Formation of anti-platelet factor 4/heparin antibodies after cardiac surgery: Influence of perioperative platelet activation, the inflammatory response, and histocompatibility leukocyte antigen status

Domenico Paparella, MD,^a Giuseppe Scrascia, MD,^a Antonella Galeone, MD,^a Maria Coviello, BSc,^b Giangiuseppe Cappabianca, MD,^a Maria Teresa Venneri, BSc,^b Biagio Favoino, BSc,^c Michele Quaranta, MD,^b Luigi de Luca Tuppiti Schinosa, MD,^a and Theodore E. Warkentin, MD^d

Background: Anticoagulation therapy with heparin induces antibodies that recognize multimolecular complexes of platelet factor 4 bound to heparin (anti-platelet factor 4/heparin antibodies). Considering that cardiac surgery induces an intense platelet activation and proinflammatory response, we examined the relationship between formation of anti-platelet factor 4/heparin antibodies and plasma levels of platelet factor 4 and interleukin 6. We also examined the relationship between anti-platelet factor 4/heparin seroconversion and the histocompatibility leukocyte antigen system.

Methods: In 71 patients undergoing cardiac surgery, anti-platelet factor 4/heparin antibody levels were evaluated by means of enzyme-linked immunosorbent assay preoperatively and 14 days postoperatively. Platelet serotonin release assays were performed to assess the platelet-activating potential of the antibodies. Plasma levels of platelet factor 4 and interleukin 6 were assayed at prespecified time points. Histocompatibility leukocyte antigen status was assessed preoperatively in all patients and was compared with that of 6156 healthy subjects.

Results: Thirty-seven (52%) patients had anti-platelet factor 4/heparin antibodies with an OD value of 0.45 or greater in 1 or more of the assays. Applying strict seroconversion criteria (>2-fold increase in Optical Density), only 16 (22.5%) patients had evidence of anti-platelet factor 4/heparin antibody seroconversion after the operation. Neither the presence of anti-platelet factor 4/heparin antibodies nor seroconversion influenced postoperative outcomes. The CW4 allele was significantly more frequent among seroconverted patients (46.9% vs 19.1%, $P = .002$). Platelet factor 4 levels did not influence seroconversion. Patients with anti-platelet factor 4/heparin levels of 0.45 OD units or greater 14 days after the operation had significantly higher interleukin 6 levels measured 1 hour after protamine administration.

Discussion: Patients with a greater amount of perioperative inflammation could be more likely to have anti-platelet factor 4/heparin antibodies 1 to 2 weeks later. We provide additional evidence that the histocompatibility leukocyte antigen CW4 confers genetic susceptibility in an acquired inflammatory disorder that includes the anti-platelet factor 4/heparin immune response.

Immune heparin-induced thrombocytopenia (HIT) is an immune-mediated complication of anticoagulation therapy with heparin. HIT is caused by platelet-activating antibodies that recognize multimolecular complexes of platelet factor 4 (PF4), a member of the C-X-C subfamily of chemokines, when PF4 is bound to heparin (anti-PF4/heparin [anti-PF4/

H] antibodies).¹ In patients with clinical HIT, multimolecular complexes of heparin, PF4, and IgG form on platelet surfaces, producing “strong” platelet activation through clustering of the platelet Fcγ receptors, with an associated decrease in the platelet count. HIT and its attendant thrombotic complications affect only a relatively small proportion of patients after cardiac surgery who form very high levels of platelet-activating anti-PF4/H antibodies.^{2,3}

The factors that influence the anti-PF4/H immune response are poorly understood. One possible factor is the histocompatibility leukocyte antigen (HLA) system, which modulates the immune response through its influence both in antigen recognition and cellular effector action. HLA molecules are glycoproteins expressed on the cell membranes of many different cell types.⁴ Few and conflicting data exist regarding whether the HLA system influences anti-PF4/H seroconversion.^{5,6} Other nongenetic factors could also influence the anti-PF4/H immune response. Considering that cardiac surgery induces intense platelet activation⁷ and

From the Division of Cardiac Surgery,^a Department of Emergency and Organ Transplant (D.E.T.O.), University of Bari, Bari, Italy; the Department of Experimental Oncology, Laboratory Unit,^b Oncology Hospital, IRCCS, Bari, Italy; Tissue Typing Laboratory,^c Policlinico of Bari, Bari, Italy; and the Department of Pathology and Molecular Medicine,^d Michael G. DeGroot School of Medicine, McMaster University, Hamilton, Ontario, Canada.

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Address for reprints: Domenico Paparella, MD, Dipartimento d'Emergenza e Trapianti d'Organo (D.E.T.O.), Division of Cardiac Surgery, University of Bari, Piazza Giulio Cesare 11, 70100 Bari, Italy (E-mail: dpaparella@cardiochir.uniba.it).

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Abbreviations and Acronyms

ACT	= activated clotting time
anti-PF4/H	= anti–platelet factor 4/heparin
CPB	= cardiopulmonary bypass
ELISA	= enzyme-linked immunosorbent assay
HIT	= heparin-induced thrombocytopenia
HLA	= histocompatibility leukocyte antigen
IL-6	= interleukin 6
LMWH	= low-molecular-weight heparin
OD	= optical density
PF4	= platelet factor 4
SRA	= serotonin release assay
UFH	= unfractionated heparin

a proinflammatory response,⁸ it is possible that the magnitude of these events can also influence the risk of antibody formation. Despite PF4 being released from activated platelets and being a component within the antigen complex of HIT, the relationship between perioperative plasma levels of PF4 and formation of anti-PF4/H antibodies is unknown. Moreover, the possible correlation between the perioperative inflammatory response and anti-PF4/H antibody formation has not been explored previously. We hypothesized that the anti-PF4/H immune response could reflect the interaction of a patient's genetic status (HLA antigens), as well as PF4 concentrations (related to perioperative platelet activation and associated PF4 release), a costimulatory effect of perioperative inflammation (as assessed based on interleukin-6 [IL-6] plasma levels), or both. By investigating these 3 parameters in one study, we have the potential to determine whether any one (or more) of these is of greater relevance in determining the anti-PF4/heparin immune response.

MATERIALS AND METHODS

Seventy-one elective cardiac surgery patients undergoing either cardiopulmonary bypass (CPB; n = 56) or off-pump cardiac surgery (n = 15) in our institution between January 2004 and June 2006 were enrolled in this prospective observational study. Exclusion criteria consisted of 1 or more of the following: urgent or emergency operation, requirement for deep hypothermia during CPB, preoperative thrombocytopenia ($<100 \times 10^9/L$), known preoperative coagulopathy, chronic inflammatory disease, and preoperative corticosteroid treatment. The institutional ethics committee approved the protocol, and informed consent was obtained from all patients entered into the study.

Operative Technique

The surgical approach was always a median sternotomy. Heparin was administered (300 U/kg), and CPB was performed at moderate hypothermia (34°C) with noncoated open systems and membrane oxygenators (Dideco, Sorin Group, Italy). Cardiac arrest was induced and maintained with cold blood cardioplegia. Intraoperative heparin monitoring was performed by using standard activated clotting time (ACT; Hemochron 8). Additional heparin boluses (5000 U) were administered if the ACT values were less than

400 seconds. Protamine was administered to reverse heparin (1 mg of protamine per milligram of total heparin given before and during CPB). For patients undergoing off-pump operations, anesthesia management was not different from that used for patients undergoing CPB. Heparin was administered at the same dose (300 U/kg), and additional boluses were administered only if ACT values decreased to less than 250 seconds. Tranexamic acid was used in all patients undergoing valve surgery and in those patients undergoing coronary artery bypass grafting in whom the risk of perioperative bleeding was considered high.

Definitions

Systemic hypertension was defined as blood pressure exceeding 140/90 mm Hg, a history of high blood pressure, or need for medications. Those having a history of diabetes regardless of the duration of disease or the need for antidiabetic agents were considered diabetic. *Hypercholesterolemia* was defined as a fasting cholesterol level of greater than 200 mg/dL. *Smoking history* was defined as any current or past form of tobacco use. *Chronic obstructive pulmonary disease* was defined as a forced expiratory volume in 1 second of less than 75% of predicted value or the need for pharmacologic therapy for the treatment of chronic pulmonary compromise. Ongoing refractory angina that required the use of intravenous nitrate therapy for control was regarded as *unstable angina*. The Euroscore was calculated by using the additive method.

Death within the same hospital admission, regardless of cause, was defined as *in-hospital mortality*. *Low cardiac output syndrome* was defined as the need for postoperative inotropic support for more than 12 hours or an intra-aortic balloon pump for more than 30 minutes to maintain systolic blood pressure greater than 90 mm Hg, mean blood pressure greater than 60 mm Hg, or cardiac index greater than $2.2 \text{ L} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$, despite sufficient volume substitution. Extubation criteria were hemodynamic stability, absence of surgical bleeding, fully rewarmed state, consciousness, and optimal blood gases with a fraction of inspired oxygen of 0.3 or less. Acute cerebrovascular events were regarded as any postoperative transient ischemic accident, reversible ischemic neurologic deficit, or stroke.

Blood and blood products were transfused to patients showing hypovolemia according with the following criteria. Packed red blood cells were transfused if the hemoglobin value was less than 8 g/dL. Fresh frozen plasma was infused if the prothrombin time (PT) value after protamine administration was at least 1.5 times the baseline in presence of significant bleeding, and platelet concentrates were transfused with platelet counts of less than $50,000/\text{mm}^3$ in the presence of significant bleeding.

Serologic Analysis

Blood samples were collected preoperatively on the morning of the operation (T0), 1 hour after the beginning of CPB (2 hours after the beginning of the operation for off-pump cases, T1), 1 hour after protamine sulphate administration (T2), and 14 days after the operation (T3). Blood samples were centrifuged for 30 minutes at 3500 rpm and frozen at -80°C until assayed.

PF4 (a component of the PF4/heparin antigen complex) levels were measured at T0, T1, and T2. Blood was collected into previously cooled tubes containing a solution of citric acid, theophylline, adenosine, and dipyridamole. Plasma PF4 levels were assayed with an enzyme-linked immunosorbent assay (ELISA; Diagnostica Stago, Asniers, France).

Inflammatory system activation was evaluated by measuring IL-6 levels at T0, T1, T2, and T3. Serum IL-6 levels were assayed with an ELISA (Immunotech, Beckman Coulter Company, Marseille, France).

All ELISAs were performed in duplicate, and the mean value was used for analysis. Results were corrected for hemodilution according to the following formula:

$$[\text{Corrected assay}]Tx = [\text{Assay}]Tx \times [\text{Hematocrit}]T0 / [\text{Hematocrit}]Tx.$$

Anti-PF4/H ELISAs

Assessment of anti-PF4/H antibody levels was performed at T0 and T3. All samples were tested with ELISAs for anti-PF4/H antibodies of IgG, IgA, and IgM classes, as previously described.⁹ The positive antibody cutoff was 0.45 OD units. Any sample that produced an OD of less than 0.45 was considered negative. All samples that produced absorbance (quantitated as units of optical density [OD] ≥ 0.45) were repeated in the presence and absence of high heparin doses (100 U/mL final). A patient sample set was considered as indicating a positive seroconversion event for anti-PF4/H antibodies (by any of the 3 immunoglobulin classes: IgG, IgA or IgM) if all of the following criteria were met: (1) the follow-up T3 sample was positive (≥ 0.45 OD); (2) the follow-up T3 sample had an OD value of at least double that of the baseline T0 sample; and (3) the follow-up T3 sample was inhibited by more than 50% by the addition of 100 U/mL heparin.

Platelet Serotonin Release Assay

We screened in the serotonin release assay (SRA) any patient sample that produced an OD of 0.45 or greater for anti-PF4/H IgG antibodies by testing the serum at 3 concentrations of heparin (ie, the 3-point SRA screen) known to produce optimal platelet activation (0.1 and 0.3 U/mL for unfractionated heparin [UFH] and 0.2 U/mL for low-molecular-weight heparin [LMWH]). Any sample that did not produce 10% or greater release in this screening phase was considered negative. Any sample that produced 10% or greater release at any of the 3 concentrations tested underwent repeat testing in the "full-screen SRA," in which testing was performed in all of the following reaction conditions: final concentrations of 0, 0.1, 0.3, 100, and 0.1 plus Fc-blocking monoclonal antibody (IV.3) and 0.1 plus 8 U/mL hirudin for UFH and final concentration of 0.2 U/mL for LMWH. The mean release was reported for those 4 reaction conditions in which reactivity was expected for a positive serum: UFH final concentrations of 0.1, 0.3, and 0.1 plus 8 U/mL hirudin and LMWH final concentration of 0.2 U/mL. The SRA result was considered positive if all the following criteria were met: (1) the mean release of these 4 reactions was 20% or greater; (2) the release was less than 10% with 100 U/mL UFH and 0.1 U/mL UFH in the presence of the Fc-blocking monoclonal antibody; and (3) the sample seroconverted from a negative baseline T0 sample. These criteria were chosen to ensure a high specificity for detecting definite seroconversion of heparin-dependent platelet-activating antibodies.

HLA Tissue Typing

Evaluations were performed preoperatively (T0). Patients were typed for HLA class I (loci B and C) by using the serologic complement-dependent cytotoxicity method.¹⁰ Allelic frequencies of seroconverted and nonseroconverted patients were compared with those of the 6156 healthy subjects residing in the same geographic region.

Statistical Analysis

All categorical variables were presented as absolute numbers and percentages and were compared with Fisher's exact test. Continuous variables, if normally distributed, were presented as means $\pm$ standard deviations and were compared by using the *t* test. Nonnormally distributed variables were presented as medians (interquartile ranges) and were compared by using the Mann-Whitney test in unpaired comparisons or the Wilcoxon signed-rank test in paired comparisons.

To quantify the whole amount of IL-6, PF4, and platelet levels through the study period, areas under the curve were calculated for each of these parameters by using the trapezoidal rule. Linear regressions were used to evaluate the influence of preoperative anti-PF4/H antibody levels on postoperative IL-6 levels. In HLA association analysis, significant *P* values were corrected according to the Bonferroni method. The peak IL-6 level after CPB was the end point used to calculate the sample size for this study. A previous article on the release of inflammatory markers after CPB¹¹ was used to assess the average release of IL-6 after CPB. A 50% anti-PF4/H

antibody-positive status (OD > 0.45) 2 weeks after cardiac surgery was hypothesized; thus 2 equal groups (anti-PF4/H antibody positive vs negative) were assumed. On this basis, a sample size of 70 patients had a power of 0.8 ($\alpha = .05$) to identify a 1.65-fold increase in post-CPB IL-6 peak levels in patients with a positive anti-PF4/H antibody status. Statistical analyses were carried out with Statview for Windows version 5.0 (SAS Institute, Inc, Cary, NC).

RESULTS

Patients underwent different types of operations, the majority being coronary artery bypass grafting procedures (Table 1). HLA association analysis (Table 2) demonstrated that the CW4 allele was significantly more frequent among patients demonstrating anti-PF4/H antibody seroconversion compared with nonseroconverted patients (46.9% vs 19.1%, *P* = .002), even after Bonferroni adjustment. In contrast, for the B50 (9.4% vs 0.9%, *P* = .03), A2 (34.4% vs 19.1%, *P* = .09), and B35 (31.3% vs 15.5%, *P* = .07) alleles, differences in frequency between the seroconverted and nonseroconverted patient groups were also seen, but these did not maintain a significant *P* value after Bonferroni adjustment. Allelic frequencies of the healthy population were different from those of seroconverted patients for the CW4, B35, and B50 alleles, although only the CW4 difference remained significant after the Bonferroni correction. Instead, allelic frequencies of nonseroconverted patients were very similar to those of the healthy population, except for Cw7 frequency. Because the 3 alleles, CW4, B35, and B50, appeared the most relevant, they were also examined within the univariate comparison of preoperative characteristics (Table 1).

Serologic analysis for the presence of anti-PF4/H antibodies showed that 37 (52%) patients had an OD value of 0.45 or greater in 1 or more of the assays. Applying the seroconversion criteria previously described, however, only 16 (22.5%) patients had evidence of anti-PF4/H antibody seroconversion after the operation. Eleven patients had positive results for IgG antibodies (9/11 with positive results for IgG only and 2/11 with positive results for IgG and IgA). Four patients had positive results for IgA alone, and 1 patient had positive results for IgM alone.

Preoperative patient characteristics, preoperative pharmacologic treatment, perioperative variables, and HLA status were analyzed to identify predictors of anti-PF4/H antibody seroconversion. By means of univariate analysis (Table 1), the only factors associated with seroconversion were the presence of the B50 allele on locus B (*P* = .03) and the presence of the CW4 allele on locus C (*P* = .02). Operation type, CPB time, off-pump procedures, and length of the operation did not influence anti-PF4/H antibody formation. Intraoperative heparin doses and time of heparin exposure during the operation were recorded, and these factors also did not influence seroconversion.

Three patients had positive SRA results (2 positive results for IgG only and 1 positive result for IgG and IgA). They had

TABLE 1. Preoperative and intraoperative variables

	All patients	No seroconversion	Seroconversion	P value
No. of patients	71	55	16	
Preoperative characteristics				
Male sex	53 (74.6%)	42 (76.4%)	11 (68.8%)	.53
HLA-B35	23 (32.3%)	16 (29.1%)	7 (43.7%)	.36
HLA-B50	4 (5.6%)	1 (1.8%)	3 (18.7%)	.03
HLA-Cw4	25 (35.2%)	15 (27.2%)	10 (62.5%)	.02
Age (y)	65.1 ± 8.5	65.9 ± 8.3	62.5 ± 8.9	.16
BMI (kg/m ²)	26.8 ± 3.4	26.7 ± 3.4	27.1 ± 3.5	.68
Systemic hypertension	49 (69%)	37 (67.3%)	12 (75%)	.76
Diabetes mellitus	29 (40.8%)	25 (45.5%)	4 (25%)	.16
Hypercholesterolemia	43 (60.6%)	34 (61.8%)	9 (56.3%)	.77
Smoke	42 (59.2%)	30 (54.5%)	12 (75%)	.16
Previous AMI	41 (57.7%)	32 (58.2%)	9 (56.3%)	.99
LVEF (%)	48.9 ± 10	48.3 ± 8.8	50.6 ± 9.4	.38
TIA/stroke	9 (12.7%)	6 (10.9%)	3 (18.8%)	.41
PVD	9 (12.7%)	6 (10.9%)	3 (18.8%)	.41
Intraoperative data				
Operation with CPB	56 (78.9%)	44 (80%)	12 (75%)	.73
CABG	49 (69%)	39 (70.9%)	10 (62.5%)	.54
Valve surgery	7 (9.9%)	5 (9.1%)	2 (12.5%)	.65
CPB duration (min)	117.1 ± 42.1	113.3 ± 30.1	131.3 ± 65.7	.19
Clamp duration (min)	66.9 ± 26.1	67.4 ± 27.1	65.4 ± 23.5	.81
OPCAB	15 (21.1%)	11 (20%)	4 (25%)	.73
Grafts per patient	3.1 ± 0.9	3.1 ± 0.8	3.1 ± 1.02	.82
Skin-to-skin time (min)	5.3 ± 1.2	5.2 ± 1.2	5.5 ± 1.2	.27
Heparin (UI)	22,218 ± 4320	22,364 ± 4094	21,718 ± 5141	.6
Heparin time (min)	160 ± 49	157 ± 42	169 ± 68	.41
Tranexamic acid	37 (52.1%)	27 (49.1%)	10 (62.5%)	.4
Tranexamic acid (mg)	1.64 ± 1.68	1.56 ± 1.72	1.93 ± 1.56	.43

Continuous variables were presented as means ± standard deviations. *BMI*, Body mass index; *AMI*, acute myocardial infarction; *LVEF*, left ventricular ejection fraction; *TIA*, transient ischemic accident; *PVD*, peripheral vascular disease; *CPB*, cardiopulmonary bypass; *CABG*, coronary artery bypass grafting; *OPCAB*, off-pump coronary artery bypass graft.

an uneventful clinical course without evidence of HIT with or without thrombosis.

One study patient died (overall mortality, 1.8%). The death occurred on postoperative day 7 as a result of low cardiac output syndrome without thrombocytopenia and without anti-PF4/H antibodies detectable by means of ELISA at T0. One other patient, who underwent successful mitral valve repair, experienced a platelet count decrease of greater than 50% (compared with the preoperative value), which began 7 days after the operation, and anti-PF4/H antibody seroconversion (but without a positive SRA result) was detected in this patient. No signs of thrombosis were apparent; however, the clinical outcome was complicated by respiratory insufficiency and prolonged intensive care unit length of stay, and the thrombocytopenia was considered to be secondary to critical illness.

Neither the presence of preoperative anti-PF4/H antibodies nor that of postoperative seroconversion (by 2-fold increase in OD levels) influenced postoperative outcome; in particular, no episodes of arterial or venous thrombosis occurred. The incidence of postoperative complications ac-

cording to anti-PF4/H antibody seroconversion is described in Table 3.

PF4 plasma levels increased significantly (from nondetectable levels at T0) after the operation (Figure 1). However, no differences in PF4 levels were observed between patients with anti-PF4/H antibody seroconversion versus those who did not exhibit seroconversion, even when comparing the areas under the curve.

IL-6 levels increased significantly (from nondetectable levels at T0) after the operation; no differences were observed between patients undergoing operations with or without CPB. IL-6 levels were similar in patients who had anti-PF4/H antibody seroconversion versus levels in those who did not exhibit seroconversion. However, 33 patients who had an OD value of 0.45 or greater for anti-PF4/H antibody at the postoperative (T3) time point had higher IL-6 levels compared with 37 anti-PF4/H antibody-negative patients, a finding that was significant at T2 ($P = .002$). Even excluding those patients ($n = 14$) who had an OD value of 0.45 or greater for anti-PF4/H antibody preoperatively (T0), IL-6 levels were significantly higher compared with those seen in patients with negative results ($P = .006$, Figure 2).

TABLE 2. Association between locus B and locus C alleles and anti-PF4/H antibody seroconversion status and comparison with allelic frequencies of 6156 healthy subjects

Alleles	No seroconversion	Seroconversion	<i>P</i> value*	Healthy population	<i>P</i> value†	<i>P</i> value‡
B7	6 (5.45%)	4 (12.5%)	.23	757 (6.15%)	.99	.13
B8	7 (6.36%)	0 (0%)	.34	397 (3.22%)	.09	.62
B13	4 (3.64%)	2 (6.25%)	.61	583 (4.74%)	.82	.66
B18	10 (9.09%)	4 (12.5%)	.51	1403 (11.4%)	.54	.78
B27	3 (2.73%)	0 (0%)	.99	170 (1.38%)	.19	.99
B35	17 (15.45%)	10 (31.25%)	.07	1792 (14.55%)	.78	.02
B38	1 (0.91%)	1 (3.13%)	.4	496 (4.03%)	.98	.99
B39	6 (5.45%)	0 (0%)	.33	247 (2.01%)	.02	.99
B44	15 (13.64%)	2 (6.25%)	.36	943 (7.66%)	.03	.99
B45	2 (1.82%)	0 (0%)	.99	70 (0.57%)	.13	.99
B49	4 (3.64%)	0 (0%)	.57	515 (4.18%)	.99	.64
B50	1 (0.91%)	3 (9.38%)	.03	304 (2.47%)	.52	.04
B51	9 (8.18%)	1 (3.13%)	.45	1046 (8.5%)	.99	.51
B55	2 (1.82%)	1 (3.13%)	.53	229 (1.86%)	.99	.45
B58	2 (1.82%)	0 (0%)	.99	138 (1.12%)	.35	.99
B60	2 (1.82%)	0 (0%)	.99	172 (1.4%)	.66	.99
B62	2 (1.82%)	2 (6.25%)	.21	260 (2.11%)	.99	.14
Cw1	5 (4.55%)	0 (0%)	.58	289 (2.35%)	.19	.99
Cw2	5 (4.55%)	0 (0%)	.58	611 (4.96%)	.99	.4
Cw3	5 (4.55%)	3 (9.38%)	.37	575 (4.67%)	.99	.18
Cw4	21 (19.09%)	15 (46.88%)	.002	1915 (15.55%)	.29	<.0001
Cw5	6 (5.45%)	3 (9.38%)	.42	422 (3.43%)	.28	.09
Cw6	15 (13.64%)	6 (18.75%)	.57	1619 (13.15%)	.88	.3
Cw7	43 (39.09%)	5 (15.63%)	.02	2894 (23.51%)	<.0001	.4

Data are analyzed as alleles (number of subjects $\times$ 2). Uncorrected significant *P* value $\leq .05$; Bonferroni-corrected significant *p* value $\leq (.05/24) = .002$. *Anti-PF4/H*, Anti-platelet factor 4/heparin. *Comparison between seroconverted versus nonseroconverted patients. †Comparison between the healthy population and nonseroconverted patients. ‡Comparison between the healthy population and seroconverted patients.

As expected, platelet counts decreased after the operation (T0 vs T2, $P < .0001$), and after 14 days, they were significantly higher than preoperative levels (T0 vs T3, $P < .0001$). No difference in platelet count levels was found between seroconverted and nonseroconverted patients, however.

DISCUSSION

We prospectively evaluated the incidence of seroconversion to anti-PF4/H antibodies in patients undergoing cardiac

surgery. Several previous studies have shown a high frequency of anti-PF4/H antibodies after cardiac operations (between 22% and 61% of the patients).^{6,12-14} Indeed, in our study approximately half of the patients (52%) had an OD value of 0.45 or greater in one or more of the assays, although somewhat less than half of these patients (22% overall) met our more stringent criteria for a seroconversion response after the operation. Heparin, a polyanion, has a high affinity for the cationic chemokine PF4, which is

TABLE 3. Clinical outcome according to anti-PF4/H antibody seroconversion status

	All patients	No seroconversion	Seroconversion	<i>P</i> value
Patients	71	55	16	
In-hospital mortality	1 (1.4%)	1 (1.8%)	0 (0%)	.99
Mechanical ventilation (h)	10.9 $\pm$ 11.2	9.7 $\pm$ 5.3	15.4 $\pm$ 21.8	.08
ICU stay (h)	34 $\pm$ 30.1	34.2 $\pm$ 31.1	33.4 $\pm$ 31.1	.93
IABP	1 (1.4%)	0 (0%)	1 (6.3%)	.22
Blood loss (mL)	663.7 $\pm$ 403.7	664.6 $\pm$ 428.8	660.6 $\pm$ 311.2	.97
Blood units	1.6 $\pm$ 1.8	1.6 $\pm$ 1.9	1.7 $\pm$ 1.5	.73
FFP (U)	0.2 $\pm$ 0.8	0.2 $\pm$ 0.7	0.07 $\pm$.2	.48
Platelets (U)	0.2 $\pm$ 1	0.22 $\pm$ 1.16	0	.46
Acute renal failure	6 (8.5%)	4 (7.3%)	2 (12.5%)	.61
Atrial fibrillation	16 (22.5%)	13 (23.6%)	3 (18.8%)	.99
Sternal wound infection	5 (7%)	3 (5.5%)	2 (12.5%)	.31

Anti-PF4/H, Anti-platelet factor 4/heparin; *ICU*, intensive care unit; *IABP*, intra-aortic balloon pump; *FFP*, fresh frozen plasma.

FIGURE 1. Box plot of platelet factor 4 (PF4) levels according to seroconversion. Data are presented as medians (interquartile ranges). In the entire population, a significant increase in PF4 levels was observed between T0 and T1 ($P < .0001$). However, no differences were seen in PF4 levels between seroconverted and nonseroconverted patients.

found within platelet α -granules and on the endothelium. Antibodies of IgG class reactive against the multimolecular PF4/H complexes can cause platelet activation through the platelet Fc γ IIa receptors.^{1,15}

It is well established that cardiac surgery, either with or without CPB, induces platelet activation with the release of their granule content (PF4, β -thromboglobulin, adenine, and guanosine nucleotides): CPB-induced platelet “stress,” heparin administration, and hypothermia are considered the main triggers of platelet activation in this context.⁷ Considering that seroconversion after cardiac surgery occurs much more frequently than other clinical settings with heparin exposure,² we sought to test the hypothesis that the intense platelet activation induced by cardiac operations, with the resulting release of PF4 from platelets facilitating formation of the immunogenic PF4/heparin complexes, could be responsible for anti-PF4/H antibody seroconversion. Recently,

Rauova and colleagues,¹⁶ in a murine experimental model, demonstrated that the development and severity of thrombocytopenia depends on the surface PF4 and total PF4 levels. Although our data indicate that significant amounts of PF4 are released in association with cardiac surgery, we found no difference in perioperative plasma PF4 levels between the patients who showed anti-PF4/H antibody seroconversion and those who did not. We also found no difference in anti-PF4/H antibody seroconversion frequencies between patients undergoing operations with or without CPB, a finding that confirms the observations of Francis and associates.¹⁷

In our study the presence of anti-PF4/H antibodies did not influence clinical outcome. It is worth noting that several large clinical studies have recently suggested that the presence of anti-PF4/H antibodies (measured as a combined IgG/IgA/IgM immune response) confers a risk of adverse

FIGURE 2. Box plot of interleukin 6 (IL-6) levels according to anti-platelet factor 4/heparin antibody OD levels (≥ 0.45 or < 0.45) measured at T3 (excluding 14 patients with OD levels ≥ 0.45 preoperatively).

outcomes among certain groups of heparin-treated patients, even in the absence of clinical HIT. In patients with acute coronary syndrome, anti-PF4/H antibodies were independent predictors of 30-day death and myocardial infarction.¹⁸ Two large studies demonstrated that the presence of anti-PF4/H antibodies before surgical intervention is an independent predictor for death, prolonged hospital stay, and organ dysfunction after adult cardiac surgery.^{19,20} Interestingly, none of these studies found evidence of clinical HIT among these antibody-positive patients, despite the apparent adverse prognostic implications of the antibodies. In our study we investigated the individual immunoglobulin classes (IgG, IgA, and IgM) that underlie the anti-PF4/H immune response and tested for functionality of the antibodies using a platelet activation assay (SRA). However, we did not identify any patients who had the clinical features of HIT (thrombocytopenia, thrombosis, or both) together with the expected serologic profile (positive anti-PF4/H IgG-ELISA result and positive SRA result).

Because the anti-PF4/H immune response is observed commonly after cardiac surgery and can have adverse prognostic implications, we investigated for a possible relationship between the presence of anti-PF4/H antibodies (a surrogate marker for the HIT immune response) and perioperative inflammation. Cardiac surgery is known to induce an acute-phase reaction that has been implicated in the pathogenesis of several postoperative complications. Different proinflammatory and anti-inflammatory mediators (cytokines and adhesion molecules) are involved.²¹ An intense release of IL-6 has been demonstrated during and after the operation (with and without CPB),⁷ and our study confirms that an activated inflammatory state persists for several days after the operation. However, no differences in serum IL-6 levels during the perioperative and postoperative periods were observed between the patients who had anti-PF4/H antibody seroconversion versus those who did not have seroconversion. Nevertheless, our data did demonstrate that patients with positive anti-PF4/H antibody levels at T3 had higher IL-6 levels during the early postoperative period (Figure 2), possibly signifying that the intense proinflammatory response provoked by cardiac surgery facilitates the immune response against the PF4/heparin complex. Considering that the amount of inflammatory response provoked by cardiac operations is higher than any other clinical condition, this finding might help to clarify the reason why cardiac surgical patients experience higher anti-PF4/H antibody seroconversion rates compared with other clinical settings with heparin exposure.²

Increased inflammation itself could be the underlying cause of adverse prognosis described in the presence of anti-PF4/H antibodies, despite the absence of clinically evident HIT.^{19,20} However, this speculation that arises from the results of our study needs to be confirmed by specifically designed experimental and clinical studies.

Finally, our data demonstrate that patients with the HLA class I CW4 antigen have a higher risk of anti-PF4/H antibody seroconversion after heparin exposure during cardiac operation. Patients with the HLA B35 and B50 antigens have also a higher risk of seroconversion, but the association did not remain significant after the Bonferroni correction. Two previous studies have been published with the aim of identifying an HLA involvement in HIT immune reactions. Greinacher and Mueller-Eckhart⁵ did not find any association between HLA class I and class II antigens and immune HIT, which was confirmed in 47 patients by means of a platelet activation assay. Our group, in a prospective evaluation of 69 patients undergoing CPB surgery, found a significantly higher incidence (without the Bonferroni correction) of the HLA class II DRB1*03 allele among 10 patients with anti-PF4/H antibodies (absorbance values, ≥ 0.40 OD units) in at least one of the collected samples.⁶ Several factors render the present study more reliable: stronger statistical evidence obtained with the Bonferroni correction, strict criteria used to identify seroconversion, better sampling time to evaluate postoperative seroconversion (14 days after heparin exposure, the time when anti-PF4/H antibodies usually reach peak levels, rather than 7 and 21 days after), and more advanced HLA tissue-typing techniques. We consider the present finding interesting because the same alleles have been identified in association with several (auto)immune disorders, such as rheumatoid arthritis,²² thyroiditis,²³ and AIDS.²⁴ Our experience provides additional evidence that the HLA-CW4 antigen confers genetic susceptibility in acquiring acute or subacute inflammatory disease, in this case the anti-PF4/H immune response.

Some limitations of our study should be discussed. Our results do not rule out a possible influence of platelet-associated PF4 levels, nor do they rule out the possibility that differing quantities of multimolecular PF4/heparin complexes formed on cell surfaces could influence the risk of seroconversion. Moreover, our sample size is not adequate to provide information into whether adverse clinical outcomes are associated with an anti-PF4/H immune response. Our study also was not designed to evaluate whether HLA status influences the postoperative inflammatory response.

In summary, our study provides evidence that both genetic factors (HLA status) and acquired factors (perioperative inflammation, as assessed based on IL-6 levels) might influence the anti-PF4/H immune response. Because several pharmacologic, device-related, and technical strategies exist to attenuate inflammatory response after cardiac surgery, future studies should clarify whether a reduced inflammatory response might attenuate the risk of anti-PF4/H antibodies seroconversion after cardiac surgery.

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