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Prevalence of Heparin-Induced Antibodies in Patients With Chronic Renal Failure Undergoing Hemodialysis

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Heparin-induced thrombocytopenia (HIT) type II is a serious complication of heparin therapy. It presents initially as thrombocytopenia, and is associated with thrombosis in 20–50% of the cases. HIT is related to the presence of heparin-induced antibodies (HIA), which show specificity for the PF4-heparin (PF4-H) complex. The FcγRIIa receptor has been suggested to participate in the pathogenic mechanism of HIA. Since patients undergoing chronic hemodialysis (HD) are exposed repeatedly to heparin, we studied the prevalence of HIA and their eventual relationship with thrombocytopenia and/or thrombosis, and the possible participation of the FcγRIIa polymorphism. We studied 207 patients with chronic renal failure (CRF) undergoing HD. As a control we included 130 blood donors and 28 patients with CRF without HD. The HIA patients were studied with the use of a PF4-H ELISA. Additionally, in some positive cases for the previous test, a ^{14}C- serotonin release assay (^{14}C-SRA) was performed. The polymorphism FcγRIIa H/R131 was studied by polymerase chain reaction (PCR) with allele-specific primers. Thirty-seven patients (17.9%) undergoing HD presented with HIA. The majority of these antibodies were IgG, IgM, and IgA. The HIA investigated presented specificity against the PF4-H complex, but not against PF4 alone ($P < 0.001$). Twelve out of 22 (54.5%) PF4-H antibodies were positive when tested with the ^{14}C-SRA. The distribution of the FcγRIIa polymorphism in patients and healthy controls was 42.6% and 41.6% for H/H131, 41% and 48.9% for the H/R131 isoform, and 16.4% and 9.5% for the R/R131 isoform, respectively. No statistically significant difference in the FcγRIIa isoform distribution was found. Twenty-nine out of 156 patients (18.5%) presented thrombocytopenia, and 21/207 (12.4%) had thrombosis of the native vein arterio-venous fistula (AVF). We did not find any statistically significant between HIA and thrombocytopenia or thrombosis. An important proportion of patients with CRF undergoing HD developed HIA, but these cases were not associated with thrombocytopenia or thrombosis of AVF. The frequency of the FcγRIIa polymorphism did not statistically differ between HIT type II and normal controls. J. Clin. Lab. Anal. 19:189–195, 2005.

Key words: heparin-induced antibodies; FcγRIIa; hemodialysis

INTRODUCTION

Heparin-induced thrombocytopenia (HIT) type II is a complication of therapy with heparin that occurs in 5–10% of patients treated with this anticoagulant [1,2]. It is recognized clinically by thrombocytopenia or a reduction in the platelet count to 30–50% of basal value [3]. It has been reported that 20–50% of HIT cases are associated with venous and/or arterial thrombosis (HIT-thrombosis (HITT)) [4].

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HIT is related to the presence of heparin-induced antibodies (HIA), which show specificity for the PF4-heparin (PF4-H) complex [5,6]. In addition, these antibodies may bind directly to activated endothelial cells, which may explain some of the specific clinical manifestations of HIT [7].

It has been suggested that the FcγRIIa receptor participates in the pathogenesis of HIT [8]. There are two functional isoforms of FcγRIIa in humans. A single-base substitution (G → A) in the codon for amino acid 131 results in an arginine (R) to histidine (H) change in the extracellular domain of the receptor, altering its binding affinity for human IgG [9]. The FcγRIIa-H131 isoform binds human IgG with higher affinity than FcγRIIA-R131. The effect of this dimorphism on the platelet response to HIA is controversial. It has been reported that platelets with the FcγRIIa-H/H131 genotype may be responsive to HIT [10]. Yet HIT has been reported to occur more commonly, if not exclusively, in donors who show at least one histidine allele at the 131 position [11]. Other studies found that platelets with the FcγRIIa-H/H131 phenotype were unresponsive to HIT, whereas platelets with the Arg/Arg 131 phenotype responded well [12].

The prevalence of HIA in patients with chronic renal failure (CRF) undergoing hemodialysis (HD) has been reported to be 1–12% [13–16]. Some of these patients present with thrombosis of the native vein arteriovenous fistula (AVF) and/or thrombocytopenia. Given that patients undergoing HD are exposed repeatedly to heparin, the main goal of this work was to study the prevalence of HIA and its eventual relationship with thrombocytopenia and/or thrombosis. Considering the controversial role of FcγRIIa polymorphisms, we took this opportunity to further investigate the participation of FcγRIIa polymorphisms in the pathogenesis of HIT.

MATERIALS AND METHODS

Patients

We studied 207 patients (54% women, 46% men; 53 ± 18 years old) with CRF who had undergone HD for 35 ± 29 months (Table 1). The etiologies of the condition included diabetic nephropathy (20.3%), nephrosclerosis (15.4%), chronic glomerulonephritis (14.5%), polycystic kidney disease (2.9%), interstitial nephritis (0.5%), other (27.0%), and unknown (19.4%).

All patients included in the study were treated with native AVF, unfractionated heparin, and a filter with a Cuprofan membrane (Terumo Medical Corp., Tokyo, Japan). Thrombosis of the AVF was clinically evaluated by phlebography. As controls we included 130 blood donors and 28 patients with CRF who had not received dialysis. We assessed the subjects for risk factors for thrombosis, including diabetes mellitus, hypercholesterolemia (>200 mg/dL), and obesity (body mass index (BMI) ≥ 31). This study was approved by our institutional review board.

TABLE 1. Demographic data and cause of end stage renal disease in 207 patients in chronic hemodialysis

Age (years)	53 ± 18
Gender	112 females (54%)
	95 males (46%)
Hemodialysis duration (months)	35 ± 29
Cause of end stage renal disease	42 (20.3%)
Diabetic nephropathy	42 (20.3%)
Nephrosclerosis	32 (15.4%)
Chronic Glomerulonephritis	30 (14.5%)
Polycystic kidney disease	6 (2.9%)
Interstitial Nephritis	1 (0.5%)
Other	56 (27.0%)
Unknown	40 (19.4%)

Methods

Blood was collected immediately before HD in tubes with EDTA (BD Vacutainer, Franklin Lake, NJ) for platelet count and DNA extraction, and without anticoagulant for the HIA study. To obtain serum, the blood was collected in tubes without anticoagulant, left to clot, and then centrifuged for 10 min at 3,500 *g*. The supernatant (serum) was then stored at –70°C until analysis.

Platelet count

A phase-contrast microscope was used for the platelet count. Briefly, the blood, which was checked visually for clotting, was diluted (1:100) with ammonium oxalate 1%. The mixture was placed in a hemacytometer and incubated in a humid chamber for 20 min at room temperature. The platelets were then counted with the phase-contrast microscope, and all samples were counted twice. Thrombocytopenia was defined as a platelet count of $< 140 \times 10^3$ platelets/μL.

PF4-heparin ELISA

For the investigation of HIA, a PF4-heparin (PF4-H) ELISA was used [5,17]. Briefly, microtiter plates (Immulon 4, Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with a 50-μL solution of phosphate-buffered saline (PBS) 10 mM, pH 7.4, containing PF4 10 μg/mL, and unfractionated sodium heparin 0.5 UI/mL (Laboratorio Chile S.A.).

After the samples were washed with PBS 0.01 M (pH = 7.4) containing 0.05% Tween-20 (PBS-Tw), the

plates were blocked with 1% bovine serum albumin (BSA; GIBCO, BRL) in PBS-Tw for 1 hr at room temperature (RT; 22–25°C). After washing with PBS-Tw, 50 µl of serum samples (1:10 dilution in PBS-Tw-BSA), negative or positive controls, were added to the plates in duplicate. After 1 hr of incubation at RT the plates were washed with PBS-Tw, and 50 µl of alkaline phosphatase-conjugated anti-human IgG, IgM, or IgA (Sigma, St. Louis, MO) were added to the plates. After 1 hr of incubation at RT and washing with PBS-Tw, 100 µl of p-nitrophenylphosphate substrate (Merck, Darmstad, Germany) in a diethanolamine buffer (pH = 9.8) were added and then incubated for 1 hr at 37°C. The plate was read at 405 nm in a StatFax-2100 microplate reader (Awareness Technology Inc., Palm City, FL). The positive samples were tested twice to confirm the results. The activity of the antibodies was expressed as the ratio of the optic density (OD) of the patient sample to the OD of the cutoff (mean+4 SD of control group). A ratio >1.0 was considered positive. To study the specificity of HIA, positive and normal sera were tested by ELISA with PF4 alone (10 µg/mL), BSA (10 µg/mL), and PF4-H in the same concentration as previously described. The PF4 used was purified from normal serum using a sepharose-heparin column [18].

^{14}C-serotonin release assay (^{14}C-SRA)

To assess the functional activity of the HIA, positive sera for these antibodies (4 IgG, 11 IgM, and 7 IgA) were studied with ^{14}C-SRA [19]. Briefly, 20 µL of test inactivated serum were mixed with 5 µL of sodium heparin (Laboratorio Chile SA.) concentrations (0.1 and 100 U/mL), and 75 µL of normal ^{14}C-serotonin-labeled platelets (2×10^5/µL). The platelet mixture was incubated in microtiter wells with a magnetic stir bar. Following a 60-min incubation at 22°C on the magnetic stir plate set at slow speed, 100 µL of 0.5% EDTA in saline were added to each well to stop the release reaction. The platelet mixture was centrifuged for 5 min at 1,500 *g*, and 100 µL of supernatant fluid were removed and counted in an automatic liquid scintillation counter (TAURUST, model 3600; ICN Biomedical, Huntsville, AL). The percent release was calculated as follows: release of test sample – background/total radioactivity – background × 100. The background was defined as the supernatant fluid radioactivity from platelets that were handled the same way the test platelets were except that a serum was substituted for buffer. A sample was considered positive with a release percentage >20% of the total incorporation of the platelets (after having subtracted from it the value liberated by normal plasm) with a concentration of 0.1 U/mL and not with 100 U/mL of heparin. Negative and positive controls were included.

Genotyping of the FcγRIIa

The FcγRIIa H/R131 polymorphism was determined by modified polymerase chain reaction (PCR) with allele-specific primers [20]. Genomic DNA was extracted from the blood samples [21]. The principal reactives were as follows: PCR buffer and dNTP (Gibco BRL), Taq polymerase (Fisher Biotech, Pittsburgh, PA), and H131-specific sense primer (5′-ATCCCA-GAAATTCTCCCA-3′) from the second extracellular domain R131-specific sense primer (5′-ATCCCA-GAAATTCTCCCG-3′), and common antisense primer from an area of downstream intron where the sequence for FcγRIIa, FcγRIIb, FcγRIIc diverge (5′-CAATTTTGCTGCTATGGGC-3′) (all primer oligonucleotides were synthesized by Bios Chile). As an internal control we used human growth hormone (hGH)-I primer (5′-TGCCTTCCCAACCATTCCCTTA-3′) and hGH-II primer (5′-CCAC TCACGGATTTCTGTTGT GTTTC-3′). The genotyping of FcγRIIa was done for 190 of the 207 patients.

Statistical Analysis

In the statistical analysis we utilized the mean, standard deviation (SD), Student's *t*-test, χ^2 test, or Fisher's test. The significance level was established at 5%.

RESULTS

Prevalence of HIA

Thirty-six out of 207 (17.4%) patients undergoing HD presented HIA. These antibodies were not found in patients with CRF without HD or in normal controls. The isotype distribution showed that of the 36 HIA cases, 20 (54.1%) were IgM, 11 (29.7%) were IgG, and five (13.5%) were IgA (Table 2). The activity of the antibodies was expressed as the ratio of the OD of the patient sample to the OD of the cutoff, as shown in Table 2. The prevalence of HIA demonstrated a tendency (albeit not statistically significant) to increase as the length of time the patients were on HD increased: 11.8%, 15.6%, 19.1%, and 24.5% of patients on HD for

TABLE 2. Isotypes and activity of heparin-induced antibodies in patients undergoing in hemodialysis*

Isotype	N	%
IgG	11 (1.42±0.27)	29.7
IgM	20 (1.47±0.30)	54.1
IgA	5 (1.58±0.20)	13.5

*The activity (OD_{405} patient/OD_{405} cutoff) of antibodies is in parentheses. Heparin-induced antibodies: 36/27 = 17.4%.

<12, 12–35, 36–59, and >60 months, respectively, presented with HIA. The duration of HD did not correlate with any specific isotype.

Specificity of HIA

When the specificity of 34 HIA was studied, a strong reaction in the presence of the PF4-H complex, independently of isotype, was observed (Fig. 1). However, 3/17 (17.6%) IgG and 3/12 (25%) patients with IgM HIA showed only slight activity for PF4 alone. On the other hand, the normal controls did not show any activity in either of the two situations, and HIA and normal sera did not react to BSA.

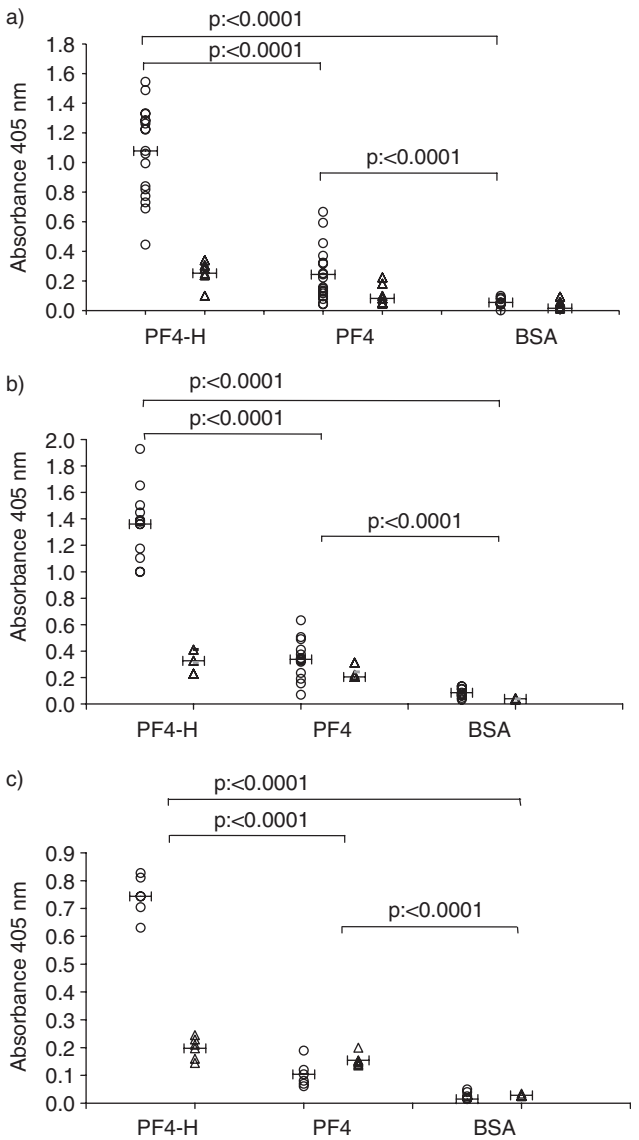

Fig. 1. Binding of HIA to PF4-H and PF4 in solid phase. ELISA was used to quantitate binding of IgG (**a**), IgM (**b**), and IgA (**c**) HIA detected in patients undergoing HD (○) (n = 17, 12, and 5, respectively), and normal individuals (δ) to microtiter wells coated with PF4-H, PF4, and BSA.

TABLE 3. Functional activity of heparin-induced antibodies

Isotype	PF4-H ELISA positives (n)	^{14}C-Serotonine release positives (n)	^{14}C-Serotonine release positives (%)
IgG	4	3	75.0
IgM	11	6	54.5
IgA	7	3	42.8

Functional Studies of Antibodies

Studies of functional activity with ^{14}C-SRA showed that 12/22 antibodies (54.5%) presented some degree of activity: 3/4 (75%) IgG, 6/11 (54.5%) IgM, and 3/7 (42.9%) IgA antibodies (Table 3).

FcγRIIa Polymorphism

The distribution FcγRIIa genotypes in the healthy controls was H/H131 41.6%, H/R131 48.9%, and RR131 9.5%, and the allele frequencies were H131 0.66 and R131 0.34. On the other hand, the distribution of the FcγRIIa genotypes in the patients undergoing HD was H/H131 42.6%, H/R131 41.0%, and R/R131 16.4%, and the allele frequencies were H131 0.62 and R131 0.38 (Table 4). No statistically significant difference in the FcγRIIa isoform distribution between patients and controls was found. As regards an eventual relationship between the polymorphism FcγRIIa R/H131 and HIT or HITT development, 8.6% of the patients with HIT/HITT presented the H/H131 polymorphism, 16.6% presented H/R131, and 16.6% presented R/R131.

HIA and Thrombocytopenia

Twenty-nine out of 127 patients undergoing HD (18.5%) presented thrombocytopenia ($<140 \times 10^3$ platelets/μL) at the time of the study. The platelet count in patients undergoing HD who were HIA-positive (n = 29) was $182 \pm 82 \times 10^3$ platelets/μL, and in HIA-negative patients (n = 127) it was $202 \pm 64 \times 10^3$ platelets/μL. These differences are not statistically significant. Table 5 shows that only 7/29 (24.1%) patients who presented thrombocytopenia were also HIA-positive. This percentage is similar to that observed in non-thrombocytopenic patients (19.6%). The samples were collected before the process of HD (in the absence of heparin) was initiated. To study the effect of HIA on the platelet count in the presence of the drug in eight HIA-positive patients and 10 HIA-negative patients, the

TABLE 4. Distribution of FcγRIIa genotypes and allele frequencies in patients in hemodialysis and thrombocytopenia and/or thrombosis, and control group*

		FcγRIIa allele frequency		FcγRIIa genotype		
	n	R131	H131	R/R131	R/H131	H/H131
Patients	134[a]	0.38	0.62	22 (16.4)	55 (41.0)	57 (42.6)
Thrombocytopenia (T)	29	0.33	0.67	4 (13.8)	11 (37.9)	14 (48.3)
Thrombosis AVF (T')	16	0.28	0.72	3 (18.8)	3 (18.8)	10 (62.4)
Without T and T'	89	0.40	0.60	15 (16.8)	41 (46.1)	33 (37.1)
Healthy controls	137	0.34	0.66	13 (9.5)	67 (48.9)	57 (41.6)

*Numbers in parentheses are to percentages.
[a]n patients with information: FcγRIIa polymorphism, platelet count and information about AVF.
AVF, arterio-venous fistula.

TABLE 5. Heparin-induced antibodies, and thrombocytopenia and thrombosis*

	HIA (+)	HIA (–)
Thrombocytopenia[a] (+)	7/29	22/29
Thrombocytopenia (–)	25/127	102/127
Thrombosis AVF (+)	3/21	18/21
Thrombosis AVF (–)	34/186	152/186

*Patients, with count platelets (n = 156) and information about thrombosis AVF (n = 207), P = NS.
[a] $< 140 \times 10^3$ platelets/μL.

platelet count was carried out pre- and post-HD. In the HIA-positive patients, the pre- and post-HD platelets counts were 206 ± 56 vs. $199 \pm 48 \times 10^3$ platelets/μL, and in the HIA-negative cases the counts were 186 ± 36 vs. $181 \pm 38 \times 10^3$ platelets/μL, respectively (P = N.S; Table 5).

HIA and Thrombosis of AVF

Twenty-one of 207 patients undergoing HD (10.1%) presented thrombosis of the AVF. Three of these patients (14.3%) and 34/186 of patients without thrombosis of the AVF (18.3%) were HIA-positive (P = N.S; Table 5).

DISCUSSION

The occurrence of HIT is the most important complication of heparin therapy, with a prevalence ranging between 5% and 10% of patients [1,2]. The purpose of this study was to determine the prevalence of HIA in patients in CRF undergoing HD, and its eventual association with thrombocytopenia and/or thrombosis, as well as to analyze the possible role of the FcγRIIa receptor polymorphism in HIT.

Prevalence of HIA

The prevalence found (17.9%) was higher than that observed in other series (2–12% in Refs. 13–16). However, most of those studies determined only IgG HIA. In our study the prevalence of IgG was 5.4% (11/207), which is similar to that found in the above-mentioned studies, in which IgG_1 was the most frequent isotype found (88%) [9]. Similar findings regarding the high prevalence of HIA and the relative paucity of clinical manifestations have been reported in patients undergoing cardiovascular surgery [22,23]. Approximately 80% of patients who develop HIT present IgG HIA, and the remaining 20% present IgM and/or IgA [24]. Another study of CRF patients found that 50% of the samples were positive for IgG, 45% were positive for IgM, and 37% were positive for IgA [25].

The HIA-positive sera studied reacted mainly with the PF4-H complex. Visentin et al. [6] observed the same results in patients treated with heparin. Six out of 17 (35.7%) of the IgG and 3/12 (25%) of the IgM antibodies that reacted with PF4-H complex also reacted weakly with PF4 alone. In this respect, it has also been observed that affinity-purified IgG HIA bound to polystyrene adsorbed PF4 without heparin, but not soluble PF4. The same antibodies bind to the PF4-H complex [26].

The concentration of PF4 that is available for binding to heparin or to the HIT-related antibodies is essential and critical for platelet aggregation induced by HIT antibodies [27].

Twelve out of 22 (54.5%) of PF4-ELISA positive sera were positive for ^{14}C-SRA. We found that a majority of positive sera (3/4, 75%) for ^{14}C-SRA corresponded to the IgG isotype. The IgG isotype is the principal mediator of platelet activation in patients with HIT. IgA and IgM play a less significant role in the pathophysiology of this syndrome [28].

From the laboratory standpoint, the diagnosis of HIT is based on two types of tests: functional assays (e.g., ^{14}C-SRA) and immunoassays (e.g., PF4-H ELISA). Neither of the two types of tests allows for diagnosis of HIT in 100% of the cases, and a discrepancy has been reported in 10–20% of patients [17]. The most commonly used functional assays are those that study the ability of the HIA IgG to activate platelets, and the ^{14}C-SRA test is highly sensitive and specific ($>90\%$) [29]. The PF4-H ELISA can detect weak antibodies that are not detected by functional assays (i.e., IgM and IgA). This immunoassay was more sensitive than the functional test, since about half of the ELISA-positive samples were also in the ^{14}C-SRA.

HIA and Thrombocytopenia

Thrombocytopenia ($<140\times10^3$ platelets/μL) was present in 18.5% of the patients at the time of this study, but this was not associated with HIA. Luzzatto et al. [16] also found no association with thrombocytopenia. Among other causes of thrombocytopenia in this setting, antiplatelet and antiphospholipid antibodies were not ruled out.

HIA and Thrombosis

Thrombosis is a complication of HIT. In the patients with CRF undergoing HD we did not find a relationship between HIA and thrombosis, a result that has been observed by other investigators [30].

FcγRIIa Polymorphism

The FcγRIIa H/R131 allele frequencies and genotype were similar between our patients and healthy donors. Apparently the genotype FcγRIIa HH131 is more frequent in healthy Chilean controls (41.6%) than in the Caucasian population (19–28%) [9,12,31]. Carlsson et al. [31] found that FcγRIIa HH131 was more frequent in patients with HIT (31.1%) than in healthy blood donors (27.7%).

The presence of HIA is not necessarily associated with HIT, and therefore some patients are asymptomatic. In vivo, other factors besides the presence of HIA are required for the production of HIT or HITT. Platelet activation by HIA is characterized by its marked interindividual variability, which may be explained by the number of FcγRIIa/platelets [32] and/or the FcγRIIa phenotype. With regard to the latter, the results are contradictory: some studies have reported a strong association of HIT with the HH131 FcγRIIa receptor [8] and RR131 FcγRIIa [31]. However, Arepally et al. [9] did not find differences in the H/R131 FcγRIIa polymorphism distribution between patients with HIT or HITT and normal controls.

Apparently the FcγRIIa polymorphism alone does not explain the absence of a relationship. Platelet activation by HIA could be caused by independent mechanisms of the FcγRIIa, particularly in the case of IgM and IgA.

Patients with CRF undergoing HD exhibit an increased tendency to develop thrombosis and accelerated atherosclerosis [33,34]. HIA with or without HIT may be one of the contributory factors to this phenomenon. However, despite the high prevalence of HIA in these patients, our results indicate that the effect of HIA on the platelet count and associated thrombosis is not important.

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