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 contraceptive drugs.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 russeli), 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 twice at 1500 g for 10 minutes at 4°C.

Normal human plasma (NHP) was a pool of citrated plasma from six healthy male donors.
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 μM 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° 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 1/4 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

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-
TABLE 1  Xa Inhibitory Activity in the Low Salt and High Salt Assay Systems

<table>
<thead>
<tr>
<th>Sample</th>
<th>Xa inhibitory activity (% NHP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low salt</td>
</tr>
<tr>
<td>NHP</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit plasma</td>
<td>6500*</td>
</tr>
<tr>
<td>Human antithrombin III</td>
<td>422</td>
</tr>
<tr>
<td>Rabbit antithrombin III</td>
<td>220</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 qual-

\[ \text{Figure 3} \] 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.

\[ \text{Figure 4} \] 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.
The elution position of the Xa inhibitory activity found in the first peak, after Sephadex G-200 chromatography of rabbit plasma, was unchanged upon rechromatography of the peak fraction. The Xa inhibitory activity eluting in the first peak (Figs. 3 and 4), from either human or rabbit plasma, was not increased by heparin.

These chromatographic experiments thus confirm the existence in rabbit plasma of an inhibitor to activated factor X other than antithrombin III and also demonstrate the existence of a similar inhibitor in human plasma. The activity of this inhibitor is decreased under physiological assay conditions. Although the total Xa inhibitory activities of human and rabbit plasma as determined in the high salt assay system are similar, the contributions of the specific inhibitory activities are not equivalent.

**THE EFFECT OF HEPARIN ON Xa INHIBITORY ACTIVITY**

The effect of heparin on Xa inhibitory activity of human and rabbit plasma and purified antithrombin III samples was determined using both assay systems (Table 2). A 2-minute preincubation period facilitated the measurement of Xa inhibitory activity in the presence of heparin. Xa inhibitory activity increases with increasing heparin concentration for human plasma in both assay systems. This behavior is also observed, although to a greater extent, with purified human and rabbit antithrombin III. In each instance the increase in activity is more marked in the high salt than in the low salt assay. Such a finding is not unexpected, since it has been shown that heparin augmentation of antithrombin III activity is greatly increased by NaCl at or near physiological concentrations.16

In contrast to human plasma and purified human and rabbit antithrombin III, rabbit plasma exhibits a slight inhibition of Xa inhibitory activity at low heparin concentrations in the low salt assay system. As the heparin concentration is increased, Xa inhibitory activity increases to levels slightly above normal. In the high salt system, however, a continuous increase in Xa inhibitory activity with increasing heparin concentration is obtained, similar to that obtained with human plasma and both purified antithrombin III preparations.

The major Xa inhibitory activity of rabbit plasma measured in the low salt assay system is not dependent on antithrombin III. Since heparin acts as a cofactor only for antithrombin III, even a large increase in the Xa inhibitory activity of antithrombin III in the presence of heparin will result in an insignificant rise in the total Xa inhibitory activity of rabbit plasma in the low salt assay system. The observed decrease in Xa inhibitory activity of rabbit plasma in the presence of heparin in the low salt assay system may be caused by heparin binding to Xa.17

In the high salt assay the Xa inhibitory activity associated with antithrombin III contributes a substantial portion of the total Xa inhibitory activity. Thus, an increase in the Xa inhibitory activity of antithrombin III in the presence of heparin reflects an increase in the total Xa inhibitory activity of rabbit plasma.

We have used the observed Xa inhibitory activity of heparinized human plasma in the high salt system as well as the distribution of this activity in rabbit plasma chromatographed on Sephadex G-200 and assayed in the high salt system to construct an empirical formula relating human and rabbit plasmas containing heparin: Total activity plasma (%) = 65% + 0.35 x human plasma (%). This expression can be stated in the following manner: the total Xa inhibitory activity of rabbit plasma (in percent) at a specific heparin concentration is equal to: 65% [the amount of activity of rabbit plasma (high salt system) which is unaffected by heparin] plus 0.35 [the fraction of the total activity of rabbit plasma (high salt assay) which is identical with antithrombin III and which will increase in the presence of heparin] multiplied by the total Xa inhibitory activity of human plasma (in percent) which contains the same concentration of heparin. Table 3 presents the observed and calculated Xa inhibitory activity for four heparinized rabbit plasma samples using this formula and the observed activity of human plasma at the same heparin concentrations. The correlation coefficient, r², for the observed and calculated values is 0.998 and indicates an excellent fit of the data to the empirical formula.

**Discussion**

The recent report identifying a species-dependent difference in antithrombin III, expressed as Xa inhibitory activity, between human and rabbit plasma in the presence of heparin raised doubts concerning the use of a rabbit animal model to study the Xa-antithrombin III reaction.

The data presented in this communication confirm and extend the qualitative and quantitative differences be-

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**Table 2 The Effect of Heparin on Xa Inhibitory Activity**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Xa inhibitory activity (±SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 unit</td>
</tr>
<tr>
<td>Human plasma, low salt</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Human plasma, high salt</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>Rabbit plasma, low salt</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Rabbit plasma, high salt</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Human antithrombin III, low salt</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Human antithrombin III, high salt</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>Rabbit antithrombin III, low salt</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Rabbit antithrombin III, high salt</td>
<td>100 ± 6</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.
The inhibitory activity in human and rabbit plasma when Xa inhibitory activity is determined in a low salt assay. The difference 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 Xa inhibitory activity to Xa neutralization in human and rabbit plasma.

### Table 3

<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>110</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 ± SD.

* 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


### Acknowledgments

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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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