Heparin-induced Thrombocytopenia: Confirmation of Diagnosis With In Vitro Methods

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Profound thrombocytopenia developed in a patient during treatment with heparin for venous thrombosis. The platelet count increased toward normal when heparin administration was stopped, but fell abruptly when the drug was again given. Platelet aggregation occurred when heparin was added to the patient's platelet-rich plasma, or to normal platelets plus the patient's serum. This serum also effected release of 3H-serotonin from normal platelets. This pattern of aggregation was clearly different from that occasionally caused by heparin in a control population. The data is consistent with an effect of heparin on platelets, possibly mediated by an immune mechanism.

A DECREASE in platelet count may result from administration of heparin. This is a reproducible phenomenon in the dog and the hamster and is associated with platelet aggregation. The thrombocytopenia is usually associated with the administration of large doses of heparin. One suggested mechanism proposes an interaction of heparin with the platelet surface or with some other protein, such as fibrinogen. This effect is not specific for heparin and may be produced by other macromolecules, e.g., dextran. A similar, although more modest effect may be observed in man, though it is less reproducible and the mechanism is uncertain.

Recently, two patients were reported whose heparin-associated thrombocytopenia was considered to be on an immune basis.

We report here a patient who developed profound thrombocytopenia as a result of heparin administered for treatment of venous thrombosis. The patient's platelets aggregated in vitro in response to a very small dose of heparin. Normal platelets underwent aggregation and release in the presence of the patient's serum and heparin, while either heparin or patient's serum alone were unreactive. The patient is presented as a case of drug-induced thrombocytopenia with identification of the mechanism and the responsible agent.

MATERIALS AND METHODS

Blood for coagulation studies was drawn through a 19-gauge needle into a plastic syringe and anticoagulated with 40% sodium citrate (1 part to 99 parts blood). Citrated platelet-rich plasma (PRP) was separated by centrifugation at 1000 g for 3 min at 24°C. Further centrifugation at 3500 g for 10 min yielded platelet-poor plasma (PPP). The platelet count in PRP was adjusted to approximately 300,000/cu mm by dilution with PPP. Serum was obtained from whole blood which had been allowed to clot in glass tubes for 2 hr. The serum was heated for 30 min at 56°C prior to use in aggregation or release studies.

Platelet aggregation was measured using a Chrono-Log Aggregometer with a Linear Instruments Corporation linear chart recorder. PRP (0.4 ml) was placed in a flat-bottomed cuvette,
stirred constantly, and maintained at 37°C. Additional reagents (0.02-0.1 ml) were then added and the change in optical transmission recorded.

Control studies were done on 27 subjects, ages 23-52, 17 women and 10 men. None of these had any prior history of hemostatic abnormality, and none had ingested aspirin within 72 hr.

The ability of platelets to aggregate in response to ADP and epinephrine was ascertained for each batch of PRP at the outset of an experiment. The use of platelet 3H-serotonin release to detect immunologic damage to platelets and the criteria for positive and negative tests have been described by Hirschman et al.5 Coagulation assays were performed using standard procedures and reagents6 (fibrin split products were determined by tanned red cell hemagglutination inhibition).

The following commercial reagents were employed: adenosine diphosphate (Sigma), epinephrine hydrochloride (Parke-Davis), sodium heparin U.S.P. from beef lung (Upjohn), sodium heparin from hog mucosa (Panheparin, Abbott), sodium heparin from hog mucosa, 170 U/mg (Sigma). Protamine sulfate (Upjohn) was obtained as a desiccated preparation and reconstituted with saline immediately before use to give a stock solution of 10 mg/ml.

Platelet counts were performed by an automated technique utilizing a Coulter Model B.7 Very low platelet counts were verified by phase microscopy.

CASE REPORT

A 66-yr-old black male retired engineer developed pain and swelling of the right lower extremity subsequent to mild trauma to this area. He was admitted to the NIH Clinical Center on March 20, 1973 with a diagnosis of deep femoral vein thrombosis.

Except for mild, late onset of diabetes mellitus, he was well until 1972, when diagnosis of a chromophobe adenoma was made and he was treated with 4500 rads of sellar radiation. He then required hormonal supplementation for panhypopituitarism and insulin therapy for diabetes mellitus. Subsequently, he progressively lost the sight of both eyes. The etiology of his visual loss was not determined with certainty, but it was felt to be a postradiation change. In December 1972, a right central retinal artery occlusion developed and was treated with heparin. This had no effect on the progressive visual loss, and platelet counts were not determined. Clinical hemorrhagic problems were not observed. No other symptoms accompanied the onset of blindness, and no new medications were introduced. However, the patient's ability and desire to ambulate decreased sharply, and he spent most of his time in bed.

Physical examination on admission revealed a black man who appeared well and complained of pain in his right leg. Blood pressure was 150/88; pulse, 80 and regular; respirations, 16; and temperature, 36.3°C. Skin and lymph nodes were normal. Bilateral total blindness with only minimal light perception was present. Optic fundi were normal except for some pallor of the right disc. The lungs were clear; there were no cardiac murmurs or gallops. The abdomen was soft; the liver and spleen were not palpable. The right leg was markedly erythematous and swollen with edema extending to the mid-thigh. Calf tenderness, a palpable cord, and a positive Homan's sign were present in the right leg. The circumference of the right thigh was 8 cm greater than the left and that of the right calf 7 cm greater than the left. Arterial pulses were normal.

Initial laboratory data: hemoglobin, 15.2 g/100 ml; hematocrit, 45%; WBC, 15,900/cu mm with 89% neutrophils; platelet count, 138,000/cu mm; reticulocytes, 0.9%; urinalysis, 4+ glucose, otherwise normal. Bilirubin was 0.8 mg/100 ml, alkaline phosphatase, 48 IU; SGOT, 19 U; LDH, 320 U. Total serum protein was 7.0 g/100 ml, with 3.6 g albumin, 3.4 g globulin; protein electrophoresis was normal. The prothrombin time was 13.3 sec (control, 11.9); partial thromboplastin time, 22.8 sec (control, 30.1); fibrinogen, 492 mg/100 ml.

Therapy was begun with intravenous heparin (Upjohn U.S.P. sodium heparin from beef lung) 10,000 U every 6 hr, and this dose adjusted between 5000 and 10,000 U to maintain the partial thromboplastin time at 45-60 sec. The patient improved clinically until 6 days after admission, when the platelet count fell and spontaneous purpura was observed.

Course

The relationship between clinical status, drug therapy, and platelet count is shown in Fig. 1. Fibrinogen was 492 mg/100 ml on day 1, 428 mg/100 ml on day 7, 464 mg/100 ml on day 16, and
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Fig. 1. Clinical severity of thrombosis in relation to platelet count and medication. (++, moderate edema of lower leg; ++++, marked edema of leg; +++++, bilateral edema of legs).

205 mg/100 ml on day 19 (no relationship was noted between fibrinogen level and heparin administration). On the second day, factor VIII was 105%, factor V was 110%, euglobulin clot lysis time greater than 3 hr, fibrin split product titer 1:16 (control, 1:4). Bone marrow aspiration revealed normocellular marrow with adequate megakaryocytes.

Antinuclear antibody titer and LE cell preparations were repeatedly negative, and the serum complement level was normal. Since the thrombocytopenia was thought to be on an immune basis, prednisone was increased and other drugs changed or discontinued. The right leg began to show signs of improvement; however, on day 8, when the platelet count was 4000/cu mm, heparin was discontinued because of its suspected role in the thrombocytopenia. Heparin was reinstituted on day 13 when the patient complained of pain in the left leg and both lower extremities were tender and swollen. The platelet count, which had risen to 38,000/cu mm, fell to 5400/cu mm. Heparin was again discontinued, and anticoagulant therapy was changed to warfarin. The platelet count gradually increased and reached normal on hospital day 24. The role of heparin was confirmed by the in vitro tests described below. The course was further complicated by pulmonary embolization, demonstrated by lung scanning with 131I-macroaggregated albumin. The platelet count remained at normal levels, and the patient was discharged on hospital day 66.

RESULTS

Platelet Aggregation Studies (Patient)

Platelet aggregation was observed when heparin was added to patient's PRP alone or serum plus normal platelets. Representative aggregometer tracings are shown in Fig. 2. Increase in light transmission coincided with formation of gross flocculation. When examined by phase microscopy, the material appeared as large aggregates of platelets.

The heparin used in the experiment illustrated in Fig. 2 was derived from beef lung and was produced by the same manufacturer, in the same lot, as that given to the patient. Aggregation was observed at a final concentration of 0.025 U/ml, but not at 0.012 U/ml. The characteristics of aggregation (lag phase, per cent transmission) were similar at all concentrations with which aggregation occurred under these experimental conditions, i.e., it appears to be an all or none phenomenon. A double wave of aggregation was not observed. Different lots of beef lung heparin yielded similar results. Heparin derived from
Heparin

Platelet aggregation responses to addition of 3.20 U/ml, 20 U/ml lung heparin to:
(1) 0.4 ml patient PRP from 29th day;
(2) 0.2 ml normal PRP plus 0.2 ml patient PPP from 29th day;
(3) 0.3 ml normal PRP plus 0.1 ml patient serum from 9th day;
(4) 0.4 ml normal PRP; (5) 0.3 ml normal PRP + 0.1 ml normal serum.

Platelet Aggregation Studies (Normal)

Two of the normal subjects demonstrated aggregation upon addition of heparin. The form of the aggregation curve was similar to that seen with the patient, i.e., a lag phase of 2-4 min and a single wave. This effect was not constant, and its presence was not correlated with any other parameters (including source of the heparin). Minimum final concentration of heparin required was in the range of 0.5–1.0 U/ml.

Inhibition by Protamine

Protamine sulfate was added to PRP and stirred for 2 min before further additions. Protamine sulfate at a final concentration of 0.2 mg/ml inhibited the effect of heparin (final concentration, 17 U/ml of hog mucosa preparation, 170 U/mg). The same concentration of protamine sulfate caused no aggregation of itself, nor did it block aggregation by ADP. A decreased amount of protamine sulfate (0.005 mg/ml) did not interfere with aggregation by 17 U/ml of heparin. Prior addition of protamine sulfate to heparin blocked the effect on platelet aggregation.

Time Course of Active Factor

Heparin was last administered on hospital day 19. Studies with patient's PRP drawn on hospital days 23, 29, and 30 demonstrated similar response to heparin. PRP from hospital day 38 showed maximum amplitude approximately one-half that obtained previously using the same heparin dose. No later samples were obtained.

Aggregation With Normal Platelets and Patient's Serum

Patient's serum, drawn on hospital days 8, 9, and 19, was available for testing. When 0.1 ml of serum was mixed with 0.3 ml normal PRP, platelet aggre-
**Table 1. Aggregation With Normal PRP Plus Patient Serum.**

<table>
<thead>
<tr>
<th>PRP</th>
<th>Serum</th>
<th>Heparin (final conc.) (U/ml)</th>
<th>Maximum Aggregation (% transmission)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 normal</td>
<td>0.1 ml patient (day 9)</td>
<td>0.5*</td>
<td>70</td>
</tr>
<tr>
<td>0.3 normal</td>
<td>0.1 ml patient (day 10)</td>
<td>0.5*</td>
<td>75</td>
</tr>
<tr>
<td>0.3 normal</td>
<td>0.1 ml patient (day 19)</td>
<td>0.1*</td>
<td>65</td>
</tr>
<tr>
<td>0.3 normal</td>
<td>0.1 ml patient, 1:10 (day 9)</td>
<td>5.0</td>
<td>70</td>
</tr>
<tr>
<td>0.3 normal</td>
<td>0.1 ml patient, 1:20 (day 9)</td>
<td>5.0</td>
<td>45</td>
</tr>
<tr>
<td>0.3 normal</td>
<td>0.1 ml patient, 1:40 (day 9)</td>
<td>5.0</td>
<td>&lt;5</td>
</tr>
<tr>
<td>0.3 normal</td>
<td>0.1 ml normal</td>
<td>10.0</td>
<td>&lt;5</td>
</tr>
<tr>
<td>0.3 normal</td>
<td>0.1 ml patient</td>
<td>0</td>
<td>&lt;5</td>
</tr>
<tr>
<td>0.3 normal</td>
<td>0.1 ml normal</td>
<td>0</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

*Minimum concentration of heparin causing aggregation.

Aggregation studies performed as described in Fig. 2. Where undiluted serum was employed, the lowest active concentration of heparin is shown; at concentrations one-fifth of these, aggregation was less than 25%.

Aggregation could be induced by heparin. Serum from hospital day 9 was used to determine minimum concentrations of heparin or serum required for aggregation (Table 1).

Progressive decrease in amplitude was observed as serum titer decreased, but there was no coincident change in the lag phase. Serum derived from a pool of normal donors did not aggregate platelets when heparin was added.

**Serotonin Release**

Normal platelets were labeled with 3H-serotonin, then incubated with patient or control serum. No heparin was added to these mixtures. Patient's serum from hospital day 8 effected significant release of serotonin, as compared with a pool of control serum, but that from day 9 did not (heparin administration ceased on day 8). Serum from hospital day 19, when heparin was being administered, also caused release.

**DISCUSSION**

The possible causes of thrombocytopenia in this patient were disseminated intravascular coagulation, ITP, and drug-related thrombocytopenia. Some degree of disseminated intravascular coagulation was undoubtedly present. The patient's clinical status was consistent with its occurrence, and the fibrin degradation product titer was elevated. Klein and Bell recently reported two patients who developed signs of disseminated intravascular coagulation during a course of heparin, indicating that the drug is not uniformly protective. In the present case, however, factor V and factor VIII levels were normal, and plasma fibrinogen did not change significantly in response to heparin therapy. On two occasions, the platelet count decreased while on heparin and increased when the drug stopped. When all factors are considered, it seems quite unlikely that disseminated intravascular coagulation played a significant role in the etiology of the thrombocytopenia.

Since the relationship between heparin and thrombocytopenia was clear, ITP and platelet effect by other drugs seemed unlikely. It then remained to explain the mechanism of this heparin-related thrombocytopenia. The in vitro
studies demonstrate platelet aggregation induced by heparin in the presence of patient’s plasma or serum. Thrombocytopenia could result if such aggregation were induced in vivo.

Aggregation of platelets by heparin has been noted in man as well as in other animal species. The constant and reproducible aggregation of human platelets by various commercial heparin preparations, as reported elsewhere, was not observed in our laboratory. Our study of 27 volunteers (10 men, 17 women) revealed two whose platelets demonstrated a collagen-like aggregometer response to various types of heparin. This response was not constant in either subject, and its significance is unclear. The minimum dose of heparin required was greater than that required in the patient (a 6- to 40-fold difference). This mechanism could not explain the aggregation and thrombocytopenia in the patient. There is no evidence for a direct toxic effect of heparin, and mediation of aggregation by the patient’s serum makes such a possibility very unlikely.

The prior exposure to heparin, the time course of the thrombocytopenia, and the return of the platelet count toward normal with discontinuation of drug all suggest an immune mechanism. This is further supported by the presence of a serum factor which mediates the heparin effect with normal platelets. More definitive evidence, such as characterization of the serum factor, could not be obtained due to limited supply of material. Both aggregation and platelet release have been used as indicators of immune damage to platelets. The aggregometer has also been employed to quantitate the lytic response of platelets to quinidine in the presence of serum from patients sensitized to this drug. The anticoagulant properties of heparin prevent use of certain tests. For example, the platelet factor 3-release test, dependent upon a coagulation endpoint, could not be employed with heparin addition.

Heparin is not a potent antigen, and true immunologic reactions to it are very unusual. A number of reactions which seemed to be immunologically mediated have been reported, but it is difficult to determine whether these are due to heparin itself or to a protein contaminant. Thrombocytopenia has not been noted in these cases, but there are descriptions of dermal reactions, anaphylaxis, and precipitating antibody with species specificity (i.e., elicited with heparin of bovine but not porcine origin). This does not help differentiate sensitivity to heparin itself from that to a contaminating protein. The lack of species specificity in the present case favors immunologic recognition of a similar structure in all preparations used. How this is related to the heterogeneous macromolecular entity with the biologic properties of heparin cannot be answered.

The relationship between the antiheparin factor (? antibody) and the extensive venous thrombosis is not clear, but the following speculation is offered. Heparin is a chemically heterogeneous, complex polysaccharide. The material used for clinical purposes is isolated from bovine lung or porcine intestinal mucosa. Although human origin of identical material is uncertain, polysaccharides of similar structure are found in human tissue. Heparin is a potent cofactor for inhibitors of activated coagulation factors. If there is some nonspecificity of the antiheparin antibody, it would tend to interfere with the endogenous polysaccharide and thus with control of activated coagulation fac-
tors. This could result in the loss of normal control of activated coagulation factors and accelerated thrombus formation.

The use of heparin as a prophylactic antithrombosis agent at surgery may result in its increased clinical use. Thrombocytopenia may complicate the course of patients repeatedly treated with heparin. Heparin must be added to the list of drugs capable of causing thrombocytopenia in man. Aggregometry can be used to document its role.

REFERENCES

Heparin-induced thrombocytopenia: confirmation of diagnosis with in vitro methods

JC Fratantoni, R Pollet and HR Gralnick