thermocycler (Perkin-Elmer) programmed for 3 minutes at 94°C, followed by 30 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 40 seconds at 72°C. A final extension step was performed at 72°C for 7 minutes. PCR products (6 μL) were then incubated for 16 hours at 60°C with 10 U of BstUI (Biolabs) in 50 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.9), 10 mmol/L MgCl2, and 1 mmol/L DTT at a final volume of 10 μL. Digestion products were separated by 6% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. Expected lengths of fragments were 366 bp for undigested PCR products, 343 bp for H/H(31), 322 bp for R/R(31), and 343 and 322 bp for R/H(31).

### [14C]Serotonin Release Assays

SRAs were performed as previously described, with 80 of the 83 plasma samples containing antibodies to H-PF4. Every plasma sample was tested in duplicate with platelets from 2 different donors having either the FcγRIIA-H-H(31) or the FcγRIIA-R-R(31) isoform. A test result was defined as positive if release > 20% was measured at 0.1 or 1 IU/mL of heparin, with complete inhibition at 10 IU/mL. The result was defined as negative if the release was < 20% or not inhibited in the presence of 10 IU/mL of heparin. SRA was also performed on 80 control sera in which no antibodies to H-PF4 were detected, obtained from groups 1 and 2 (40 from each).

### Statistical Analysis

Cumulative data (platelet counts, levels of antibodies to H-PF4) were analyzed as mean±SEM, and the unpaired t test was used for comparisons between groups. ANOVA with repeated measures was used to compare the evolution of platelet counts. Fisher’s exact test was used to compare the frequency of events between patient groups (eg, thrombocytopenia, development of antibodies to H-PF4), the distribution of FcγRIIA isoforms, and the classes/subclasses of antibodies. A value of P ≤ 0.05 was required for statistical significance.
Results

Development of Antibodies to H-PF4 Complexes After CPB

Significant levels of antibodies to H-PF4 \( (A_{492}=0.5) \) were present in 3 patients in group 1 before surgery (Figure 1). Four additional patients (2 in each group) developed antibodies between day 3 and day 5, but in all cases except 1, levels were stable and remained at \(<1.0\) absorbance unit.

After 1 week of postoperative treatment, 83 patients (ie, 25.3%) had significant titers of antibodies to H-PF4. Forty-six received UFH (29% of 157 patients) and thirty-seven LMWH (21% of 171 patients), but the difference was not significant \( (P=0.12) \). Absorbance values measured before CPB in subjects who later developed antibodies to H-PF4 were higher than those of patients with consistently negative ELISA results \( (P<0.0001, \text{Table 2}) \). However, even in these “negative” patients, absorbance values increased significantly in the second week of heparin treatment \( (P<0.0001 \text{ compared with baseline values}) \).

Evolution of Platelet Counts and FcyRIIA Genotype

Before surgery, 7 patients had thrombocytopenia \(<100\times10^9/\text{L}\) ), but none developed antibodies to H-PF4, and all recovered a normal platelet count. When patients were considered together, the platelet count fell from \(212\pm3\times10^9/\text{L} \) (mean\(\pm\)SEM) before surgery to \(100\pm3\times10^9/\text{L} \) during CPB. It then remained stable during the first 2 postoperative days, increased to \(146\pm3\times10^9/\text{L} \) on days 3 to 4, and in most patients was completely normal on days 5 to 7.

Mean platelet counts were lower in patients of group 1 as early as the end of CPB \( (131\pm3.4\times10^9/\text{L}) \) compared with those of group 2 \( (149\pm3.9\times10^9/\text{L}, P=0.001) \), and this difference increased on days 3 to 4 \( (126\pm3.1\times10^9/\text{L} \text{ versus } 165\pm3.7\times10^9/\text{L}) \). In addition, 83 patients in group 1 had thrombocytopenia \(<100\times10^9/\text{L}) \) 3 to 4 days after surgery or a decrease in platelet count >40%, compared with only 26 in group 2 \( (P<0.0001) \). Mean platelet counts reached values >\(200\times10^9/\text{L}\) in both groups after day 8 after surgery, with an evolution that was not significantly

| TABLE 2. Levels of Antibodies to H-PF4 \( (A_{492}, \text{Mean}\pm\text{SD}) \) After CPB in Patients With or Without Significant Levels of Antibodies 7 to 10 Days After CPB |
|-----------------------------------------------|-----------------|-----------------|-----------------|
|                                              | Before CPB      | Days 3 to 5     | Days 7 to 10    |
| Patients without antibodies to H-PF4         | 0.091\pm0.070   | 0.115\pm0.068   | 0.242\pm0.124   |
| Patients with antibodies to H-PF4            | 0.160\pm0.164   | 0.215\pm0.222   | 1.225\pm0.074   |

Figure 1. Development of antibodies to H-PF4 after CPB. Antibodies to H-PF4 before and after CPB in patients receiving UFH (●) or LMWH (○) postoperatively. \( A_{492}=0.5 \) was taken into account (dotted line). The 8 patients with antibodies and positive SRA are numbered. Arrows indicate patients with HIT.
different in patients with or without antibodies to H-PF4 ($P>0.1$, repeated-measures ANOVA) (Figure 2). On days 8 to 10, however, patients in group 1 with antibodies to H-PF4 had lower platelet counts than those without antibodies ($P=0.03$, unpaired $t$ test).

HIT was suspected in 6 patients in group 1 (platelets $<100 \times 10^9/L$ or decrease $>40\%$ on days 8 to 10) and then confirmed by positive H-PF4 ELISA and positive SRA (Table 3). All 6 patients received UFH, and in 2 cases, platelet counts dropped below $50 \times 10^9/L$, particularly in

**TABLE 3.** Patients Treated With UFH or LMWH After CPB With Antibodies to H-PF4 and Positive Results by SRA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Platelet Counts</th>
<th>AβG2</th>
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<td></td>
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<td>DB-10, $\times 10^9/L$</td>
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patient 2, who had severe pulmonary embolism. This patient was then treated with danaparoid sodium but died on day 13. Thrombosis did not occur in the other patients, and there was a favorable outcome after heparin withdrawal and initiation of oral anticoagulants (warfarin).

The distribution of FcγRIIA isoforms was studied in patients with and without antibodies to H-PF4 (Figure 3). The H/H<sup>131</sup>, H/R<sup>131</sup>, and R/R<sup>131</sup> alleles were found in 18.5%, 54.5%, and 27%, respectively, of patients who did not develop antibodies to H-PF4. The corresponding values were 19.7%, 42.2%, and 38.1%, respectively, in patients with antibodies, and the differences observed were not statistically significant. The mean platelet counts in patients with antibodies to H-PF4 were significantly lower on days 8 to 10 of the postoperative period only in those with the R/R<sup>131</sup> genotype (Figure 3, P<0.018), and this difference remained significant, even when the values of the 4 patients with HIT were excluded. By comparison, the evolution of platelet counts in patients without antibodies was identical whatever their FcγRIIA genotype.

Class and Subclass of Antibodies to H-PF4 and Platelet Activation

The classes of immunoglobulins specific for H-PF4 were determined in 79 patients. Significant levels of IgG antibodies were measured in 48 patients (24 from each group), with IgA and/or IgM in 29 samples. In 31 patients, only IgA and/or IgM antibodies to H-PF4 were detected. The prevalence of IgG and IgA antibodies and plasma levels were similar in both groups, whereas IgM isotype was more common in group 1 (31 versus 14 in group 2, P=0.003), and titers were higher (0.546±0.049 versus 0.339±0.035, P=0.01).

The predominant subclass of IgG antibodies was IgG1, which was present in 40 of 45 patients (88.8%) (Table 4). An IgG3 response was present in 12 patients, with IgG1 in 8 of them, and no IgG2 or IgG4 antibodies were detected.

SRA was positive in 8 of 80 samples containing antibodies to H-PF4 and negative for all 80 control sera without antibodies. Six samples were from patients treated with UFH, and they all had HIT with antibodies that also cross-reacted with LMWH (dalteparin) on SRA. The other 2 positive samples on SRA were from patients 7 and 8, who were treated with dalteparin (Table 3), and platelets remained >250×10<sup>9</sup>/L in these subjects despite high levels of antibodies to H-PF4. The 8 positive samples contained high levels of antibodies (mean A<sub>492</sub>=2.43±0.271), with IgG1 in all patients and IgG3 in 2. Interestingly, the R<sup>131</sup> gene was expressed by each of these patients, 4 being R<sup>131</sup>/R<sup>131</sup> homozygotes and 4 being H<sup>131</sup>/R<sup>131</sup> heterozygotes. Moreover, SRA was positive with both R<sup>131</sup>/R<sup>131</sup> and H<sup>131</sup>/H<sup>131</sup> [<sup>14</sup>C]serotonin-labeled platelets. The maximum release induced by antibodies was slightly higher with R<sup>131</sup>/R<sup>131</sup> platelets (74%; range, 54% to 93% versus 58%, 23% to 90% with H<sup>131</sup>/H<sup>131</sup> cells, cumulative data of 2 separate experiments), but the differences were not significant (P=0.075).

Discussion

In this study, we evaluated the prevalence of antibodies to H-PF4 complexes in patients undergoing cardiac surgery, and we determined the predisposing factors that could favor the development of HIT. Antibodies to H-PF4 were detected in 29% of patients treated with UFH throughout the postoperative period (group 1) and in 21% of those receiving LMWH (group 2). These results are in accordance with those recently reported, but 2 previous studies found a higher prevalence of antibodies to H-PF4 after CPB (51% and 61%). This difference is probably related to the presence of antibodies before surgery in both studies (in 19% and 22% of patients, respectively), whereas only 3 subjects of our cohort had above-normal antibody titers when admitted for cardiac surgery. All our patients had been exposed to heparin 1 to 3 months before CPB, and mean preoperative absorbance levels were higher in those who then developed heparin-dependent antibodies. An anamnestic response to heparin was likely, because 54% (12/22) of patients with absorbance values

| TABLE 4. IgG Subclass Assays in Patients With and Without (Controls) Significant Levels of IgG Antibodies to H-PF4 Complexes |
|------------------|------------------|------------------|------------------|------------------|
|                  | IgG1             | IgG2             | IgG3             | IgG4             |
| Controls (n=37)  | 0.060±0.040      | 0.013±0.027      | 0.067±0.048      | 0.041±0.03       |
| Group 1 (n=21)   | 18               | 0                | 8                | 0                |
|                  | (0.20–2.84)      |                  | (0.35–2.70)      |                  |
| Group 2 (n=24)   | 22               | 0                | 4                | 0                |
|                  | (0.19–1.44)      |                  | (0.56–1.88)      |                  |

The mean absorbance levels at 492 nm±SD in controls are shown for each subclass assay. The number of positive assays in patients is indicated, with the range in parenthesis.
between 0.25 and 0.5 before CPB later developed antibodies to H-PF4, compared with 22.4% of those with preoperative $A_{P2}$ values $<0.25$ ($68/303$, $P=0.001$).

CPB was always performed with UFH, and this could explain why the prevalence of heparin-dependent antibodies was not different between patients who received UFH or LMWH in the postoperative period. However, it is not certain that the exclusive use of LMWH during and after CPB is less immunogenic than UFH.$^{16,17}$ PF4 was undoubtedly released in every case during CPB, potentially enhancing the production of heparin-dependent antibodies whether patients were then treated with LMWH or with UFH. In the patients in group 1, most of whom underwent valve replacement, platelet counts were lower after CPB than in those with CABG (group 2), and platelet activation was possibly more intense as a result of longer extracorporeal circulation. In the postoperative period, the patients in group 1 were treated by UFH before warfarin was introduced, because LMWHs are not currently authorized in France after CPB for valve replacement. Most patients in group 2 underwent CABG and received LMWH only for prophylaxis of venous thromboembolism. The patients in groups 1 and 2 were thus different, and in addition to heparin, the underlying conditions might also have enhanced the pathogenicity of heparin-dependent antibodies and hence thrombocytopenia and thrombosis.

Nevertheless, our experience supports the hypothesis that in the presence of LMWH, antibodies to H-PF4 are less likely to induce platelet activation and to provoke HIT. Indeed, HIT occurred only in patients who received UFH postoperatively (incidence of 3.8%), thus explaining why platelet counts were significantly reduced on days 8 to 10, when antibodies to H-PF4 were present only in group 1. In contrast, thrombocytopenia never occurred after 8 days of LMWH in group 2, although 2 subjects also had high levels of IgG to H-PF4, with positive results on SRA. On the other hand, in agreement with the findings of Suh et al.$^{18}$ the antibodies to H-PF4 that activated platelets were preferentially IgG1, which existed at high plasma levels. However, in 1 patient with definite HIT (patient 6), the antibodies were almost exclusively IgG3, and this also supports the pathogenicity of this subclass. IgG was the most frequent antibody isotype (61%), but IgM (57%) and IgA (31.6%) were also often detected. However, the pathogenicity of IgG and IgM, which has been discussed in previous reports,$^{11,14,18}$ is probably low, because no thrombocytopenia was observed when they were present alone, and SRA results were negative. High titers of IgM were more frequent in group 1, suggesting a primary antibody response to H-PF4. This is unlikely, however, because all patients had previously been challenged with UFH, and none of the cases with preoperative IgM antibodies later developed IgG to H-PF4.

We also investigated whether the FcγRIIA polymorphism of FcγRIIA did not play a role in the immune response to heparin and PF4. Second, when antibodies to H-PF4 were present, mean platelet counts were significantly lower only in FcγRIIA R/R$^{131}$ patients, although their antibody titers were identical to those of H$^{131}$/R$^{131}$ and H$^{131}$/H$^{131}$ patients, and this supports the hypothesis that heparin-dependent IgG1 preferentially activates R/R$^{131}$ platelets.$^8$

Several studies have been performed on the distribution of FcγRIIA polymorphism and the development of HIT, but with debatable results.$^9,10,19–22$ In 3 studies, the homozgyous H/H$^{131}$ genotype was shown to be more frequent than R/R$^{131}$ in patients with HIT.$^9,10$ In addition, Denomme et al.$^{20}$ found that IgG1 antibodies to H-PF4 produced higher amounts of membrane-derived microparticles from H/H$^{131}$ platelets, and it was suggested that platelets with this polymorphism should be chosen for functional assays for HIT.$^21$ Our observations do not agree with these conclusions, because the His$^{131}$ gene was not overrepresented in patients with HIT, and antibodies to H-PF4 activated both H/H$^{131}$ and R$^{131}$/R$^{131}$ platelets as assessed by SRA.

Furthermore, our results are in accordance with a recent study of a large population of 389 patients with HIT showing that FcγRIIA-R/R$^{131}$ was overrepresented, particularly in subjects with thrombotic complications.$^{22}$ The FcγRIIA-R$^{131}$ allele is also a recognized risk factor in systemic lupus erythematosus, enhancing the pathogenicity of antibodies by reducing the phagocytosis and clearance of immune complexes.$^{23}$ Such a process could contribute to HIT, but the platelet activation induced by heparin-dependent antibodies in vivo involves several components, such as ADP receptors, other than FcγRIIA receptors.$^{24}$ In addition, functionally significant mutations in human Fcγ receptor sequences other than those concerning the amino acid at position 131 could also explain differences in platelet susceptibility to antibodies.$^{25}$

It is thus possible that, together with acquired factors related to the underlying disorders, other genetic parameters play a role in the development of heparin-dependent antibodies, their pathogenicity, and the occurrence of HIT.

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References


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