Sustained Antithrombotic Activity of Hirudin After its Plasma Clearance: Comparison With Heparin

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Thrombus extension in patients with venous thromboembolism is due to the accretion of fibrin onto existing thrombi. Extension is promoted by both circulating and thrombus-bound thrombin, which convert fibrinogen to fibrin. Heparin is an effective antithrombotic agent, but it requires continuous administration to achieve persistent inhibition of thrombus extension. Heparin is highly effective in inhibiting fluid phase thrombin, but is a relatively ineffective inhibitor of thrombus-bound thrombin. Hirudin, unlike heparin, inactivates both circulating and fibrin-bound thrombin and, therefore, has the potential to prevent thrombus extension even after a short course of treatment. The aim of this study was to evaluate the time course of the accretion of new fibrin onto preexisting rabbit jugular vein thrombi after a 3-hour infusion of saline, heparin, and hirudin. Heparin and recombinant (r)-hirudin (CSP 39399) were infused at doses that doubled the activated partial thromboplastin time (aPTT). At the end of the 3-hour infusions in rabbits treated with saline, heparin (0.75 mg/kg), or r-hirudin (1.25 mg/kg), accretion of 125I-fibrinogen was 59 ± 5 µg, 34 ± 4 µg, and 21 ± 2 µg, respectively (heparin and r-hirudin vs saline, P < .01; r-hirudin vs heparin, P < .01). Three hours after the end of the infusions, the accreted 125I-fibrinogen in the saline-, heparin-, and hirudin-treated animals was 89 ± 6 µg, 51 ± 7 µg, and 23 ± 3 µg, respectively; 9 hours after the end of the infusions, the accreted 125I-fibrinogen was 112 ± 9 µg, 82 ± 7 µg, and 25 ± 3 µg, respectively. aPTT and thrombin clotting time (TCT) returned to the baseline value 90 minutes after the end of the heparin or r-hirudin infusion. During in vitro experiments, human fibrin clots previously incubated in human plasma containing r-hirudin did not promote fibrinopeptide A (FPA) generation when washed and then incubated in human plasma in the absence of thrombin inhibitors. This persistent inhibition of FPA production was not observed after incubation in human plasma of human plasma clots preincubated with heparin. We conclude that heparin is effective in inhibiting thrombus extension while it is present in the circulation, but that this effect is rapidly lost after its plasma clearance. In contrast, the antithrombotic activity of r-hirudin is sustained beyond its plasma clearance, presumably because of its ability to inactivate thrombus-bound thrombin. Our findings indicate that r-hirudin might be an effective antithrombotic agent even when used for short periods.

Heparin is an effective treatment for venous thromboembolism; it reduces the risk of clinically important recurrent venous thromboembolism and prevents extension of thrombosis in the majority of patients. However, recurrence is common when heparin is discontinued prematurely. These observations suggest that, in many patients with venous thrombosis, the stimulus to thrombosis is suppressed during heparin treatment, but becomes expressed when it is discontinued.

Recent in vitro studies provide a possible explanation for the high risk of recurrent thrombosis when heparin is prematurely stopped. When fibrinogen is converted to fibrin during thrombogenesis, thrombin binds to the fibrin, where it is protected from the inactivation operated by its naturally occurring plasma inhibitors. The fibrin-bound thrombin is enzymatically active and has the potential to induce extension of thrombosis through two separate mechanisms. These are (1) by cleaving circulating fibrinogen and converting it to fibrin; and (2) by activating factors V and VIII and so resulting in the autocalytic generation of high concentrations of free thrombin in the vicinity of the thrombus. Of these two mechanisms, the second probably makes a much greater contribution to thrombus growth. Further, there is experimental evidence that heparin is relatively limited in its ability to inactivate clot-bound thrombin, but is an extremely efficient inhibitor of free thrombin. In contrast, a number of antithrombin III–independent thrombin inhibitors, including hirudin, are effective at inhibiting both clot-bound and free thrombin.

These clinical and experimental observations are consistent with the hypothesis that, while in the circulation, heparin prevents thrombus extension by inhibiting the autocalytic generation of free thrombin by clot-bound thrombin, but that it does not inactivate clot-bound thrombin, which is then able to generate more thrombin and induce thrombus extension when heparin therapy is discontinued. In contrast, unlike heparin, hirudin inactivates both free and clot-bound thrombin, and so has the potential to inactivate clot-bound thrombin and, therefore, prevent thrombus growth even after it is cleared from the circulation.

In the present study, we performed experiments designed to compare the ability of heparin and recombinant (r)-hirudin to prevent thrombus extension both during their administration and after they are discontinued and cleared from the circulation.

MATERIALS AND METHODS

Materials. Human 125I-fibrinogen (FIBI-125 C, 90% clottable, specific activity 7.1 MBq/pg) was purchased from Sorin Biomedica, Saluggia, Italy. Bovine thrombin (RTH 19 lot no. 506015 L) was obtained from the Istituto Behring, L'Aquila, Italy, and human thrombin (Ortho Q.F.A. lot no. QFT157) from Ortho, Milan, Italy. The chromogenic substrates (S2238, H-D-Phe-Pip-Arg-NH-NOZ-Cl, lot no. 89833 51 and S-2222, Bz-Ile-Glu(-OR)-Gly-Arg-pNA-HCl, lot no. 89012 51), human thrombin, and factor Xa used in the anti-IIa and anti-Xa assays were also obtained from Ortho. The
chromogenic substrate (Chromozym TH, lot no. 163712) used in the hirudin assay was obtained from Boehringer Mannheim, Mannheim, Germany. The activated partial thromboplastin time (aPTT) reagent and the bovine thrombin, used to perform the thrombin time, were obtained from Ortho. Sodium pentobarbital was obtained from Farmitalia Carlo Erba, Milan, Italy. All animal studies were performed with white New Zealand rabbits and conformed to the guidelines of the American Physiological Society and of the International Committee on Thrombosis and Haemostasis. The study was performed in compliance with the animal welfare regulations of the University of Perugia, Italy.

Plasma levels of fibrinopeptide A (FPA) were measured using an enzyme immunoassay kit produced by Diagnostica Stago (Asseychrom FPA) and obtained from Boehringer Mannheim Italia, Milan, Italy.

**Thrombus growth model.** The effectiveness of heparin and r-hirudin in preventing thrombus growth was assessed by comparing their ability to inhibit the accretion of 125I-fibrinogen onto autologous nonradioactive thrombi pre-formed in the jugular vein of rabbits. Preliminary studies demonstrated that venous thrombi could be formed with bovine thrombin, rather than human material, and that radiolabeled human fibrinogen could be used in place of rabbit fibrinogen to monitor extension. Standard-size nonradioactive thrombi were produced in the external jugular veins of white New Zealand rabbits of both sexes (weight, 2.5 ± 0.1 kg) as previously described. Rabbits were anesthetized by the injection of sodium pentobarbital, 30 mg/kg, via the marginal ear vein. Additional pentobarbital was administered when needed to maintain anesthesia. The left jugular vein and the right carotid artery were exposed through a paramedial incision of the neck. The right carotid artery was then cannulated with a 1.5-mm diameter polyethylene cannula for blood sampling. The left jugular vein was isolated and cleared over a length of 2 cm and small side branches were ligated. The jugular segment was emptied of blood, and flow was temporarily occluded both proximally and distally by two surgical clamps, 1.5 cm apart. A 10-cm long 3.0 braided polyester suture, presoaked in a collagen solution, was introduced lengthwise in the lumen of the isolated jugular vein for a length of 4 cm to avoid embolization of the induced thrombus. Citrated rabbit blood (150 µL) was then aspirated in a 1-mL syringe prefilled with 50 µL (5 IU) of a thrombin solution and 10 µL of a CaCl₂ solution (0.25 mol/L). This mixture was quickly mixed and injected in the isolated venous segment of the jugular vein via a 25-gauge needle. Injection of air bubbles was carefully avoided. In all instances, the thrombi formed within less than 1 minute. Thrombi were allowed to age for 30 minutes, and then both vessel clamps were removed and blood flow restored. Fifteen minutes after the thrombi were formed, each animal was injected with 10 µCi of 125I-fibrinogen. Five minutes later, heparin, r-hirudin, or saline was infused for 3 hours. At the end of the infusion, the venous segment containing the thrombus was tied off, opened longitudinally, and the remaining thrombus removed and washed 10 times in saline. The circulating fibrinogen radioactivity was estimated from the mean of the blood samples collected at hourly intervals throughout the infusion. The ratio of the radioactivity of the thrombus to the circulating fibrinogen radioactivity was used to quantify the thrombus growth, which was then expressed as micrograms of 125I-fibrinogen accreted onto the thrombus. In other experiments, 19-gauge butterfly needle, blood was collected from the antecubital vein of healthy volunteers into plastic syringes prefilled with 1/10 vol of 3.8% trisodium citrate. After thorough mixing with the

with a specific activity of 11,496 anthrombin units (ATU)/mg, was manufactured by Ciba-Geigy and Plantorgan, Werk, Germany, and obtained from Ciba-Geigy, Basel, Switzerland. The antithrombotic effects of heparin and r-hirudin were initially compared at doses that doubled the aPTT. To establish aPTT equivalence, increasing doses of heparin and r-hirudin were infused over a 3-hour period by a constant-rate infusion pump and the aPTT was measured every 30 minutes. A steady prolongation of the aPTT was achieved for each anticoagulant after 2 hours. The aPTT was prolonged by twice the control with a heparin dose of 0.75 mg/kg and with a r-hirudin dose of 1.25 mg/kg infused over 3 hours. In other experiments, the dose of heparin was doubled to 1.5 mg/kg. A comparative control group of animals was infused with 20 mL of 0.9% saline. The infusions were given over a 3-hour period into the right marginal ear vein (contralateral to the side with the induced thrombus). Thirty-two rabbits were included in each treatment group.

**Experimental procedure.** Treatment with either heparin, r-hirudin, or saline was commenced 50 minutes after induction of the experimental thrombus and 5 minutes after injecting the radioactive fibrinogen.

Thrombus growth during treatment was assessed by measuring the amount of radioactive fibrin that accumulated onto the experimental thrombi at the end of the 3-hour infusion. Subsequent thrombus growth was assessed by measuring the additional radioactivity that accumulated onto the thrombi 1 hour, 3 hours and 9 hours after treatment had been discontinued. Fibrin accumulation was measured at these times by euthanizing different groups of rabbits at the designated times. Each series of experiments was performed on batches of 32 rabbits, which were divided into four groups, to provide eight measurements of accumulated radioactive fibrin at each time point.

In a separate series of experiments, designed to assess the extension of thrombi formed after the exposure of rabbits to heparin or r-hirudin, experimental thrombi were produced in both jugular veins; the left-sided thrombi were produced 30 minutes before the 3-hour infusion was started and thrombus growth was assessed 9 hours later, as in the previous experiments. The right-sided thrombi were produced 9 hours after the 3-hour infusion was discontinued (that is, after the antithrombins had been cleared from the circulation), and thrombus growth was assessed 3 hours later. The experimental treatments were infused through the femoral artery in the same dosages as in the previous experiments.

**Coagulation tests.** Blood samples were drawn for coagulation testing before treatments were begun and then hourly during the period of treatment. Samples of arterial blood of 4.5 mL were drawn into 0.5 mL trisodium citrate (final concentration, 0.012 mol/L) for determination of aPTT, thrombin clotting time (TCT; bovine thrombin, 1.25 U/mL), anti-Xa and anti-IIa chromogenic assays (heparin-treated rabbits), and hirudin plasma level (r-hirudin–treated rabbits). The blood samples were centrifuged at 8,800 x g for 10 minutes at 4°C. The resultant platelet poor plasma was then stored at −70°C in aliquots until it was assayed. aPTT and TCT were performed by standard methods. The chromogenic antifactor Xa and antifactor IIa assays were performed by the method of Teien et al. Plasma levels of hirudin were measured by a chromogenic assay as described by Spannagl et al. Standard curves were constructed by adding to pooled rabbit citrated plasma increasing concentrations of heparin in the anti-Xa and anti-IIa assays, and of r-hirudin in the hirudin concentration assay.

**In vitro FPA generation by thrombin bound to fibrin.** Using a 19-gauge butterfly needle, blood was collected from the antecubital vein of healthy volunteers into plastic syringes prefilled with 1/10 vol of 3.8% trisodium citrate. After thorough mixing with the
anticoagulant, the red blood cells were sedimented by centrifugation at 1,700 × g for 15 minutes at 4°C, and the platelet-poor plasma was harvested, divided in 250-μL aliquots, and transferred to polypropylene tubes. Fibrin clots were formed around wire hooks by the addition of CaCl₂ (final concentration, 25 mmol/L). The clots were aged for 60 minutes at 37°C with constant agitation, and then washed 10 times with 1-mL aliquots of 0.1 mol/L NaCl buffered with 0.05 Tris-HCl, pH 7.4 (TBS), over the course of 24 hours to eliminate FPA trapped within the clots. The efficiency of washing was determined by assaying for FPA aliquots taken from each of the washes. Washed clots were incubated in 1-mL aliquots of fresh citrated plasma for 60 minutes at 37°C in the presence of heparin or r-hirudin or an equal volume of saline. At intervals, 100-μL aliquots were removed for FPA assay. At the end of the 1-hour incubation, the clots were removed, extensively washed, and immersed again in 1 mL of fresh citrated plasma for 60 minutes at 37°C in the absence of thrombin inhibitors. Again, at intervals, 100-μL aliquots were removed for FPA assay. In control experiments, the clots were incubated in TBS instead of plasma. As a control, FPA was also measured in plasma remaining at 37°C for the duration of the previous experiments.

The concentrations of r-hirudin and heparin used in these studies were 10 μmol/L and 10 anti-Xa U/ml, respectively. These concentrations were chosen because in preliminary studies we found that they produced greater than 90% inhibition of FPA generation.

FPA assay. FPA assay was performed in plasma aliquots removed from plasma in which the clots were immersed. The unreacted fibrinogen was precipitated by the addition of 300 μL chilled ethanol, followed by centrifugation at 15,000g for 5 minutes. The ethanol supernatants were then evaporated to dryness in a vacuum concentrator (Hetovac; Intermed, Birkerød, Denmark), reconstituted to original volume with distilled water, and assayed for FPA. FPA assay was performed by an immunoenzymatic method.

Statistical analysis. The results are expressed as the mean ± SD. Statistical analysis was performed by two-way analysis of variance (ANOVA). In the presence of significant F values, Student's t test for unpaired values was adopted, using two-tailed significance tables and taking P < .05 to indicate a significant difference.

RESULTS

Rabbit thrombus growth inhibition studies. The results of studies assessing the effects of heparin or r-hirudin on thrombus growth are shown in Fig 1. At the end of the 3-hour infusion, fibrin accretion (mean ± SD) was 59 ± 5 μg for saline-treated rabbits; 34 ± 4 μg for rabbits treated with 0.75 mg/kg heparin; and 21 ± 2 μg for rabbits treated with 1.25 mg/kg r-hirudin. The differences between the saline-treated group and either the heparin- or r-hirudin-treated rabbits were statistically significant at P < .01. The difference between the r-hirudin- and heparin-treated rabbits was statistically significant at P < .01. In saline-treated rabbits, there was progressive fibrin accretion on the thrombi over the 3-hour infusion, while in heparin- and r-hirudin-treated rabbits, approximately 70% of the radioactivity present at 3 hours had accumulated by 1 hour (data not shown); it is likely that a large proportion of the accumulated radioactivity occurred in the heparin-treated and hirudin-treated animals during the 30- to 60-minute period between injection of the radioactive fibrinogen and the attainment of steady-state anticoagulant levels. One hour after treatment had been discontinued, accumulated radioactivity for saline-, heparin-, and r-hirudin-treated animals was 76 ± 6 μg, 38 ± 4 μg, and 22 ± 2 μg, respectively. The difference in the increase in radioactivity between heparin- and hirudin-treated rabbits was statistically significant (P < .01). Three hours after the end of the infusion period, radioactive fibrin accumulation was 89 ± 6 μg, 51 ± 7 μg and 23 ± 3 μg for the saline-, heparin-, and hirudin-treated animals, respectively (P < .01 heparin v r-hirudin). Nine hours after the end of the infusion period, the accreted radioactive fibrinogen was 112 ± 9 μg, 82 ± 7 μg, and 25 ± 3 μg for the saline-, heparin-, and hirudin-treated animals, respectively (P < .01 heparin v r-hirudin). In rabbits treated with the higher dose of heparin (1.5 mg/kg), 23 ± 3 μg of radioactive fibrin accumulated over the 3-hour infusion period, an amount similar to that observed with r-hirudin. However, after the higher dose of heparin was discontinued, there was a rapid accumulation of radioactive fibrin, which occurred at a rate similar to that observed after the lower dose of heparin had been discontinued (28 ± 3 μg, 39 ± 4 μg, and 53 ± 4 μg of radioactive fibrin at 1, 3, and 9 hours, respectively).

The results of the aPTT (Fig 2) and TCT measurements returned to baseline values 90 minutes after the heparin and r-hirudin infusions were discontinued. The antifactor
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Fig 3. Time course of (A) anti-Xa and (B) anti-IIa plasma levels in heparin-treated rabbits, and (C) hirudin plasma level in hirudin-treated rabbits.

Xa and antifactor IIa levels became undetectable 1 hour after the heparin infusion was discontinued (Fig 3). The plasma hirudin levels became undetectable 1 hour after the r-hirudin infusion was discontinued (Fig 3).

Experiments with the bilateral thrombi were performed to determine whether thrombus formation or thrombus growth was influenced by prior exposure of the rabbits to heparin or r-hirudin. The left-sided thrombi (the control group), which were harvested 9 hours after the 3-hour infusions had been discontinued, showed a similar level of accumulation of new radioactive fibrin as was demonstrated in the previous series of experiments: 106 ± 9 μg with saline, 89 ± 8 μg with heparin, and 29 ± 3 μg with r-hirudin. These findings confirm the results of the previous set of experiments and indicate that thrombi that are exposed to r-hirudin, but not to heparin, are prevented from accumulating new fibrin. In contrast, neither r-hirudin nor heparin influenced thrombus formation or thrombus growth of thrombi formed after the two agents had been cleared from the circulation. Thus, accumulation of radioactive fibrin onto the right-sided thrombi, which had been formed 9 hours after 3-hour infusion had been discontinued, was similar for the three treatment groups: 52 ± 4 μg, 49 ± 6 μg, and 56 ± 5 μg for the saline-, heparin-, and r-hirudin-treated animals, respectively, at 3 hours after thrombus formation.

FPA generation by clot-bound thrombin. The effects of heparin and r-hirudin on FPA generation by clot-bound thrombin was studied in vitro in human plasma. The results are shown in Fig 4. Baseline FPA levels of 14 ± 4 ng/mL were obtained when human citrated plasma was incubated at 37°C for 3 hours without the addition of a clot. The

Fig 4. Comparison of the inhibitory effect of heparin and hirudin against FPA production by clot-bound thrombin. Clots were first incubated in plasma containing heparin and hirudin (first incubation), and then in plasma in absence of these agents (second incubation). (□) Saline; (■) heparin; (●) r-hirudin.
human plasma clots contained some trapped FPA, because when they were immersed in PBS for 1 hour they yielded FPA levels of 28 ± 4 ng/mL. Similar levels of FPA were obtained when the clots were removed and re-immersed for 1 hour in a fresh solution of phosphate-buffered saline (PBS). When human plasma clots were immersed in plasma, there was a sharp increase in FPA levels to 498 ± 89 ng/mL at 30 minutes and 712 ± 108 ng/mL at 1 hour. The generation of FPA was inhibited by both heparin 10 antiXa U/mL and r-hirudin 10 µmol/L. Thus, when the clots were immersed in heparin-containing plasma, the FPA levels were 89 ± 9 ng/mL at 30 minutes and 112 ± 14 ng/mL at 1 hour. When the clots were immersed in r-hirudin-containing plasma, the FPA levels were 74 ± 8 ng/mL at 30 minutes and 61 ± 9 at 1 hour. To determine whether the inhibitory effect of the anticoagulant was persistent, clots were incubated for 60 minutes in plasma containing heparin 10 antiXa U/mL, hirudin 10 µmol/L, or an equal volume of saline. The clots were then removed and incubated for 60 minutes in plasma free of these anticoagulants. The clots that had been exposed to control (saline) plasma generated 511 ± 69 ng/mL of FPA; clots exposed to heparinized plasma generated 419 ± 59 ng/mL of FPA; and clots exposed to the plasma containing r-hirudin generated 68 ± 6 ng/mL of FPA. Thus clot-bound thrombin was not inhibited by prior exposure of the clots to heparin, but it was inhibited by prior exposure of the clots to r-hirudin.

DISCUSSION

Hirudin, a 65–amino acid polypeptide, is a naturally occurring selective inhibitor of thrombin originally isolated from the leech (Hirudo medicinalis) salivary glands11 and currently obtained through the cloning and expression of a cDNA coding and processing in Escherichia coli and yeast. Hirudin is a direct inhibitor of thrombin, as it does not require antithrombin III or any other plasma cofactor. CGP 39393 is a recombinant desulfated hirudin differing from natural hirudin, as it lacks the sulfate group on Tyr 63. The antithrombotic properties of CGP 39393 have recently been reviewed.13 Heparin exerts its anticoagulant effect by catalyzing the ability of antithrombin II to inactivate thrombin, factor Xa, and other coagulation enzymes.14 The inactivation of thrombin by heparin requires that heparin binds to both antithrombin III and the clotting enzyme to form a ternary complex. Recent experiments performed in vitro comparing the anticoagulant effects of heparin and r-hirudin have demonstrated marked differences in the ability of these anticoagulants to inhibit fibrin-bound thrombin.3,15 Thus, approximately 20-fold higher concentrations of heparin are required to inactivate thrombus-bound thrombin than fluid-phase thrombin.3 Further, when tested in therapeutic concentrations (0.3 U/mL), heparin does not inhibit fibrin-bound thrombin, probably because the heparin-binding site of thrombin is masked when the enzyme is bound to fibrin.3 In contrast, r-hirudin is able to inactivate fibrin-bound thrombin almost as effectively as fluid-phase thrombin.3 Based on these observations in vitro, we performed experiments to determine whether these two thrombin inhibitors have different effects on limiting thrombus growth after they were cleared from the circulation. Thrombus growth was assessed by measuring the accumulation of radioactive fibrinogen onto thrombi that were produced by a combination of stasis and increased coagulability.

There was a progressive and approximately linear increase in thrombus growth over a 12-hour period in the control (saline-treated) animals. Both heparin and r-hirudin limited thrombus growth during the 3-hour infusion period when they were administered in concentrations that doubled the baseline aPTT. As previously reported,16 r-hirudin was significantly more effective than heparin, although it was possible to produce an equivalent antithrombotic effect with heparin by doubling its dose. A marked difference in the pattern of thrombus growth was observed between the animals treated with heparin and r-hirudin in the 9 hours after the treatments were discontinued. Animals treated with both doses of heparin exhibited a progressive increase in radioactive fibrin accumulation, which paralleled the increase observed in the control animals. In contrast, thrombus growth was virtually abolished in the animals treated with r-hirudin. These findings are consistent with previous observations made in vitro and indicate that the inhibitory effect of r-hirudin on clot-bound thrombin persists for at least 9 hours after the thrombin inhibitor is cleared from the circulation. Presumably, because of its high affinity for thrombin, r-hirudin remains bound to clot-bound thrombin for hours after it is cleared from the circulation. In contrast, the inhibitory effect of heparin on clot-bound thrombin is lost as soon as the anticoagulant is cleared from the circulation. The effect observed with r-hirudin is not caused by binding to endothelium or blood cells, since r-hirudin did not have a lasting antithrombotic effect when it was cleared from the circulation before the experimental thrombi were produced.

The explanation proposed for the sustained antithrombotic effect of hirudin observed in vivo is supported by the experiments performed in vitro with human plasma clots. Thus, when plasma clots were incubated in citrated plasma containing heparin, the ability of the clots to generate FPA was partly inhibited, but this protective effect of heparin was lost when the clots were removed and immersed in plasma free of heparin. In contrast, when plasma clots were incubated in citrated plasma containing r-hirudin, FPA generation was inhibited and the inhibitory effect was sustained after the clots were removed and immersed in plasma free of added r-hirudin.

Our findings demonstrate that r-hirudin can counteract the thrombogenic effect of venous thrombi for a number of hours after the inhibitor is cleared from the circulation. Evidence from this and other studies suggests that r-hirudin achieves this effect by binding to and inhibiting clot-bound thrombin. Although r-hirudin is a reversible thrombin inhibitor, it has a very high affinity for thrombin and a slow off-time. This inhibitory effect could be sustained clinically after the drug is discontinued. Whether this observation will allow the length of anticoagulant therapy in patients with venous thrombosis to be reduced by replacing heparin with hirudin will have to be tested in a formal clinical trial.
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