Identification of a fibrinolytic enzyme in *Schistosoma mansoni* eggs and modulated blood fibrinogen metabolism in *S. mansoni*-infected mice

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SUMMARY

Aqueous extracts of *Schistosoma mansoni* eggs have been shown to have fibrinolytic activity inhibitable by a serine protease inhibitor. Fibrinolytic activity was not present in extracts of either adult worms or cercariae. A 27 kDa enzyme that was proteolytically active on fibrinogen in zymography and that degraded fibrinogen in a pattern similar to that of plasmin, is presumed to be responsible for the schistosome egg fibrinolytic activity. Anti-human fibrinogen antisera were shown to have antibodies that cross-reacted with mouse fibrinogen in Western immunoblots. Electroblotted sera from *S. mansoni*-infected and control uninfected mice displayed different antigenic profiles when probed with the cross-reactive anti-human fibrinogen antibodies, suggesting an alteration in mouse host fibrinogen metabolism as a result of the parasitic infection. We discuss the possibility that modulation of fibrinogen metabolism is a factor in a recently discovered anti-atherogenic effect exerted by schistosomes.

Key words: *Schistosoma mansoni*, eggs, fibrinogen, fibrinolysis, serine protease.

INTRODUCTION

Schistosoma mansoni eggs have been shown to be potent agonists of blood platelet aggregation and activation and it was suggested that interactions between platelets and eggs may be required for extravasation of the eggs in the early stages of excretion from the host (Ngaiza & Doenhoff, 1990). It has long been known that activation of platelet aggregation is prothrombotic and causes generation of fibrin from fibrinogen (De Gaetano & Cerletti, 2002). The question therefore arises whether there is a mechanism to counteract what might be excessive generation and subsequent intravascular deposition of fibrin by schistosome egg-activated platelets. We describe here the existence in *S. mansoni* eggs of a plasmin-like enzyme with fibrino(gen)olytic activities that may constitute such a counteractive mechanism.

MATERIALS AND METHODS

A Puerto Rican isolate of *S. mansoni* was used as the source of schistosome-derived material. Cercariae, adult worms and eggs and their soluble extracts were prepared as described by Doenhoff *et al*. (1981). Mice were infected with cercariae percutaneously as described by Smithers & Terry (1965).

Tests for fibrinolytic activity were performed on fibrin films prepared by adding 10 units of thrombin (Sigma, Poole, UK) to a solution of 50 mg human fibrinogen (KabiVitrum AB, Stockholm, Sweden) in 10 ml of phosphate-buffered saline (PBS), pH 7·2, and pouring the mixture into a 10 cm plastic Petri dish. After the fibrin film had stabilized, aliquots of schistosome extracts were placed on the surface and after 4 h incubation at 37 °C the plates were photographed. The fibrinolytic potency of *S. mansoni* egg extracts was estimated by comparing the area of lysis given by the parasite-derived material with that given by aliquots containing different concentrations of purified human plasmin (Sigma, Poole, UK).

Separation of proteins by polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS–PAGE) was performed on a Mini Protean II apparatus (Bio-Rad Laboratories Ltd, Hemel Hamstead, Herts, UK) using an adapted method (Laemmli, 1970). Zymography to test for fibrinogenolytic activity was performed by adaptation of the methods of Lockwood *et al*. (1987). One hundred μg of protein from *S. mansoni* eggs isolated from mouse livers and 100 μg protein from uninfected mouse liver homogenate were subjected to SDS–PAGE in 12% acrylamide gels containing 0·05% (w/w) purified human fibrinogen. Following electrophoresis the gels

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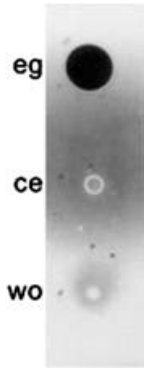

Fig. 1. Fibrinolytic activity in *Schistosoma mansoni* eggs. Ten μl aliquots, each containing 10 μg bovine serum albumin-equivalent soluble proteins from, respectively, *S. mansoni* eggs (eg), cercariae (ce) or adult worms (wo) were placed on a fibrin film, incubated for 4 h at 37 °C and photographed over a dark background. The black circle represents an area of fibrinolysis developed by the egg extract after incubation. No such activity was evident in the other two extracts. The same result was obtained with 2 further batches of each of the 3 extracts prepared on different occasions.

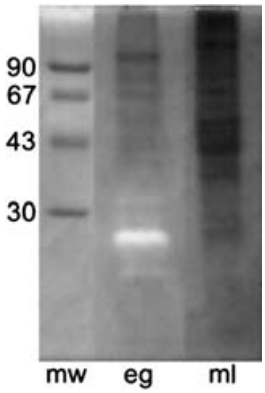

Fig. 2. Zymograph of fibrinogenolytic activity in *Schistosoma mansoni* egg extracts. 100 μg of *S. mansoni* egg protein (eg) and uninfected mouse liver homogenate (ml) were subjected to PAGE in gels containing fibrinogen, followed by incubation in buffers that allowed reconstitution of enzymatic activity. mw, Molecular weight standards.

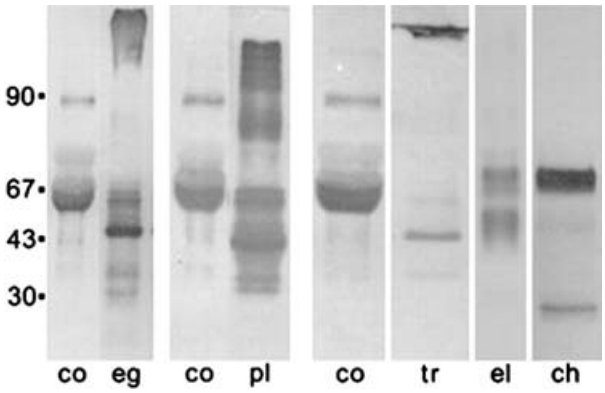

Fig. 3. Western immunoblot showing fibrinogenolytic activity in extracts of *Schistosoma mansoni* eggs. Human fibrinogen was incubated *in vitro* with *S. mansoni* egg extract (eg), human plasmin (pl), trypsin (tr), elastase (el) and chymotrypsin (ch) or without any addition (co). After 10 min incubation at 37 °C an aliquot from each incubate was electroblotted and probed with antibodies specific for human fibrinogen.

were incubated for 1 h at 37 °C in 2% Triton X-100 in PBS, followed by 16 h at 37 °C in PBS. Proteins remaining in the gel were stained with Coomassie blue. Proteolytic activity against the fibrinogen substrate was visible as a clear area in dark (blue) background.

In an alternative test for the presence of fibrinogenolytic activity in *S. mansoni* eggs, 100 μl aliquots, each containing 100 μg purified human fibrinogen in PBS, were incubated for 15 min at 37 °C with equal volumes of PBS alone (control), or PBS that contained an estimated 120 μg of bovine serum albumin-equivalent of *S. mansoni* egg protein. Controls were 100 μg fibrinogen incubated with 0·25 units human plasmin, 100 μg porcine pancreatic trypsin, 100 μg porcine pancreatic elastase, or 100 μg bovine pancreatic chymotrypsin. The human fibrinogen and purified proteolytic enzymes were purchased from Sigma (Poole, UK). After incubation for 10 min, 10 μl aliquots from each of the mixtures and respective controls were subjected to SDS–PAGE under reducing conditions. The electrophoresed proteins were electroblotted onto nitrocellulose paper and probed with antisera as previously described (Towbin, Staehelin & Gordon, 1979). A rabbit anti-human fibrinogen antiserum purchased from Sigma (Poole, UK) was used as the source of primary antibodies for Fig. 3, and a goat anti-human fibrinogen serum from Cambio (Cambridge, UK) was used for Fig. 4. Enzyme conjugated goat anti-rabbit IgG and rabbit anti-goat IgG antibodies were used as the secondary antibodies for Figs 3 and 4 respectively.

To test for antigenic cross-reactivity between mouse and human fibrinogen purified mouse fibrinogen (Sigma, Poole, UK) was heated at 100 °C for 4 min in PBS and 6 μg protein was electrophoresed by SDS–PAGE under non-reducing conditions, electrotransferred to NCP and probed with a goat anti-human fibrinogen antiserum.

To display fibrinogen and its derivatives in mouse blood, samples of blood were taken from superficial veins of individual *S. mansoni*-infected or uninfected mice. The blood was allowed to clot at 37 °C for 1 h and at 4 °C for 4 h, centrifuged (Micro Centaur, MSE Scientific Instruments, Crawley, UK) and the serum removed. Within 2 h the serum samples were heated to 100 °C for 30 sec (Fig. 4B) or for 4 min (Fig. 4C) and a 1 μl aliquot of serum from each mouse was separately subjected to SDS–PAGE, electroblotted onto NCP and probed with a goat anti-human fibrinogen antiserum.

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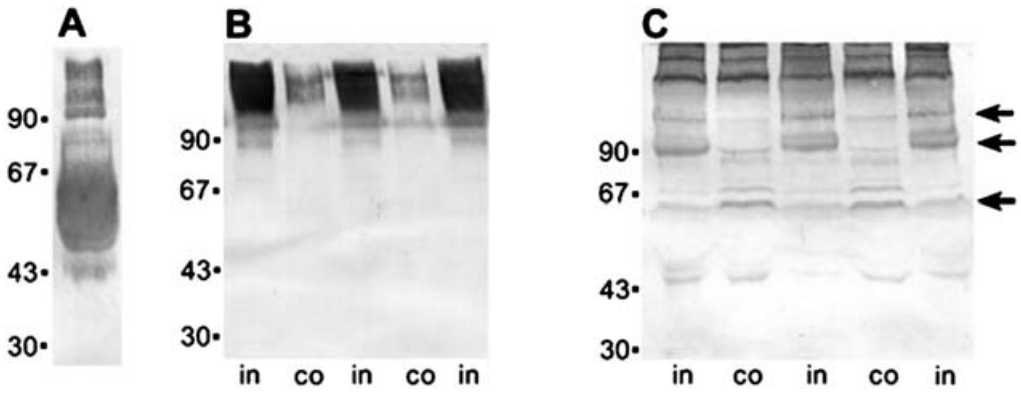

Fig. 4. Fibrinogen in serum of *Schistosoma mansoni*-infected mice. (A) Western immunoblot of mouse fibrinogen probed with primary antibodies specific for human fibrinogen. (B and C) Western blot immunoreactivity of sera from *S. mansoni*-infected mice (in) or uninfected control mice (co). The infected mice had been given percutaneous infections of 150 *S. mansoni* cercariae 6 weeks previously. Serum samples from individual mice were subjected to 100 °C for either 30 sec (B) or 4 min (C) before electrophoresis in polyacrylamide gel.

RESULTS

Fig. 1 shows that extracts of homogenized *S. mansoni* eggs possess fibrinolytic activity that can be demonstrated on a fibrin film: no such activity was found in extracts of *S. mansoni* adult worms or cercariae. The fibrinolytic activity in egg extracts was inhibited by treatment with 1 mM concentrations of the serine protease inhibitor phenylmethylsulphonylfluoride. Comparative studies indicated that 1 mg of protein in the *S. mansoni* egg extract had fibrinolytic activity equivalent to 0·01 units of commercially-obtained purified plasmin.

Fibrinogenolytic activity was detected in extracts of *S. mansoni* eggs isolated from livers of infected mice by zymography in polyacrylamide-substrate gel electrophoresis, with no such activity present in extracts of uninfected mouse liver, the host tissue from which the eggs were extracted (Fig. 2). The predominant fibrinogenolytic activity was associated with a molecule of approximately 27 kDa.

When human fibrinogen was incubated with *S. mansoni* egg extract the pattern of degradation of the protein substrate showed some similarity with that which occurred after incubation of the blood clotting protein with purified human plasmin (Fig. 3). The patterns of degradation of fibrinogen found after incubation with 3 other mammalian serine proteases, trypsin, chymotrypsin and elastase, was distinct from that given by the schistosome egg extract and plasmin (Fig. 3).

Fig. 4A shows that human and mouse fibrinogens are antigenically cross-reactive and the goat anti-human fibrinogen antiserum could therefore be used to detect mouse fibrinogen in Western immunoblots of mouse serum. Fig. 4B and C show the reactivity of the anti-human fibrinogen antibodies against heat-denatured serum from 3 representative *S. mansoni*-infected and 2 representative uninfected control mice. After 30 sec of heat treatment (Fig. 4B) the sera from infected animals had more intensely reacting fibrinogen-related material of relatively high molecular weight than the sera from uninfected controls. Analysis of sera more extensively denatured by heating for 4 min showed the infected and uninfected animals to be different with respect to several circulating fibrinogen-related proteins (identified by arrows in Fig. 4C).

DISCUSSION

The results show that extracts of *S. mansoni* eggs possess fibrinolytic activity that is not present in extracts of either cercariae or adult worms. Fibrinolytic activity is also released from *S. mansoni* eggs that have been freshly isolated and placed intact on the surface of a fibrin film (Curtis, 1991), a process that may also occur *in vivo*. A proteolytic enzyme of 27 kDa with fibrinogenolytic activity was found in egg extracts subjected to SDS–PAGE and in the absence of evidence of other enzymatic activity against the fibrinogen substrate it seems probable that the 27 kDa enzyme was responsible for the hydrolysis of fibrin. The results indicate the fibrinogenolytic activity of this enzyme is more similar to that of plasmin than to that of 3 other serine proteases. By extrapolation, the same enzyme may be responsible for the changes observed to host blood fibrinogen. Results not shown here have indicated that the differences between the sera of infected and uninfected mice in this respect became apparent only after the infections in the former animals had become patent.

A search of current literature databases has not identified any other reports of serine protease activity in schistosome eggs.

We propose that the fibrin(ogen)olytic activity demonstrated here in *S. mansoni* eggs balances the thrombogenic potential that the intact egg surface has previously been shown to possess (Ngaiza & Doenhoff, 1990). We have found little published work concerned with fibrinogen metabolism in

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human schistosomiasis, except that hyperfibrinolysis associated with decreased plasminogen activator inhibitor-1 (PAI-1) has been observed in patients with hepatosplenic disease (El-Bassiouni *et al*. 1996).

Elsewhere we have shown that schistosome infections in mice exert an anti-atherogenic effect (Doenhoff *et al*. 2002). Blood fibrinogen has been identified as a risk factor for atherogenisis in humans (Bini & Kudryk, 1995; Tribouilloy *et al*. 1998) and may potentiate the atherogenic effect of hyperlipidaemia (Levenson *et al*. 1997). In mice the evidence linking fibrinogen with atherogenesis is less firm than in humans (Koopman *et al*. 1997; Xiao *et al*. 1998), but we consider further investigation of the fibrin(ogen)olysis occurring during the course of schistosome infection and its possible connection with atherogenesis to be worthwhile.

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