Generation of Anti-Hirudin Antibodies in Heparin-Induced Thrombocytopenic Patients Treated With R-Hirudin

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**Background**—Hirudin is a small protein with strong thrombin inhibition that may be antigenic. The generation and disappearance of anti-hirudin antibodies were investigated in patients with heparin-induced thrombocytopenia who were treated with recombinant hirudin (r-hirudin) for ≥5 days.

**Methods and Results**—The IgA, IgE, IgG, and IgM isoatypes of anti-hirudin antibodies were determined by ELISA before and after the start of r-hirudin therapy. A total of 56% of patients (13 of 23) developed ≥1 antibody isotype during therapy. No IgE antibodies were generated. IgA, IgG, and IgM antibodies were detected in 30% (7 of 23), 52% (12 of 23), and 17% (4 of 23) of patients, respectively. Four patients generated only IgG, 2 patients developed either IgM or IgG and IgM, 5 patients IgG and IgA, and 2 patients IgG, IgM, and IgA antibodies. IgM antibodies disappeared within 8 days of the cessation of r-hirudin. IgA and IgG antibodies disappeared within 1 year in all but 1 patient. Binding of purified IgG to r-hirudin in IgG antibody–positive patients (n=7) was demonstrated by competitive ELISA for r-hirudin. Of the 7 IgG antibody samples, 1 each neutralized or enhanced the anticoagulant activity of r-hirudin.

**Conclusions**—R-hirudin may be antigenic in patients with heparin-induced thrombocytopenia. More comprehensive investigations will be required to determine the biological relevance of this and to establish the antibody-generation pattern in other diseases. (*Circulation. 1999;100:1528-1532.*)

**Key Words:** antibodies ■ anticoagulants ■ proteins ■ thrombosis

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Hirudin is a protein containing 65 to 66 amino acids and having a molecular weight of ≈7000 Da. It is secreted from the salivary glands of the leech *Hirudo medicinalis* and is characterized as a specific, tight-binding, thrombin inhibitor. Today, hirudin is produced by recombinant DNA technology and is used for anticoagulant and antithrombotic therapy. Recombinant hirudin (r-hirudin) is almost as effective and safe as heparin for the prophylaxis of venous thrombosis in total hip replacement and for the treatment of myocardial infarction, unstable angina, and deep vein thrombosis. It is the drug of choice for the anticoagulant treatment of patients with heparin-induced thrombocytopenia (HIT) and thromboembolic complications because it is a non-heparin anticoagulant with specific antithrombin potency, which thus enables rapid anticoagulation. As polypeptides, hirudins may elicit an immunological response in humans; this may also be due to the long phylogenetic distance from invertebrates. Animal and clinical studies have shown, however, that it has a low immunogenic potential and is well tolerated.

Although r-hirudin is a protein with low immunogenicity, anti-hirudin antibodies may appear during treatment in HIT patients; this should be investigated in detail. Therefore, we developed ELISA methods to detect the isotypes of anti-hirudin antibodies. A prospective study was performed in 23 HIT patients who received r-hirudin therapy for ≥5 days. A high level of generated anti-hirudin antibodies was found.

**Methods**

**Patients**

HIT type II was diagnosed in 23 patients (aged 22 to 89 years) according to the following criteria: (1) heparin treatment; (2) thrombocytopenia (platelet count <100×10⁹/l or decrease of >50% in initial platelet count); (3) objectively documented thromboembolic complications during heparin therapy; (4) positive antibody detection using ¹⁴C-serotonin release assay, hirudin-induced platelet activation assay, or heparin-induced IgG antibodies; and (5) recovery of thrombocytopenia after heparin withdrawal. There were more women (n=17) than men (n=6) in the study (no significant difference).

After a diagnosis of HIT, patients received 0.1 mg/kg body weight r-hirudin as a bolus (Lepirudin) followed by a continuous intravenous infusion of 0.1 mg·kg body weight⁻¹·h⁻¹. The daily dose was adjusted using activated partial thromboplastin time (aPTT) values (target range, 1.5- to 2.0-fold prolongation). Twenty patients received r-hirudin intravenously; in 6 of these, r-hirudin was switched to twice-daily treatment with 25 mg SC. Three patients received only twice-daily treatment with 25 mg of r-hirudin SC per day for 7 to 12 days. Antibodies to r-hirudin were generated regardless of whether...
patients received r-hirudin intravenously, subcutaneously, or both (intravenously followed by subcutaneously). No statistically significant differences in age, r-hirudin daily dose, duration of treatment, or intravenous dosage were observed between the patients with and without generation of anti-hirudin antibodies.

Plasma and serum samples were collected from 23 HIT patients at different intervals before, during, and after r-hirudin treatment. Plasma was obtained by collecting 9 volumes of free-flowing blood by clean puncture of a cutaneous vein in 1 volume of 3.8% trisodium citrate solution in 10-mL polystyrene tubes (Sarstedt). The samples were centrifuged within 30 minutes at 1800g for 20 minutes, and plasma was aspirated and stored at −80°C after shock-freezing in liquid nitrogen. Serum was obtained from separate blood samples in plastic tubes containing kaolin (blood coagulation for 30 minutes); it was processed in the same way as plasma.

Control subjects included 20 healthy volunteers (20 to 48 years of age) who had not taken any medication for at least 2 weeks, 16 non-HIT patients (35 to 60 years of age) with no r-hirudin treatment; and the 23 HIT patients before r-hirudin treatment. The period of observation was from October 1996 to July 1998. Approval of the protocol was obtained from the ethical committee of the Faculty of Clinical Medicine II of the University of Heidelberg.

Materials

R-hirudin for the in vitro studies was provided by Knoll AG, Ludwigshafen, Germany. Bovine serum albumin (BSA; Tween-20; peroxidase-labeled goat anti-human antibodies to IgA (α-chain specific; working dilution, 1:10,000), IgE (ε-chain specific; 1:2,000), IgG (Fc-fragment specific; 1:50,000), and IgM (μ-chain specific; 1:15,000)); chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB); dimethyl sulfoxide; 3% H₂O₂; and 95.5% H₂SO₄ were purchased from Sigma. Rabbit polyclonal IgG antibodies to r-hirudin were donated by Knoll AG. Biotin-conjugated anti-rabbit IgG (whole molecule) and streptavidin-peroxidase were from Sigma. All other reagents were obtained from Merck and were of analytical grade.

ELISAs for Anti-Hirudin Antibody Isotypes

The 4 ELISAs for the 4 Ig isotypes of anti-hirudin antibodies were optimized using standard methods. PBS (pH 7.2) contained 0.01 mol/L Na₂HPO₄, 0.145 mol/L NaCl. PBS-Tween was prepared by mixing PBS with 0.1% Tween-20. Carbonate/bicarbonate coating buffer (pH 9.2) contained 0.1 mol/L Tween-20. Sodium acetic buffer contained 0.1 mol/L sodium acetate and 0.1 mol/L acetic acid, and it had a pH of 4.9. Peroxidase substrate solution was freshly prepared by adding 1.88 mg of TMB and 15 μL of 3% H₂O₂ to 10 mL of the sodium acetic buffer.

The 4 ELISAs were performed using different microtiter plates (U-form, Greiner Microlon) for each immunoglobulin. Samples were analyzed in triplicate. Normal pooled plasma was analyzed in all assays as a negative control. The amount of reaction product of the ELISA was detected at an optical density (OD) of 450 nm on a microtiter-plate reader linked to a Biolinx software program (MR 7000, Dynex). The amount of r-hirudin in the coating solution, the dilution of plasma, and the incubation times were optimized in preparatory experiments. With increasing concentrations of r-hirudin in the coating solution, the optimal dose plateau was reached at 5 μg of r-hirudin, which resulted in maximum absorbances ranging from 2.0 to 2.5 at 450 nm for the different Ig isotypes. The amount of r-hirudin bound to the surface of the wells was 700 ng of 5 μg, as measured by competitive r-hirudin ELISA using 1 μg/mL rabbit IgG anti-hirudin antibody and increasing free r-hirudin concentrations. A total of 10% of the bound r-hirudin retained its biological activity (anti-thrombin S2238 chromogenic substrate assay). The purified 10 μg/mL human IgG antibody was displaced >98% by 1 mg/mL rabbit antibody. A 1:50 dilution of the plasma samples decreased unspecific binding to an optimal extent (Δ absorbance at 450 nm=positive sample−negative control). The dilution also led to a 50-fold lower concentration of free r-hirudin and r-hirudin−containing immune complexes in plasma. A total of 0 to 25 mg/mL human immunoglobulins (Pentaglobin) did not influence the effects of r-hirudin in normal or patient plasma on IgA, IgE, IgG, and IgM anti-hirudin antibodies. In contrast, IgA, IgG, and IgM antibodies bound dose-dependently to immobilized r-hirudin. IgE antibodies were negative in all patients and, therefore, could not be tested (data not shown).

The final ELISA procedures were run as follows. Wells were coated with 100 μL of 5 μg of r-hirudin/mL in 0.1 mol/L carbonate buffer (pH 9.2) and incubated for 24 hours at 4°C. After blocking with 200 μL of 1% BSA in PBS per well for 1 hour at 37°C, wells were washed 3 times with PBS-Tween. Then, 100 μL of plasma diluted 1:50 in PBS was added and incubated for 1 hour at 37°C. After washing 3 times with PBS, 100 μL of peroxidase-labeled goat anti-human immunoglobulin was incubated for 1 hour at 37°C (working dilutions prepared with 0.1% BSA in PBS) and then washed 3 times with PBS; 100 μL of TMB peroxidase substrate solution was added and incubated for 10 minutes at 37°C. Enzyme reaction was stopped with 50 μL of 2 mol/L sulfuric acid. Hirudin-specific antibody binding (Δ absorbance at 450 nm) was calculated by raw data (absorbance at 450 nm) minus normal plasma (absorbance, 450 nm) using an Excel 6.0 spreadsheet.

The means±3SD of the samples of the healthy subjects, the non-HIT patients, and the 23 HIT patients at baseline before r-hirudin treatment were used to determine the upper limit of the normal range for each respective of r-hirudin antibodies. Results of test samples above the respective cutoff values were defined as positive. No significant differences were observed between serum and plasma samples in a series of pilot control experiments.

Coagulation Assays and R-Hirudin ELISA

Pooled normal fresh plasma was obtained from 20 healthy subjects who had not taken any medication for 2 weeks; 100 μL of plasma was incubated with 25 μL of purified IgG from volunteers or patients for all experiments. Plasma contained no r-hirudin, 250 ng/mL r-hirudin (S2238 assay and ELISA), or 500 ng/mL r-hirudin (aPTT and ecarin clotting times [ECT]). All analyses were run in duplicate.

The aPTTs (pathrombtin, Behringwerke AG; normal, 55 s) and ECTs (ecarin reagent from Knoll AG; normal, <55 s) were performed using a KC110 coagulometer (Amelung). Coagulation times were expressed as the ratio between the coagulation time(s) with and without r-hirudin. Thrombin inhibition was measured using the chromogenic substrate S2238 assay and individual blanks for each sample on microtiter plates. Plasma was spiked with 25 to 1000 ng of r-hirudin/mL to obtain a standard curve, and the OD was plotted against the r-hirudin concentration to calculate the amount of r-hirudin in the samples. Values were expressed as a ratio between the measured and added r-hirudin concentration for the in vitro studies.

The competitive ELISAs for r-hirudin (lower limit of detection, 80 ng/mL) were performed according to standard methods. In brief, the ELISA was performed as follows: 200 μL of r-hirudin (1 μg/mL) was incubated overnight at 4°C in wells of microtiter plates (see above). The wells were washed 3 times with 300 μL of PBS-Tween and then incubated with 300 μL of 1% BSA for 60 minutes at 25°C and washed 3 times, as described above; 100-μL samples diluted 1:20 in PBS and 100 μL of rabbit-anti-hirudin antibody (1.5 μg/mL) were incubated overnight at 4°C and washed 3 times with 300 μL of PBS and 100 μL of biotin-conjugated anti-rabbit-IgG diluted 1:2500 in PBS was added and incubated for 4 hours at 25°C and washed 3 times with PBS. Wells were incubated with 200 μL of streptavidin-peroxidase complex diluted 1:10 000 in PBS for 30 minutes at 25°C and washed 3 times with PBS; 100 μL of the substrate (60 mmol/L TMB in dimethyl sulfoxide) was added, and the reaction was stopped by adding 100 μL of 2 mol/L H₂SO₄. The absorbance was measured at 450 nm within 60 minutes against a blank containing all reagents except the substrate in the microtiter-plate reader (see above). The concentration of r-hirudin was calculated from a standard curve obtained with normal pooled plasma spiked with 50 to 7000 ng/mL r-hirudin using the Biolinx and Microsoft Excel software programs (see above). The ELISA detected the 2 r-hirudins used (Knoll AG and Hoechst Marion Rousell) at
concentrations between 50 and 2500 ng/mL. (n=60; y=0.9312; x=0.0236; r=0.9769).

**Purification of IgG**
The total IgG fractions were affinity-isolated from 1 mL of sera from 5 normal healthy volunteers, 5 HIT patients without anti-hirudin antibodies, and 7 HIT patients with IgG anti-hirudin antibodies using staphyloccocal protein A–sepharose (Sigma). The purity of the IgG preparations was confirmed by analytical SDS-PAGE with Coomasie brilliant blue staining, and it was higher than 95%. The protein concentrations of the IgG preparations were measured by Lowry assay with bovine albumin as the standard (total protein assay kit, Sigma). The effects were estimated by adding 25 µL of 7.5 mg/mL purified IgG to 100 µL of fresh plasma containing 500 ng/mL r-hirudin. The anticoagulant activities of the samples were analyzed by aPTT, ECT, and the anti-thrombin S2238 assay, and the r-hirudin ELISA between the 3 groups was performed using ANOVA. The Wilcoxon test for paired samples was used, and P<0.05 was the level of significance. Values are given as mean±SD. NA indicates not applicable.

**Results**

**Characterization of the Anti-Hirudin ELISAs**
The normal ranges (OD) for the immunoglobulins are given in Table 1 for healthy volunteers, non-HIT patients, and HIT patients before receiving r-hirudin; the pooled data are given as mean±SD. No significant differences existed between the 3 groups of subjects (Table 1). Immunoglobulin antibodies were defined as positive when absorbances were higher than mean±3SD for each immunoglobulin in 2 blood samples taken on different days.

The intra-assay coefficients of variation for positive antibodies were as follows: IgA (n=13), 6.59%; IgG (n=8), 2.9%; and IgM (n=9), 2.83%. For positive samples, they were as follows: IgA (n=7), 20.05%; IgG (n=12), 12.11%; and IgM (n=7), 12.94%. Normal distribution of the values was not rejected using the Kolmogoroff-Smirnoff test.

**Generation and Disappearance of Anti-Hirudin Antibody Isotypes**
Antibodies were in the normal range in all patients before starting r-hirudin therapy. IgA, IgG, or IgM antibodies were found in 13 of 23 HIT patients (56%) after they receiving r-hirudin for ≥5 days. IgA, IgG, and IgM antibodies were found in 30% (7 of 23), 56% (12 of 23), and 17% (4 of 23) of patients, respectively. No IgE antibodies were found. Among the 13 positive patients, 4 generated only IgG, 2 had either IgM or IgG and IgM, 5 had both IgA and IgG, and 2 had IgA, IgG, and IgM anti-hirudin antibodies. Table 1 shows the highest OD of the anti-hirudin antibodies in negative and positive patients. The OD in the IgA, IgG, and IgM antibody-positive patients was significantly higher than in the patients negative for anti-hirudin antibodies after r-hirudin treatment.

The cumulative appearance of the anti-hirudin antibodies throughout the treatment period is depicted in Figure 1. Immunoglobulins were generated in a typical pattern. The disappearance of the anti-hirudin antibodies was followed for up to 490 days after the cessation of r-hirudin. IgM antibodies normalized in all (4 of 4) patients within 8 days; IgA anti-hirudin antibodies in 6 of 7 patients and IgG anti-hirudin antibodies in 11 of 12 patients also normalized within this period.

The time course of the anti-hirudin antibodies from 1 patient is shown in Figure 2 during 30 days of treatment with r-hirudin. IgA, IgG, and IgM antibodies increased at days 9, 18, and 20 after the start of the treatment. Compared with Figure 1, the onset of the generation of the 3 antibodies in this patient was not typical, but this patient generated all 3 anti-hirudin antibodies. After withdrawal of r-hirudin, IgA and IgM antibodies normalized after 3 days, and IgG antibodies decreased over 40 days and disappeared after 6 months.

**Influence of Purified Anti-Hirudin IgG Antibody on the Anticoagulant Activities of R-Hirudin**
No statistically significant differences existed for aPTT, ECT, S2238 assay, and the r-hirudin ELISA between the 3 groups after the addition of 25 µL of protein A–purified IgG from the
subjects to pooled plasma containing 250 or 500 ng/mL r-hirudin (Table 2).

No significant differences existed in aPTT, ECT, and S2238 assays before and after r-hirudin administration in the anti-hirudin antibody–positive patients. The r-hirudin ELISA showed significantly higher concentrations in this set of experiments, indicating competitive binding of the human-IgG antibody and the rabbit anti-hirudin antibody in the ELISA ($P < 0.05$). Purified IgG from 1 patient reduced the ratios of the ECT and S2238 assays (thrombin inhibition) to normal or below normal, indicating biologically relevant inhibition of r-hirudin and competition with the rabbit anti-hirudin antibody in the ELISA. The IgG of patients 1 and 2 caused the S2238 assay to normalize; the IgG in patient 6 caused the ratio of thrombin inhibition in the S2238 assay to increase. The latter was not the case in the aPTT and ECT assays (Table 2).

The clinical course of the patients was uneventful after switching from heparin to r-hirudin therapy. No clinical side effects or resistance to r-hirudin were observed during or after the treatment.

**Discussion**

In the present study, we report on the generation and disappearance of anti-hirudin IgA, IgE, IgG, and IgM antibodies in 23 patients with HIT who were treated with r-hirudin. The effects of purified IgG antibodies on anticoagulant activity were evaluated. No antibodies were detected in the controls or in the 23 HIT patients before r-hirudin therapy, whereas after starting r-hirudin, 56% of patients developed IgA, IgG, and/or IgM isotypes of anti-hirudin antibodies, predominantly of the IgG isotype (52%). No clinical symptoms of intolerance or resistance to r-hirudin were observed. In a study of 263 immunocompetent volunteers with no known previous exposure to anticoagulants, subcutaneous r-hirudin had weak allergen effects, inducing cutaneous skin reactions in 1% of volunteers, with associated hirudin-specific IgE antibodies but no IgG, IgM, or IgA antibodies. However, no local allergic reactions were seen in >1500 patients given subcutaneous r-hirudin for the prophylaxis of thromboembolism after elective hip replacement. In the present study, patients with heparin-induced thrombocytopenia were treated with r-hirudin. None of the patients developed local allergic reactions or IgE antibodies to r-hirudin. Whether this is due to the low number of patients or a different immunological response remains unknown.

The generation of IgG antibodies has been reported in 47% of HIT patients after r-hirudin therapy. In the present study, IgA and IgM anti-hirudin antibodies were also detected.
in 30% and 17% of patients, respectively, and they were usually found together with IgG antibodies, except for 1 patient in whom only IgM anti-hirudin antibodies were found. HIT is regarded as an immune-mediated complication of heparin therapy with production of antibodies, predominantly of the IgG class.\textsuperscript{17} After stopping r-hirudin therapy, antibodies normalized in 22 of 23 patients within 490 days. The significance of the presence of antibodies may be of special importance in patients before re-exposure to r-hirudin.

Our findings raise the important question as to whether the generation of anti-hirudin antibodies interferes with the anticoagulant activities of r-hirudin. The r-hirudin ELISA demonstrated binding of the human anti-hirudin IgG antibody to r-hirudin. Analysis of the biological effect of purified anti-hirudin IgG antibodies showed a lack of influence on aPTT, ECT, and thrombin inhibition in all but 1 of 7 patients (patient 3) in whom the antibody abolished the in vitro effects of r-hirudin on the coagulation tests. However, in 1 of 7 patients (patient 6), the r-hirudin ELISA showed a high concentration of soluble r-hirudin, indicating an interaction of the human with the rabbit r-hirudin antibody in the assay. The antibody of this patient, however, inhibited anticoagulant activity only on the thrombin. The fact that r-hirudin was bound to the surface of microtiter plates to analyze the anti-hirudin antibodies must be considered. A total of 10% of the bound r-hirudin retained its biological activity. This implies that some epitopes have been lost, and the presentation of neo-epitopes, which may be recognized by the antibodies, cannot be excluded. This may explain why these antibodies seem to have little effect on the activity of soluble r-hirudin. These data assume that the human response is polyclonal and unlikely to be exclusively epitope-specific, resulting in some individuals having neutralizing, no, or enhancing antibodies. No conclusion, therefore, can be drawn at present on a relationship between the antibodies and thrombotic or hemorrhagic events.

In summary, our findings indicate an influence of anti-hirudin immune complexes on the metabolism of r-hirudin, which means that anticoagulation must be closely followed-up in HIT patients. The clinical relevance of these findings regarding the incidence of thrombotic or hemorrhagic complications during r-hirudin treatment remains to be investigated.

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References
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