Heparin-Induced Thrombocytopenia: Use of Indirect Immunofluorescence

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ABSTRACT

Sera from 14 patients who developed heparin-induced thrombocytopenia were tested by an indirect platelet immunofluorescent test in an attempt to characterize the serologic reactions between platelets, heparin, and the antibody. Positive results were observed in seven cases with variable patterns of reactions in the tests when performed in the presence or the absence of the offending drug. It was possible to absorb out the antibody in the presence of heparin and recover the antibody in the eluate. Neither complement nor the presence of immune complexes were found to play a role in the thrombocytopenia induced by heparin.

Introduction

Thrombocytopenia has been well recognized as a complication of heparin administration. The frequency of this adverse effect has been variably reported as occurring in one to thirty percent of patients receiving heparin. The importance of confirming the diagnosis of heparin induced thrombocytopenia (HIT) is associated with the fact that a small number of these thrombocytopenic patients may develop spontaneous thrombosis. In this report, the indirect platelet immunofluorescent test (IFT) has been utilized in the study of HIT ranging from tests that demonstrate changes in platelet functions to those that recognize the presence of immunoglobulins on the platelet surface. In this report, the indirect platelet immunofluorescent test (IFT) has been utilized in the study of HIT ranging from tests that demonstrate changes in platelet functions to those that recognize the presence of immunoglobulins on the platelet surface. In this report, the indirect platelet immunofluorescent test (IFT) has been utilized in the study of HIT ranging from tests that demonstrate changes in platelet functions to those that recognize the presence of immunoglobulins on the platelet surface.

The mechanism underlying HIT is not well understood. Several investigations have provided evidence that in most cases HIT is immunologically mediated, and a heparin-dependent platelet antibody has been demonstrated in those cases. A variety of laboratory techniques has been utilized in the study of HIT ranging from tests that demonstrate changes in platelet functions to those that recognize the presence of immunoglobulins on the platelet surface. In this report, the indirect platelet immunofluorescent test (IFT) has been utilized in the study of HIT ranging from tests that demonstrate changes in platelet functions to those that recognize the presence of immunoglobulins on the platelet surface. In this report, the indirect platelet immunofluorescent test (IFT) has been utilized in the study of HIT ranging from tests that demonstrate changes in platelet functions to those that recognize the presence of immunoglobulins on the platelet surface.

Procedure and Methods

Sera from 14 patients who developed an episode of thrombocytopenia while on
heparin were collected over a one-year period. Immune complexes in the sera were precipitated out by an equal volume of four percent polyethanol glycol (PEG) in phosphate buffered saline (PBS) by the method of Chia and the sera were extensively dialyzed in tyrode buffer. Two sera were also dialyzed and tested in identical manner, without PEG treatment. Platelet aggregation tests were carried out in an aggregometer* using 0.140 ml of normal platelet rich plasma (PRP), 0.220 ml of heat-inactivated patient's sera, and bovine lung heparin (BLH), 0.04 ml heparin sodium,† diluted to yield a final concentration of one μ per ml in the reaction mixture. Aggregation was monitored for 15 minutes. Normal sera was substituted for patient sera in control studies.

For various testing procedures, normal platelets from group “O” platelet concentrates were separated by albumin density gradient as described by Walsh and resuspended in tyrode solution. The platelets were washed three times in a PBS-EDTA buffer containing 0.1 percent bovine serum albumin (BSA) and adjusted to a final concentration of 1–5 × 10⁸ per ml.

The indirect IFT was performed according to Borne et al.² Equal volumes of the normal platelet suspension as described in the previous paragraph and patient or control sera were incubated for 30 minutes at room temperature. The mixture included either BLH in a final concentration of 10 μ per ml or buffer in equal volume. After three washes with PBS-EDTA-BSA buffer, the platelets were incubated with FITC labeled goat-antihuman IgG‡ diluted 1:50 (as determined to be optimum by checkerboard titration) for 30 minutes. After three more washes, the platelets were suspended in 0.1 ml of a one percent solution of paraformaldehyde in PBS and examined with a fluorescent microscope. A positive control consisting of polyspecific rabbit-antihuman platelet antibody prepared in our laboratory, and normal human serum were tested at the same time. The positive control was stained with goat antiserum to rabbit IgG.§ The results of the indirect IFT were scored from 0 to 4⁺.

Absorbntion and elution experiments were performed with the sera from two patients. Equal volumes of serum and normal platelet suspension (1–5 × 10⁸) were incubated in the presence of buffer or heparin, one μ per ml, for one hr at 37°, then overnight at 4°C. The mixture was centrifuged and the supernatant removed. Eluates were prepared from sensitized platelets, the eluting buffer consisting of a citric acid solution, pH 2.8 (0.1M trisodium citrate in 1.0M NaCl added to a solution of 1.0 M citric acid and 1.0 M NaCl) according to Brown et al.³ The pH of the supernatant was raised to 7.2 by the drop-wise addition of a neutralizing solution (0.04M NaOH in 0.14 M NaCl).

The absorbed sera as well as the resulting eluates were tested by the indirect IFT simultaneously with the patients’ sera. The eluted platelets were also tested by immunofluorescence to determine whether immunoglobulin or complement remained on the platelet surface.

Complement binding was determined in the 14 patients by the use of an FITC IgG fraction of goat antihuman C3.|| The patients’ heat inactivated sera were mixed with normal fresh sera as a source of complement in a 1:1 ratio prior to testing. The fluorescent tagged anti-sera was diluted 1:10, as determined to be

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‡ Southern Biotechnology Associates, Birmingham, AL.
§ Hyland Laboratory, Costa Mesa, CA.
|| Cooper Bio-Medical, Malvern, PA.
optimum by titration studies. Positive and negative controls were tested simultaneously.

Results

The clinical data indicated that all but one of the patients tested were admitted to the coronary care unit for accelerated angina. The mean patient age was 67 years. All the patients had platelet counts above \(150 \times 10^3/\text{mm}^3\) on admission. One patient manifested a drop in platelets following heparin therapy to \(136 \times 10^3/\text{mm}^3\); eight patients dropped their platelet counts below \(100 \times 10^3/\text{mm}^3\) and five patients developed severe thrombocytopenia below \(20 \times 10^3/\text{mm}^3\). The interval between the initiation of heparin therapy and the development of thrombocytopenia varied from six to 20 days. The heparin administered consisted of beef lung heparin (four patients) and intestinal mucosal heparin (10 patients). The heparin was administered either as a bolus or by continuous infusion. None of the patients demonstrated evidence of disseminated intravascular coagulation or bleeding; one patient developed a pulmonary embolus and two other patients had deep venous thrombosis associated with the HIT.

Sera from all 14 patients were able to aggregate normal platelets in the presence of heparin. The reaction, when tested in three of these sera, could be abolished by pre-incubation of the serum with goat anti-human IgG* for 10 minutes at 37°C, the optimum concentration of sera and IgG being predetermined by micro titration experiments.

In six patients, the indirect anti-IgG IFT was found to be weak to moderately positive as summarized in table I. In all but two of those sera, the reaction was enhanced by the presence of heparin. None of the sera was able to bind complement. In one serum, the reaction remained unchanged; in the other serum, the result of the test was positive in the presence of buffer and absent with heparin. The sera treated with PEG did not manifest different results from the untreated sera.

The positive sera of two patients (2 and 5, table I) became negative following absorption with normal platelets when tested by indirect immunofluorescence staining and in aggregation tests. The eluate recovered from these absorbed sera showed 1+ reactions by IFT with normal donor platelets (table II). The eluates were found to be negative when tested in aggregation studies.

Comment

Laboratory confirmation of HIT is important in patients receiving heparin.
because, in these patients, thrombocytopenia may be associated with arterial or venous thrombosis. In such cases, heparin therapy should be discontinued. Two clinical forms of HIT have now been recognized. One form manifests itself as a mild, asymptomatic thrombocytopenia early in the course of heparin therapy. It is usually transient with a mild to moderate decrease in the platelet count. Some patients may, in addition, demonstrate platelet dysfunctions that might predispose to bleeding. A second type, occurring late in the course of heparin therapy, appears to have an immune etiology. It is usually associated with severe thrombocytopenia, and, in one to two percent of these cases, HIT may be complicated by thrombosis.

The immune mechanism responsible for platelet aggregation in HIT remains unknown. It appears to be a drug-dependent phenomenon,—the responsible factor being found in the IgG fraction of the serum. Utilizing direct immunofluorescence, Stead et al reported the presence of elevated platelet-associated IgG in five patients, two of whom demonstrated, in addition, platelet-associated complement. Kelton has similarly shown elevated levels of platelet-associated IgG in 14 out of 16 patients by means of a direct antiglobulin consumption assay. Direct assays for platelet-associated IgG appear, in general, to be more sensitive than indirect methods. The patients under our care were unable to be tested by a direct assay and it was necessary to rely on frozen stored sera. Using indirect methods, others have shown agglutination of normal platelets in the presence of heparin in nine out of fourteen sera from patients with HIT. The agglutinated platelets, furthermore, showed positive immunofluorescent staining for both IgG and C3. Although heparin antibodies were shown by another study to fix complement, this has not been a general finding and platelet lysis has not been reported as yet. Likewise, it was not possible for us to demonstrate by immunofluorescence the presence of platelet-associated C3 in the sera of the 14 patients tested.

In six out of 14 patients, IgG heparin dependent antibodies were shown by indirect immunofluorescence. Four out of five cases showed enhanced staining following the addition of heparin. This enhanced effect by heparin has been commented on by other investigators, in spite of their finding elevated base line levels of platelet IgG in the absence of the drug. In one case, the reaction was positive in the presence of buffer alone; in another case, both buffer and heparin gave similar reactions. The variable patterns obtained in our patients are of interest, in view of the findings of Pfueiler et al that suggest different platelet antigenic specificities of the heparin antibody. These findings also support that antibody is not clearly a heparin-dependent in vitro induced phenomenon.

The immunofluorescent intensity was generally weak, suggesting that the level of heparin-platelet antibodies may be low and not readily detected by indirect methods. It seems unlikely that the presence of immune complexes were responsible for the positive reactions, as there was not apparent difference in reactivity in immune complex free sera following treatment with PEG as compared to the non-treated sera. This finding tends to suggest that the serologic reaction in HIT is not mediated by the presence of immune complexes, although those may still be present, not having been precipitated out from the serum in spite of addition of PEG. In one experiment, the sera from two patients were absorbed with normal platelets. The absorbed sera manifested a decrease in staining as compared to the unabsorbed sera. The recovered eluates were, in addition, shown to stain positively when reacted with normal platelets in the presence of
heparin. When tested by the usual method, however, neither absorbed sera nor eluates caused platelets to aggregate in the presence of heparin. Of interest is the fact that one serum sample belonged to a patient who developed a pulmonary embolus as an additional complication of heparin administration.

These findings confirm that HIT is mediated via an immune mechanism. Sera from patients with HIT may show variability of reactions. The antibody can be absorbed out of the serum, usually in the presence of the offending drug and can be subsequently recovered in the eluate. Although immune complex formation as a mechanism for the action of heparin has not been entirely ruled out, it seems unlikely to be the responsible factor. It seems more likely that heparin acts as a hapten that binds to certain platelet membrane glycoproteins or that it induces antigenic alterations in certain glycoproteins which lead to antibody binding by the platelets.

References