Anticoagulant Activity

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Although heparin has been used clinically for prophylaxis and treatment of thrombosis, it has suffered from problems such as short duration within compartments in vivo that require long term anticoagulation. A covalent antithrombin-heparin complex has been produced with high anticoagulant activity and a long half-life relative to heparin. The product had high anti-factor Xa and antithrombin activities compared with noncovalent mixtures of antithrombin and heparin (861 and 753 units/mg versus 209 and 198 units/mg, respectively). Reaction with thrombin was rapid with bimolecular and second order rate constants of 1.3 × 10^6 M⁻¹ s⁻¹ and 3.1 × 10^5 M⁻¹ s⁻¹, respectively. The intravenous half-life of the complex in rabbits was 2.6 h as compared with 0.32 h for similar loads of heparin. Subcutaneous injection of antithrombin-heparin resulted in plasma levels (peaking at 24–30 h) that were still detectable 96 h post-injection. Given the increased lifetime in these vascular and intravascular spaces, use of the covalent complex in the lung was investigated. Activity of antithrombin-heparin instilled into rabbit lungs remained for 48 h with no detection of any complex systemically. Thus, this highly active agent has features required for pulmonary sequestration as a possible treatment for thrombotic diseases such as respiratory distress syndrome.

Fibrin deposition in the intravascular system causes significant morbidity in the form of deep vein thrombosis, pulmonary embolism, stroke, and myocardial infarction. Heparin is an anticoagulant that, when administered systemically, is effective for both the prevention and treatment of intravascular thromboembolic events (1). Fibrin deposition also occurs outside the intravascular system in a variety of organs and contributes to the morbidity of several diseases (2, 3). One example is neonatal respiratory distress syndrome (RDS), which is characterized by fibrin deposition in the extravascular, interstitial, and intra-alveolar spaces (4, 5). Neonatal RDS is a complex disease that probably has multiple pathologic mechanisms contributing to its severity, and the subsequent development of the chronic lung disease, bronchopulmonary dysplasia (BPD) (6–8). The potential importance of fibrin to the severity of neonatal RDS and BPD is supported by the pathologic presence of fibrin in the lung in neonatal RDS, fibrin monomer inhibition of surfactant function (7), and fibrin promotion of fibroblast proliferation (6). The potential role of anticoagulants such as heparin for the prevention of extravascular fibrin deposition in neonatal RDS has not been assessed previously.

A significant limitation for the potential benefit of anticoagulants in most disease states characterized by extravascular fibrin deposition is that anticoagulants cannot be administered locally. One exception is the lung, where anticoagulants can be administered directly into the airways. Heparin has been administered to adult patients via the lung for the purposes of systemic anticoagulation for intravascular thrombosis (9). Unfortunately, heparin itself is not an ideal anticoagulant to administer into the lung in newborns with RDS because of heparin’s rapid entry into the intravascular system and its dependence on antithrombin (AT) for activity, which would probably not be present in the intra-alveolar space in the early stages of neonatal RDS. Although purified AT is available, it is a relatively poor inhibitor of thrombin in the absence of heparin (10).

Heparin covalently linked to AT would probably be retained in the lung because of its size. If its anticoagulant activities were retained, heparin complexed to AT could function locally as an anticoagulant. Heparin has previously been covalently linked to AT for the purposes of prolonging its half-life when administered intravascularly (11, 12). The major difficulty encountered in earlier attempts to conjugate AT to heparin was that the introduction of reactive groups for linkage, on AT or heparin, led to complexes with reduced anticoagulant activities. Additionally, the presence of excess unblocked linkage groups in the products could have had unwanted interactions with other molecules or surfaces in vivo.

Recently, we described the synthesis of a unique covalent complex (ATH), formed from AT and standard commercial heparin, which required no modification of either the protein or glycosaminoglycan (GAG) prior to conjugation. Production of ATH was accomplished by taking advantage of the fact that subpopulation of heparin molecules contain aldose termini and the concept that, during prolonged incubations, a Schiff base

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‡ The abbreviations used are: RDS, respiratory distress syndrome; BPD, bronchopulmonary dysplasia; AT, antithrombin; H, heparin; ATH, antithrombin-heparin; GAG, glycosaminoglycan; FPR, phenyl-lalanyl-prolyl-arginyl; PAGE, polyacrylamide gel electrophoresis; BAL, bronchoalveolar lavage; HASH, high affinity standard heparin.

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may form spontaneously between AT lysyl ε-amino and heparin aldose aldehyde groups. Amadori rearrangement could then occur, if there were a free hydroxyl group on C2 of the terminal aldose aldehyde groups. Amadori rearrangement could then be eluted with 0.02M phosphate, pH 7.0, and dialyzed against 0.01 M to butyl-agarose beads while heparin did not. The bound material was previously.2 The relative quantities of AT and heparin to be used in the agarose/DEAE) procedure.

Complex Preparation—Production of ATH has been described previously.7 The relative quantities of AT and heparin to be used in the incubations were determined from an estimate of the fraction of heparin molecules containing both a pentasaccharide (13) and an aldose (14) at their termini (Table I). In brief, a covalent complex between AT and heparin was prepared by incubating 0.5–3 mg/ml AT with 50–70 mg/ml heparin in buffer (0.3 M phosphate, 1 M NaCl, pH 8.0; or 0.02 M phosphate, 0.15 M NaCl, pH 7.3) for 3–14 days at 40 °C. Subsequently, 0.1 ml of 0.5 M NaBH4/CN in buffer/ml of reaction mixture was added at 37 °C and incubated for an additional 5 h, allowing reduction of any Schiff base not stabilized by Amadori rearrangement.

Complex Purification—Subsequently the covalent ATH product was purified by gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden) using 2 ml NaCl for elution. A high molecular weight fraction containing covalent ATH complex was isolated and was essentially void of free AT. The ATH fraction was further purified by electrophoresis on a 7.5% polyacrylamide gel at pH 8.8, using nondenaturing conditions. The section of gel that contained only the ATH complex was cut out and the ATH eluted by incubation in buffer (3.0 g/liter Tris, 14.4 g/liter glycine, pH 8.8) at 23 °C. Purification of ATH was also accomplished on butyl-agarose in one step. Adjustment of the (NH4)2SO4 concentration from 2.5 to 1.8 M allowed pure ATH to be eluted from the beads while AT remained bound. Large quantities of ATH were purified from the reaction mixture by a two-step procedure involving hydrophobic chromatography on butyl-agarose (Sigma) followed by anion exchange chromatography on DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden). In 2.5 M (NH4)2SO4, 0.02 M phosphate, pH 7.0, ATH and AT bound to butyl-agarose beads while heparin did not. The bound material was eluted with 0.02 M phosphate, pH 7.0, and dialyzed against 0.01 M Tris-HCl, pH 8.0 buffer, then mixed with pre-equilibrated DEAE Sephacrose beads. After washing the beads with 0.2 M NaCl in pH 8.0 buffer to remove bound AT, ATH was eluted with 2 ml NaCl in pH 8.0 buffer. The purified ATH was concentrated at 4 °C by dialysis, with a M, 12,000–14,000 cut-off, under nitrogen pressure (1 atmosphere). All further analyses were carried out using ATH purified by the two-step (butyl-agarose/DEAE) procedure.

Analysis of ATH Complex—The ATH complex was subjected to heparin degradation by incubation at 37 °C with 0.01 unit/ml heparinase, 0.001 ± CaCl2, 0.001 M sodium acetate, 0.15 M NaCl. Protease digestion of the ATH complex involved reaction at 37 °C for 96 h in 0.5 M Tris-HCl, pH 8.0, with the general protease P-5417 (Sigma), which contained no heparin-degrading activity. P-5417 was pH 7.5 initially, followed by additions of another 2 mg/ml every 24 h. Following SDS-PAGE as per Laemmli (15), the GAG portion of ATH was stained sequentially with alcian blue and silver (16). The AT portion of ATH was stained using Coomassie Blue. To estimate the molar ratio of AT to H in ATH, SDS-PAGE gels of heparinase-treated ATH (with multiple AT standards), and protease-treated ATH (with multiple samples of known heparin mass), were prepared. They were stained for protein and GAG, respectively. The relative mass of the ATH components was determined by comparing laser densitometry readings against the similarly quantitated standards. The AT to heparin molar ratio was calculated using a molecular weight of 59,000 for AT and an average molecular weight of 15,000 for heparin. Western immunoblots of ATH employed standard techniques (17) with a sheep anti-human AT antibody (Affinity Biologicals, Hamilton, Ontario, Canada). Anti-factor Xa and antithrombin activities were determined on an ACL300 using kits (Stachrom anti-Xa, Diagnostica Stago, Asnières, France; IL test anti-IIa, Instrumentation Laboratory, Milano, Italy). The method for measuring activity involved incubation of the sample with excess enzyme (factor Xa or thrombin) for 30 s in buffer (containing, in some cases, exogenous AT), followed by addition of a chromogenic peptide substrate to determine the residual activity. The amount of enzyme activity inhibited during the reaction was compared with a standard curve, constructed from reactions with plasma samples containing standard heparin (from Diagnostica Stago) of known concentrations (units/ml). The units/ml of the sample was divided by the mg/ml of heparin present to obtain specific activities for the compound. To check that antithrombin activity was due to covalent binding of thrombin, SDS-PAGE of ATH reacted with 125I-thrombin (labeled by chloramine T; Ref. 18) was performed followed by autoradiography. Fluorescence intensity measurements of buffered (phosphate-buffered saline) solutions of ATH, AT + heparin, and AT + exogenous heparin were made using a Perkin-Elmer LS50B luminescence spectrometer. AT protein concentrations in rabbit plasma and bronchoalveolar lavage (BAL) samples were measured using an antibody that did not cross-react with rabbit AT (Affinity Biologicals).

Kinetics of Thrombin Inhibition—The methods used to determine the bimolecular and second order rate constants were the same as those used by Hoylaerts et al. (10). Constants for the hypothetical kinetic mechanism shown below (Reaction 1) were determined.

\[

t_1 \quad \text{Ila} + \text{ATH} \quad \text{Ila} \cdot \text{ATH} \quad \text{Ila-ATH}
\]

\[
\begin{align*}
& k_1 \\
& k_2
\end{align*}
\]

REACTION 1

Semilog plots of remaining thrombin(Ila) activity versus time were used to calculate apparent rate constants (k_{app} = \ln t_{1/2}, where \( t_{1/2} \) is the time required for disappearance of half of the starting thrombin activity). Double-reciprocal plots of k_{app} versus [ATH] allowed determination of the bimolecular rate constant as the ratio kJK, where k_1 is the 1/ordinate intercept and K_1 is the abscissa intercept. Use of FPR-thrombin as a competitive inhibitor in the reaction of thrombin + ATH permitted calculation of the IC_{50} (\text{Ila-ATH}). The second order rate constant, k_1, could then be calculated from the equation K_2 = \frac{k_1}{k_2}, where K_2 is the equilibrium constant for disappearance of the thrombin-ATH intermediate complex.

In Vivo Pharmacokinetics—Rabbits were injected either intravenously or subcutaneously with different amounts of ATH, AT, H, or AT + H in 1–2 ml of 0.15 M NaCl, 0.02 M phosphate, pH 7.3. At predetermined times, 0.5-ml samples of blood were taken into 3.8% sodium citrate (volume blood:citrate = 9:1), centrifuged at 3000 × g for 15 min, and the supernatant plasma stored at −60 °C. Plasma samples were analyzed for anti-factor Xa activity and AT protein concentrations as described previously. Half-lives of the various species were calculated by nonlinear least squares fitting to single- or double-exponential equations.

The biodistribution of ATH, after introduction into the rabbit lung, was determined. Anesthetized rabbits were orally intubated (polypropylene endotracheal tubes). ATH in 0.15 M NaCl (0.5–1.5 ml/kg) was instilled sequentially into each lung through the tube by alternating lateral positioning of the rabbit. In a subsample of animals, immediate suction was applied to the tube to retrieve the instilled fluid. All rabbits

### Table I

| Property                  | Estimated fraction of commercial heparin |
|---------------------------|------------------------------------------|
| Contains a pentasaccharide| 33                                       |
| Contains an aldose        | 10                                       |
| Pentasaccharide at C1 terminus | 4                         |
| Pentasaccharide at aldose terminus | 0.4                      |
Kinetics of Covalent Antithrombin-Heparin Complexes

RESULTS

Purification of Product—Fig. 1A shows the SDS-PAGE of fractions collected during gel filtration on Sephadex G-200 under high ionic strength conditions of the reaction mixture of AT plus H following incubation. Polydisperse high molecular mass ATH complexes separated in early fractions followed by a mixture of unseparated lower molecular mass ATH, unreacted AT, while addition of exogenous heparin to the ATH solution had no effect. Specific anti-factor Xa and antithrombin activities of ATH were extubated and recovered from anesthesia. Blood was obtained over time for plasma samples as described above. After 48 h, the rabbits were again anesthetized and intubated, and BAL was performed with 2 ml of 0.15 M NaCl.

Physicochemical Properties—The AT to H ratio in the ATH complex is 0.9 or approximately 1 to 1. The molecular mass of ATH complexes ranged from 69 to 100 kDa. An analysis of the purified product and its constituent components following enzyme degradation is shown in Fig. 2. Subsequent gel filtration of the heparin from protease degraded ATH complex showed it to have slightly larger average molecular mass (18 kDa) compared with the starting heparin (15 kDa). Heparinase-degraded ATH complex appears as one to three blurred bands, migrating at slightly higher molecular mass compared with starting AT (Fig. 2, lane 4) due to the one to three disaccharide units that remained linked to the AT despite heparinase. This was not seen with heparinase treatment of noncovalent mixtures of AT and heparin. Western immunoblots of ATH, and ATH pretreated with heparinase, shown in Fig. 3, provide further evidence that ATH contains AT, covalently linked to heparin, which is released only after heparin degradation.

In Vitro Anticoagulant Activity—Incubation of 125I-labeled thrombin with ATH complex, followed by SDS-PAGE and autoradiography, demonstrated ATH-thrombin complex formation (Fig. 4). Titration of ATH with purified factor Xa or thrombin showed that >98% of the molecules were active. Thus, 0.98–1 mol of either factor Xa or thrombin were inhibited for each mole of ATH used. Furthermore, anti-factor Xa assays of fractions from Sephadex G-200 chromatography of ATH demonstrated that a significant amount of the conjugate (~10%) had high activity (>200 units/mg of heparin) and contained a low molecular mass heparin component (~2000 to ~4000 Da, determined by gel filtration of the heparin released by protease treatment). Relative fluorescence intensity measurements of 100 nM ATH showed it to be elevated compared with 100 nM AT. Addition of a >5-fold molar excess of heparin (saturating amounts) was required to obtain a similar fluorescence with AT, while addition of exogenous heparin to the ATH solution had no effect.

Specific anti-factor Xa and antithrombin activities of ATH are shown in Table II. When exogenous AT was added, a more than 15-fold increase in capacity of ATH to inhibit either factor Xa or thrombin was observed. Thus the ATH compound had catalytic in addition to noncatalytic activities. Additionally, the activities of ATH, when exogenous AT was present, were approximately 4 times and 2 times greater than the correspond-
ing values for standard heparin and high affinity standard heparin (HASH), respectively. This result indicated that, during ATH formation, AT had selected for heparin molecules enriched with AT binding sites (and thus anticoagulant activities) compared with heparin prepared by affinity chromatography (HASH).

In vitro kinetics of the reaction of ATH with thrombin are shown in Fig. 5. The bimolecular rate constant and second order rate constants obtained for ATH inhibition of thrombin were $1.3 \times 10^9 \pm 2 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ ($n = 3$) and $3.1 \times 10^9 \pm 4 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ ($n = 3$ $k_{-1}$ determinations) respectively. In comparison, the bimolecular rate constant for noncovalent mixtures of AT + standard heparin was $1 \times 10^8 \text{M}^{-1} \text{s}^{-1}$. The addition of exogenous heparin at molar concentrations greater than 5 times the [ATH] inhibited the thrombin + ATH reaction (Fig. 6).

In Vivo Pharmacokinetics—The intravascular clearance of human AT, heparin, noncovalent mixtures of AT + heparin, and ATH from plasma after intravenous injection into rabbits are shown in Fig. 7. Heparin with or without coinjection of AT was rapidly cleared. The clearance of AT followed an exponential decay pattern. If the plasma curves of heparin, heparin coinjected with AT, AT, and ATH were all analyzed as single-exponential decays (simple two-compartment model) half-lives
of 0.32, 0.41, 13, and 2.6 h, respectively, were obtained. Use of a double-exponential model for AT and ATH gives half-lives for the β phase of 69 and 13 h, respectively. The plasma clearance of ATH more closely resembled AT than heparin in the rabbit model.

Subcutaneous injection into rabbits of ATH, AT, H, and AT_1H showed ATH reached peak plasma concentration at 24–30 h post-injection, with significant amounts observed up to 96 h post-injection (Fig. 8). Heparin was rapidly absorbed, with peak activity by 1 h, and disappearance by 4 h. AT pharmacokinetics were similar to ATH; however, the maximum concentration of ATH in plasma after subcutaneous injection was only 3–5% of that for AT.

Results from tracheal instillation of ATH are shown in Fig. 9. Approximately 50% of the anti-factor Xa activity of the starting fluid could be recovered in fluid extracted immediately after instillation (Fig. 9A). A similar amount of ATH was detected by mass (Fig. 9B). BAL taken 48 h after the introduction of ATH, still contained measurable anti-factor Xa activity (~2% of instillation fluid) and human AT (~3% of instillation fluid) in all animals, regardless of whether initial suction had been performed. Plasma samples taken throughout the lung experiments revealed that, in all animals, no detectable ATH (either by anti-factor Xa assay or AT enzyme-linked immunosorbent assay) was present in the circulation.

DISCUSSION

Fibrin deposition in the lung is a consistent feature of neonatal RDS (4, 5). A variety of observations suggest that fibrin may be contributing both to the severity of neonatal RDS and subsequent development of BPD (6, 8, 19). The local administration of an anticoagulant that could prevent fibrin formation in the lung, but not be absorbed systemically, could be beneficial. The properties of an anticoagulant that may be successful locally within the alveolar space include a large molecular weight, to prevent systemic absorption, and the capacity to inhibit thrombin in the absence of any other components of the coagulation system. To develop such an agent, we explored the possibility of conjugating heparin to the natural thrombin inhibitor, AT. Taking advantage of unique properties of AT and heparin, the complex ATH was formed. ATH was of large molecular weight, was retained within the lung when administered locally, and had potent antithrombin activity.

Heparin is a highly sulfonated polymer containing repeating uronic acid-glucosamine disaccharides (20). Endogenously, heparin is produced and secreted by mast cells in a proteoglycan form containing long heparin GAG chains linked glycosidically to serine residues of a core protein (21). Commercially, heparin is prepared from intestinal mucosa or lung as a degradation product consisting of GAG molecules that have no polypeptide attached. Most of these heparin GAG fragments are still linked to a serine, but approximately 10% have a free aldose terminus (14). The free aldose aldehyde group was used to produce a novel covalent complex of AT and heparin with high anti-factor Xa/IIa activities and a prolonged half-life when administered intravenously or locally in the lung. Purification of the ATH conjugate was optimally achieved by a two-step procedure consisting of hydrophobic chromatography (butylagarose) followed by anion exchange chromatography (DEAE-Sepharose). The final product contained AT and heparin linked together by either Amadori rearrangement or reduction of the Schiff base between an AT lysyl ε-amino group and a heparin aldose aldehyde group. In the ATH complex, the AT to heparin molar ratio was close to 1:1. Due to heparin’s polydispersity, the molecular mass ranged from 69 to 100 kDa. ATH exhibited characteristics of a covalent conjugate, which showed no signs of dissociation on SDS-PAGE after heating at 100 °C in β-mer-
captoethanol- and detergent-containing buffer (Fig. 2). The average molecular mass of the heparin component of ATH was slightly higher than the starting heparin (18 kDa compared to 15 kDa), indicating that AT had selected for larger heparin molecules during conjugation.

Since complex formation could occur by simple incubation of unmodified AT and heparin, it was not surprising that a high activity compound was produced. Under these conditions, binding of heparin by AT would initially occur via non-covalent interactions with high affinity binding sites (pentasaccharide sequences; Ref. 22). When a heparin molecule was bound to AT via a pentasaccharide sequence that was in close proximity to an aldosel terminus on the heparin molecule, covalent attachment occurred. Evidence for this mechanism is apparent by the fact that preparations contained highly active ATH molecules where the pentasaccharide sequence was close to the AT component due to the short heparin chain length (~2000 to ~4000 Da). AT in the conjugate had an elevated fluorescence characteristic of the active conformation (23), probably due to direct interaction with the heparin in the complex, since similar mixtures of AT + heparin had significantly reduced values.

The specific anti-factor Xa and antithrombin activities of ATH were found to be significantly and unexpectedly increased, compared with either standard heparin or HASH, when exogenous AT was added (Table II). The anti-factor Xa activity of ATH is comparable to the value obtained by Rosen-berg et al. (24) for a subfraction of commercial heparin, which contained two AT binding sites/molecule. If the heparin moiety in ATH contained, on the average, more than 1 AT binding pentasaccharide/molecule, this may explain how the conjugated heparin could catalyze inhibition by exogenous AT while still being linked to the AT at the aldosel terminus. Selection for the very small percentage of multi-pentasaccharide, very high affinity, heparin present in standard heparin (~1–3%; Ref. 24) was possible, given the long incubations with a high hepa-рин:AT molar ratio (200:1).

Kinetically, the velocity of reaction with thrombin was about 1 order of magnitude faster for ATH than for AT + standard heparin. The second order rate constant of $3.1 \times 10^9$ M$^{-1}$ s$^{-1}$ is one of the highest ever reported and is probably diffusion rate-limited (25). There are several potential reasons for the very high rate of reaction. Unlike noncovalent mixtures of AT and heparin, there is no initial binding of serpin and GAG required, which is the rate-determining step (26). In addition, AT may have selected for heparins with higher average anionic density, which would enhance the interactions of thrombin with ATH since its binding is a charge-dependent phenomenon (27). All of the heparin molecules in ATH contain the high affinity AT binding site, and, since the average heparin chain length in the conjugate was greater than the starting heparin, thrombin would have a shorter mean path distance for contacting the inhibitor complex. The geometry of heparin in the ATH complex was optimal, since addition of exogenous heparin inhibited the reaction with thrombin (Fig. 6).

ATH has several important differences compared with previous covalent AT-heparin complexes. AT conjugated to nitrous acid-treated heparin via reduction of the Schiff base between a lysyl ε-amino and anhydromannose aldehyde had significantly lower anti-factor Xa activity (140 units/mg of heparin) and essentially no anti-thrombin activity (28). Joining of AT to heparin by hexamethylenediamine spacer arms between AT lysyl ε-amino groups and heparin uronic acid carboxyl groups resulted in a heterogeneous product composed of molecules with variable numbers of AT joined to heparin chains, as well as significant amounts of unreacted linkage groups remaining on the complex (12). Attachment of hexamethylenediamine linkage groups to high affinity heparin reduced the specific anti-factor Xa activity from 250 units/mg to 162 units/mg, and addition of exogenous heparin to the spacer arm bonded complex doubled the rate of inhibition of Xa (12), indicating that significant amounts of the modified heparin had lost activity. In contrast, added heparin had no effect on the reaction of ATH with Xa (data not shown). The second order rate constant determined for reaction of the hexamethylenediamine-linked AT and heparin with thrombin was $6.7 \times 10^9$ M$^{-1}$ s$^{-1}$ (10). This is 4–5 times lower than that for ATH reported here using the same method of measurement. Covalent bonding of AT and heparin using other methods (29, 30) has given products with decreased activity.

The clearance of ATH, when administered intravenously, was significantly decreased compared with free heparin. The intravenous half-life of ATH was at least 8 times longer than that for heparin, and its disappearance profile approached that of human AT in the rabbit (Fig. 7). Given the increased size of ATH compared with heparin, its elimination via glomerular filtration through the kidneys would be limited. It is possible that the covalently linked AT in ATH may have also have inhibited binding of the heparin component with cell surface receptors involved in clearance. Although subcutaneous administration resulted in prolonged in vivo activity, the bioavailability was greatly reduced (Fig. 8). The poor absorption of ATH into the blood stream, from a subcutaneous injection, suggested that it might be retained in the lung and, if so, may have a role as an anticoagulant for neonatal RDS.

RDS is characterized by increased lung permeability, which leads to intra-alveolar coagulation and the formation of a hyaline membranes that contain fibrin (4, 5). The hyaline mem-brane inhibits gas exchange (8), causes recruitment of fibro-blasts resulting in fibrosis (6), and assists in the evolution toward BPD (19). As thrombin inhibitors are not likely to be present in consistently high levels in the air space, it would be highly useful to have a permanently activated AT molecule available to prevent thrombin-mediated fibrin deposition.

Results from the introduction of ATH into the lungs of rabbits showed that significant activity, as well as AT concentra-tion, could be recovered in BAL taken 48 h after the start of the experiment (Fig. 9). Heparin delivered intratracheally in dogs is readily lost from the lungs into the circulation, with plasma values peaking at 6 h (9). Conversely, ATH was retained in the lungs for over 48 h without detectable ATH in plasma by a highly sensitive AT enzyme-linked immunosorbent assay. Thus, ATH is retained with high antithrombin activity in the lung following local instillation. These properties are critically important to prevent fibrin deposition in the lung. The potential benefits of ATH in neonatal RDS warrant evaluation in an animal model and potentially in newborns.

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