In vitro effect of larval stages of Ascaris lumbricoides on human blood clotting

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ABSTRACT
The effects of larval stages of Ascaris lumbricoides on human blood clotting was studied in vitro. Extracts and excretory/secretory products of third-stage larvae (L₃) and late third-stage larvae (LL₃) cultured from ova obtained from infected patients were analysed for anti-coagulant activity. Prothrombin time (PT) was prolonged by the addition of either whole extract of L₃/LL₃ or ES products of L₃/LL₃ as compared to controls. Partial thromboplastin time with kaolin (PTTK) was also prolonged on the addition of either extracts/ES products of L₃/LL₃. The prolongation of PTTK was significantly higher with extracts/ES products of L₃ when compared to the extracts/ES products of LL₃ (p<0.005). Thrombin time (TT) was prolonged by extracts of L₃/LL₃ and their ES products.

KEY WORDS: Ascaris lumbricoides, larvae, anticoagulant

INTRODUCTION
Blood dwelling parasites such as Schistosoma mansoni and Dirofilaria immitis have been shown to possess anticoagulant properties. This prevents stimulation of the coagulation system of the host and parasites thus escape being entrapped in a thrombus (Crawford et al., 1982). Ascaris lumbricoides, a nematode parasite of man, spends part of its life-cycle in the blood circulation of the infected host. The hatched larvae actively migrate through the intestinal wall and pass into the portal circulation of the liver, right heart and through the pulmonary vessels to the intra-alveolar tissue of the lungs. Since these developmental stages of Ascaris lumbricoides which pass through the host’s circulatory system are a foreign body, they could stimulate the coagulation system. Although adult Ascaris suum have been shown to possess antithrombotic activity (Crawford et al., 1982 and Howse et al., 1984), the literature does not reveal any information regarding the antithrombotic activity of the larvae, the stage which passes through the blood stream of the host. The present study reports the effects of larval stages of Ascaris lumbricoides on the human blood clotting system in vitro and the possible mechanism of action thereof.

MATERIALS AND METHODS
Parasite and its products
Stool samples were collected from patients with ascariasis and Ascaris eggs were concentrated by the saturated salt flotation technique. The eggs were separated from the associated debris by layering over 40% sucrose and centrifuged at 100 g for 10 min. The interface contained the purified eggs. The purified eggs (200/ml) were allowed to develop and hatched by a modified liquid medium technique as detailed by Wattal et al. (1985, 1986). The larvae were pressed free of the infective eggs between a microscope slide and cover slip and identified as third-stage larvae (L₃) and advanced third-stage larvae (LL₃) as described by Beaver et
al. (1984). The culture material was then layered over 20% sucrose followed by centrifugation (100 g for 10 min) to separate the larvae from egg shells and unhatched eggs. The larvae were homogenized in phosphate buffered saline (pH 7.2) in a tissue homogenizer followed by sonication. The material was centrifuged at 400 g for 30 min. The supernatant fluid was collected and labelled as L3 and LL3 extract and stored at -20°C till use.

Excretory/secretory (ES) products were collected by incubating L3/LL3 larvae in RPMI-1640 medium (Sigma Chemicals) at 37°C for 72 h in the presence of antibiotics followed by centrifugation at 100 g for 10 min and stored at -20°C till use. Protein concentration of L3/LL3 extracts and ES products were determined by the method of Lowry et al. (1951).

Plasma
Platelet-poor plasma was prepared from eight healthy volunteers who had no known defects of platelet function (Crawford et al., 1982).

Coagulation studies
Prothrombin (PT), thrombin time (TT) and partial thromboplastin time with kaolin (PTTK) were measured as detailed by Crawford et al. (1982). Briefly, 0.1 ml of whole extract/ES product of L3/LL3 (protein concentrations L3-5.457 mg/ml (diluted to 1 mg/ml), LL3-10.857 mg/ml (diluted to 1 mg/ml), L3ES-147 µg/ml, LL3ES-272 µg/ml) was added to 0.1 ml of plasma in a separate set of experiments. The above mixture was incubated at 37°C in waterbath for 5 min and 0.1 ml of this mixture was discarded. To this solution, for assay of PT, 0.1 ml of brain extract thromboplastin (Sigma Chemicals) was added to each tube keeping 2 min intervals between the tubes. After 1 min incubation, 0.1 ml of M/40 calcium chloride (BDH Chemicals Ltd.) was added and the formation of a fibrin clot was observed. The time from this addition to the clot formation was measured.

For TT assay, 0.1 ml of kaolin was added and the formation of a fibrin clot was observed. The time from this to the clot formation was measured.

For PTTK assay, 0.1 ml of kaolin was added to each tube keeping 3 min intervals. After incubation in water bath at 37°C for 9 min and 30 seconds, 0.1 ml of Cephalin (Sigma Chemicals) was added. After exactly 10 sec., 0.1 ml of M/40 calcium chloride (BDH Chemicals Ltd.) was added in rapid succession and the time from this addition to clot formation was measured.

Control experiments using heat inactivated (100°C for 15 min) L3/LL3 extracts/ES products were also set up (Tseng, et al., 1977). PT, TT and PTTK were also measured on corresponding blood samples without use of L3/LL3 extracts or ES products and recorded as normal readings. The parameters were also measured on all the blood samples with addition of RPMI/PBS instead of L3/LL3 extracts or ES products.

Determinations of the parameters for all the samples were performed in duplicate and the mean readings were recorded. The values obtained were subjected to statistical analysis using paired t-test.

RESULTS

Prothrombin time (PT)
The PT was significantly prolonged in test samples compared to controls (p<0.001). There was a significant difference between prolongation of the PT by L3
### TABLE 1. Effect of larval stages of *A. lumbricoides* on prothrombin time.

<table>
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<tr>
<th>Sample No.</th>
<th>Normal</th>
<th>RPM1</th>
<th>(Time in seconds)</th>
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<th>L₁ ES</th>
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Mean±SD  
15.87±5.1±2.46  18.75±1.16  18.875±1.642  33±4.342  21.375±2.133  90.875±9.062  24.25±2.659

Normal vs L₁  p<0.001  Normal vs LL₁  ES  p<0.001  L₁ vs LL₁  p<0.001  L₁ ES vs LL₁ ES  p<0.05
RPM1 vs L₁  p<0.001  Normal vs LL₁ ES  p<0.001  L₁ ES vs LL₁ ES  p<0.05
PBS vs L₁  p<0.001
Normal vs LL₁  p<0.001
RPM1 vs LL₁  p<0.001
PBS vs LL₁  p<0.001
TABLE II. Effect of larval stages of *A. lumbricoides* on thrombin time.

<table>
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Mean±SD 13.25±1.035 18.75±3.57 19.5±1.069 33.25±3.882 45.875±1.959 102±11.338 44.375±6.128

Normal vs L<sub>3</sub>, p<0.001 RPMI vs L<sub>3</sub>, p<0.001 PBS vs L<sub>3</sub>, p<0.001 Normal vs L<sub>3</sub> ES, p<0.001 Normal vs LL<sub>3</sub> ES, p<0.001 L<sub>3</sub> ES vs LL<sub>3</sub> ES, p<0.05
TABLE III. Effect of larval stages of *A. lumbricoides* on partial thromboplastin time with kaolin.

<table>
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<tr>
<th>Sample No.</th>
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<th><em>L</em>₁ ES</th>
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Mean±SD 42±2.618 53±5.264 55.625±2.326 110.125±2.948 80.125±13.632 158.25±39.037 152.5±12.432

Normal vs *L*₁ *p*<0.001
RPMI vs *L*₁ *p*<0.001
PBS vs *L*₁ *p*<0.001
Normal vs LL*₃ *p*<0.001
RPMI vs LL*₃ *p*<0.001
PBS vs LL*₃ *p*<0.001
*L*₁ vs LL*₃ *p*<0.001
*L*₁ ES vs LL*₃ ES *p*<0.05
FIG. 1 Effect of larval stages of *Ascaris lumbricoides* on prothrombin time (PT), thrombin time (TT) and partial thromboplastin time with kaolin (PTTK).

and LL₃ extracts (p<0.001). It was observed that L₃/LL₃ ES products also prolonged PT significantly as compared to controls (p<0.001). However no highly significant difference was observed between prolongation of PT by L₃ ES and LL₃ ES (p<0.05) (Table I).

**Thrombin time (TT)**

There was a significant difference in the prolongation of TT in test samples as compared to controls (p<0.001) and also in the prolongation of TT by L₃ and LL₃ extracts (p<0.001). However there was no highly significant difference between the prolongation of TT by L₃ and LL₃ ES products (p<0.05) (Table II).

**Partial thromboplastin time with kaolin (PTTK)**

PTTK was significantly prolonged by L₃ and LL₃ extracts as well as L₃ and LL₃ ES products as compared to controls (p<0.001). There was also a significant difference between prolongation of PTTK by L₃ vs LL₃ extracts (p<0.005) and L₃ ES vs LL₃ ES products (p<0.001) (Table III). LL₃ extract had maximum effect on the prolongation of PT, TT and PTTK (Fig. 1). All the three parameters studied with heat killed extracts showed no significant difference when compared to normal readings.

**DISCUSSION**

The present study has indicated that larval stages of *Ascaris lumbricoides* var
Effect of *A. lumbricoides* larvae on human blood clotting

hominis possess antithrombotic activities which may be involved in evasion of the host defence system.

The statistically significant differences in the prolongation of PT by the L₃/LL₃ extracts as well as with ES products as compared to controls is in contrast with the reported observations on *S. mansoni* (Tsang et al., 1977) and *A. suum* adult worm (Crawford et al., 1982). However, DeWitt & Warren (1959) have reported significantly prolonged PT in mice at 6 weeks continuing to the 16th week after infection with 125 *S. mansoni* cercariae. These workers have not considered this observation in relation to haemostasis but rather as one of the methods of measurement for liver dysfunction in studies of hepatosplenic schistosomiasis in mice. The present study indicated that larval stages of *A. lumbricoides* var hominis produce an inhibitor for the extrinsic blood coagulation pathway as well which is not produced by the adult worms. Alternatively, there may be different components produced which may be acting through two different pathways and subsequently in the adult stage concentration of the component acting through the extrinsic pathway may be reduced. Further there is a possibility that the concentration of an inhibitor is related to the association of the parasite with the host and levels are substantially reduced in adult stages as levels of metabolic products have been reported to differ in developmental and adult stages of *Ascaris* (Mishra et al., 1982).

The present study further indicates that L₃ ES and LL₃ ES may be producing different anticoagulant components as PTTK was significantly prolonged by LL₃ ES as compared to L₃ ES. However no significant difference was observed in the prolongation of PT and TT by the LL₃ and L₃ ES.

The PTTK was also significantly prolonged by L₃/LL₃ extracts as well as ES products which indicates the presence of an inhibitor of the intrinsic blood coagulation pathway. The significant anticoagulant effects of ES products indicate that the anticoagulant is not localized but may be diffusely spread in the parasite as also reported earlier (Crawford et al., 1982).

Further, the anticoagulant component appears to be thermolabile as heat killed extracts had an effect similar to that of the controls.

In summary, the present work revealed that larval stages and their ES products of *A. lumbricoides* var hominis produce thermolabile anticoagulant components which may be acting through both the intrinsic and extrinsic blood coagulation pathway and thus evade the host defence system. Further, this observation is in contrast with the earlier studies conducted on adult worms where the anticoagulant component has been shown to act on the intrinsic blood coagulation pathway alone.

REFERENCES


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