ORIGINAL ARTICLE

Protamine-induced immune thrombocytopenia

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BACKGROUND: Protamine is widely used to reverse the anticoagulant effects of heparin. Although mild thrombocytopenia is common in patients given protamine after cardiac procedures, acute severe thrombocytopenia has not been described. We encountered a patient who experienced profound thrombocytopenia and bleeding shortly after administration of protamine and performed studies to characterize the responsible mechanism.

STUDY DESIGN AND METHODS: Patient serum was studied for antibodies that recognize protamine, heparin-protamine complexes, and platelets (PLTs) treated with protamine using flow cytometry, enzyme-linked immunosorbent assay, and serotonin release from labeled PLTs.

RESULTS: A high-titer immunoglobulin G antibody was detected in patient serum that recognizes protamine in a complex with heparin or PLT surface glycosaminoglycans (GAGs) and activates PLTs treated with protamine at concentrations achieved in vivo after protamine infusion. The antibody is distinctly different from those found in patients with heparin-induced thrombocytopenia on the basis of its failure to recognize heparin in a complex with PLT factor 4 (PF4) and to release serotonin from labeled PLTs in the absence of protamine.

CONCLUSIONS: Findings made suggest that the patient's antibody is specific for conformational changes induced in protamine when it reacts with heparin or a PLT surface GAG. Development of severe thrombocytopenia after treatment of this patient with protamine defines a previously undescribed mechanism of drug-induced immune thrombocytopenia. Patients given protamine who produce this type of antibody may be at risk of experiencing thrombocytopenia if given the drug a second time while antibody is still present.

Protamine sulfate, a mixture of 5- to 10-kDa cationic DNA-binding proteins derived from salmon sperm[1,2] is commonly used to reverse the effects of heparin after cardiac surgery involving cardiopulmonary bypass. A modest decrease in platelet (PLT) levels almost invariably follows cardiopulmonary bypass.[3,4] Various studies have suggested that PLT levels sometimes decrease further when protamine is given,[3] possibly because protamine-heparin complexes bind to PLTs and cause them to be sequestered transiently in the lungs.[3,5] Infusion of protamine alone to normal subjects caused a 50% decrease in PLT levels lasting about 30 minutes in one study.[5] However, severe, sustained thrombocytopenia after protamine infusion has not been reported. Here, we describe a patient who experienced profound thrombocytopenia and bleeding symptoms shortly after protamine was given to counteract heparin. Laboratory studies revealed a high-titer antibody that reacted with protamine-coated PLTs and with heparin-protamine complexes. Thrombocytopenia in this case appears to be mediated by a previously undescribed mechanism involving antibody recognition of

ABBREVIATIONS: CS = chondroitin sulfate; GAG(s) = glycosaminoaminoglycan(s); HIT = heparin-induced thrombocytopenia; PF4 = platelet factor 4.

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neoepitopes induced in the positively charged protamine molecule when it binds to negatively charged glycosaminoaminoglycans (GAGs) expressed on the PLT surface.

MATERIALS AND METHODS

Flow cytometry

The method has been described in detail previously.[6] In brief, 1×10^{-7} washed group O PLTs were incubated with 40 µL of test serum and protamine sulfate (Sigma-Aldrich, St Louis, MO) at various concentrations in a total volume of 75 µL. After washing in buffer containing protamine at the same concentration as in the primary mixture, PLT-associated immunoglobulins were detected by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA) using fluorescein isothiocyanate (FITC)-tagged anti-human immunoglobulin (Ig)G (Fc-specific; Jackson ImmunoResearch, West Grove PA). A positive reaction was defined as one in which median PLT fluorescence intensity was at least twice that obtained with the same serum sample in the absence of drug. Reactions of this strength always exceeded control values by at least three standard deviations.

^{14}C-serotonin release assay

The ^{14}C-serotonin release assay was performed with slight modifications according to the procedure described by Sheridan and colleagues.[7]

Detection of antibodies recognizing heparin-protamine complexes

Heparin was incubated with protamine at various ratios of the two substances for 1 hour and aliquots of the resulting complexes were plated in the wells of a microtiter plate as described previously for complexes of heparin and PLT factor 4 (PF4) used to detect antibodies found in patients with heparin-induced thrombocytopenia (HIT).[8,9] Patient or normal control serum (50 µL) diluted 1:50 in phosphate-buffered saline was incubated in the wells for 1 hour at room temperature followed by washing. Bound antibodies were detected by adding 100 µL of a 1:8000 dilution of horseradish peroxidase–labeled goat anti-human IgG Fc (Jackson ImmunoResearch) and incubating for 1 hour at room temperature, followed by washing and addition of substrate. Optical density (490 nm) was measured in each well using an enzyme-linked immunosorbent assay (ELISA) plate reader.

Case report

A 75-year-old woman was admitted to the Cleveland Clinic in 2011 because of shortness of breath and chest discomfort. She had been taking aspirin and clopidogrel, but no other antithrombotic agents. Past medical history included a diagnosis of scleroderma with pulmonary fibrosis. She had undergone coronary artery bypass grafting in 1994, stent grafting and angioplasty of the thoracic aorta in 2005, and coronary artery bypass grafting and homograft replacement of the aortic valve in 2006. Complete blood count performed at the time of this hospital admission (2011) showed a hemoglobin (Hb) level of 10.7 g/dL, white blood cell count of 7.8×10^9/L, and PLT count of 201×10^9/L. Cardiac catheterization revealed severe stenosis of the subclavian artery and the origin of the left internal mammary artery at its origin. Stenting of the subclavian and the origin of left internal mammary artery was successfully performed. During the procedure, a bolus of 5000 IU unfractionated heparin was administered. After the procedure, she was given 20 mg of protamine (APP Pharmaceuticals, Schaumburg, IL) intravenously (IV). A blood count done 2 hours later showed that the Hb level was 10.6 g/dL and PLT count had decreased to 23×10^9/L. She was transferred to the intensive care unit. The next morning the PLT count was 3.0×10^9/L and petechial hemorrhages were noted in the skin and buccal mucosa. A fresh hematoma was noted in the left shoulder area. A solid-phase assay for heparin-dependent antibodies (PF4 ELISA) was negative. Repeat blood count in citrate again revealed a PLT level of 3.0×10^9/L and no PLT clumps were observed on the blood film, ruling out pseudo-thrombocytopenia. Transfusion of 5 units of pooled PLTs increased the PLT count to 50×10^9/L. She was discharged 2 days later. The PLT count had stabilized in the normal range and the shoulder hematoma had resolved 6 days later when she was seen as an outpatient.

RESULTS

A protamine-dependent, PLT-binding antibody was identified in the patient's serum

When normal PLTs were incubated with patient serum and protamine (12.5 µg/mL) and then washed in buffer containing protamine at the same concentration, a strong IgG, PLT-reactive antibody was demonstrated using flow cytometry (Fig. 1). No reaction was obtained with patient serum alone or with normal serum plus protamine. Weaker reactions were obtained when protamine was used at concentrations higher or lower than 12.5 µg/mL (Fig. 2). When protamine was omitted from the buffer in which sensitized PLTs were washed, positive IgG reactivity was no longer detected (data not shown). Testing of patient serum at serial dilutions showed that protamine-dependent reactions were obtained with dilutions as high as 1:800 (Fig. 3). No protamine-dependent, PLT-reactive antibodies were identified in serum from any of 50 unselected normal subjects (data not shown).

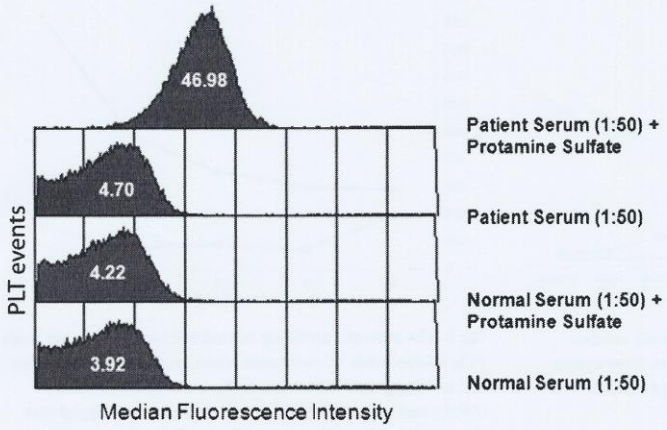

Fig. 1. An IgG antibody present in the patient's serum reacted with normal group O PLTs when protamine is present. Results shown (flow cytometry) are typical of four independent studies that gave comparable results. Protamine sulfate was used at 12.5 µg/mL in both the primary reaction and the buffer used for washing before adding FITC-labeled secondary antibody.

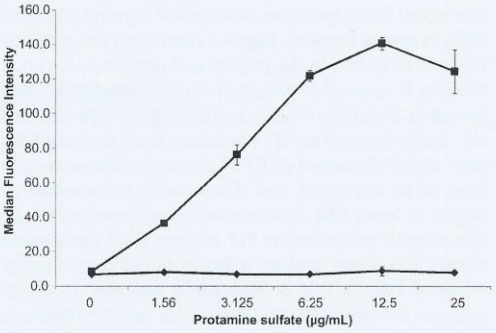

Fig. 2. A protamine concentration of about 12.5 µg/mL was optimal for protamine-dependent binding of patient's antibody to PLTs (■). Results with normal serum and protamine were negative (◆). Values shown (flow cytometry) are averages of triplicate determinations. Brackets denote 1.0 SD.

The patient's antibody reacted with protamine-heparin complexes in a solid-phase assay and induced PLT activation via FcγRIIa when incubated with PLTs in the presence of protamine

Patients with HIT have antibodies that recognize the basic PLT alpha granule protein PF4 in a complex with heparin.[10,11] In preliminary studies using an in-house assay, we confirmed that this type of antibody was not present in patient serum. However, findings shown in Figs. 1-3 suggested that the patient might have an antibody analogous to those found in HIT that was specific not for PF4, but for the highly basic protamine molecule in a complex with heparin or another GAG. To test this possibility, we prepared complexes of heparin and protamine sulfate at different ratios, fixed them by passive absorption to wells of a microtiter plate,[8] and studied their reactions with the patient's antibody. As shown in Fig. 4, strong reactions were obtained with complexes produced in a mixture containing 31 µg/ml protamine and 9 units/mL heparin and weaker binding was seen at other ratios. Assuming a mean molecular weight for protamine of 5000 Da (manufacturer specifications), heparin specific activity of 175 units/mg and a mean molecular weight of 12,000 Da for unfractionated heparin,[8] the molar ratio of protamine to heparin in complexes that were optimal for antibody detection was about 1:1.

A second property of HIT antibodies is that they activate washed PLTs in the presence of low concentrations of heparin (0.1-0.3 units/mL), leading to release of serotonin from PLTs labeled with ^{14}C-serotonin.[7,11] The patient's serum tested negative in the standard[7] serotonin release assay using heparin at a 0.1 and 100 units/mL. However, when labeled PLTs were incubated with patient serum and protamine at various concentrations, dose-dependent release of serotonin occurred (Fig. 5). No release was obtained with normal serum tested similarly. Protamine-dependent release of serotonin by patient antibody was completely inhibited when PLTs were treated with 1.0 µg of monoclonal IV.3 specific for the FcγRIIa receptor (data not shown), indicating that the patient's antibody, like those from patients with HIT,[7] induces release by activating PLTs via FcγRIIa.

DISCUSSION

Our findings show that the patient studied had a high-titer IgG antibody that reacted with normal PLTs only when protamine was present. Acute, severe thrombocytopenia occurred shortly after an IV infusion of 20 mg protamine. Assuming uniform distribution of the drug throughout a plasma volume of approximately 3000 mL, the peak concentration of protamine after injection could have been as high as 7 µg/mL. As shown in Fig. 3, binding of antibody to PLTs was detectable at protamine concentrations as low as 1.5 µg/mL even when patient serum was used at a dilution of 1:50. Assuming that the antibody level in the test sample obtained several days after the thrombocytopenic episode is representative of the level when protamine was administered, it is clear that the amount of protamine infused

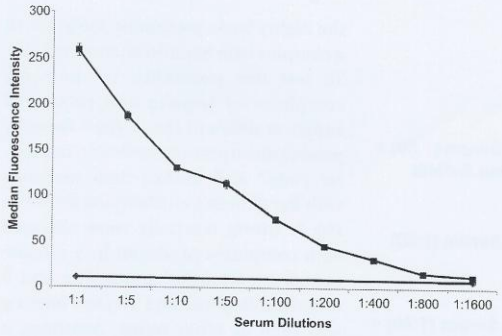

Fig. 3. The protamine-dependent patient antibody reacted with PLTs at dilutions up to 1:800. (■) Reactions (flow cytometry) obtained with protamine at 12.5 µg/mL; (◆) reaction in the absence of protamine.

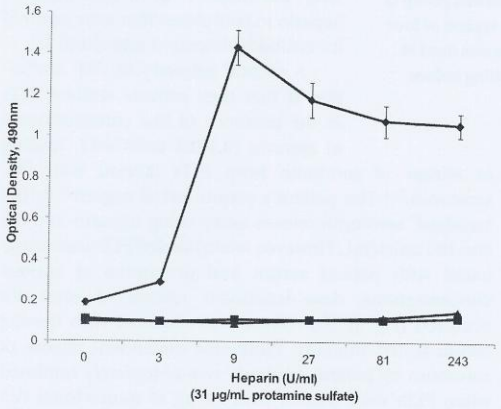

Fig. 4. Reactions of patient's antibody (diluted 1:50) with complexes of heparin:protamine prepared at various ratios. The strongest reactions were obtained with complexes prepared using 31 µg/mL protamine sulfate and 9 IU/mL heparin (◆). No reactions were obtained with either of two normal sera (■ and ▲). Values shown are means of triplicate determinations. Brackets denote 1.0 SD.

would have been sufficient to promote antibody binding to the patient's PLTs in vivo. Together, these observations strongly suggest that acute thrombocytopenia was caused by a protamine-dependent, PLT-reactive antibody. Our patient had a likely exposure to protamine when she underwent cardiac surgery several years before her episode of thrombocytopenia and it seems possible that her antibody persisted from that time, explaining why thrombocytopenia developed acutely after protamine administration.

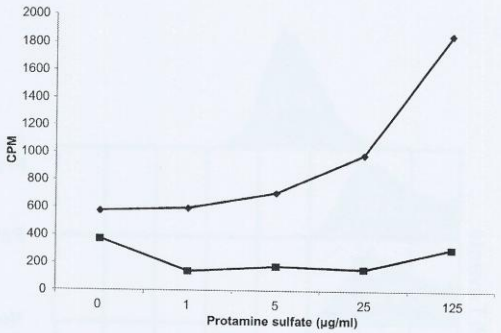

Fig. 5. The patient's antibody caused release of serotonin from PLTs labeled with ^{14}C-serotonin when protamine was present at 5 to 125 µg/mL. ^{14}C-release is shown as counts/minute (CPM), and the equivalent percentage release obtained with the normal serum (■) was 0%. The equivalent percentage release obtained with the patient's serum (◆) ranged from 6% (0 µg/mL protamine) to 43% (125 µg/mL protamine). Results shown are typical of two independent studies.

Findings shown in Figs. 2, 4, and 5 show that protamine-dependent reactions of the patient's antibody resembled PF4-dependent reactions of a typical HIT antibody in several respects. Figure 2 shows that the antibody binds to PLTs only in the presence of protamine and that binding is optimal at protamine concentrations ranging from 6 to 25 µg/mL. Rauova and colleagues[12] showed that HIT antibodies and an HIT-like monoclonal antibody KKO react with PLTs treated with PF4 at concentrations ranging from 50 to 100 µg/mL and that binding is decreased at higher or lower PF4 concentrations and after enzymatic degradation of the major PLT surface GAG chondroitin sulfate (CS). Their findings indicate that at certain molar ratios of PF4:CS, PF4 is structurally modified to create epitopes for which HIT antibodies are specific. Findings shown in Fig. 2 suggest that when protamine binds to CS, it is similarly modified to create an epitope or epitopes recognized by the patient's antibody. A second characteristic of HIT antibodies is that they recognize complexes of heparin and PF4 produced at PF4-heparin molar ratios about 2:1 but react less well with complexes formed at higher or lower ratios.[8] Figure 4 illustrates that protamine-heparin complexes formed at a molar ratio about 1:1 were optimal for detection of the patient's antibody. Finally, HIT antibodies release serotonin from PLTs by a mechanism dependent on the FcγRIIa receptor.[7] Although the serotonin release assay is usually performed by adding low-dose heparin (0.1 U/ml), strong HIT antibodies often cause release without added heparin, apparently because they recognize small quantities of PF4 in a complex with CS on the PLT surface.[13-15] Protamine-dependent release of serotonin by the patient's antibody (Fig. 5) that was

blocked by the FcRγIIa-specific monoclonal IV.3 closely resembles the behavior of this type of HIT antibody, the difference being recognition of protamine on the PLT surface rather than PF4. Together, these considerations make it likely that the antibody identified in this patient is specific for structural changes induced in protamine when it binds to CS or to another GAG. One major difference is that after PLTs are washed in buffer, the patient's antibody remains bound to protamine-treated PLTs in quantities readily detected by flow cytometry, provided that protamine is kept present in the wash solutions (Figs. 1 and 2).

To the best of our knowledge, thrombocytopenia caused by a protamine-dependent, PLT-reactive antibody of the type described here has not been described previously. However, in a recent report, Chudasama and colleagues[16] showed that mice immunized with protamine-heparin complexes produce antibodies that recognize complexes formed in a mixture of 31 μg/mL protamine and 4 units/mL heparin. This ratio is similar to the one we found to be optimal for detecting our patient's antibody in a solid-phase assay (31 μg protamine, 9 units heparin/mL; Fig. 4). Moreover, Lee and coworkers[17] recently studied a large series of patients given protamine after cardiac surgery and found that, at 30 days, 29% have IgG antibodies specific for protamine-heparin complexes. Studies performed with a subset of these antibodies showed that they induce protamine-dependent release of serotonin from PLTs. The findings of Chudusama and coworkers[16] and Lee and coworkers[17] show that heparin-protamine complexes are immunogenic in mice and humans and that the resulting antibodies are capable of activating PLTs in the presence of protamine. Findings made in our patient suggest that reexposure of a patient who has this type of antibody to protamine can cause thrombocytopenia. Studies are therefore indicated to determine how long protamine-dependent, PLT-reactive antibodies persist in patients given protamine to define the window period during which they may be at risk for thrombocytopenia if the drug is administered a second time.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in connection with this publication.

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