SECRETION OF A PROTEOLYTIC ANTICOAGULANT BY *ANCYLOSTOMA* HOOKWORMS*

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Human hookworm disease, a clinical condition caused by *Ancylostoma duodenale* or *Necator americanus* infection, affects up to 630 million people in the developing world (1). Using their buccal cavities and hooklike teeth, the adult parasites attach themselves to villi in the small intestine. Each worm can then extract up to 0.20 ml of blood per day causing intestinal blood loss and ultimately iron-deficiency anemia and hypoalbuminemia (2, 3).

To date the biochemical mechanism by which hookworms prevent blood coagulation while feeding remains unexplained. Previous studies have shown that extracts of the dog hookworm *Ancylostoma caninum* can prolong prothrombin time (PT)[1] (4–6), with variable effects on partial thromboplastin time (PTT) (4–6), and interfere with collagen- or ADP-induced platelet aggregation, as well as inhibit the action of factor Xa (6).

The recent finding of a proteolytic enzyme with anticlotting properties from the giant leech *Haementeria ghilianii* (7) led us to examine whether a similar proteolytic anticoagulant exists in the *Ancylostoma* hookworms. Data presented here suggests that the *Ancylostoma* hookworms secrete a 36,000 dalton protease which both interferes with fibrin clot formation and promotes fibrin clot dissolution. This proteolytic enzyme could be critical for continuous exsanguination from villous capillaries and therefore represents a potential target for immunological intervention.

Materials and Methods

Hookworms. Third-stage infective filariform (L_3) larvae of *A. duodenale* were the gift of Dr. Gerhard Schad, University of Pennsylvania, School of Veterinary Medicine, Philadelphia (8). Briefly, 1,000–1,500 L_3 larvae were administered to 10-wk-old beagles reared helminth-naive (White Eagle Laboratories, Doylestown, PA) and immunocompromised with a daily oral dose of 5 mg prednisolone. 42 d after infection, the entire length of the small intestine was removed, slit longitudinally, and suspended in 0.85% NaCl at 37°C. Within 2 h the majority of adult worms released their grasp and were collected at the bottom of the cylinder. The living worms were individually rinsed in saline and were either used immediately or stored at −80°C.

L_3 larvae of *A. caninum* were initially the gift of Dr. G. Schad, but were later cultured from embryonated eggs in the feces of infected pups (9). The L_3 larvae (1,500–2,000) were administered to mongrel pups, aged 2–12 mo. The pups were sacrificed 20–30 d after infection when their hematocrits fell below 25%, indicating heavy infections with *A. caninum.* Alternatively,

* Supported by grant RF-81060 from the Rockefeller Foundation.

[1] *Abbreviations used in the paper:* AMC, 7-amino-4-methylcoumarin; ES products, excretory/secretory products; meosucc-ala-ala-pro-val-AMC, methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-4-methylcoumarinyl-7-amide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PT, prothrombin time; PTT, partial thromboplastin time; SDS, sodium dodecyl sulfate; and TES, *N*-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid.

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Volume 157 May 1983 1594–1603

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adult *A. caninum* were obtained from naturally infected pups from the School of Veterinary Medicine, Auburn University, AL. The adult worms were collected by the same procedure as for *A. duodenale*.

Supernatants from Hookworm Homogenates. Approximately 100 human or dog hookworms were suspended in 1.0 ml of 0.1 M Tris-HCl buffer (pH 8.1), ground in a Tenbroeck tissue grinder (A. H. Thomas Co., Philadelphia), and centrifuged at 1,000 rpm for 10 min at 4°C in an IEC centrifuge (International Equipment Co., Boston, MA). The supernatant (homogenate) was removed and stored at −20°C. The homogenate had a protein content of 4.0–8.5 mg/ml when measured according to the method of Bradford (10).

Excretory/Secretory (ES) products. ES products from adult *A. caninum* were isolated using a modified procedure of Day et al. (11). Approximately 100–1,000 adult worms were incubated in phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin sulfate at 37°C for 22 h. The viability was 100% (as determined by motility) for the first 12 h, but began to slowly decline thereafter. At 22 h ~40–75% were viable. The fluid was removed from the settled worms and spun at 1,000 rpm for 10 min at 4°C in an IEC centrifuge to remove any debris, and stored at −80°C. The ES products had a protein content of 0.05–0.20 mg/ml when measured according to the method of Bradford (10). Alternatively, aliquots were taken at various times to assess the proteolytic activity of the secreted material. Because of the difficulty in obtaining the human hookworm in quantity, ES products were not obtained from *A. duodenale*.

Clotting Times. PT were measured at 37°C with 0.1 ml of citrated plasma incubated with various concentrations of either homogenates or ES products. 0.20 ml of Simplastin Automated (General Diagnostics Warner Lambert Co., Morris Plains, NJ) was added to the mixture and the time for clot formation to occur was noted.

PTT were measured at 37°C with 0.1 ml of citrated plasma, 0.1 ml of Automated APTT (General Diagnostics Warner Lambert Co.), and various concentrations of *A. duodenale* homogenates. Clotting was initiated by adding 0.10 ml of 0.025 M $CaCl_2$ and the time for clot formation to occur was noted.

Electrophoretic Separation of Proteolytic Activities. The protein composition of the homogenates and ES products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12) after silver staining (13). Proteolytic activity in the gels was visualized by a modified procedure of Granelli-Piperno and Reich (14). Aliquots of hookworm homogenates or ES products were added to 20 μl of a buffer containing 10% glycerol, 3% SDS, and 0.0625 M Tris-HCl buffer, pH 6.8, and placed in an 80°C bath for 30 s. Samples were loaded onto 10% SDS-polyacrylamide slab gels not more than 0.75 mm in thickness and subjected to electrophoresis. After electrophoresis the gel was gently rocked in 2.5% aqueous Triton X-100 for 40 min at 22°C, rinsed thoroughly with distilled water, and then rocked in distilled water for an additional 30 min. At this time the gel was removed and overlayed onto an agar plate containing casein (5.2 ml of 0.1 M Tris-HCl buffer, pH 8.1, 3.6 ml of 2.5% agar, and 2.0 ml of 8% Carnation Instant Milk [boiled 10 minutes in PBS]) and incubated at 37°C. Depending on enzyme concentration, bands of lysis corresponding to proteolytic activity appeared in 4–12 h. These bands were visualized better by staining with amido black, and then destaining with a solution of methanol, acetic acid, and water (70:10:20).

^{125}I-Fibrinogen Plates. Multi-well Linbro plates (Linbro Scientific, Inc., Hamden, CT) coated with ^{125}I-fibrinogen were prepared by the method of Unkeless et al. (15). Radioactivity in solution was determined with a Packard Auto-Gamma Scintillation Spectrometer (model 3002; Packard Instrument Co., Downers Grove, IL).

Electrophoretic Separation of Fibrinogen Fragments. A solution of bovine fibrinogen (6.7 mg/ml) was incubated at 37°C in the presence of ES products (0.2 mg/ml). As controls, an equal amount of fibrinogen was incubated either alone or with plasmin. At indicated times aliquots were removed, added to buffer containing 10% glycerol, 3% SDS, and 0.0625 M Tris-HCl buffer, pH 6.8, and boiled for 5 min. The samples were subjected to SDS-PAGE.

Anticoagulant Activity of ES-generated Fibrinogen Degradation Products. Bovine fibrinogen (6.7 mg/ml) was incubated either alone or with 0.01 vol of ES products (0.20 mg/ml protein) for 12–24 h at 37°C. PT were measured with various concentrations of citrated plasma and ES-generated fibrinogen degradation products or fibrinogen alone.

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Electrophoretic Separation of Plasminogen Fragments. Plasminogen was purified from human plasma by affinity chromatography (16). The plasminogen (2.3 mg/ml) was incubated at 37°C in the presence of ES products (0.05 mg/ml protein). As controls, an equal amount of plasminogen was incubated either alone or with 1.0 mU urokinase. At indicated times aliquots were removed, added to buffer containing 10% glycerol, 5% 2-mercaptoethanol, 3% SDS, and 0.0625 M Tris-HCl buffer, pH 6.8, and boiled for 5 min. The samples were subjected to SDS-PAGE.

Assessment of Elastolytic Activity. Elastolytic activity of hookworm homogenates and ES products was determined using a synthetic peptide substrate covalently linked to a fluorescent leaving group (17). The rate of hydrolysis of methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-4-methylcoumarinyl-7-amide (meosucc-ala-ala-pro-val-AMC) (Vega Biochemicals, Tucson, AR) was measured spectrofluorimetrically in a Perkin-Elmer 204 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Various concentrations of either hookworm homogenates or ES products were added to a cuvette containing the substrate (10–50 μM) in buffer containing 0.05 M *N*-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), 0.50 M NaCl, 0.03–0.10 M $CaCl_2$ at pH 7.0 with 10% vol/vol dimethyl sulfoxide at 22° or 37°C. The initial rate of increase in the 7-amino-4-methylcoumarin (AMC) concentration was monitored at excitation and emission wavelengths of 370 and 460 nm, respectively.

Results

Clotting Times. To determine whether *Ancylostoma* hookworms had an effect on fibrin clot formation, homogenates of *A. duodenale* were added to samples of normal citrated human plasma that were then assayed for PT and PTT. The addition of aliquots of the homogenates prolonged PT and PTT in a concentration-dependent manner (Fig. 1). A prolongation of PT was also observed with ES products of *A. caninum* (data not shown).

Electrophoretic Separation of Proteolytic Activity. The composition of *A. caninum* homogenates were analyzed by separation on SDS-PAGE (Fig. 2A). In lane a, ~40 major bands appeared after silver staining. Since it was suspected that *Ancylostoma* hookworms might secrete their anticoagulant, ES products were also analyzed. Compared with the crude homogenates, ES products contained fewer proteins (Fig. 2A, lane b). All 12 of the secreted proteins could be identified in the homogenates of the adult worms.

The previous finding of an anticlotting protease from the leech (7) led us to investigate the possibility that some of the protein bands in Fig. 2A might have proteolytic activity. To analyze these proteolytic components, both *A. caninum* homogenates and ES products were separated on SDS-PAGE and overlayed onto casein agar (Fig. 2B). Examination of proteolytic activity in the homogenates revealed seven bands (lane a) including three major components at 31,000, 36,000, and 40,000 daltons. Smaller amounts of proteolytic activity were associated with molecular mass bands at 21,000, 71,000, 91,000, and 102,000 daltons. Homogenates of *A. duodenale* showed a similar pattern of proteolytic activity (data not shown).

In contrast, only a single band of proteolytic activity was found in the ES products of *A. caninum* (Fig. 2B, lane b). The apparent molecular mass of this protease was 36,000 daltons.

Anticlotting Properties of ES Products. A number of experiments were undertaken to determine the specificity of the proteolytic activity which could account for the anticlotting effect. Using multi-well plates coated with ^{125}I-fibrinogen, both homogenates and ES products of *A. caninum* were observed to degrade radiolabeled fibrinogen coated on plates (Fig. 3). The amount of fibrinogen degraded was proportional to the amount of homogenate or ES products added, and showed no significant amplification

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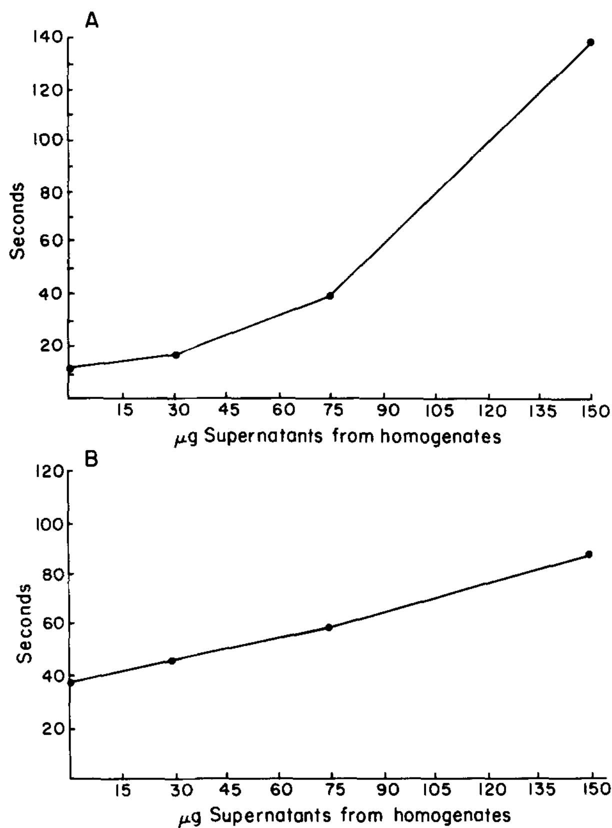

FIG. 1. The influence of *A. duodenale* homogenates on PT (A) and PTT (B) of normal human plasma (see Materials and Methods).

by the addition of plasminogen (data not shown). This latter experiment rules out the possibility that the protease was acting as a plasminogen activator.

The ability of ES products to degrade fibrinogen was also demonstrated using SDS-PAGE under nonreduced conditions (Fig. 4). ES products (lane b) catalyzed the degradation of fibrinogen (Fig. 4, lane a) to five major components of molecular mass 223,000, 204,000, 156,000, 122,000, and 80,000 daltons (lanes d and e), and a minor component at 61,000 daltons (lane e). The molecular mass of fibrinogen alone incubated at 37°C remained unchanged throughout the experiment, and the molecular mass of the fibrinogen degradation products was different then those observed with plasmin-catalyzed degradation of fibrinogen (data not shown).

The fibrinogen degradation products resulting from ES digestion, by themselves increased PT. When 50 μl of fibrinogen (6.7 mg/ml), which had been previously incubated for 12–24 h at 37°C with 0.01 vol of ES products, were added to citrated plasma, the PT was prolonged 80% as compared with a 30% prolongation with 50 μl of fibrinogen (6.7 mg/ml) incubated alone under similar conditions.

In addition to direct fibrinogenolysis, ES products also catalyzed the cleavage of plasminogen (Fig. 5). After a 1 h incubation with plasminogen, two polypeptides of 40,000 and 58,000 daltons were formed (Fig. 5, lane b). The molecular mass of the smaller fragment is similar to that reported for mini-plasminogen (18) which is formed

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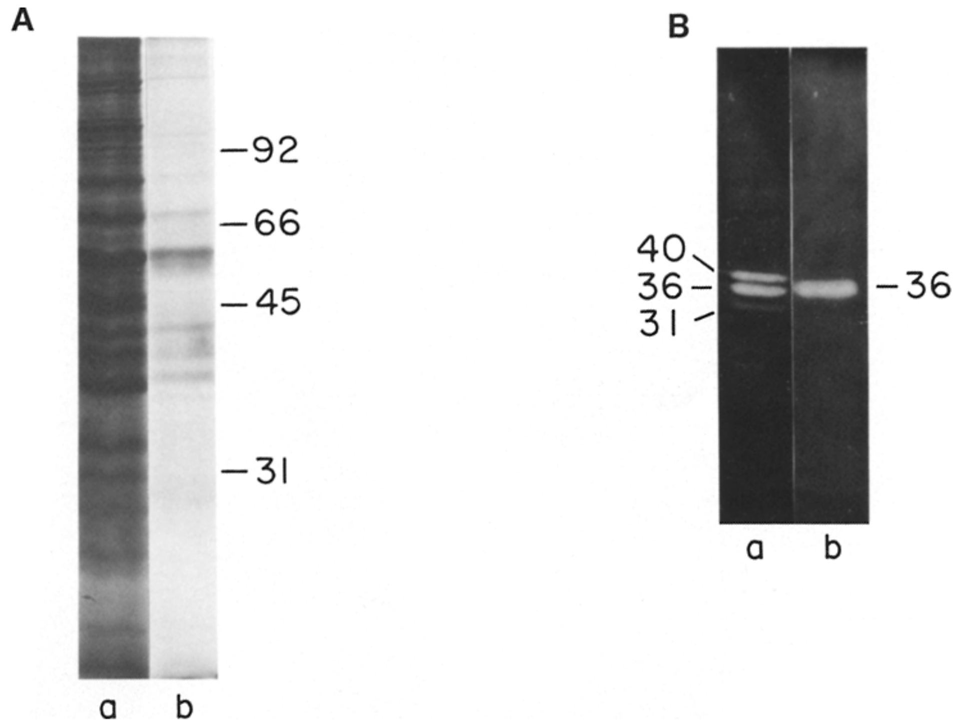

FIG. 2. (A) SDS-PAGE analysis of hookworm protein composition. Homogenates (80 μg of protein) or ES products (2 μg of protein) were electrophoresed under reduced conditions in SDS-polyacrylamide (10%) slab gels. Lane a, *A. caninum* homogenates; lane b, *A. caninum* ES products (see Materials and Methods). (B) Identification of hookworm proteases after SDS-PAGE. Homogenates (85 μg of protein) or ES products (4 μg of protein) were electrophoresed under nonreduced conditions in SDS-polyacrylamide (10%) slab gels. Lane a, *A. caninum* homogenates; lane b, *A. caninum* ES products. The figure is a photograph of the amido-black stained casein agar indicator gel (see Materials and Methods). Molecular masses × 10^3.

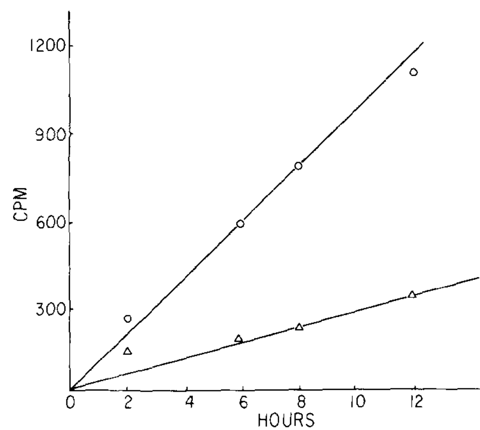

FIG. 3. Fibrinogenolytic activity of hookworm proteins. Homogenates (200 μg of protein) or ES products (2.5 μg of protein) were incubated at 37°C in 300 μl of 0.1 M Tris-HCl buffer, pH 8.1 (Materials and Methods). (○) *A. caninum* homogenates; (△) *A. caninum* ES products.

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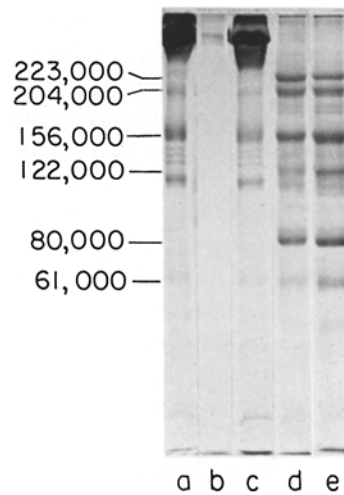

FIG. 4. SDS-PAGE of bovine fibrinogen incubated with *A. caninum* ES products. 60 µl of fibrinogen (6.7 mg/ml) in PBS and 60 µl of ES products (0.20 mg/ml) were incubated at 37°C for 0–6 h. 20-µl aliquots of the mixture were taken at 0, 3, and 6 h and added to a 3% SDS solution (Material and Methods) to stop the reaction. Digests were analyzed in 6.5% polyacrylamide gels under nonreduced conditions. (a) fibrinogen alone (10 µl); (b) ES products alone (10 µl); (c) 0 h digest; (d) 3 h digest; (e) 6 h digest.

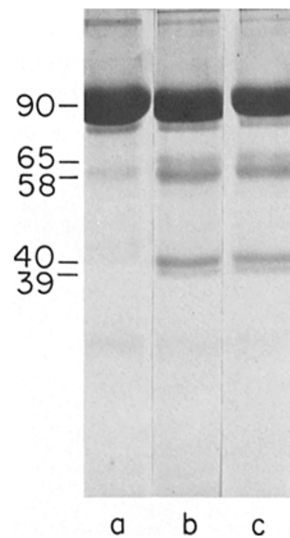

FIG. 5. SDS-PAGE of purified human plasminogen incubated with *A. caninum* ES products. 30 µl of plasminogen (2.3 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.1, and 90 µl of ES products (0.05 mg/ml) were incubated at 37°C for 0–90 min. 40-µl aliquots of the mixture were taken at 0, 60, and 90 min and added to a buffer containing 3% SDS and 5% 2-mercaptoethanol (Materials and Methods) to stop the reaction. Digests were analyzed in 10% polyacrylamide gels under reduced conditions. (a) 0 h digest; (b) 60 min digest; (c) 90 min digest. Molecular masses × 10^3.

when leukocyte elastase cleaves plasminogen. This 40,000 dalton fragment was observed by the casein lysis technique to have proteolytic activity (data not shown). In addition to the two major bands at 40,000 and 58,000 daltons, two minor bands appeared at 39,000 and 65,000 daltons. This heterogeneity may reflect the finding

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that human plasminogen contains two major components, plasminogen a and b, which have slightly different molecular masses (19). This cleavage by ES products was in contrast to the incubation of plasminogen with urokinase which resulted in two fragments of 68,000 and 28,000 daltons, corresponding to the heavy and light chain of plasmin, respectively (data not shown). Plasminogen alone showed no degradation during this incubation.

Elastolytic Properties of ES Products. The catalytic cleavage of plasminogen to a mini-plasminogen-like fragment suggested that ES products might have a proteolytic activity with elastolytic properties. Both hookworm homogenates and ES products could hydrolyze the synthetic substrate meosucc-ala-ala-pro-val-AMC, which has specificity for elastolytic enzymes (17). The specific activity for the hydrolysis of the substrate (20 μM) at 37°C was 0.02 nmol of AMC released/min/mg protein and 0.21 nmol of AMC released/min/mg protein for the homogenates and ES products, respectively. This is comparable to 21 nmol of AMC released/min/mg protein using commercially purified porcine elastase (Elastin Products Co., Pacific, MS). The low activity observed for the purified enzyme reflects the suboptimal synthetic substrate concentrations (17) and the pH conditions (pH 7.0 instead of the optimal pH 8.8) used for the experiment.

The time course of secretion in vitro by *A. caninum* of the elastolytic-like protease was followed using the synthetic substrate (Fig. 6). *A. caninum* hookworms secrete the protease in linear fashion during the first 9 h in vitro. Subsequently, the amount of protease released decreases, probably reflecting a decrease in the viability of the worms. This increase in elastolytic activity with time in vitro was paralleled by increasing intensity of the zone of lysis at 36,000 daltons on SDS-PAGE with casein agar (data not shown).

Discussion

Evidence has been presented that the *Ancylostoma* hookworms secrete a 36,000 dalton proteolytic enzyme. It is hypothesized that this secreted protease may have a role

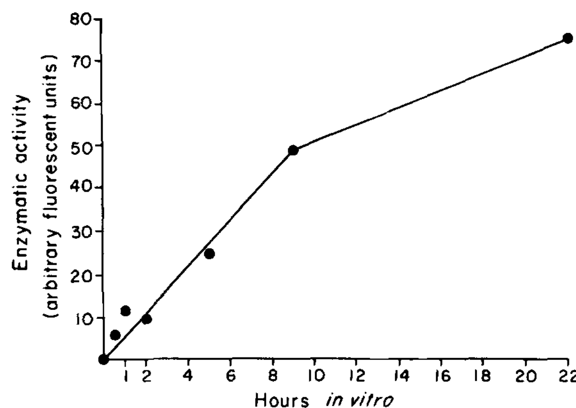

FIG. 6. Time course of protease secreted by *A. caninum* in vitro. 100 adult worms were incubated at 37°C in PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin sulfate. 60-μl aliquots were taken at 0.5, 1, 2, 5, 9, and 22 h and stored at −80°C. All samples were thawed and added to 3.0 ml of buffer containing 0.05 M TES, 0.5 M NaCl, 0.1 M $CaCl_2$, and 25 μM meosucc-ala-ala-pro-val-AMC, and incubated at 22°C for 12 h. Fluorescence was monitored at excitation and emission wavelengths of 370 and 460 nm, respectively.

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related to the antihemostatic mechanism of the hookworm. Recently, anticoagulant activity from the giant leech *Haementeria ghilianii* (7) and the bacterium *Streptococcus faecalis* (20) has been attributed to a proteolytic enzyme that not only inhibits the clotting of plasma, but also dissolves previously formed fibrin clots. We examined the possibility that *Ancylostoma* ES products function in a similar manner. In this way proteolysis may act concurrently with previously reported anticoagulating properties of hookworm extracts, namely the inhibition of platelet aggregation (6) and factor Xa activity (6), to ensure free blood flow into the buccal cavity and alimentary canal of the parasite. In addition to the protease described above, other proteases with anticlotting activity might also be present in the ES products. This possibility exists since the casein-lysis technique utilized for the identification of the protease only detects proteolytic enzymes that are reactivated by removal of SDS.

The anticoagulant effects of hookworm ES products on plasma can be explained, in part, by a direct action on fibrinogen to produce nonclottable derivatives. When fibrinogen was incubated with ES products for various times, breakdown products ranging between 80,000 and 223,000 daltons were observed with SDS-PAGE. These products did not resemble the well characterized fragments observed with plasmin degradation (fragments X, Y, D, and E). Degradation of fibrinogen was also observed when ^{125}I-fibrinogen coated on multi-well plates was incubated with ES products. These fibrinogen degradation products also increased PT.

In addition to direct fibrinogenolysis, ES products cleaved plasminogen to two fragments of 58,000 and 40,000 daltons. This smaller fragment resembles mini-plasminogen, a product formed by leukocyte elastase digestion of plasminogen, which in turn can be activated by urokinase to form mini-plasmin. Mini-plasmin is not readily inhibited by α_2-antiplasmin, the major physiologic inhibitor of plasmin in plasma (18, 21), and may represent an asset to the hookworm whose survival necessitates destruction of a fibrin clot.

This catalytic conversion of plasminogen to a mini-plasminogen-like fragment led us to investigate whether the ES protease had elastolytic properties. The protease was found to hydrolyze meosucc-ala-ala-pro-val-AMC, a synthetic peptide substrate for elastolytic enzymes (17). The quantity of elastolytic-like activity released in vitro by the hookworms increased with time.

The elastolytic-like properties of the ES protease may be important in preventing clotting just as the analogous leukocyte elastase may have important anticoagulant properties (18, 21–24). Leukocyte elastase converts plasminogen to mini-plasminogen (18), inactivates α_2-antiplasmin and $C\bar{1}$ inactivator (21), degrades procoagulants (factors II, V, VII, VIII, XII, and XIII) (22), and breaks down fibrinogen (23, 24). Furthermore, the elastase-catalyzed products of fibrinogen digestion can also inhibit the thrombin-catalyzed conversion of fibrinogen of fibrin (24). A biochemical comparison of leukocyte elastase and the ES protease from hookworms is currently in progress.

From a medical and veterinary standpoint, the proteolytic anticoagulant of the hookworm represents a unique feature available to natural and induced immunological intervention. In support of this is the fact that dogs with repeated infections became immune to *A. caninum* infection (25), and sera from these dogs neutralized proteolytic enzyme activity in esophageal extracts from the parasite (26, 27). Presumably, during feeding ES products are introduced into the host and elicit protective

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antibodies. It is possible that these protective antibodies inhibit the 36,000 dalton ES protease, block the antihemostatic mechanism, allow clot formatin, and starve the parasite. This possibility is under investigation.

Summary

Hookworms of the genus *Ancylostoma* secrete an anticoagulant that both inhibits the clotting of human plasma and promotes fibrin clot dissolution. This anticoagulant activity is attributable to a 36,000 dalton proteolytic enzyme. The protease can degrade fibrinogen into five smaller polypeptides that intrinsically have anticoagulating properties, covert plasminogen to a mini-plasminogen-like molecule, and hydrolyze a synthetic peptide substrate with specificity for elastolytic enzymes. It is hypothesized that the parasite uses this enzyme to prevent blood clotting while feeding on villous capillaries.

We would like to thank Dr. Nguyen Le Trang for his helpful discussions and advice. In addition, we would like to acknowledge the advice and assistance of Drs. Tom Donnelly, Angela Granelli-Piperno, Rafael Mira y Lopez, Jay Valinsky, Edward Reich, Peter Harpel, and Daniel Rifkin. We would especially like to thank Dr. Gerhard Schad for his continued advice in the establishment and maintenance of dog hookworm infections, and Dr. Guy Pidgeon for his help in obtaining dogs with natural hookworm infections. The technical help of Mr. Mark Wilson is also appreciated. Finally, we thank Drs. Daniel Rifkin, Alan Fairlamb, and Steven Meshnick for help in preparing this manuscript.

Received for publication 4 January 1983.

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