

A Diagnostic Test for Heparin-Induced Thrombocytopenia

By David Sheridan, Cedric Carter, and John G. Kelton

Heparin-induced thrombocytopenia can be a serious and difficult-to-diagnose complication of heparin therapy. Serum from patients with heparin-induced thrombocytopenia can cause heparin-dependent platelet aggregation, but the low sensitivity and specificity of this test limit its clinical usefulness. In this report we describe an assay for heparin-induced thrombocytopenia that is both sensitive and specific. The improvement in the assay was accomplished by measuring platelet release instead of aggregation and by measuring platelet release at two heparin concentrations. The rationale for the use of two heparin concentrations was that sera from patients with heparin-induced thrombocytopenia caused release at therapeutic but not at high concentrations of heparin. Twenty-eight sera samples from patients suspected of having heparin-induced thrombocytopenia and 573 controls were coded and tested in the assay. The patients with possible hepa-

rin-induced thrombocytopenia were ranked according to the likelihood of having this disorder by using prospectively defined criteria. The test had a high specificity (99%); only one of 573 controls showed a positive result. The test was also very sensitive, and the likelihood of a positive test result was directly correlated with the clinical likelihood of the patient having heparin-induced thrombocytopenia. Six of six patients with definitive heparin-induced thrombocytopenia had positive test results, whereas zero of four patients in whom the diagnosis was unlikely had positive test results. The two-point test for heparin-induced thrombocytopenia represents a sensitive and specific test for this disorder. This test may be useful not only in confirming the diagnosis of this disorder but also may provide information about its pathogenesis.

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HEPARIN IS AN EFFECTIVE anticoagulant for the prevention and the treatment of venous thrombosis. Thrombocytopenia develops in approximately 5% to 10% of patients treated with heparin.¹⁻⁴ Usually this complication has little clinical impact, but thrombocytopenia in association with arterial thromboses develops in a small proportion of patients treated with heparin.² This adverse reaction, unlike isolated heparin-induced thrombocytopenia, results in considerable morbidity, including the occlusion of the arteries supplying the heart, brain, and extremities. In these circumstances it is necessary to discontinue heparin therapy as quickly as possible.²

Many of the patients receiving heparin have comorbid conditions that predispose them to thrombocytopenia resulting from other causes, and it can be difficult to be certain of the diagnosis of heparin-induced thrombocytopenia. Yet it is important to be as certain as possible of the diagnosis of heparin-induced thrombocytopenia since discontinuation of the heparin often requires the institution of other antithrombotic therapies such as defibrination or venous ligation that by themselves can result in morbidity. Patients with heparin-induced thrombocytopenia have elevated levels of platelet-associated IgG, but the low specificity of this finding makes a positive result of limited value.^{5,6} Previous investigators have demonstrated a heparin-dependent platelet-aggregating factor in sera of patients with heparin-induced thrombocytopenia,⁷ and it was suggested that this test could be used to diagnose heparin-induced thrombocytopenia. However, in a prospective study evaluating the usefulness of testing for heparin-dependent platelet aggregation, we found that this response does not occur in every patient.⁶ Furthermore, the result of the test is positive in some patients without heparin-induced thrombocytopenia. Problems with both sensitivity and specificity seriously compromise the diagnostic usefulness of any test.

During the performance of our previous study, we made an unexpected observation: sera from patients with heparin-induced thrombocytopenia would initiate platelet aggregation at therapeutic concentrations of heparin, but this response did not occur at high concentrations of heparin. We

also observed that ¹⁴C-serotonin platelet release was a more sensitive test of platelet activation than was aggregation. These two observations were exploited to develop a sensitive and specific test for confirming the diagnosis of heparin-induced thrombocytopenia.

MATERIALS AND METHODS

Materials. The following materials were used: porcine heparin (Hepalean, Harris Laboratories, Toronto); 5-hydroxy-¹⁴C-tryptamine creatinine sulphate (Amersham Corp, Oakville, Canada); calcium- and albumin-free Tyrode's solution containing apyrase, pH 6.2; albumin-free Tyrode's solution, pH 7.4; aqueous counting scintillant (ACS; Amersham Corp).

Sample collection. Serum and plasma samples were obtained from patients in the Hamilton area. The samples from the thrombocytopenic patients receiving heparin were collected within two days of development of the thrombocytopenia. These samples were obtained from subsamples that were collected as part of routine patient management. All samples were heat inactivated for 60 minutes at 56 °C. Plasma samples were centrifuged for five minutes at 8,000 g after heat inactivation to remove the fibrin gel, and the supernatant serum was removed. All samples were stored at -70 °C until use.

Platelet preparation. Whole blood from healthy individuals was collected into ACD (acid-citrate-dextrose, 1:6, vol:vol). The platelet-rich plasma (PRP) was obtained by centrifugation for 15 minutes at 250 g. The PRP was incubated with ¹⁴C-serotonin for 45 minutes at 37 °C (50 μCi ¹⁴C-serotonin per mL of PRP). Approximately 50% of

From the Departments of Medicine and Pathology, McMaster University Medical Centre and The Canadian Red Cross Blood Transfusion Service Hamilton Centre, Ontario.

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Dr J. Kelton is a Research Associate of the Stroke and Heart Foundation of Ontario.

Address reprint requests to Dr J.G. Kelton, Room 2N34, McMaster University Medical Centre, 1200 Main St West, Hamilton, Ontario L8N 3Z5, Canada.

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the ^{14}C -serotonin bound to the washed platelets, giving a final specific activity of $1.84 \times 10^{-4} \mu\text{Ci}$ of serotonin per 10^6 platelets. The platelets were washed once in calcium- and albumin-free Tyrode's solution and were resuspended in albumin-free Tyrode's solution. The final platelet count was adjusted to $300,000/\mu\text{L}$.

Serotonin release assay. Twenty microliters of test serum was mixed with $5 \mu\text{L}$ of one of two heparin concentrations (0.1 and 100 U/mL, final) and $75 \mu\text{L}$ of ^{14}C -serotonin-labeled platelets. The platelet mixture was incubated in microtiter wells that contained a magnetic stir bar. Following a 60-minute incubation at 22°C on a magnetic stir plate set at a slow speed, $100 \mu\text{L}$ of 0.5% EDTA in saline was added to each well to terminate the release reaction. The platelets were centrifuged for five minutes at 1,500 g, and $50 \mu\text{L}$ of the supernatant fluid was removed. This aliquot was added to 10 mL of ACS, and the samples were counted in a liquid scintillation counter. The percent release was calculated as follows:

$$\text{percent release} = \frac{\text{release of test sample} - \text{background}}{\text{total radioactivity} - \text{background}} \times 100.$$

The background was defined as the supernatant fluid radioactivity from platelets that were handled identically to the test platelets except that buffer was substituted for serum. The total platelet radioactivity was the radioactivity of the test platelet sample. On the basis of preliminary studies, a test result was defined as positive if both of the following criteria were fulfilled: greater than 20% release at 0.1 U/mL heparin and less than 20% release at 100 U/mL heparin.

Preliminary studies. Sera from three patients with definite (see subsequent criteria) heparin-induced thrombocytopenia and three normal controls were tested for heparin-dependent ^{14}C -serotonin release. The concentrations of heparin used for these preliminary experiments ranged from 0 to 1,000 U/mL. None of the normal control samples tested induced serotonin release at any of these concentrations, whereas the heparin-induced thrombocytopenia patient samples induced maximum release at therapeutic heparin concentrations (Fig 1).

Patients with possible heparin-induced thrombocytopenia. Sera or plasma from 28 patients with possible heparin-induced thrombocytopenia were available. To categorize these patients, we developed clinical criteria to estimate the likelihood of a particular patient having heparin-induced thrombocytopenia (Table 1). Each patient's hospital record was reviewed by a single individual and the

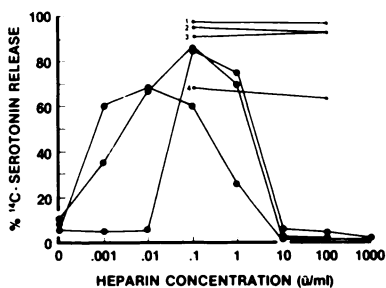


Fig 1. The relationship between the percent release of ^{14}C -serotonin from platelets obtained from healthy individuals (ordinate) and the final concentration of heparin (abscissa). Each series of points is the result obtained with serum from a different patient with definite heparin-induced thrombocytopenia. The lines numbered 1 to 4 represent four different sera samples not from patients with heparin-induced thrombocytopenia. Those samples induced release at 0.1 and 100 U/mL of heparin. Numbers 1 and 2 are sera from two patients with ITP. Number 3 is the serum from a hospitalized control, and number 4 is heat-aggregated IgG.

Table 1. Clinical Criteria for Estimating the Likelihood That a Patient Has Heparin-Induced Thrombocytopenia

Definite heparin-induced thrombocytopenia
Thrombocytopenia develops in patient while receiving heparin, and other causes of thrombocytopenia are excluded. Thrombocytopenia recurs when the patient is rechallenged with heparin; or
Thrombocytopenia plus acute arterial thrombosis, with other causes of arterial thrombosis excluded.
Probable heparin-induced thrombocytopenia
Thrombocytopenia develops in patient while receiving heparin; and
Other causes of thrombocytopenia excluded, ie, blood culture is negative; other drugs excluded.
Possible heparin-induced thrombocytopenia
Patient fulfills second criteria, but the thrombocytopenia resolves during continued heparin therapy; or
Patient fulfills second criteria, but thrombocytopenia does not recur with subsequent administration of heparin (negative challenge).
Heparin-induced thrombocytopenia is unlikely
Thrombocytopenia develops in patient while receiving heparin, but another cause for the thrombocytopenia is found; or
The thrombocytopenia does not resolve after the heparin is discontinued.

patient classified according to the criteria of Table 1. The patients were then grouped according to their classification, and the results of the assay were compared among groups.

Controls. The controls consisted of 15 healthy laboratory personnel, 408 unselected hospitalized patients not receiving heparin, 50 hospitalized nonthrombocytopenic patients receiving heparin, and 100 unselected thrombocytopenic patients not receiving heparin. After the test was completed, a positive result was noted in one of the heparin controls, and a review of this individual's chart demonstrated that he had become thrombocytopenic on the day of testing.

Heat-aggregated human IgG served as a positive control for platelet release. Human IgG (Cohn II) was heat aggregated at 63°C for 20 minutes. This was then incubated with the platelets in place of the serum, and the percent release measured in the usual way.

The patient and control samples were tested at the two heparin concentrations by a single individual who was blinded as to their origin. The result was considered positive for heparin-induced thrombocytopenia only if release (greater than 20%) occurred at a heparin concentration of 0.1 U/mL and did not occur at 100 U/mL of heparin.

RESULTS

When attempting to diagnose a relatively uncommon disease, test specificity is of primary importance. The test described in this report was highly specific for heparin-induced thrombocytopenia (Table 1). Only one of 573 control samples showed positive results. The controls included healthy controls ($n = 15$), nonthrombocytopenic heparin controls ($n = 50$), hospitalized patients not receiving heparin ($n = 408$), and patients with thrombocytopenia not resulting from heparin ($n = 100$). There was only one false-positive result (release at therapeutic concentrations of heparin, 0.1 U/mL, but not at high concentrations of heparin, 100 U/mL). This occurred in a 12-year-old girl with idiopathic thrombocytopenic purpura (ITP) who had never received heparin. One heparin control showed positive test results, but

this patient was found to be thrombocytopenic on the day of testing.

The importance of testing for release at high- and low-heparin concentrations is illustrated by the observation that had we defined a positive result only as requiring ^{14}C -serotonin release at a therapeutic heparin concentration (0.1 U/mL), then 21 of the controls would have been misclassified as having heparin-induced thrombocytopenia (Table 2). Many of these patients had ITP, and their sera induced ^{14}C -serotonin release even without the addition of any heparin. These patients clearly represented a group separate from the heparin-induced thrombocytopenia patients since only one of these patients demonstrated inhibition of release at the 100-U/mL concentration of heparin.

The test was sensitive for the detection of heparin-induced thrombocytopenia, and there was a strong relationship between the likelihood of a patient having heparin-induced thrombocytopenia and a positive result in the assay. Six of six patients with definite heparin-induced thrombocytopenia (Table 3) had a positive result, whereas none of the four patients in the fourth group (heparin-induced thrombocytopenia unlikely) and only two of ten in the third group were positive.

The test was positive regardless of whether the patient was receiving heparin at the time of testing or not. Three patients who were classified as having definite heparin-induced thrombocytopenia were receiving heparin at the time the test was performed, and positive results occurred in all. The sera samples from these patients induced platelet release without the addition of heparin, although the addition of 1.0 U/mL of heparin to the test serum did not inhibit the release reaction, as could be predicted from Fig 1.

Platelet release induced by immune complexes (heat-aggregated IgG) was not affected by either concentration of heparin and ranged from 60% to 90% (Fig 1).

The level of platelet-associated IgG was measured at the time of thrombocytopenia in seven of the patients with suspected heparin-induced thrombocytopenia and was elevated in six.

DISCUSSION

Heparin-induced thrombocytopenia is a complication of heparin therapy that can result in morbidity and occasionally death.¹⁻⁴ The diagnosis remains one of exclusion because

Table 2. The Results of the In Vitro Test for Heparin-Induced Thrombocytopenia for 28 Thrombocytopenic Patients Who Were Receiving Heparin

Classification	Number	Number With Positive In Vitro Test Results
Definitive heparin-induced thrombocytopenia	6	6
Probable heparin-induced thrombocytopenia	8	5
Possible heparin-induced thrombocytopenia	10	2
Heparin-induced thrombocytopenia unlikely	4	0

Table 3. Results of Measuring Heparin-Dependent Serotonin Release in the Various Control Populations

Pattern of Serotonin Release	Heparin Concentration (Final)	Number of Various Control Subjects			
		Healthy Controls	Hospital Controls	ITP Patients	Heparin Controls (Nonthrombocytopenic)
<20%	0.1 U	15	395	92	40
<20%	100 U				
>20%	0.1 U	0	13	7	1
>20%	100 U				
>20%*	0.1 U	0	0	1	1†
<20%*	100 U				

*Includes those controls with a positive test (as defined in the text) for heparin-induced thrombocytopenia.

†This patient was discovered to be thrombocytopenic at the time of testing.

there is no highly sensitive or specific diagnostic test for this disorder.^{5,6} Therefore, clinical decisions must be based on diagnostic probabilities. The implications of continuing or discontinuing heparin therapy are considerable, and it would be useful to have a test that could confirm or exclude heparin-induced thrombocytopenia. The in vitro test described in this report represents such a test and is both sensitive and specific for confirming the diagnosis of heparin-induced thrombocytopenia.

The technique is based upon two observations: (1) heparin-dependent platelet ^{14}C -serotonin release occurs at therapeutic concentrations of heparin, and (2) very high concentrations of heparin inhibit the release reaction. Others have reported, and we have confirmed, that sera from some patients with heparin-induced thrombocytopenia can induce platelet aggregation in the presence of heparin.^{6,7} But, this in vitro test is neither sensitive nor specific.⁶ The method described in this report represents an improvement in both sensitivity and specificity. To increase the sensitivity we measured ^{14}C -serotonin release instead of platelet aggregation since immunologic stimuli can induce release without aggregation.⁸ The problem with the use of a single concentration of heparin and measuring platelet release as a test for heparin-induced thrombocytopenia is its low specificity. Sera from patients with other thrombocytopenic disorders also can induce platelet release. This difficulty was overcome by assessing the release reaction at two heparin concentrations. Preliminary studies showed that heparin-dependent platelet release was unimodal (Fig 1). Sera from patients with heparin-induced thrombocytopenia caused platelet release at therapeutic concentrations of heparin, but high concentrations of heparin inhibited the reaction. Subsequent studies, not described in this report, have demonstrated that the plasma factor that causes heparin-dependent platelet release is IgG. The unimodal pattern of release may provide insight into the spatial orientation of the binding of the antibody-heparin complex to the platelet. For example, a similar unimodal reaction was observed with malaria-induced thrombocytopenia in which the binding of IgG was directed at platelet-bound antigens.⁹ However, unimodal binding also

characterizes certain immune complex interactions. Consequently, the characterization of the precise type of binding of IgG-heparin to the platelet surface probably will require the use of immunoglobulin digests.

The inclusion of the high-heparin concentration was the primary factor explaining the increased specificity. Using this test modification, none of the patients that were classified clinically as having definite heparin-induced thrombocytopenia had negative results; however, 20 of 21 control patients whose sera induced release with low concentrations of heparin continued to cause release at high-heparin concentrations. The high specificity of the test for heparin-induced thrombocytopenia was confirmed by testing many other sera samples from patients without this disorder. However, it is more difficult to evaluate test sensitivity because there is not a diagnostic "gold standard" for heparin-induced thrombocytopenia. For this reason, we related the *in vitro* results to a clinical estimate of the likelihood that the patient had heparin-induced thrombocytopenia. We found that those patients with the highest clinical probability of having heparin-induced thrombocytopenia also had the highest positivity

rate in the assay. And as the clinical probability of a patient having heparin-induced thrombocytopenia decreased, so did the likelihood of a positive test. It is theoretically possible that other heparin concentrations could be used in the diagnostic test. For example, Fig 1 indicates that a no-heparin-added test in addition to the 0.1-unit test could be as sensitive as the 0.1-unit and the 100-unit test in detecting heparin-induced thrombocytopenia. However, this approach is not always clinically practical since it requires the cessation of heparin therapy to perform the test.

In summary, this study indicates that the measurement of heparin-dependent platelet release at two heparin concentrations can be used as a specific diagnostic test for heparin-induced thrombocytopenia. It is possible that this test also can be used to provide insight into the pathophysiology of heparin-induced thrombocytopenia.

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