IN VIVO ACTIVITY OF MICROBIAL HEPARINASE

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Heparin, prepared from mammalian intestinal mucosa, is the most widely used anticoagulant. It has been estimated, however, that the use of heparin leads to complications such as bleeding 8-33% of the time. Of all prescription drugs, heparin is the drug responsible for the greatest number of deaths in otherwise healthy patients.

For these reasons, it would be extremely useful to have a method to control blood heparin levels. We propose that this might be accomplished by using heparinase, an enzyme which specifically degrades the anticoagulant activity of heparin. In this report, we discuss the in vivo activity of both free and immobilized heparinase.

METHODS

Enzyme Production. Heparinase was produced from Flavobacterium heparinum and was purified by cell sonication, nucleic acid precipitation via protamine sulfate, and hydroxylapatite chromatography. At this stage of purification, heparinase has a specific activity of 150 mg heparin degraded/mg protein-h and is catalytically pure (free from contaminating sulfatases and glucuronidases).

Enzyme Immobilization. Heparinase was immobilized using a variation (4) of the procedure of March et al. to sepharose beads which were washed to remove noncovalently bound enzyme. There was no detectable leaching, as judged by protein concentration, of heparinase from the support over a one month period at 25°C. Radiolabeled heparinase was also made by using 3H-histidine in the fermentation broth. When this radiolabeled heparinase was immobilized and washed in human blood for 24 hrs, no leakage of heparinase was detected. When immobilized heparinase was used in animal studies, a 100 ml reactor was employed in which the immobilized enzyme was continuously recirculated to prevent packing of the sepharose beads. The design of this reactor is described in reference 8.

Activity of Free Heparinase. In these experiments New Zealand White rabbits weighing approximately 2 kg were injected with free heparinase. Prior to injection, the rabbits were anesthetized with rompun 0.45 mg/kg subcutaneously and ketamine 0.45 mg/kg intramuscularly. The right external jugular vein was catheterized and the blood samples obtained were diluted 1:10 with 3.8% sodium citrate. Plasma was prepared by centrifugation at 2000 rpm, 4°C, for 15 mins. Beef lung heparin, USP sodium salt for injection from the Upjohn Company, was administered through the catheter. Activated partial thromboplastin time (aPTT) was performed with the Platelet Activator Assay Kit from General Diagnostics. Enzyme linked immunosorbent assays, ELISA, were used to determine antibody formation in the rabbit studies.

Activity Tests of Immobilized Heparinase. Dogs weighing 11 kg were anesthetized with pentobarbital and the carotid artery and internal jugular vein were cannulated with a Scribner shunt connected to silastic tubing joining directly to the heparinase reactor as described previously. Porcine heparin, sodium salt USP (450 U/kg), was injected intravenously and blood was sampled as it exited the filter before it was returned to the dog. Three heparin assays — aPTT using Thrombostabile activating reagent, whole blood recalcification time (WBRT), and Azure were used. Azure metachromatically detects heparin. Ouchterlony precipitin tests were used to determine if antibodies to heparinase were formed in these experiments.

RESULTS

Several experiments were conducted, each using 3 rabbits, to determine if free heparinase injected intravenously would destroy heparin's anticoagulant activity. Figure 1a is a control showing the anticoagulant activity in rabbit serum following an injection of 100 units heparin/kg. Figure 1b shows the results of an experiment in which the same dose of heparin (100 U/kg) was given at one minute and 0.5 mg of heparinase at 2 mins. It is clear that injection of heparinase substantially decreased aPTT when compared to the control. In another experiment, rabbits were pretreated with 0.5 mg of heparinase at one minute and then 100 units heparin/kg was injected at 2 mins (Figure 1c). No substantial anticoagulant activity was observed. These experiments demonstrate that injected heparinase destroys the anticoagulant activity of heparin in vivo. All rabbits were healthy and lived following the experiment. Initial studies show the injected heparinase is antigenic; however, serum containing antibodies to the heparinase preparation did not inactivate heparinase as judged by aPTT.

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Figure 1. Effect of injected heparinase on blood heparin levels, a) control - 100 units heparin/kg, b) 100 units heparin/kg at one minute, followed by 0.5 mg of heparinase at 2 mins, c) 0.5 mg of heparinase at one minute followed by 100 units heparin/kg at 2 mins.

Figure 2. In vivo deheparinization of blood from an 11 kg female dog which received 450 units heparin/kg. Blood was drawn from the carotid artery through a peristaltic pump to the immobilized heparinase filter. Blood exiting the filter flowed through a sampling septum, then through a bubble trap containing a dacron mesh thrombus filter and returned to the jugular vein. Samples were assayed for activity by activated partial thromboplastin time (aPTT), whole blood recalcification time (WBRT), and metachromasia by Azure A.
Langer et al. Heparinase for heparin removal

To test the in vivo activity of immobilized heparinase, blood from a dog, heparinized at 450 U/kg, was passed through the filter. As shown in Figure 2, within 2 min or one pass through the filter nearly all of heparin's anticoagulant activity by aPTT was destroyed; WBRT gave identical results. To demonstrate that the change in aPTT was due to a decrease in heparin concentration and not a nonspecific effect of immobilized heparinase on coagulation factors, the Azure A assay was used to chemically detect heparin. This assay also demonstrates a loss of heparin. (This same assay has been performed for the free enzyme in the case of the rabbits and gives identical results.) The reason that the Azure A assay does not show zero heparin activity is that Azure detects only heparin fragments of hexasaccharide or larger. Fragments this size are present (we have determined this by both HPLC and gel permeation chromatography2 in addition, digestion of heparin by heparinase in buffer also demonstrates this phenomena) and these fragments are detected by Azure A but do not possess anticoagulant activity as measured by aPTT17.

At the end of the experiment, blood taken from the dogs showed no decrease in hematocrit, a 30% decrease in white blood cell count, and a 70% decrease in platelet count. These values are typical of those obtained for tests of extracorporeal circuits in dogs1,18-20. Blood was taken from the dogs one and 2 mos following the experiments and no antibodies to heparinase were detected.

**DISCUSSION**

It is possible that the use of heparinase may lead to a more specific and possibly less toxic8 means of controlling blood heparin levels than current procedures employing protamine sulfate. While microbial heparinase was used in the current study, mammalian heparinases have also been isolated21. A human heparinase should not produce the antigenic effects displayed by the bacterial enzyme. Until a human heparinase is purified and the genetic information identified, however, it will not be possible to use microbial techniques to produce the necessary quantities of the human enzyme needed for testing. Nonetheless, the antiserum to bacterial heparinase did not inactivate the enzyme so it is possible that antibodies may have been made against impurities in the preparation and that a more purified heparinase4 may not be antigenic. In addition the results obtained with microbial heparinase are encouraging in that they show a decrease in heparin's activity in rabbit serum can be caused rapidly by a heparinase injection. Heparinase has also been shown to decrease aPTT of heparinized human plasma and human blood in vitro6,22.

Another approach to preventing immunogenicity is to immobilize the enzyme to a solid support material. The results obtained with immobilized heparinase also showed a rapid clearing of heparin's anticoagulant activity. No antibodies to the immobilized enzyme were detected.

While the immobilized enzyme caused some blood damage, this damage was typical of that caused by extracorporeal circuits in dogs. To improve blood compatibility, other supports and the use of a smaller filter will be examined. The latter is possible because heparinase has been purified to specific activities 70 times greater (with low recovery) than the enzyme used in the current experiment4.

An immobilized heparinase filter could be used either at the end of a clinical procedure (e.g., renal dialysis, pump-oxygenator) to eliminate heparin without the toxic effects of protamine or continuously to prevent high levels of heparin from ever entering the patient. Blood filters (in some cases as large as 2 L) are often used at the effluent of extracorporeal devices to remove microemboli formed during these procedures. Heparinase might be bonded to the biomaterials of these filters. An attractive feature of the application of immobilized heparinase is that it could be used in procedures where blood must enter extracorporeal circulation and where existing biomaterials already interface with blood at the desired location (i.e., at the end of the extracorporeal circuit). Thus, the eventual application of this process may not require any additional apparatus or invasive procedure.

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**REFERENCES**