Platelet Factor 4/Heparin Antibodies in Blood Bank Donors

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Abstract

Platelet factor 4 (PF4)/heparin antibody, typically associated with heparin therapy, is reported in some heparin-naive people. Seroprevalence in the general population, however, remains unclear. We prospectively evaluated PF4/heparin antibody in approximately 4,000 blood bank donors using a commercial enzyme-linked immunosorbent assay for initial and then repeated (confirmatory) testing. Antibody was detected initially in 249 (6.6%; 95% confidence interval [CI], 5.8%-7.4%) of 3,795 donors and repeatedly in 163 (4.3%; 95% CI, 3.7%-5.0%) of 3,789 evaluable donors. “Unconfirmed” positives were mostly (93%) low positives (optical density [OD] = 0.40-0.59). Of 163 repeatedly positive samples, 116 (71.2%) were low positives, and 124 (76.1%) exhibited heparin-dependent binding. Predominant isotypes of intermediate to high seropositive samples (OD >0.6) were mostly (93%) low positives (optical density [OD] = 0.40-0.59). The marked background seroprevalence of PF4/heparin antibody (4.3%-6.6%) with the preponderance of low (and frequently nonreproducible) positives in blood donors suggests the need for further assay calibration, categorization of antibody level, and studies evaluating clinical relevance of “naturally occurring” PF4/heparin antibodies.

Heparin-induced thrombocytopenia (HIT) is an immune-mediated disorder caused by antibodies that recognize a complex of 2 naturally occurring antigens: platelet factor 4 (PF4), which is a platelet-derived protein, and heparin, which is a member of the glycosaminoglycan family. HIT occurs in approximately 1% to 5% of patients treated with unfractionated heparin (UFH) and 0.2% to 0.8% of patients treated with low-molecular-weight heparin (LMWH). The immune response to PF4/heparin characteristically begins 4 days after initial exposure to UFH or LMWH. With few exceptions, all cases of HIT are acquired in the wake of heparin exposure.

Diagnosis of HIT is based on clinical criteria and laboratory demonstration of PF4/heparin antibodies by immunologic or functional assays. At most medical centers, immunoassays are preferred over functional assays because of their technical simplicity, high sensitivity (>99%), and rapid turnaround time. The specificity of immunoassays, however, is compromised by the high rates of asymptomatic PF4/heparin seroconversions that occur in various clinical settings. Asymptomatic seroconversions are seen in approximately 8% to 17% of patients receiving UFH, 2% to 8% receiving LMWH, and 1% to 2% receiving fondaparinux. For unknown reasons, exceptionally high rates of seroconversion (approximately 27%-61%) are documented in patients undergoing cardiac surgery. The clinical significance of asymptomatic seroconversions is presently unknown.

Until recently, it was believed that the specificity of immunoassays was principally affected by the occurrence of “false-positive” antibodies during heparin treatment and that PF4/heparin antibodies do not occur in healthy subjects. This perception was based on studies of healthy subjects performed by the manufacturers of commercial immunoassays (eg, PF4...
bodies (APLAs) or systemic lupus erythematosus.20 Last, in bodies among patients diagnosed with antiphospholipid anti-
describes the occurrence of false-positive PF4/heparin anti-
elisa), heparin dependency, and isotype. Including relative titer (ie, optical density [OD] result from
determined the serologic features of PF4/heparin antibodies detected,
the occurrence of false-positive PF4/heparin antibodies among patients diagnosed with antiphospholipid anti-
odies (APLAs) or systemic lupus erythematosus.20 Last, in

Materials and Methods

Study Design

This seroprevalence study of PF4/heparin antibody (IgG, IgM, IgA) was conducted using samples from people who
donated blood to the American Red Cross (ARC) between June 2008 and May 2009. Based on our previous retrospec-
tive estimate of 1% seroprevalence in healthy subjects by GTI
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tiva estimate of seroprevalence.

Imprecision (coefficient of variation [CV]) associated with duplicate tests was calculated as follows: (100% × mean
SD) for an evaluatable sample result, according to the ELISA manufacturer, OD readings from duplicate
tests should fall within 20% of the mean OD. However,
because higher CVs may occur at lower ODs despite negligi-
able absolute well-to-well differences (eg, well 1 OD = 0.063,
well 2 OD = 0.038, absolute difference = 0.025, average OD
= 0.051, SD = 0.018, and CV = 35%), we refined this quality
acceptance criterion as follows: (1) well-to-well CV less than
20%; or (2) well-to-well CV more than 20%, where [mean
OD + (3 × SD)] is less than 0.40. Samples that failed the cri-
ria were considered nonevaluable.

Samples were tested initially using one kit lot
(061808X45, GTI), and then initially positive samples were
retested (confirmed) using a second kit lot (120908X45,
GTI). Although the manufacturer does not require such
retesting, we applied this approach to support a conservative
estimate of seroprevalence.

Characterization of PF4/Heparin Antibody

Heparin Dependence

For repeatedly positive samples, heparin-dependent bind-
ing was assessed using the GTI ELISA procedure according
to the manufacturer’s directions. Briefly, the ELISA is per-
formed as described in the preceding section, but with the
initial incubation step done in the absence or presence of 100
U/mL of UFH (Heplock, Elkins-Sinn, Cherry Hill, NJ). The
percentage of inhibition of binding by excess heparin is calcu-
ated as follows: \[1 - \left(\frac{\text{OD}_{\text{sample with heparin}} - \text{OD}_{\text{negative control}}}{\text{OD}_{\text{sample without heparin}} - \text{OD}_{\text{negative control}}}\right) \times 100\%\]. Heparin-
dependent binding is considered present if the percentage of
inhibition is 50% or more.
Isotype

For repeatedly positive samples of high or intermediate positivity, qualitative information on the presence or absence of anti-PF4/heparin IgG, IgM, or IgA was obtained by using the GTI ELISA modified for detection of human isotypes. We replaced the manufacturer’s polyclonal antihuman IgG/IgA/IgM antibodies with alkaline phosphatase–conjugated antihuman IgG (dilution 1:2,000; Sigma, St Louis, MO), antihuman IgM (dilution 1:5,000; Sigma), or antihuman IgA (dilution 1:1,000; Rockland, Gilbertsville, PA) and increased the duration of color development by 30 minutes, according to experimental conditions optimized in our laboratory. Because limited sample volumes precluded quantifying each isotype, we estimated relative amounts of isotype in a sample using OD ratios, eg, IgG/IgM or IgA/IgM. A ratio of 2 or more was considered evidence of a predominant expression pattern, and a ratio of less than 2 was considered indeterminate.

Data Analyses

The percentage (and associated 95% CI) of initially positive samples and of repeatedly positive samples among evaluable samples was calculated. The proportion of repeatedly seropositive samples demonstrating heparin-dependent binding was compared among the high, intermediate, and low OD groups using the χ² test. Interassay imprecision, reported as CV (100% × mean OD/SD), for GTI ELISA lot 061808X45 was calculated by using results from the manufacturer’s negative and positive control samples and 2 in-house positive control samples. OD results from the initial and repeated tests of initially positive samples were compared by using linear regression. The effect of sample age on seropositivity was assessed by using the Mann-Whitney test (repeatedly seropositive samples vs other samples) or the Kruskal-Wallis test (among repeatedly seropositive samples grouped by OD).

Results

Sample Disposition

A total of 4,339 blood units from unique donations were collected, of which 330 were excluded owing to inadequate sample quality (eg, hemolysis), insufficient volume, or storage issues. We thus tested 4,009 donor units for PF4/heparin antibody using 1 lot of the GTI ELISA. Assay quality criteria (see the “Materials and Methods” section) were not met in 214 samples (5.3%), and reagent limitations precluded their reanalysis using the same kit lot. Therefore 3,795 donor units composed the evaluable sample population.

PF4/Heparin Antibody in Blood Bank Donors

Of 3,795 donor units, 249 (6.6%; 95% CI, 5.8%-7.4%) had PF4/heparin antibodies, using the ELISA manufacturer’s OD cutoff value of 0.40. Overall, 198 samples (5.2% of evaluable samples; 79.5% of seropositive samples) had a low positive result (OD = 0.40-0.59), 40 samples (1.1% of evaluable samples; 16.1% of seropositive samples) had an intermediate positive result (OD = 0.60-0.99), and 11 samples (0.3% of evaluable samples; 4.4% of seropositive samples) had a high positive result (OD ≥1.0). The highest OD was 1.99.

Repeated confirmatory testing using a different lot of the ELISA was conducted on 243 of 249 initially positive samples; insufficient volume precluded the repeated testing of 6 initially low-positive samples. All results met quality acceptance criteria. Of 243 initially positive samples, 80 (32.9%) were not confirmed (including 74 initially low-positive samples and 6 initially intermediate-positive samples), and 163 (67.1%) were repeatedly positive.

Excluding 6 initially positive samples that could not be retested, the repeatedly positive seroprevalence rate in blood bank donors was 4.3% (163/3,789, 95% CI, 3.7%-5.0%). Of 163 repeatedly seropositive samples, 116 (71.2%) samples (3.1% of 3,789 evaluable samples) had a low-positive result, 34 (20.9%) samples (0.9% of evaluable samples) had an intermediate-positive result, and 13 (8.0%) samples (0.3% of evaluable samples) had a high-positive result

Characterization of PF4/Heparin Antibodies

Heparin Dependence

HIT antibodies display heparin-dependent binding to the PF4/heparin complex, with increased binding at low heparin concentrations and reduced binding at higher heparin concentrations. On the other hand, antibodies with nonspecific
binding to PF4/heparin complexes, such as autoantibodies from patients with APLA or systemic lupus erythematosus, lack heparin-dependent binding. To determine if PF4/heparin antibodies from blood bank donors display heparin-dependent binding characteristic of HIT antibodies, we tested the repeatedly positive samples for reduction of binding in the presence of 100 U/mL of heparin. Heparin dependence of antibody binding occurred in 124 (76.1%) of 163 repeatedly positive samples, and no difference was detected in the percentage of samples with heparin-dependent antibody among the high-, intermediate-, or low-positive groups (84.6%, 85.7%, and 72.1%, respectively; \( P = .20 \)).

**Isotype**

Naturally occurring antibodies are generally of the IgM isotype, isotype class switching (IgG or IgA) results from affinity maturation with further antigen exposure. To determine if PF4/heparin antibodies in blood bank donors are “naturally occurring” or show evidence of isotype switching, we tested the repeatedly positive samples for the presence of IgG, IgM, and IgA using an in-house isotype assay. Owing to the reduced sensitivity of the isotype-specific assays, only samples with high or intermediate seropositivity (and sufficient volume) were evaluated. Overall, of 39 repeatedly positive samples tested, IgG predominated over IgM in 20 (51%), IgM predominated over IgG in 9 (23%), and predominance was indeterminate in 10 (26%), with comparable results obtained for the high- (\( n = 12 \)) and intermediate- (\( n = 27 \)) positive samples [Figure 2].

**Analytic Considerations**

**ELISA Reproducibility**

Assay imprecision (CV) during initial testing (single kit lot) of all samples was 35.0% for the manufacturer’s negative control sample (mean OD = 0.188; \( n = 106 \) runs), 25.1% for the manufacturer’s positive control sample (mean OD = 2.90; \( n = 106 \) runs), 32.8% for in-house positive control sample A (mean OD = 1.51; \( n = 74 \) runs), and 23.9% for in-house positive control sample B (mean OD = 2.90; \( n = 32 \) runs).

[Figure 3] shows the comparison of the OD results from initial vs repeated testing (different kit lots) of 243 initially positive samples. By linear regression, results were strongly correlated \( (r = 0.80) \), without a proportional bias (slope = 1.00; SE = 0.048) or constant bias (intercept = –0.037; SE = 0.028). However, some differences in individual results were apparent \( (r^2 = 0.65) \), including near the OD positivity cutoff of 0.40, and this translated to a 67% repeated positivity rate (as described in the “PF4/Heparin Antibody in Blood Bank Donors” section).

**Sample Age as Preanalytic Variable**

We investigated the effect of sample age on seropositivity for 2,562 samples in which age could be determined. Sample age did not differ \( (P = .14) \) between repeatedly seropositive samples (median, 11 days; range, 2-52 days; \( n = 101 \)) and the other samples (median, 9 days; range, 0-57 days; \( n = 2,461 \)). Among 101 repeatedly seropositive samples with available data, the median (range) age was 14.5 (4-34) days for 8 high
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positives, 12 (5-48) days for 22 intermediate positives, and 11 (2-52) days for 71 low positives (P = .64).

Cutoff Value

We calculated a new cutoff value as an upper limit of a nonparametric 95% reference interval based on the distribution of OD in our sample of 3,792 evaluable donor units. This cutoff value was determined to be 0.51 (bootstrap 90% CI, 0.49-0.53). Notably, the 90% CI does not include the cutoff value of 0.4 recommended by the manufacturer.

Discussion

PF4 is an endogenous platelet protein that becomes immunogenic on binding to heparin or other glycosaminoglycans. Recent studies indicate that some people develop PF4/heparin autoantibodies in the absence of heparin exposure. We undertook this prospective study to determine the prevalence of PF4/heparin antibodies in the general population using blood bank donors as a surrogate pool for healthy people. By surveying this group of donors using a commercial immunoassay for screening (initial) and confirmatory (repeated) testing, we found a 4.3% (95% CI, 3.7%-5.0%) seroprevalence of PF4/heparin antibodies in the general population, with most (71%) repeatedly seropositive donors expressing antibody associated with a low-positive OD result (0.40-0.59) and most (76%) exhibiting heparin-dependent antibody binding. Although not required by the assay manufacturer, we chose to retest initially positive donors to obtain a more conservative seroprevalence estimate. In the absence of this repeated testing, seroprevalence was initially greater (6.6%, 95% CI, 5.8%-7.4%), and the vast majority (93%) of “unconfirmed” positive samples were initially of low positivity.

Although the high seroprevalence of PF4/heparin antibodies in this study is comparable to the frequency of autoantibodies such as APLA (5%-6.5%) in the general population, it exceeds previous estimates of PF4/heparin antibody seropositivity in healthy subjects and also seroconversion rates seen in certain clinical settings of LMWH exposure. Of note, 80% of PF4/heparin antibody seropositivity in donors during initial testing could be attributed to results in the low-positive range (OD, 0.40-0.59), and among the low-positive samples, more than one third lacked repeated seropositivity. Fluctuations around the cutoff value (as shown in Figure 3), owing in part to inherent assay variability (eg, interassay CVs, 24%-35%), could significantly impact the outcome of the assay. These findings suggest that some low-positive results may merely reflect nonspecific binding or background “noise” of the assay rather than true seropositivity. While it is possible that the anticoagulant used for blood collection or prolonged storage could have increased the assay’s background noise by promoting nonspecific antibody binding, we do not suspect that these preanalytic variables contributed significantly to our findings. We have previously shown that the GTI ELISA is generally insensitive to sample conditions, including type of anticoagulant and storage conditions. In the present study, we found that antibody signals do not vary significantly with sample age. In addition, a putative effect of anticoagulant or sample condition would likely be attenuated through dilutional effects because the sample is diluted 50-fold before assay.

The cutoff value of 0.40 or more in the GTI ELISA was calculated based on the distribution of OD values obtained for 120 healthy people and calculating a nonparametric 95% reference interval with a 90% confidence to determine the upper limits of normal. Based on these calculations, one would predict a 2.5% to 5% seroprevalence among healthy donors depending on how the 95% reference interval was calculated (1-sided vs 2-sided reference range). While a 4.3% seropositivity rate, as seen in our study, may be within an acceptable abnormal range for a laboratory assay, the difficulty with this particular assay is the implication of a positive PF4/heparin antibody. Positive values, even low-positive values, are construed by most clinicians as evidence of heparin sensitization. Evidence of heparin sensitization could preclude some patients from vital interventions or procedures, such as cardiac bypass surgery, or could lead to use of an alternative (and more costly) anticoagulant. In other cases in which laboratories do not quantify the results of the ELISA OD but merely report qualitative findings (ie, as a positive or negative result), a seropositive value could contribute to a false diagnosis of HIT. Finally, the high seroprevalence in the general population could affect the evaluation of the immunogenicity of new heparin-like drugs, particularly if baseline values are not taken into consideration at the time of drug exposure.

Our finding of high background seroprevalence in the general population compounds the problem of decreased specificity associated with PF4/heparin immunoassays. Recent studies suggest that the specificity of PF4/heparin immunoassays can be considerably improved through refinement of the cutoff value for positivity. Indeed, several recent studies indicate that antibodies with high ODs (≥1.0-1.4) are more likely to cause platelet activation and have a higher likelihood of causing disease. These observations, taken together with our findings that 5.2% of donors in our study were seropositive by low OD (0.40-0.59) on initial testing and that many of these same donors were seronegative on repeated testing, suggest that a higher OD cutoff (0.51) could perhaps improve the specificity of the immunoassay. Additional prospective studies are clearly needed to validate these recommendations. At a minimum, future serologic studies reporting on the laboratory
diagnosis of HIT or on heparin sensitization should provide baseline testing of subjects and quantitative information on the OD values associated with seroconversion.

Approximately 30% of repeatedly seropositive donors in this study had antibodies of intermediate or high positivity (OD >0.60). To what extent the seropositivity in these donors is due to prior heparin exposure or the presence of naturally occurring PF4/heparin autoantibodies is not known. Owing to sample anonymity, we were unable to ascertain heparin exposure in the donor population. Although the ARC does not have explicit criteria to exclude donors who have been recently hospitalized, donors are excluded if they have had angina, myocardial infarction, angioplasty, or bypass surgery within the previous 6 months or organ transplantation within the previous 12 months. People who have had recent surgery are reviewed on a case-by-case basis to determine eligibility. These restrictive donor criteria, as well as our observation that 23% of donors expressed a predominant IgM isotype, suggest the presence of naturally occurring PF4/heparin autoantibodies in a subset of donors. These findings are in keeping with the findings by Jaax and colleagues30 and Krauel et al,31 whose studies of nonspecific binding of PF4 to bacteria or RNA/DNA complexes suggest that the immune system may be “primed” to PF4/heparin antibody formation even in the absence of heparin exposure. Finally, additional studies need to be performed on the transfusion safety of seropositive units because recipients receiving plasma or products containing high levels of PF4/heparin antibodies could be theoretically at risk for developing HIT with subsequent heparin exposure.

Limitations

The limited sample volume of donor segments and lack of donor access precluded characterization of several relevant biologic properties of PF4/heparin antibodies. Specifically, we were unable to document the platelet activating effects of PF4/heparin antibodies or ascertain the antigen specificity (binding to antigens in addition to PF4/heparin) owing to limited sample volume. Donor anonymity precluded studies of the temporal aspects of the PF4/heparin immune response in seropositive donors. Because information was not available on the blood donors, the possibility exists that a person may have donated on multiple, separate occasions during the study period. Future prospective studies should circumvent these study limitations and provide further insights into mechanisms predisposing to HIT antibody development.

Conclusions

Among blood bank donors, the seroprevalence of PF4/heparin antibody (by repeated positivity in the GTI ELISA) is 4.3% (95% CI, 3.7%-5.0%), with most (71%) antibodies being of low positivity (OD, 0.40-0.59) and with most (76%) expressing heparin-dependent binding. Antibodies of intermediate to high positivity (OD >0.60) typically were of the IgG (51%) or IgM (23%) isotype. The occurrence of high-positive PF4/heparin antibodies in blood bank donors, albeit an overall small percentage (0.3%), raises theoretical concerns regarding the safety of transfusing plasma from these seropositive donors. Prospective studies, perhaps using healthy donors, are warranted for future extensive characterization of the risk factors, incidence, and clinical significance of PF4/heparin antibodies occurring in the absence of heparin exposure.

References