mission of other arboviruses, particularly those transmitted by long-feeding ticks, needs to be explored.

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10. Donor ticks were infected at the nymphal stage by feeding on viremic hamsters inoculated with either the prototype IIA or Sicilian SiAr 126 THO virus isolates and then reared to the adult stage; average titers for males and females were 2.0 and 3.0 for IIA and 2.5 and 3.0 for SiAr, $\log_{10}$ PFU per tick, respectively.
11. Adults were assayed individually for virus (Table 1) on the day they completed engorgement (0), nymphs individually on days 0 and 12, and larvae in three pools of 10 on day 0 and in three pools of 30 on day 10 [days 12 and 10 were the times of maximum virus titers, respectively (*2*)]. Mean virus titers for donor females after feeding were 4.1 (range, 2.0 to 5.4), and 2.6 (1.7 to 3.7) $\log_{10}$ PFU per tick for donor males; recipient females were 3.9 (1.1 to 5.4), recipient males, 2.2 (1.3 to 3.7), recipient nymphs, 2.2 (1.3 to 3.0) on day 0 and 4.3 (3.4 to 4.9) on day 12, and recipient larvae <1.0 on day 0 and 1.4 (0.9 to 1.8) $\log_{10}$ PFU per tick on day 10 after engorgement. There was no difference in virus titers of recipient ticks that became infected with either IIA or SiAr THO virus.
12. We thank J. M. Murdock, J. S. Porterfield, and D. H. L. Bishop for their help.

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Heparin Promotes the Inactivation of Antithrombin by Neutrophil Elastase

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Heparin is an acceleratory cofactor for antithrombin, a circulating inhibitor of blood coagulation enzymes. The presence of heparin on blood vessel walls is believed to contribute to the nonthrombogenic properties of those surfaces. In apparent opposition to this function, heparin was found to greatly accelerate the in vitro inactivation of antithrombin by neutrophil elastase. Inactivation rates in solution were potentiated several hundredfold by specific heparin fractions with anticoagulant activity. Although the data suggest that a heparin-antithrombin complex is essential for the inactivation by elastase to occur, the enzyme itself interacts tightly with heparin. These results suggest a mechanism which, if operating in vivo, could lead to a localized neutralization of the anticoagulant function of heparin at the endothelial surface.

THE BLOOD COAGULATION SYSTEM IS a series of linked proteolytic reactions that, in the presence of appropriate stimuli, can rapidly lead to clot formation. A number of natural anticoagulant mechanisms are present to guard against the undesirable deposition of thrombi within normal blood vessels. Recent evidence suggests that the luminal surface of the blood vessel, the endothelium, is itself actively involved in the regulation of thrombosis (*1*). When the normally nonthrombogenic nature of the endothelium is lost, as in certain inflammatory conditions, a hypercoagulable state can result. The current study suggests a mechanism by which the normal functioning of the endothelial heparin-antithrombin system might be modified in inflammatory disease.

Antithrombin (also referred to as antithrombin III or AT-III) is the primary plasma inhibitor of blood coagulation enzymes and is a member of a large superfamily of related proteins that includes several serine proteinase inhibitors (serpins) (*2*). These proteinase inhibitors share a common mechanism by which a covalent, essentially irreversible complex is formed with the target enzymes (*3*). It is interesting that several of these inhibitors are themselves the target of attack by proteinases of mammalian and nonmammalian origin. In the case of antithrombin, neutrophil elastase catalyzes a specific and inactivating cleavage near the site involved in the inhibition of coagulation enzymes (*4*). It has been hypothesized that this inactivation phenomenon contributes to the hypercoagulable state linked to neutrophil activation in inflammation and septicemia (*5, 6*).

Maintenance of plasma concentrations of antithrombin at or near the normal level of approximately 2 μM is apparently essential to avoid the thrombotic tendency associated with both hereditary and acquired deficiency states (*7*). Disease-related decreases in circulating antithrombin levels can be more than 50% in extreme cases of septicemia. Such large decreases have most often been attributed to a massive consumption of antithrombin in the course of inhibiting large amounts of activated coagulation enzymes. A possible alternative explanation was suggested by the recently described inactivation of antithrombin in vitro by neutrophil elastase (*5*). The widespread activation of neutrophils in inflammation and the potential for significant release of elastase give this mechanism some appeal. However, the extrapolation of this observation to the physiological setting must take into account the very high plasma levels of a specific and fast-acting elastase inhibitor. This inhibitor, α-1 proteinase inhibitor, normally prevents expression of elastolytic activity in plasma (*3*). To address this conceptually problematic situation, we investigated the influence of heparin on the elastase-antithrombin interaction.

The anticoagulant heparin is a functionally and chemically heterogeneous sulfated carbohydrate with the ability to bind to antithrombin and accelerate its rate of inhi-

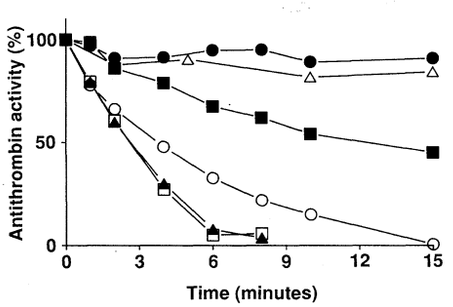

Fig. 1. Effect of heparin on the rate of antithrombin inactivation by neutrophil elastase. A reaction mixture that contained purified human antithrombin (*10*) at a concentration of 2 μM and various concentrations of heparin in 0.15M NaCl and 0.02M tris HCl, pH 7.5, was warmed to 37°C. Elastase was added to a concentration of 5 nM to initiate the reaction. Reactions were stopped at the indicated times by a 20-fold dilution of an aliquot of the reaction mixture into the second-phase assay buffer (0.15M NaCl and 0.05M tris HCl, pH 8.4) that contained 1 μg of α-1 proteinase inhibitor per milliliter. Antithrombin activity was then determined by a chromogenic heparin cofactor assay as described (*7*). The individual reaction mixtures were as follows: no added heparin (●); 1 μg per milliliter of inactive heparin (△); 50 (■), 100 (○), 400 (▲), or 700 (□) nanograms per milliliter of the anticoagulant fraction of heparin. The human neutrophil elastase was a gift of J. Travis or was a human sputum–derived preparation obtained commercially from Elastin Products Company.

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bition of coagulation enzymes. Heparin does not normally circulate in blood but, rather, is an integral component of vascular endothelial cell walls (*8*). The anticoagulant activity of heparin, as purified from animal tissues and administered therapeutically, derives from a limited subfraction of heparin molecules (35%) with affinity for antithrombin (*9*). Despite its central role in the normal functioning of antithrombin, a similar potentiation by heparin of the inactivation of antithrombin by elastase was not detected in earlier studies (*5*). We were prompted to reexplore this possibility in consideration of the profound influence of heparin on antithrombin inhibitory function and its potential ability to localize events at a surface in vivo.

A representative kinetic study of the inactivation of highly purified human antithrombin by neutrophil elastase is presented in Fig. 1. For these studies in vitro, we found it essential to use extremely well characterized and highly purified antithrombin preparations. This is because heparin and platelet factor 4, a heparin-neutralizing component, are frequent contaminants of antithrombin purified from pooled blood plasma by affinity chromatography on immobilized heparin supports. The absence of these contaminants was ensured in the present study by inclusion of specific separation steps (*10*) and confirmed by specific assays for these components (*11*).

Inactivation of antithrombin occurred at a negligible rate in the absence of heparin (Fig. 1). When small amounts of active anticoagulant heparin (*12*) were included, however, a progressive and eventually complete loss of the inhibitory activity of antithrombin occurred. In the curve that represents a heparin concentration of 50 ng/ml, the approximate calculated molar ratios of heparin, elastase, and antithrombin were 1:1.5:600, respectively. Rates of inactivation were dependent on heparin concentrations at low levels but reached an apparent maximum velocity at approximately 0.5 μg/ml of the active heparin species. To achieve maximal inactivation rates, then, a heparin concentration less than 3% of that required for stoichiometric equivalence with antithrombin was needed. Thus, both heparin and elastase act catalytically to inactivate antithrombin. This catalytic role for elastase distinguishes the mechanism of the present heparin-accelerated inactivation phenomenon from the heparin-accelerated inhibition of clotting enzymes by antithrombin. In the latter case, heparin catalyzes the formation of a covalent, stoichiometric complex between antithrombin and enzyme (*13*).

To determine the magnitude of the present heparin effect, we compared the rate of inactivation in the presence or absence of a saturating level of heparin (10 μg/ml) and assumed that first-order kinetics were obeyed in each case. With 5 n*M* elastase and 500 n*M* antithrombin, an approximately 400-fold rate enhancement was observed. This enhancement value derives from the ratio of velocities of 650 n*M*/min in the presence and 1.6 n*M*/min in the absence of the active heparin species. Although this calculated rate enhancement reflects the specific solution-phase conditions and reactant concentrations used, its magnitude emphasizes that heparin can greatly accelerate this inactivation phenomenon.

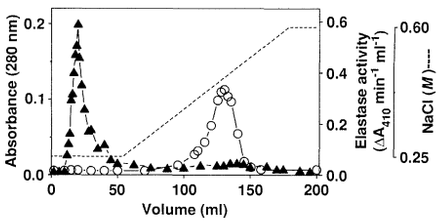

Fig. 2. Heparin-Sepharose chromatography of elastase-inactivated antithrombin. A reaction mixture containing 5 mg of antithrombin and 150 μg of elastase was initiated with a trace amount of heparin and monitored for antithrombin functional activity until inactivation was complete. Before application of the reaction mixture on the column, free heparin was removed by adsorption on QAE-Sepharose (*10*). The reaction mixture was applied to a heparin-Sepharose column (1.1 by 8.8 cm) preequilibrated in a buffer that consisted of 0.25*M* NaCl, 0.02*M* tris HCl, *p*H 7.5. Elastase-inactivated antithrombin (▲) eluted as a single unbound peak under the conditions of application. Elastase was eluted by a 120-ml linear gradient of NaCl from 0.25 to 0.6*M*. Elastase activity (○) was detected by its ability to hydrolyze the chromogenic substrate methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma) as previously described (*16*). Heparin-Sepharose was prepared as described previously (*10*).

The inactive heparin fraction failed to promote the cleavage of antithrombin by elastase (Fig. 1). Other glycosaminoglycans including dermatan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, and dextran sulfate were also without significant stimulatory effect at similar concentrations. The distinguishing characteristic of active heparin, in contrast to the inactive heparin fraction and other sulfated carbohydrates, is its ability to bind with high affinity and specificity to antithrombin. The apparently stringent requirement for the active heparin species in the present reaction strongly suggests that a heparin-antithrombin complex is the substrate for elastase attack.

Previous studies with crossed immunoelectrophoresis demonstrated that, in contrast to functional antithrombin, elastase-cleaved antithrombin had little or no binding affinity for heparin (*5*). We have confirmed this result using affinity chromatography on immobilized heparin. Elastase-inactivated antithrombin is not adsorbed to immobilized heparin at a relatively low (0.25*M*) concentration of sodium chloride (Fig. 2). This is in contrast to the tight binding of functionally active antithrombin to heparin, which serves as the basis for the affinity purification of the inhibitor from most other plasma proteins and which requires much higher ionic strengths for desorption (*10*). Also evident in Fig. 2 is a tight binding interaction between heparin and neutrophil elastase. A single peak of elastase activity eluted at a sodium chloride concentration of approximately 0.5*M*. No elastase activity was detected in the unadsorbed eluate region containing the elastase-cleaved antithrombin.

It is likely that the binding interaction between the highly cationic neutrophil elastase and the heparin affinity matrix is largely electrostatic and relatively nonspecific in nature. This is supported by the considerable affinity of neutrophil elastase for cation-exchange chromatography gels (*14*) and its ability to interact with several sulfated glycosaminoglycans (*15*). This stimulatory effect of heparin on the inactivation of antithrombin is in contrast to a more commonly observed inhibition of neutrophil elastase activity in the presence of sulfated glycosaminoglycans (*15*).

It is uncertain to what degree, if any, the interaction between heparin and elastase itself contributes to the rate of inactivation of antithrombin in solution. The inactivation clearly requires the presence of the active anticoagulant heparin fraction that binds specifically to antithrombin. Moreover, heparin exhibits an apparently tighter affinity for antithrombin than for elastase as judged by chromatographic elution from the immobilized glycosaminoglycan. Notwithstanding these considerations, an interaction between elastase and endothelial-bound heparin in vivo might localize the enzyme and sequester it away from the high circulating levels of elastase inhibitors. A further consideration is the likelihood of significant local amounts of elastase released from the activated neutrophils, which normally adhere to sites of acute inflammation. This model would suggest that an association between neutrophil elastase and endothelial heparin in vivo might contribute significantly to antithrombin inactivation and thereby facilitate the occurrence of localized clotting events.

The inhibition of coagulation enzymes by the heparin-antithrombin system is accepted as a pivotal regulatory mechanism of the clotting process. The demonstration of active anticoagulant heparin on the vascular endothelium (*8*) supports the concept that

the heparin-antithrombin system is important in the balance between procoagulant and anticoagulant pathways on the vessel wall. The results of the present in vitro study suggest, however, that under certain conditions, the binding of antithrombin by heparin could lead to a potentially opposing outcome. The participation of heparin in the inactivation of antithrombin by elastase represents an unexpected action for this anticoagulant. A similar occurrence on in vivo surface-bound heparin could lead to a localized reversal of the nonthrombogenic character of the vascular endothelial lining.

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11. Functional activity levels of heparin were assayed by a chromogenic substrate assay with factor Xa as described by A. N. Teien and M. Lie [*Thromb. Res.* **10**, 399 (1977)]. Platelet factor 4 levels were determined with a commercial competitive radioimmunoassay kit (Abbott Laboratories).
12. Active heparin refers to the subfraction of commercial porcine mucosal heparin (Scientific Protein Laboratories) that has anticoagulant activity. Active heparin was separated by adsorption on columns of immobilized human antithrombin as previously described [M. Hook, I. Bjork, J. Hopwood, U. Lindahl, *FEBS Lett.* **66**, 90 (1976)]. The anticoagulant activity of this material was approximately 550 units/mg in comparison with the 150 units/mg of the unfractionated material. The heparin fraction without anticoagulant effect was prepared by removal of the active component by adsorption on the immobilized antithrombin column and possessed less than 5 activity unit/mg.
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Stable Integration and Expression of a Bacterial Gene in the Mosquito *Anopheles gambiae*

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Foreign DNA was successfully introduced into the germline of the African mosquito vector of malaria *Anopheles gambiae*. Stable integration of genes into the germlines of insects had been achieved previously only in *Drosophila melanogaster* and related species and required the use of the P element transposon. In these experiments with *Anopheles gambiae*, the plasmid pUChsneo was used, which contains the selectable marker *neo* gene flanked by P element inverted repeats. Mosquitoes injected with this plasmid were screened for resistance to the neomycin analog G-418. A single event of plasmid insertion was recovered. Integration appears to be stable and, thus far, resistance to G-418 has been expressed for eight generations. The transformation event appears to be independent of P.

THE WORLDWIDE RESURGENCE OF malaria is due in part to drug resistance in the malaria parasite and insecticide resistance in the anopheline mosquito vectors. The development of control schemes based on genetic modification of the capacity of vector populations to transmit malaria (*1*) may eventually provide alternatives to traditional control methods. To achieve this goal, it will be necessary to develop methods for the physical introduction of genes into mosquito lines. This will make it possible to evaluate the effects of potentially parasiticidal genes (for example, diphenoloxidase) on the development of the parasite in vivo. Foreign DNA has been integrated into the germline of *Drosophila melanogaster* and related *Drosophila* species (*2*) through the use of the P element, a germline-specific transposable element of *D. melanogaster* (*3*). Similar transposons have not been found, nor has stable integration been achieved, in other insects. In this study, we developed a method for introducing DNA into the germline of the African malaria vector *Anopheles gambiae*.

To inject DNA into mosquito embryos, we initially followed the techniques described for injection of DNA into *D. melanogaster* embryos (*4*). However, a number of modifications were necessary because of the unique properties of mosquito eggs. The most important difference was the inability to remove the chorion, necessitating a needle that could penetrate the egg shell without killing the embryo.

Mosquito oviposition was controlled to provide eggs of precise age for injection (*5*). Eggs were maintained at 21° to 22°C to slow development of pole cells, the embryonic precursor of the germline. The pole cells of *A. gambiae* appear ultrastructurally similar to those of *D. melanogaster* (*6*) and form about 3 hours after oviposition at 21°C. Between 1.5 and 3 hours after oviposition, eggs floating on a drop of water were aligned and attached to double-stick cellophane tape; the other surface of the tape was attached to a slide. The water was removed from around the eggs and the eggs were dehydrated for 1 to 1.5 minutes, depending on the age of the eggs, in a desiccator until they were slightly bowed inward. Slight dehydration was required so that the eggs would not leak during injection. Excessive dehydration decreased viability and was avoided. After desiccation, prehydrated

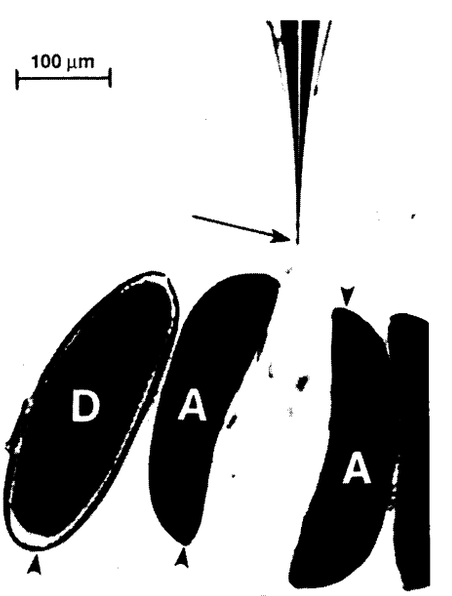

Fig. 1. Comparison of *A. gambiae* (A) and *D. melanogaster* (D) eggs. Arrowheads identify the posterior end of each egg. The end of the injection needle (arrow) is shown.

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