Respiratory distress syndrome (RDS) is a common condition in premature infants and is characterized by increased permeability, pulmonary edema, and fibrin deposition within the lung's intravascular, interstitial, and intra-alveolar spaces (2, 12, 14). Although surfactant therapy has been used with success in decreasing mortality associated with neonatal RDS, surfactant failure has led to the reduction of bronchopulmonary dysplasia (BPD) (20, 21, 23, 43), suggesting that other mechanisms are important. Some studies (9, 11, 37) suggested that fibrin deposition may contribute to the severity of RDS as well as to the development of BPD. Fibrin monomer, produced by thrombin proteolysis of fibrinogen, leads to impaired surfactant function (31, 41), increased permeability, and further fibrin formation. Fibrin stimulates fibroblast proliferation in the lung (10, 11), which may contribute to the pathogenesis of BPD.

Regulation of thrombin, a key enzyme in coagulation, is critically important to the generation of fibrin. One can hypothesize that if thrombin activity within the alveolar space can be downregulated, fibrin deposition can be decreased and can potentially reduce the incidence and severity of BPD. Heparin has been used as an anticoagulant for both the treatment and prophylaxis of intravascular thrombosis. However, when heparin was directly instilled into the lung, it was not retained within the lung (19). Furthermore, in the absence of antithrombin (AT), heparin would have no AT activity within the lung. Although plasma proteins may diffuse into the lung during RDS, these molecules may not all reach the alveolar space in the same relative proportions, such that the amount of AT present for interaction with heparin may still be insufficient. This scenario is likely because it has been shown that there is increased binding of AT to the subendothelial extracellular matrix compared with fibrinogen (45) due to the presence of proteoheparan sulfate molecules (18). To obviate these difficulties, we developed a novel AT-heparin covalent complex (ATH) with high specific AT activity. Our studies showed that ATH is retained within the rabbit lung for at least 48 h and that ATH extracted from the lung 48 h after its administration still retained AT activity (8).

Rat fetal distal lung epithelium (FDLE) provides an in vitro system that has been used to study epithelial cell regulation of thrombin (29). Previously, our laboratory (1) has shown that the FDLE surface is procoagulant and promotes thrombin generation in plasma as well as secreting factor VII (FVII)-dependent tissue factor (TF) activity. However, FDLE also expresses glycansaminoglycans that have AT catalytic activities (29) and may promote thrombin inhibition through plasma inhibitors bound to receptors on the cell surface (1). Thus, given the complicated balance of coagulant factors expressed by FDLE, it would be advantageous to regulate thrombin generation by a controlled administration of an anticoagulant such as ATH. Although we have shown that active [anti-factor Xa (FXa)] ATH could be recovered from the rabbit lung 48 h after administration, it was not determined whether ATH could inhibit thrombin generation on the alveolar surface. Because the AT and heparin components of ATH cannot dissociate, its pharmacokinetics may be different compared with free AT plus heparin due to altered binding to the FDLE plasma membrane or endocytosis. Also, the mechanism by which ATH could affect thrombin generation may be complicated because it has been shown that ATH has direct AT activity as well as the ability to catalyze inhibition of thrombin by exogenous...
AT (8). For these reasons, it was necessary to investigate the potential effect of ATH on thrombin generation on FDLE in vitro before proceeding to more complicated in vivo models of lung injury.

In this study, we have provided further proof that thrombin generation on the surface of the FDLE was TF dependent. We also showed that thrombin generation on the surface of FDLE could be suppressed by anticoagulants and that ATH was more efficient than either standard heparin (SH) or SH + AT. Some of the possible mechanisms by which ATH inhibits thrombin generation on FDLE are discussed.

**MATERIALS AND METHODS**

Cell culture. FDLE was isolated from fetal rats and grown in primary culture according to methods previously described in detail (30). In brief, lungs from fetal rats (Wistar, Charles River) of 20-day gestational age (term = 22 days) were removed, and epithelial cells were isolated and separated from fibroblasts by differential adherence. Cells were plated out in 24-well Nunc plastic plates (Gisco BRL) at 2 × 10⁶ cells/well. FDLE was grown in a 37°C incubator with a 95% humidified room air-5% CO₂ atmosphere. Previous analyses have shown that the cells cultured by this method were >90% type II epithelial with ≤5% fibroblast contamination (30). All studies were completed on the cells in primary culture before the first passage and within 3 days of harvest.

Thrombin generation. All plasmas used were from adult human donors. Thrombin generation studies were conducted with control pooled plasma from healthy adults or FVII-deficient plasma (Instrumentation Laboratory, Lexington, MA). FVII levels were ≤1% according to the manufacturer. Plasma from adults was obtained by mixing whole blood with 3.8% sodium citrate (9 parts blood to 1 part citrate) followed by centrifugation for 20 min at 3,000 g to obtain supernatant plasmas.

The method for measuring thrombin generation on FDLE has been reported previously (29). Reactions were done on FDLE monolayers in 24-well plates that were placed on a metal block on a Thermolyne drier-bath set at 37°C. After the plates were removed from the incubator atmosphere and placed on the heated block in room air, thrombin generation was not carried out until the FTC of the culture medium (measured by testing aliquots on litmus paper) had increased to 7.2 but not >7.6. Each well of confluent FDLE monolayer was washed with 2 × 1 ml of buffer (0.036 M sodium acetate, 0.036 M sodium diethylbarbiturate, and 0.145 M NaCl, pH 7.40) and was then incubated for 3 min with 100 µl of buffer and 200 µl of defibrinated plasma (prepared by incubating 500 µl of plasma with 15 µl of 6 U Ancrod/ml buffer at 37°C for 10 min, washing out the clot, and washing out any further clot formed after incubating on ice for another 10 min). A clock was started as 100 µl of 0.04 M CaCl₂ in buffer were added, and at various times, 25-µl aliquots of the reaction mixture on the surface of the FDLE were removed and mixed with 475 µl of 0.005 M Na₂-EDTA on ice. Twenty-five microliters of each EDTA sample were then mixed with 775 µl of 0.0016 M S-2238 (KabiVitum, Stockholm, Sweden) in buffer and heated at 37°C for 10 min before termination of the amidolytic reaction by the addition of 200 µl of 50% acetic acid. The absorbance at 405 nm was measured, and the concentration of thrombin was determined by comparing results to a standard curve generated with thrombin in S-2238. EDTA samples were also used to measure the concentrations of prothrombin, thrombin-AT (TAT) complexes, and thrombin-heparin cofactor II (IIa-HCII) complexes. Because thrombin bound to α2-macroglobulin (α2-M) retains activity against small substrates (5), the contribution of thrombin-α2-M (IIa-α2-M) to total thrombin activity was measured with a previously described method (29). In this case, the same method as the one described above was used for total thrombin except that 50 µl of the reaction mixture were taken at each time point and incubated with 0.007 ml of 0.15 M NaCl containing 0.5 U SH and 0.084 U AT (to inhibit any free thrombin) for 1 min on ice before mixing 25 µl of the incubate with 475 µl of Na₂-EDTA. Any thrombin activity measured was due only to α2-M-bound thrombin. Subtraction of the IIa-α2-M activity from the total thrombin activity gave the amount of free thrombin. In one set of experiments, FXa generation was estimated with the same procedure as that above for thrombin generation except that S-2222 (KabiVitum) was used as the substrate.

TF dependence of thrombin generation on FDLE. To verify that thrombin generation on the surface of FDLE was TF dependent, FVII-depleted plasma was used, and the results were compared with experiments using control plasma. To investigate the degree to which the intrinsic pathway participates in any thrombin generated with FVII-depleted plasma, the following experiments were done. Thrombin and IIa-α2-M generation were carried out in plastic tubes at 37°C as described above in Thrombin generation except that 135 µl of defibrinated plasma, 15 µl of buffer (containing, in some cases, either 4 µl of 49.9 mg anti-human factor XI (FXI) goat IgG/ml or 11.6 µl of 16.96 mg nonimmune goat IgG/ml (both from Affinity Biologicals, Hamilton, ON)) were mixed with 33 µl of TF reagent (Thromborel S human thromboplastin PT reagent; Behringwerke, Marburg, Germany) + 117 µl of 0.04 M CaCl₂ in buffer at 0 min time. The effect of the anti-FXI antibody on the thrombin generation in FVII-deficient plasma on FDLE was also investigated. Attenuation of the production of free thrombin by the blockade of FXI activation would indicate that contact activation of the intrinsic pathway was involved in thrombin generation in the absence of FVII (extrinsic pathway).

Suppression of FDLE promoted thrombin generation by ATI and other anticoagulants. To compare the AT activity of ATI (>98% free of starting AT and heparin prepared as described previously (8)) with that of SH (grade I-A, sodium salt; Sigma, Mississauga, ON) and that of AT (single band on SDS-PAGE and >90% functional AT activity; Bayer, Mississauga, ON) in SH + AT, each anticoagulant was diluted in buffer and incubated with defibrinated control plasma on the surface of FDLE. Equivalent amounts, by mass, of AT and/or SH were used in each group. The heparin and AT concentrations in the recalculated reaction mixtures were 0.128 and 0.505 µg/ml, respectively. Thrombin generation assays were measured in the presence of FDLE for buffer alone, ATI in buffer, AT + SH in buffer, and SH in buffer.

Prothrombin consumption. The EDTA time samples were used to determine prothrombin consumption during the experiments. Prothrombin concentrations were measured at each time point during the thrombin generation experiments with a commercially available ELISA (Affinity Biologicals). Control plasma with a known prothrombin concentration was used as a standard.

Thrombin inhibition (thrombin-inhibitor complexes). The EDTA time samples were used to determine the TAT and IIa-HCII complexes formed during the experiments. TATs were measured with an ELISA kit (Affinity Biologicals). Purified TATs (Affinity Biologicals) placed in control plasma were used as standards. It was verified that thrombin-ATH (IIa-ATH) could be detected by the ELISA kit from analyses of the reaction of human thrombin in rabbit plasma. Only IIa-ATH gave a positive result because rabbit AT could not be detected by the anti-human AT antibody. IIa-HCII complex was measured with an ELISA kit (Affinity Biologicals).
Purified IIa-HCII complexes (Affinity Biologicals) placed in control plasma were used as standards.

Statistics. Results are reported as means ± SE unless otherwise indicated. Comparisons among different groups were made by repeated-measures ANOVA. For time-course experiments, repeated-measures ANOVA over time was compared among groups. On finding significance with ANOVA, the ATH group was then compared with the ATSH group. Unpaired Student’s t-test was used when only two groups were compared. The rate of prothrombin consumption was obtained by calculating the slope over the first 4 min using linear regression. Values were considered statistically different for P values < 0.05.

RESULTS

Influence of FDLE on thrombin generation in control plasma and FVII-deficient plasma. In control plasma, free thrombin was generated rapidly, with peak activities achieved 4 min after the addition of calcium. However, in FVII-deficient plasma, peak thrombin generation was both delayed and decreased (Fig. 1). The total amount of free thrombin differed significantly between control plasma and FVII-deficient plasma (P < 0.001). These data confirm that TF is present on the FDLE, which accelerates thrombin generation because the absence of FVII in the plasma delayed and decreased free thrombin activity. To determine whether the intrinsic pathway was involved in the generation of thrombin when FVII was absent, experiments were carried out with an anti-human FXI antibody to block FXI activation. Thrombin generation in control plasma with calcium+TF on a plastic surface was slightly delayed when anti-FXI antibody was present in the reaction mixture (Fig. 2A). When FVII-deficient plasma was reacted with calcium+TF on plastic, FXI antibody caused thrombin generation to be significantly decreased and delayed (Fig. 2B). These effects were not seen if nonimmune IgG was used as a control. The reaction between FVII-deficient plasma+calcium and added anti-human FXI antibody on an FDLE surface resulted in no detectable generation of thrombin after 32 min. These data verify that thrombin generation in FVII-deficient plasma was likely enhanced by FXI activation through the intrinsic pathway.

Influence of FDLE on prothrombin consumption in control plasma and FVII-deficient plasma. The pattern of prothrombin consumption during thrombin-generation experiments differed dramatically between control plasma and FVII-deficient plasma. In control plasma,
after the addition of calcium, prothrombin was rapidly consumed by 4 min (65% depleted), which coincided with the peak of thrombin generated (Fig. 3). However, in FVII-deficient plasma, there was no significant consumption of prothrombin from 0.5 to 4 min, and a significant decrease in prothrombin was not apparent until 8 min (Fig. 3). The concentration of prothrombin remaining at the end of the experiments was negligible in control plasma, whereas the concentration of prothrombin remaining in FVII-deficient plasma was ~30%. These results were consistent with the delay and decreased amounts of thrombin generated in the FVII-deficient plasma compared with the control plasma and demonstrate the effect of FVII (TF) on prothrombin consumption.

Thrombin-inhibitor complex formation in control plasma and FVII-deficient plasma. Thrombin-inhibitor complex formation was significantly less in the FVII-deficient plasma compared with the control plasma (P = 0.02; Table 1). In addition, the appearance of peak concentrations of TAT and IIa-HCII complexes was delayed in the FVII-deficient plasma compared with the control plasma. These results were in agreement with the decreased conversion of prothrombin to thrombin in FVII-deficient plasma.

Suppression of thrombin generation on FDLE by ATH. The amount of thrombin generated on the surface of FDLE was statistically different (P < 0.001) among all four groups (ATH, AT + SH, SH, and buffer). ATH suppressed thrombin generation to a greater extent than did AT + SH (P < 0.001; Fig. 4). Significant amounts of free thrombin were generated 1–2 min after the start of the reaction in the AT + SH, SH, and buffer groups, whereas no thrombin activity was detected until 4 min in the ATH group. The maximum amount of thrombin generated occurred at 4 min for the AT + SH, SH, and buffer groups and at 4–8 min for the ATH group. ATH decreased the peak amount of thrombin generated by 80% (Fig. 4). An attempt was made in one set of experiments to measure free FXa generation on the FDLE. A small peak of activity against FXa substrate (S-2222) was observed at 1 min in buffer, which was inhibited by either ATH, SH, or AT + SH.

Prothrombin consumption on FDLE in the presence of ATH. Because peak amounts of thrombin occurred after 4 min, we compared the rate of prothrombin consump-

**Table 1. Thrombin-inhibitor complex formation in control and factor VII-deficient plasma on surface of rat FDLE**

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Total Inhibitor Complexes, nM</th>
<th>TAT, %</th>
<th>IIa-HCII, %</th>
<th>IIa-α2M, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VII deficient</td>
<td>186 ± 16</td>
<td>44</td>
<td>15</td>
<td>41</td>
</tr>
<tr>
<td>Control</td>
<td>356 ± 27*</td>
<td>43</td>
<td>15</td>
<td>42</td>
</tr>
</tbody>
</table>

Values are means ± SE. Each inhibitor complex is expressed as a percentage of total amount of complexes formed. FDLE, fetal distal lung epithelium; TAT, thrombin-antithrombin complex; IIa-HCII, thrombin-heparin cofactor II complex; IIa-α2M, thrombin-α2-macroglobulin complex. *Total concentration of inhibitor complexes was significantly decreased in factor VII-deficient plasma compared with control plasma, P = 0.02. Distribution of thrombin-inhibitor complexes remained constant.
that FDLE promotes the generation of thrombin andsis of fibrinogen (6). Previous work (1, 16) has shown
system, generation of thrombin, and thrombin proteoly-
Fibrin is formed after activation of the coagulation
feature of neonatal RDS, promotes fibroblast prolifera-
BPD. Fibrin deposition in the lung, a pathological
mation between the ATH group and the AT+SH group up
to that time point. The rate of prothrombin consump-
tion was significantly slower in the presence of ATH
AD compared with AT+SH (P < 0.01; Fig. 5). The concentra-
tions of prothrombin left at 32 min in the presence of
ATH were significantly increased compared with
AT+SH (P < 0.01; Fig. 5).
Thrombin-inhibitor complexes in the presence of ATH.
Concentrations of thrombin-inhibitor complexes were
significantly less in the ATH group compared with the
AT+SH group (P < 0.05; Table 2). Peak TAT was
achieved earlier in the ATH group (4–8 min) compared
with the AT+SH group (8 min) and the SH or buffer
group (>8 min). However, peak concentrations of IIa-
IIa-HCII complex occurred at the same rate for all groups
(4 min).

**DISCUSSION**

The failure of surfactant to reduce the incidence of
BPD in premature infants has stimulated the investiga-
tion of other potentially responsible pathological mecha-
nisms contributing to neonatal RDS and subsequent
BPD. Fibrin deposition in the lung, a pathological
feature of neonatal RDS, promotes fibroblast proliferation
that contributes to the development of BPD (15).
Fibrin is formed after activation of the coagulation
system, generation of thrombin, and thrombin proteoly-
sis of fibrinogen (6). Previous work (1, 16) has shown that FDLE promotes the generation of thrombin and

![Graph](http://ajplung.physiology.org/)

**Table 2. Thrombin-inhibitor complex formation on rat
FDLE in presence of anticoagulants**

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Total Inhibitor Complexes, nM</th>
<th>TAT, %</th>
<th>IIa-HCII, %</th>
<th>IIa-ATM, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>351 ± 33</td>
<td>51</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>SH</td>
<td>280 ± 24</td>
<td>51</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td>AT + SH</td>
<td>349 ± 20</td>
<td>57</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td>ATH</td>
<td>241 ± 32*</td>
<td>52</td>
<td>5</td>
<td>43</td>
</tr>
</tbody>
</table>

Values are means ± SE. Each inhibitor complex is expressed as a percentage of total amount of complexes formed. AT, antithrombin; SH, standard heparin; ATH, antithrombin-heparin covalent complex.

that the TF pathway may be important. Our results
show that the generation of thrombin in FVII-deficient
plasma on the surface of FDLE is impaired compared
with that in control plasma (Fig. 1). These data provide
further evidence for the importance of the TF pathway
in the generation of thrombin on the apical FDLE
surface. Our results also show that a novel inhibitor of
thrombin, ATH, more effectively inhibits thrombin gen-
eration on the surface of FDLE than either SH alone or
AT and SH in combination (Fig. 4).

Neonatal RDS is a complex disease that likely has
multiple pathological mechanisms contributing to its
severity and subsequent development of BPD (15). The
presence of fibrin in the lung in neonatal RDS, fibrin
monomer inhibition of surfactant function (31, 41), and
fibrin promotion of fibroblast proliferation (10, 11) all
support the concept that fibrin contributes to the
severity of both neonatal RDS and BPD. Early use of
dexamethasone and surfactant reduces the incidence of
BPD in very low birth weight infants (20, 21, 23, 43).
Dexamethasone reduces vascular permeability that
decreases protein leakage into the alveolar space (42).
Preventing leakage of coagulation proteins, particu-
larly fibrinogen, into the alveolar space will decrease
fibrin formation, which may be beneficial in neonatal
RDS.

The mechanism(s) by which fibrin is formed in the
lung is important to understand in order to develop
strategies to inhibit this process. Although blood coagu-
lation can theoretically be initiated by the contact
system or by exposure of blood to TF, the latter is the
physiologically important activation pathway (4, 26).
In this pathway, activated FVII (FVIIa) binds to TF and
subsequently activates factor X (25) as well as other
feedback loops (27, 34, 35). FXa in combination with
activated factor V, calcium, phospholipid, and prothrom-
bin form the prothrombinase complex that converts
prothrombin to thrombin (24). TF, an extracellular
lipoprotein bound to the membranes of cells that synthe-
size it (17), functions as a cell-surface receptor for
FVIIa (34). TF is produced by several cell types and is
present in almost all tissues. It has been shown that rat
adult lung epithelial cells express TF (16). Therefore, it
is likely that TF is important for thrombin generation
on the surface of FDLE and contributes to fibrin
formation.
To provide further evidence regarding the importance of TF in the generation of thrombin on the surface of FDLE, we compared FVII-depleted plasma with control plasma (which contained physiological amounts of FVII). The amount of thrombin generated (Fig. 1) and prothrombin consumed (Fig. 3) was significantly decreased in FVII-depleted plasma compared with control plasma. A significant amount of the thrombin generated in FVII-deficient plasma was likely due to activation reactions in the intrinsic pathway because addition of anti-FXa antibody caused the appearance of free thrombin to be both delayed and decreased (Fig. 2).

Prothrombin consumption was also delayed in FVII-deficient plasma relative to control plasma (Fig. 3), which agreed with the delay in appearance of peak TAT and IIa-HCII complex formation (Table 1). The initial increase in prothrombin from 0 to 0.5 min was probably due to denatured prothrombin adsorbed onto cell surfaces because there was no thrombin generated during that period of time. At times \( t \geq 4 \) min, very little prothrombin remained in the normal control plasma for conversion to free thrombin, whereas prothrombin-to-thrombin conversion was slow throughout the experiments in FVII-deficient plasma. Because little prothrombin remained in the normal plasma at \( t \geq 4 \) min, all of the thrombin that could be generated was available for conversion to inhibitor complexes, which reduced the free thrombin concentrations observed at \( t \geq 8 \) min to the levels detected in the depleted plasma. Reduction in thrombin generation and prothrombin consumption in the absence of FVII shows that the TF pathway is the predominant pathway by which thrombin is generated on the surface of FDLE.

Another factor related to TF that may be involved in thrombin generation on FDLE is the TF pathway inhibitor (TFPI). TFPI inhibits FXa directly by a calcium-independent binding of FXa through the second Kunitz domain of TFPI (13). Also, TFPI can inhibit FVIIa activity by a calcium-dependent mechanism that involves binding of the first Kunitz domain of TFPI to FVIIa either by interaction of TFPI-FXa complexes with FVIIa-TF complexes (13) or by interaction of TFPI with FXa-FVIIa-TF complexes (7). In vivo, TFPI is produced in endothelial cells (3, 44), but it is unclear whether TFPI is made by other cells (38). Although TFPI has been detected in lavage fluid from adult patients that are at risk for RDS, as well as those with RDS (36), it has not been shown whether alveolar epithelial cells produce the inhibitor. Intravascularly, TFPI is found either bound to the vessel wall (50–80% of total), circulating in plasma, or in platelets (38). TFPI in the plasma phase exists in concentrations of 50–150 ng/ml (28), but >85% is present as lipoprotein complexes with low anticoagulant activity (22). During RDS, increased lung permeability may allow for access of plasma TFPI to the alveolar space, but it is unclear how much of free plasma TFPI may appear in the lung fluid because mesenchymal and epithelial cell TFPI binding is largely unknown. Nevertheless, immunodepletion studies in rabbits have shown that TFPI is necessary to prevent disseminated intravascular coagulation elicited by low doses of TF (39). Further experiments are required to determine the possible involvement of TFPI in thrombin generation on FDLE surfaces.

Effective inhibition of thrombin generation or thrombin itself by an anticoagulant offers a potential therapeutic modality for neonatal RDS in addition to surfactant. Although an anticoagulant could be administered systemically, the concentrations within the alveolar space would likely be low, particularly before significant damage of the lung. Another route of administration is directly into the lung, with the goal of preventing thrombin generation on the surface of FDLE before significant lung damage. Physiologically, there are several processes by which thrombin formation is regulated. Therapeutically, heparin and related compounds are the most frequently used agents to regulate thrombin. Heparin is a heterogeneous compound with an average molecular weight of 15,000 and a range of 3,000–30,000. Heparin contains a pentasaccharide sequence that binds to the inhibitor AT, converting it into a rapid inhibitor of many serine proteases including thrombin (33). Heparin has been administered previously into the lung in adults and was found to be rapidly absorbed (19). Thus the direct administration of heparin alone into the lung is not likely a viable option for neonatal RDS. Purified AT is available and could be administered into the lung; however, AT is a relatively weak inhibitor of thrombin in the absence of heparin (33). We have previously produced a covalent conjugate of AT to heparin and called it ATH. In the absence of a cellular surface, Chan et al. (8) have shown that ATH has significantly greater AT activity compared with a combination of SH and AT that are not covalently linked. However, the effect of ATH on the mechanisms involved in thrombin generation were not studied. Unlike assays that measure inactivation of a limited amount of exogenous thrombin, inhibition of thrombin generation relies on the effects of the anticoagulant on inhibition of feedback activation of the coagulation cascade, as well as on catalytic capacity (32). It was unclear whether the concentration of ATH used in this study (8 nM) would be sufficient to significantly prevent feedback activation by direct, noncatalytic reaction with the initial thrombin (or FXa) generated, and the level of ATH catalytic activity (=0.1 U/ml) employed has been shown previously, in experiments with heparin, to give only modest effects on thrombin generation (40). Furthermore, although it has been demonstrated that ATH remains in the lungs of rabbits for at least 48 h, with high anti-FXa activity and essentially no leakage into the systemic circulation (8), the effect of FDLE on the capacity of ATH to inhibit thrombin generation was not determined. If ATH were sequestered by FDLE to a significant degree (either by binding to the plasma membrane or by endocytosis), the AT activities could have been diminished. Thus, although ATH had many properties that suggested that it might be an anticoagulant that could be administered prophylactically into the lungs of neonates with RDS, it was necessary to investigate thrombin generation on FDLE in the pres-
ence of ATH before proceeding to an animal model of neonatal RDS.

Our results showed that ATH was considerably more effective in regulating thrombin generation in the presence of FDLE compared with AT+SH (Fig. 5, Table 2). Because it has been shown previously that the direct inhibition of thrombin by AT is extremely rapid compared with that of AT+SH (8), the delay in the appearance of free thrombin with AT (Fig. 4) may be due to a rapid inhibition of the thrombin formed initially, which would block thrombin-mediated feedback activation of the coagulation cascade (32). This hypothesis is in agreement with the observation that although TAT concentrations were lower with ATH, peak TAT formation occurred earlier compared with the other groups (Table 2). The superior AT activity may be due to the conjugation of AT to heparin because one equilibrium step in the inhibition of thrombin by AT and heparin was not required with ATH. This property may be particularly important for the potential effectiveness of ATH in the intra-alveolar space.

Experiments are underway to test the superior activity of ATH against thrombin generation on lung epithelium in a rat damaged lung model.

In summary, we have shown that the TF pathway plays a significant role in the generation of thrombin on the surface of rat FDLE. A novel ATH was shown to effectively suppress thrombin generation on FDLE, with superior activity compared with those of AT and heparin.

We thank Sue Smith for technical assistance in animal care and LuAnn Brooker for assistance in preparation of the manuscript. This work was supported by Project 7 from the Medical Research Council of Canada Group in Developmental Lung Biology. M. Andrew holds a Career Investigator Award from the Heart and Stroke Foundation of Canada. A. Chan holds a Research Fellowship Award from the Research Institute at the Hospital for Sick Children, Toronto, Canada.

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Received 6 August 1997; accepted in final form 2 March 1998.

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