Laboratory diagnosis of heparin-induced thrombocytopenia type II after clearance of platelet factor 4/heparin complex

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Laboratory confirmation of heparin-induced thrombocytopenia (HIT) is limited by assay sensitivity. We investigated whether laboratory confirmation can be improved after antigen clearance by determining free antibody and combining the results of antigenic and biologic assays. Blood samples were collected over 5 to 6 weeks in 14 HIT patients. As an antigenic assay, the fluorescence-linked immunofiltration assay (FLIFA) was performed, and as a biologic assay, the carbon 14–labeled serotonin release assay was performed. At day 1 when heparin was stopped, 11 of 14 patients showed positive results in both assays; thus each assay had a sensitivity of 80%. The 3 patients with negative results seroconverted in one or both assays during the subsequent 7 days. Combining the positive results of the assays increased the sensitivity to 100% at day 7, regardless of whether the antigenic or the biologic assay was performed first. Both assays became negative in all patients within 5 to 6 weeks. The sensitivity of antigen and biologic assays in HIT patients increased to 100% after the time course of the heparin-induced antibody. We assume that in some HIT patients the free antibody can be detected after withdrawal of heparin and after clearance of the platelet-factor 4/heparin complex.

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Abbreviations: ELISA = enzyme-linked immunosorbent assay; FLIFA = fluorescence-linked immunofiltration assay; HIT = heparin-induced thrombocytopenia; IgG = immunoglobulin G; PF4 = platelet factor 4; SRA = serotonin release assay
in HIT without thrombosis\textsuperscript{16} but has not yet been studied in HIT with thromboembolism.

There is evidence from the literature that free antibody can be detected after clearance of the immune complex.\textsuperscript{17} Based on these observations, we assumed that in HIT patients with negative test results at day 1 of blood sampling, the antibody may appear in the first few days after disappearance of the antigen. The time course of PF4 and heparin antibodies over 50 days has been reported in 1 HIT patient,\textsuperscript{18} and another patient was monitored for 2 weeks with the \textsuperscript{14}C-labeled serotonin assay.\textsuperscript{3}

**METHODS**

**Patients.** The diagnosis of HIT was made in 14 patients during heparin treatment if all of the following criteria were fulfilled: fall in platelet count to below $100,000 \times 10^9/L$ or by $>50\%$ of the pretreatment value; occurrence of thromboembolism during heparinization; return of platelet count to pretreatment value after end of heparin therapy; exclusion of other causes of thrombocytopenia. Blood samples were taken in all patients for 5 to 6 weeks, starting immediately after the end of heparin treatment, defined as day 1.

Blood was collected from all patients into plastic tubes containing kaolin and was centrifuged within 30 minutes at 1800 $g$ for 20 minutes at room temperature. Serum was aliquotted, shock frozen in liquid nitrogen, and stored at $-80^\circ C$. The study was approved by the ethics committee of the University of Heidelberg, and informed consent was obtained from each person.

**Fluorescence-linked immunofiltration assay.** The heparin-induced IgG antibody was determined by fluorescence-labeled heparin in the presence of PF4 and the patient’s serum. The assay was performed under previously reported experimental conditions.\textsuperscript{8}

**\textsuperscript{14}C-labeled SRA.** The biologic effect of the patient’s heparin-induced IgG antibody was determined by using \textsuperscript{14}C-labeled SRA. The assay was performed as previously report-

**RESULTS**

Eight patients were men and 6 were women, with 69 years of age (range 55 to 79) and 55 to 79 kg body weight as indications for heparinization. Thirteen received unfractionated heparin, and 1 patient received low-molecular-weight heparin for prophylaxis of thromboembolism, caused by cardiovascular ($n = 1$), neurologic ($n = 2$), or gynecologic disease ($n = 3$); by carcinoma ($n = 2$); or as a postoperative complication ($n = 6$). Platelets decreased from 234 (179 to 325) $\times 10^6/mL$ to 43 (2 to 123) $\times 10^6/mL$ between days 5 and 8 during heparin treatment and normalized to 313 (100 to 643) $\times 10^6/mL$ in all patients within 9 days of stopping heparin. Eight patients developed deep venous thrombosis, 3 developed pulmonary embolism, and 1 each developed arterial thrombosis, myocardial infarction, and cutaneous necrosis during heparin therapy. After diagnosis, anticoagulation was switched to danaparoid ($n = 2$) and r-hirudin ($n = 12$).

Figs 1 thorough 4 show the different time courses of the platelet count, the FLIFA, and the \textsuperscript{14}C-labeled SRA after heparin was stopped. Fig 1 shows the results in 1 patient who had the highest HIT-IgG antibody antigen and activity levels at the time of the platelet nadir. The assay was performed under previously reported experimental conditions.\textsuperscript{8} The same positive and negative controls were run in all experiments.

**Fig 1.** The time courses (pattern 1) of the FLIFA, the \textsuperscript{14}C-labeled SRA, and the platelet count are shown in a patient with HIT after heparin treatment was stopped.
cytopenia and with negative $^{14}$C-labeled SRA during the observation period. Fig 4 shows the results in a patient in the reverse situation: with negative IgG antibody but positive $^{14}$C-labeled SRA at day 6. The time courses of the assays in Figs 1 through 4 indicate that laboratory confirmation of HIT was still able to be obtained in all 4 patients, even several days after heparin was stopped and after normalization of the platelet count. The time courses of the platelets and the two assays in the other 10 patients followed one of the four patterns presented.

The sensitivity for laboratory confirmation of HIT was 80% for both the $^{14}$C-labeled SRA and FLIFA assay at day 1 and increased to 85% at day 1, if a positive result in at least one of the assays was defined as laboratory confirmation of HIT (Fig 5). However, originally both assays were always performed on the same day. The result was not dependent on which assay was performed first on day 1.

Between days 2 and 7, 1 patient became positive at day 7 in the $^{14}$C-labeled SRA and 2 patients became positive at days 4 and 7 in the FLIFA. Combining the positive results in the two assays increased the sensitivity of the laboratory confirmation of HIT to 100% of patients over 7 days. All patients had negative HIT-IgG antigen and biologic assays 5 to 6 weeks after stopping heparin (Fig 6).

**DISCUSSION**

Investigations to combine the PF4/heparin ELISA with the heparin-induced platelet activation assay or with the platelet aggregation assay have been con-
ducted only in HIT without thrombosis. The present study confirmed reports that antigenic and biologic assays for laboratory confirmation of HIT with thrombosis are positive in about 80% of patients. After withdrawal of heparin, however, and by repeated testing for heparin-induced antibodies up to day 7, combining the results of antigenic and biologic assays increased sensitivity to 100%. The time courses of the assays in our HIT patients demonstrate that a positive result in one assay indeed confirms the laboratory diagnosis of HIT. Positive results in one of two assays can be combined if the assays used have high specificity. The two assays used in the present study both have a specificity of above 95%. To reduce the probability of a false-positive result, positive findings for heparin-induced antibodies had to be determined twice on 2 consecutive days.

When conventional assays are used, clinical HIT with thrombocytopenia and thromboembolic complications will not be confirmed by laboratory testing in 10% to 20% of patients. An inappropriate decision to continue the heparin in these patients might be made. It remains difficult to diagnose HIT type II based on clinical findings, because patients at greatest risk for HIT are severely ill with many other possible causes for thrombocytopenia or micro- or macrovascular thrombotic events. Because an ideal assay for HIT has not yet been established, a different strategy for diagnosis is necessary.

Previously an increase in free antibodies was described in immune complex diseases. On the first
exposure to the antigen, the host develops the antibody response after between a week and 10 days. The synthesized antibodies result in formation of antigen-antibody complexes, which facilitate removal of the antigen in general by cells in the reticuloendothelial system. The fate of the disappearance of preformed complexes injected into animals has been described in detail.\textsuperscript{19} The antigen disappears from the circulation in three phases: the first represents equilibration of the antigen between the intra- and extravascular compartments; the second is produced by catabolism of the antigen; the third involves the immune clearance of the antigen by newly formed specific antibody.\textsuperscript{19} As more antibodies are synthesized, the lattice structure of the immune complexes increases until the complexes reach a critical size and then are rapidly removed from the circulation by the reticuloendothelial system. The complexes are cleared within 7 days, and free antibody can be detected.\textsuperscript{16,19} For the results of the present study, this mechanism is believed to be of importance in HIT patients, because all negative results of the antigenic tests in HIT patients became positive in the following 7 days, and most were also positive in the biologic \textsuperscript{14}C-labeled SRA after clearance of the antigen. The cumulative frequency of positive antigen and biologic assays increased over 7 days while platelets normalized during this period, indicating termination of the immunologic process of the disease. This complies with the mechanisms described above for immune complex diseases, where the pathogenic process ceases after clearance of the antigen-antibody complexes and free antigen can be detected. The reason for the more rapid decrease in the normalization of the heparin-induced IgG antibody in these patients remains unclear at present as compared with the decrease in IgG antibodies in other immunologic diseases.

The present findings also agree with the observation that patients who were tested only on the day when heparin was stopped should be considered still to have a substantial risk of developing thrombotic complication.\textsuperscript{6} So far, in contrast to the platelet count, the antigen and biologic assays for laboratory confirmation of HIT have not been followed up in a larger number of patients. Thus both follow-up of 1 week after onset of the disease and the combination of two sensitive assays may improve the laboratory confirmation of HIT. Follow-up in our HIT patients revealed four patterns for heparin-induced antibodies. Pattern 1 is characterized by positive antigenic tests at the platelets’ nadir and similar time courses for the normalization of the antigen and biologic assays, while pattern 2 shows dissimilar time curves for the assays. Patterns 3 and 4 are positive in only one of the two assays. There are many possible reasons for the differences between the antigenic and biologic assay results, including the polyclonal structure of the IgG antibody and the possibility of interleukin-8 or neutrophil activating peptide as antigens.

In conclusion, repeated determination of sensitive heparin-induced IgG antibody and \textsuperscript{14}C-labeled SRAs confirmed the diagnosis in all HIT patients. This may be caused by an interruption of the antigen-antibody complex after elimination of the antigen, leading to an increase in free antibody that can then be detected in antigenic and biologic assays for laboratory confirma-

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\caption{Cumulative data on positive \textsuperscript{14}C-labeled SRA and FLIFA results (\%) are depicted over the time in patients with HIT after cessation of heparin treatment.}
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tion of HIT. Although the FLIFA, rather than a more standard ELISA, was chosen for immunologic detection of the antibody, it is reasonable to assume that the findings are applicable to the more widely used ELISA.

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REFERENCES