SUMMARY When activated factor X (Xa) inhibitory activity of serially diluted human and rabbit plasma is determined in a low salt assay, a linear plot is obtained for human, but not for rabbit plasma. When a high salt assay is used, the dilution curves for both human and rabbit plasma are linear, and qualitative as well as quantitative differences are essentially eliminated. On Sephadex G-200 chromatography Xa inhibitory activity of human and rabbit plasma appears in two peaks. With the low salt assay the first and second peaks for human plasma contain respectively 30% and 70% of the activity; whereas with rabbit plasma these values are >95% and <5% of the activity. With the high salt assay the figures for human plasma are <5% and >95%, and with rabbit plasma 65 ± 3% and 35 ± 3%, respectively. With the high salt system, rabbit plasma shows a continuous increase in Xa inhibitory activity with increasing heparin concentrations, similar to that obtained with human plasma. In the high salt system the relative contributions of antithrombin III to Xa neutralization in human and rabbit plasma are different. However, in experiments in which Xa inhibitory activity of antithrombin III is altered by heparin, a simple formula, Total activity (%) = 65% + 0.35 x human plasma (%), permits translation of rabbit data on the Xa-antithrombin III-heparin reaction to man. On the basis of these findings, the rabbit model can effectively be used to study the Xa-antithrombin III reaction.

RECENTLY there has been an explosion of information in the field of coagulation in relation to which animal models can help separate the trivia from observations of intrinsic biological importance as well as those of potential clinical value. Animal models never provide final answers but offer only approximations: for no single animal model can ever duplicate a disease in man. Thus, animal models should not be expected to be ideal, nor to be universally suited to all foreseeable uses. On the other hand, for a model to be a good one, it must provide a new insight, have relevance to a particular problem and respond predictably.1

In the past 5 years there has been increasing and provocative research concerning the role of the normal plasma inhibitors in intravascular coagulation. Antithrombin III is a naturally occurring plasma protease inhibitor, identical with the inhibitor to activated factor X (Xa) and heparin cofactor,2 that has broad specificity3 and plays an important role in maintaining the fluidity of blood.4 One of its primary physiological effects is neutralization of Xa, a biological activity of antithrombin III greatly enhanced by trace amounts of exogenous heparin.5 It is the demonstration that heparin increases the reaction rate between Xa and antithrombin III that has provided a biochemical rationale for clinical trials of low dose heparin in surgical patients,6 whereas it is the finding that estrogen retards this reaction rate that has suggested a basis for the hypercoagulability induced by estrogen-containing oral contraceptives.7 Accordingly, interest has been increased in further explorations of the Xa-antithrombin III reaction.

The rabbit has been widely employed as an in vivo model of thrombogenesis8-11 and if it is to be used effectively to explore further the effect of heparin and estrogen on the Xa-antithrombin III reaction, any substantive species differences between man and the rabbit must be identified. Failure to do so can invalidate extrapolations of results obtained in the animal model to human subjects. Experiments of specific interest are: (1) whether rabbits treated with oral contraceptives exhibit a decrease in antithrombin III reactivity which can be correlated, through the use of an animal model, with an induced hypercoagulable state and (2) correlation between increased antithrombin III reactivity and the antithrombotic effect of low doses of heparin in an animal model.

Because a marked difference has been noted in the capacity of diluted human and rabbit plasma samples to inhibit Xa in the presence of low concentrations of heparin,12 the possibility exists that the rabbit may not be a valid model with which to examine the Xa-antithrombin III reaction. It is to this issue that the present report is directed.

Materials

Russell's viper venom (Vipera russelli), BSA, rabbit brain cephalin, DEAE-cellulose, bovine factor VII and X-deficient plasma were obtained from Sigma. Sephadex G-200 was a product of Pharmacia Fine Chemicals. Citrated pooled rabbit plasma was obtained from Pel-Freeze Biologicals, Inc., and used as normal rabbit plasma. New Zealand white male rabbits were obtained from Camm Research Institute.

Rabbit blood obtained from the marginal ear vein was mixed (9 parts blood to 1 part citrate) with 3.8% trisodium citrate, pH 7.5, and the plasma harvested by centrifugation two times at 1500 g for 10 minutes at 4°C.

Normal human plasma (NHP) was a pool of citrated plasma from six healthy male donors.

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Hog mucosal heparin lot 45584 was provided by Riker Laboratories.

The stock cephalin solution was prepared by suspension of one vial of rabbit brain cephalin in 100 ml of warm physiological saline. This suspension was frozen in 2-ml aliquots and stored at -20°C.

Anticoagulant-free bovine plasma-cephalin reagent was prepared by mixing equal volumes of anticoagulant-free bovine plasma and the stock cephalin solution.

Bovine factor VII and X-deficient plasma-cephalin was prepared by mixing equal volumes of bovine factor VII and X-deficient plasma and the stock cephalin solution.

Bovine-activated factor X was obtained by activation of the barium sulfate eluate of bovine plasma with crude Russell's viper venom in the presence of 10 mM Ca²⁺. The Xa was freed from other activated clotting factors by chromatography on DEAE-cellulose, dialyzed against physiological saline, and diluted in a Tris-maleate solution, 0.02 M, pH 7.5, containing BSA, 10 mg per ml, and stored at -20°C.

Human and rabbit antithrombin III were purified by established procedures. Each protein was >95% homogeneous as determined by disc gel electrophoresis.

All other reagents were of the highest grade available.

**Methods**

**ANTITHROMBIN III QUANTITATIVE ASSAY**

The quantity of antithrombin III was determined as described previously.

**Xa INHIBITORY ACTIVITY ASSAY**

The Xa inhibitory activity of various samples was determined in a low salt (0.03 M NaCl) and in a high salt (0.13 M NaCl) assay system in the following manner: (1) The low salt assay system: to 0.1 ml of the sample was added 0.3 ml of 0.02 M Tris maleate, pH 7.5, and this mixture incubated at 37°C for 1 minute. A 0.1-ml portion of the stock Xa solution was added to the reaction mixture and a stopwatch started. A 0.1-ml fraction of this preincubation mixture was added to a test tube kept at 37°C. At 3 minutes and 45 seconds 0.1 ml of 0.03 M CaCl₂ was added to the subsample followed by 0.2 ml of the bovine factor VII and X-deficient plasma-cephalin solution and the clotting time determined. (2) The high salt assay system: to 0.1 ml of the sample was added 0.3 ml of a solution containing 0.15 M NaCl in 0.02 M Tris-maleate, pH 7.5. The remainder of the assay was performed as described in (1).

The preincubation time of 4 minutes was reduced to 2 minutes where indicated and when assaying for Xa inhibitory activity in the presence of heparin.

**ANALYTICAL SEPHADEX G-200 CHROMATOGRAPHY OF PLASMA SAMPLES**

Lyophilized rabbit and human plasma samples were reconstituted by the addition of 1/2 to 1/3 of their original volume of distilled water. The samples were centrifuged at 4°C for 20 minutes at 5000 g. A 0.6-ml sample was placed on a 0.9 x 30 cm column, packed with Sephadex G-200 which had been equilibrated with a 0.15 M NaCl, 0.02 M Tris-HCl pH 7.5 solution, and 0.7-ml fractions were collected. Fractions were assayed for the quantity of antithrombin III, and Xa inhibitory activity in the low salt and high salt assay systems.

**Results**

**THE EFFECT OF NaCl CONCENTRATION ON Xa INHIBITORY ACTIVITY**

Figure 1 shows the dilution curves obtained when the Xa inhibitory activity of serially diluted human and rabbit plasma samples are determined in the low salt assay system. A linear plot of the percent human plasma versus log clotting time is obtained, whereas with rabbit plasma serial dilutions produced a rise in Xa inhibitory activity which persists to a 25-fold dilution and then declines.

In contrast, when the Xa inhibitory activity is determined in the high salt assay system, and a clotting time of 30 seconds or less is obtained for undiluted plasma sam-

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**Figure 1** Dilution curves of human —— and rabbit ∆ — — ∆ plasma when assayed in the low salt system. The plasma samples were diluted in physiological saline. The rabbit samples were assayed with a 2-minute preincubation period.

**Figure 2** Dilution curves of human —— and rabbit ∆ — — ∆ plasma when assayed in the high salt system. The plasma samples were diluted in physiological saline. The rabbit plasma dilutions were assayed with a 2-minute preincubation period.
Table 1  Xa Inhibitory Activity in the Low Salt and High Salt Assay Systems

<table>
<thead>
<tr>
<th>Sample</th>
<th>Low salt</th>
<th>High salt</th>
<th>Antithrombin III quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHP</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit plasma</td>
<td>6500*</td>
<td>146</td>
<td>98</td>
</tr>
<tr>
<td>Human antithrombin III</td>
<td>422</td>
<td>468</td>
<td>193</td>
</tr>
<tr>
<td>Rabbit antithrombin III</td>
<td>220</td>
<td>312</td>
<td>96</td>
</tr>
</tbody>
</table>

Xa = activated factor X; NHP = normal human plasma. * Assayed after a 50-fold dilution in physiological saline.

When concentrated human plasma is chromatographed on Sephadex G-200, Xa inhibitory activity is found in two peaks. For the low salt assay, 70% of this activity, found in the second peak, coincides with antithrombin III determined by the quantitative assay (Fig. 3). For the high salt assay system, the second peak contains >95% of this activity.

When concentrated rabbit plasma is chromatographed on Sephadex G-200, Xa inhibitory activity is also found in two peaks. However, the first peak, eluted near the void volume of the column, contains >95% of the activity, when assayed with the low salt system. With the high salt assay, the first peak contains only 65 ± 3% of the total activity whereas the second peak now comprises 35 ± 3% of the activity and cochromatographs with the major peak of antithrombin III as determined by the quantitative assay (Fig. 4). The data presented above are the averages of five separate experiments. Three samples were pooled rabbit plasma, while the other two samples were from single rabbit donors. No significant differences in the qualitative activity were observed.

**Figure 3** The elution positions of (1) antithrombin III (units per ml) as determined in the quantitative assay ●—●, (2) Xa inhibitory activity (percent normal human plasma) determined using the low salt assay ○—○, and (3) Xa inhibitory activity (percent normal human plasma) determined using the high salt assay △—△ after chromatography of concentrated human plasma on Sephadex G-200.

**Figure 4** The elution positions of (1) antithrombin III (units per ml) as determined in the quantitative assay ●—●, (2) Xa inhibitory activity (percent normal rabbit plasma) determined using the low salt assay ○—○, and (3) Xa inhibitory activity (percent normal rabbit plasma) determined using the high salt assay △—△ after chromatography of concentrated rabbit plasma on Sephadex G-200.
TABLE 2  The Effect of Heparin on Xa Inhibitory Activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Xa inhibitory activity (±SD)*</th>
<th>0 unit†</th>
<th>0.025 unit†</th>
<th>0.05 unit†</th>
<th>0.10 unit†</th>
<th>0.25 unit†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human plasma, low salt</td>
<td></td>
<td>100 ± 4</td>
<td>114 ± 4</td>
<td>130 ± 6</td>
<td>170 ± 6</td>
<td>252 ± 10</td>
</tr>
<tr>
<td>Human plasma, high salt</td>
<td></td>
<td>100 ± 6</td>
<td>128 ± 4</td>
<td>180 ± 10</td>
<td>272 ± 8</td>
<td>428 ± 15</td>
</tr>
<tr>
<td>Rabbit plasma, low salt</td>
<td></td>
<td>100 ± 8</td>
<td>83 ± 5</td>
<td>94 ± 8</td>
<td>94 ± 10</td>
<td>106 ± 8</td>
</tr>
<tr>
<td>Rabbit plasma, high salt</td>
<td></td>
<td>100 ± 4</td>
<td>110 ± 5</td>
<td>128 ± 5</td>
<td>154 ± 8</td>
<td>212 ± 10</td>
</tr>
<tr>
<td>Human antithrombin III, low salt</td>
<td></td>
<td>100 ± 8</td>
<td>200 ± 12</td>
<td>245 ± 10</td>
<td>310 ± 20</td>
<td>375 ± 30</td>
</tr>
<tr>
<td>Human antithrombin III, high salt</td>
<td></td>
<td>100 ± 3</td>
<td>250 ± 15</td>
<td>335 ± 20</td>
<td>425 ± 25</td>
<td>&gt;5604</td>
</tr>
<tr>
<td>Rabbit antithrombin III, low salt</td>
<td></td>
<td>100 ± 5</td>
<td>130 ± 8</td>
<td>180 ± 10</td>
<td>290 ± 20</td>
<td>455 ± 30</td>
</tr>
<tr>
<td>Rabbit antithrombin III, high salt</td>
<td></td>
<td>100 ± 6</td>
<td>305 ± 15</td>
<td>460 ± 20</td>
<td>560 ± 30</td>
<td>750 ± 60</td>
</tr>
</tbody>
</table>

* The average of five determinations for each sample.
† Unit of heparin per ml sample; 0 unit of heparin was defined as 100% for each sample.
‡ The clotting time was prolonged to such an extent (>5 minutes) that this figure is a minimum value.
between rabbit and human plasma when Xa inhibitory activity is determined in a low salt assay. These differences can be markedly diminished when Xa inhibitory activity is determined in a reaction mixture containing NaCl near physiological concentrations.

Since a straight line dilution curve can be obtained for rabbit plasma (Fig. 2), changes in Xa inhibitory activity can be determined in plasma obtained from rabbits receiving drugs such as oral contraceptives. Although the distributions of the Xa inhibitory activity in rabbit and human plasma differ, the important question to be answered is if an increase or decrease in this activity will result in a change in the in vivo antithrombotic potential? To investigate this question an assay which can quantitate the Xa inhibitory activity of rabbit plasma is essential. This is accomplished by using the high salt assay system.

In the high salt system the relative contributions of antithrombin III to Xa neutralization in human and rabbit plasma are different. However, in experiments in which Xa inhibitory activity of antithrombin III is altered by heparin, a simple calculation permits translation of rabbit data on the Xa-antithrombin III reaction to man. The purpose for constructing the formula was limited to the goal of demonstrating that by using the high salt assay the effect of heparin on rabbit plasma is predictable and consistent with the effect of this drug on human plasma. This is demonstrated by the highly significant correlation, r² = 0.998, obtained when the measured Xa inhibitory activity of heparinized rabbit plasma is compared to the calculated value, which was obtained by using the observed Xa inhibitory activity of heparinized human plasma.

On the basis of these findings the rabbit model can be used effectively to study the Xa-antithrombin III reaction.

**Acknowledgments**

We are grateful to Riker Laboratories for generously providing hog mucosal heparin.

**Table 3: Predicted Value of Xa Inhibitory Activity of Rabbit Plasma in the Presence of Heparin**

<table>
<thead>
<tr>
<th>Heparin concentration U/ml plasma</th>
<th>Observed Xa inhibitory activity (% human plasma)</th>
<th>Calculated Xa inhibitory activity (% rabbit plasma)</th>
<th>Observed Xa inhibitory activity (% rabbit plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 6</td>
<td>100</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>0.025</td>
<td>128 ± 4</td>
<td>130</td>
<td>110 ± 5</td>
</tr>
<tr>
<td>0.05</td>
<td>180 ± 10</td>
<td>128</td>
<td>128 ± 5</td>
</tr>
<tr>
<td>0.10</td>
<td>272 ± 8</td>
<td>160</td>
<td>154 ± 8</td>
</tr>
<tr>
<td>0.25</td>
<td>428 ± 15</td>
<td>215</td>
<td>212 ± 10</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± so.

* Using the high salt assay system.

† Using the formula: Total Xa inhibitory activity (percent rabbit plasma) = 65% × 0.35 × Xa inhibitory activity (percent human plasma).

‡ Using the high salt assay system.

**References**

The rabbit as an animal model for the activated factor X-antithrombin III-heparin reaction.
S N Gitel, S Wessler and V M Medina

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