Schistosoma mansoni: anomalous immunogenic properties of a 27 kDa larval serine protease associated with protective immunity

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SUMMARY

A cationic *Schistosoma mansoni* cercarial antigen was shown to be a serine protease as it was capable of hydrolysing *N*-acetyl-DL-phenylalanine β-naphthyl ester (NAPBNE) after precipitation by immunoelectrophoresis, and this reaction was modulated by the serine protease inhibitors phenylmethanesulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP). The antigen in the immunoprecipitin arcs could also be radio-isotope labelled with tritiated DFP. The peptidolytic enzyme identified in immunoelectrophoresis with polyspecific sera and radio-isotope labelled with tritiated DFP had a relative molecular size of approximately 27 kDa in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), and evidence obtained after partial purification, SDS–PAGE and immunoblotting supported this size estimate for the enzyme. A rabbit antiserum raised against the peptidolytic antigen reacted against a doublet of antigens at 27/28 kDa in immunoelectrophoresis arcs and against an antigen of 60 kDa in Western immunoblots of crude cercarial homogenate. However, the latter serum precipitated the cationic antigen in immunoelectrophoresed cercarial homogenates only after pre-incubation of the homogenates with PMSF. Fractions containing the partially purified protease also degraded radio-isotope labelled human IgG. The reactivity of a range of polyspecific and monospecific rabbit antisera in Western blots with larval extracts indicated that antibody responses against the 27/28 kDa doublet may be modulated. When immunized with material which contained the 27 kDa enzyme as a major constituent, and which was secreted by *S. mansoni* cercariae during transformation, only 5 of 16 mice produced antibody to this antigen that was detectable in Western blots. The 5 antibody 'responder' mice were significantly ($P < 0{\cdot}001$) protected against challenge with a percutaneous infection of *S. mansoni* cercariae compared with a group of mice also immunized with CTF, but which had not produced antibodies against the 27/28 kDa doublet. The results indicate that the 27 kDa serine protease of *S. mansoni* larvae is a target that is sensitive to immunological attack.

Key words: *Schistosoma mansoni*, cercariae, mice, protease, elastase, immune-evasion, immunoglobulin degradation, vaccination.

INTRODUCTION

Proteolytic enzymes of schistosome larvae are considered to have an important role in facilitating penetration of host skin by the parasite and subsequent migration of schistosomula (Gazzinelli & Pellegrino, 1964; Dresden & Asch, 1972; Campbell *et al*. 1976; Kloetzel, 1978; McKerrow & Doenhoff, 1988). The role of these enzymes may, however, be more complex than simply to hydrolyse proteinaceous barriers which the invading larvae encounter, since larval-derived proteolytic activity has been implicated in the generation of inflammatory reactivity associated with penetrating schistosomes (Teixeira *et al*. 1993) and this inflammation, rather than being purely a mechanism of host-defence, has been shown also to enhance parasite infectivity (Fallon *et al*. 1996*b*).

Several laboratories have succeeded in purifying the seemingly relevant enzymes from *Schistosoma mansoni*. Thus, Landsperger, Stirewalt & Dresden (1982) purified a 25 kDa enzyme resembling vertebrate chymotrypsin from cercariae. McKerrow *et al*. (1985) isolated a 30 kDa serine protease, the predicted amino acid sequence of which showed a high degree of homology with rat pancreatic elastase I and II, but which had a chymotrypsin-like active site (Newport *et al*. 1988). Marikovsky, Fishelson & Arnon (1988*b*) purified proteases of 28 kDa and 60 kDa from schistosomula secretions and implicated the activities of these 2 enzymes in the release of the glycocalyx and complement-activating molecules from the larval surface (Marikovsky, Arnon & Fishelson, 1988*a*). More recently a membrane-

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bound form of a 28 kDa protease has been detected (Ghendler *et al*. 1996), and a 47 kDa serine protease has been identified and purified (Chavez-Orlortegui, Resende & Tavares, 1992).

Because of their importance in facilitating parasite invasion the larval proteases are of potential interest as possible targets of protective immune responses and of chemotherapeutic agents. Thus, a monoclonal antibody reactive against the 30 kDa cercarial elastase identified by McKerrow *et al*. (1985) was cytotoxic to *S. mansoni* cercariae *in vitro* (Pino-Heiss *et al*. 1986), and specific chemical inhibitors of the enzyme have been shown to protect mice against infection (Cohen *et al*. 1991). Pierrot *et al*. (1996) have also shown that rat antibodies that are specific for the elastase mediated macrophage cytotoxicity against schistosomula *in vitro*.

We here investigate further a cationic *S. mansoni* larval protease, the existence of which was initially detected by a combination of zymography and immunoelectrophoresis (Doenhoff *et al*. 1988). In respect of its chymotrypsin-like activity and a relative molecular size of 27 kDa, this enzyme has here been shown to resemble previously identified major serine proteases of *S. mansoni* larvae (Landsperger *et al*. 1982; McKerrow *et al*. 1985; Marikovsky *et al*. 1988*a*). It has also been shown to hydrolyse immunoglobulin, and to have irregular immunogenic properties in rabbits and mice.

MATERIALS AND METHODS

Parasite and antigens

A Puerto Rican isolate of *S. mansoni* was maintained by passage through random-bred mice and albino *Biomphalaria glabrata* snails. Production of cercarial suspensions from patent snails, and centrifuged cercarial homogenate (CH) were as previously described (Doenhoff *et al*. 1981). The supernatant (CH) was stored at −70 °C until needed, and had a protein concentration of approximately 7 mg/ml (bovine serum albumin equivalent).

Cercarial transformation fluid (CTF) was prepared by resuspending cercariae in tissue culture medium 199 (Wellcome Reagents, Beckenham, Kent) at a concentration of 2×10^5/ml immediately after they had been sedimented by cooling (i.e. while still alive) and mechanically transforming them by aspiration 14–20 times through a 19G × 1·5 inch syringe needle (Colley & Wikel, 1974). The parasites were incubated for 4 h at 37 °C, centrifuged out, and the supernatant (CTF) stored at −70 °C until required. Before use CTF was concentrated approximately 10-fold by ultrafiltration to give a protein concentration of approximately 3 mg/ml.

Immunization and challenge of mice

Inbred male CBA/Ca mice, approximately 6 weeks old, were immunized with CTF by injection of 0·2 ml of a 10% (v/v) suspension of alum adjuvant on to which had been adsorbed 100 μl of CTF containing 100 μg protein. Immunizations with CTF were repeated 5 times and antibody responses assayed in Western immunoblots and ELISA (see below). Selected mice (see text) were given a percutaneous challenge of 200 *S. mansoni* cercariae, and worm burdens determined by portal perfusion as described by Doenhoff *et al*. (1978*a*). Liver egg counts were determined after digestion of the tissue in potassium hydroxide as described by Doenhoff *et al*. (1978*b*). The Student's *t*-test was used to determine whether group mean differences were significant.

Rabbit antisera

Polyspecific antisera were raised in adult New Zealand White rabbits by repeated fortnightly injections of 0·5 ml volumes of antigenic extracts (CTF and CH) emulsified in Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for boosters. Rabbit sera specific for a cationic cercarial peptidase active on chymotrypsin substrates (Doenhoff *et al*. 1988) were prepared using 2 methods. (i) CH was immunoelectrophoresed (Dunne *et al*. 1981) with polyspecific rabbit anti-CTF serum and immunoprecipitation lines containing a cathodally migrating peptidolytic enzyme were displayed through their activity on *N*-acetyl-DL-phenylalanine β-naphthyl ester (NAPBNE; see Fig. 1 below and Doenhoff *et al*. 1988). Approximately 100 enzymatically reactive immunoprecipitin lines were excised and washed extensively in isotonic saline to remove non-precipitated material. The agar containing the immune complexes was homogenized with an equal volume (3 ml) of saline, emulsified with 6 ml of Freund's adjuvant and used to immunize rabbits as described (Goudie, Horne & Wilkinson, 1966; Dunne *et al*. 1986). (ii) CTF was subjected to SDS–PAGE and electrotransferred to nitrocellulose paper as described below. Two lanes were cut from each side of the NCP and stained non-specifically for protein. Two proteins with putative proteolytic enzyme activity banding at 27 and 28 kDa respectively were excised separately and placed in the minimum volume of dimethyl formamide (DMF) to achieve dissolution. An equal volume of isotonic saline was added and the mixture emulsified with Freund's adjuvant for immunization of rabbits. Rabbits were test bled after the fourth injections of the respective antigens, and tested for antibody in immunoelectrophoresis or immunoblotting. Immunizations and test bleeds were continued until sera were arbitrarily considered to have appropriate or workable antibody reactivity, which was generally found to be after the sixth or seventh immunization.

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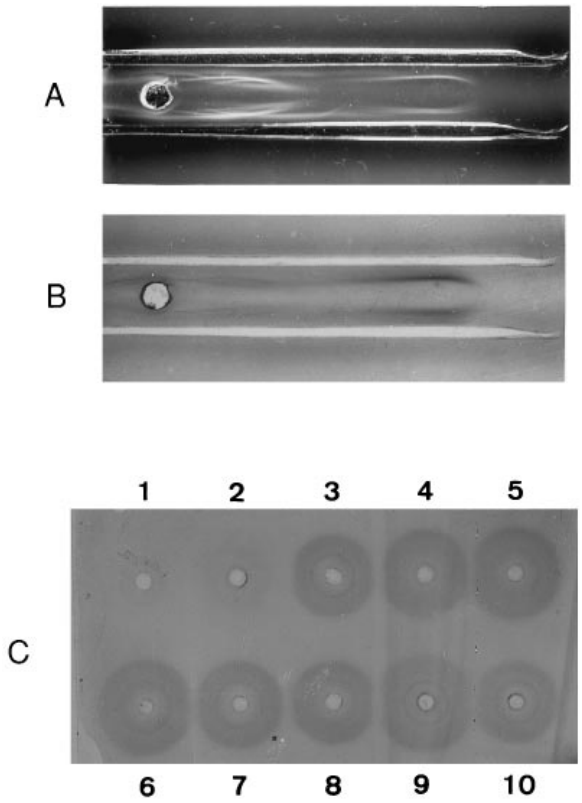

Fig. 1. Immunoelectrophoresis and radial immunodiffusion of *Schistosoma mansoni* CH with rabbit anti-CTF serum. (A and B) IEP of CH with anti-CTF serum photographed over (A) indirect light, and (B) direct light after the plate had been washed and immersed in a chromogenic substrate mixture of NAPBNE and FBB. In this and subsequent IEPs the anode is to the left. Note that chromophore has been generated by the extreme cathodal precipitin arc. (C) Radial immunodiffusion of unfractionated CH after treatment with protease inhibitors and the anti-CTF serum used in (A) and (B) above. Prior to addition to the antigen wells the CH was incubated for 30 min at 37 °C with (1) 1 mM PMSF; (2) 1 mM DFP; (3) 1 mM TLCK; (4) 1 mM TPCK; (5) 4 μg bestatin to 50 μl CH; (6) 5 mM phenanthroline; (7) 5 mM HMB; (8) 2 mM EDTA; (9) 100 μg SBTI to 50 μl CH; (10) untreated control.

Rabbit anti-infection sera were prepared by administering 5–6 percutaneous infections, each infection consisting of $10–20 \times 10^3$ *S. mansoni* cercariae, at 4-week intervals via the ear, as described by Fallon, Fookes & Doenhoff (1996*a*).

Rabbits considered to have appropriate antibody reactivity were exsanguinated by cardiac puncture and the immune sera stored at −20 °C until required.

Immunoelectrophoresis, immunodiffusion and preparative electrophoresis

Immunoelectrophoresis (IEP) was performed on 84 mm square glass plates in agar/barbitone buffer, pH 8·6, as described (Dunne *et al.* 1981). Single radial immunodiffusion (RID) was performed as described (Mancini, Carbonara & Heremans, 1965). Immunodiffusion in agar was performed as described by Ouchterlony (1958).

Chymotrypsin-like peptidolytic activity (Pearse, 1972) was identified in immunoprecipitates in agar by washing the agar films in excess isotonic saline for 24 h, followed by immersion in a chromogenic substrate mixture consisting of 5 mg NAPBNE and 10 mg Fast Blue B (FBB; Sigma) dissolved in 2 ml of DMF, which was then added to 40 ml of 0·05 M phosphate-buffered saline, pH 7·6 (PBS).

Preparative electrophoresis of CTF was performed as described previously for fractionation of *S. mansoni* egg antigens (Dunne *et al.* 1981).

Protease inhibitors and estimation of protease activity using azocoll

Protease inhibitors used in this study included: phenylmethanesulphonyl fluoride (PMSF); diisopropylfluorophosphate (DFP); *N*-tosyl-L-lysine chloromethyl ketone (TLCK); *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK); bestatin; phenanthroline; *p*-hydroxymercuribenzoic acid (HMB); ethylenediaminetetraacetic acid (EDTA); soybean trypsin inhibitor (SBTI). All were obtained from Sigma (Poole, Dorset, UK). Tritium-labelled DFP ([1,3-^{3}H]DFP) was obtained from NEN Research Products.

Proteolytic activity on azocoll was assayed as described by Dresden & Asch (1972). Thus 100 μl samples of fractions of CH obtained by preparative electrophoresis were added to 5 mg azocoll (Sigma) in 200 μl of 0·05 M PBS, pH 7·6. The mixture was incubated overnight at 37 °C, and the volume made up to 0·5 ml with distilled water. Undigested azocoll was removed by centrifugation and light absorption by the supernatants analysed at 540 nm.

Polyacrylamide gel electrophoresis

One-dimensional electrophoresis in polyacrylamide with sodium dodecylsulphate (SDS–PAGE) was performed as described (Laemmli, 1970) under non-reducing conditions as reported previously (Curtis, Fallon & Doenhoff, 1996).

Protease substrate gels, immunoblotting, autoradiography and enzyme-immunoassay

Gelatinolytic activity was detected in SDS–PAGE using a method adapted from Lockwood *et al.* (1987). SDS–PAGE gels were prepared as above with 10 mg gelatin substrate (Sigma) added to the acrylamide solution. Electrophoresis was as above for SDS–PAGE. After electrophoresis the gel was

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washed with 2·5% Triton X–100 in 0·05 M PBS, pH 7·6, and incubated overnight at 37 °C in PBS alone. Substrate gels were stained with Coomassie blue and proteolytic activity was demonstrated as a clear area in a blue background.

Western immunoblotting of antigens derived from SDS–PAGE gels was performed as described previously (Fallon *et al*. 1996*a*).

ELISA on mouse antisera was performed in microtitration plates essentially as described by McLaren *et al*. (1981) for human sera, adapted as described by Fallon *et al*. (1996*a*).

Radio-isotope labelling, immunoprecipitation with protein A and autoradiography

S. mansoni CH was radio-isotope labelled with [1,3-^{3}H]DFP following the method of Pastenack & Eisen (1985). [1,3-^{3}H]DFP in propylene glycol (3 Ci/mmol) was added to 100 μl of *S. mansoni* CH to a final concentration of 10^{-3} M. After 30 min at room temperature, the reaction was stopped by adding Tris/HCl, pH 8·1, to a final concentration of 15 mM and SDS to a final concentration of 0·1%. Labelled proteins were recovered by cold acetone precipitation and co-precipitated with rabbit antisera, or analysed in SDS–PAGE or one-dimensional IEP.

Human immunoglobulin (Dakopatts Dako Corporation, California) was dissolved in PBS and iodinated using Iodogen (Pierce Chemical Co.) according to the method of Fracker & Speck (1978). The radio-isotope labelled IgG was further purified by SDS–PAGE (Tamashiro, Rao & Scott, 1987) by subjecting a sample of IgG carrying 5×10^6 cpm in 100 μl of sample buffer to SDS–PAGE under non-reducing conditions as above. Gel slices containing radioactive material of molecular size 150–160 kDa were excised from the gel and gel pieces 1 cm square were transferred to Eppendorf tubes and macerated. The effect of *S. mansoni* CH on radio-isotope labelled IgG was determined by incubating the macerated gel samples at 37 °C for up to 18 h with 1 mg CH protein dissolved in 100 μl of 0·05 M PBS, pH 7·6. Ten μl samples removed during the course of the incubation were prepared for SDS–PAGE by boiling in sample buffer, again in non-reducing conditions, immediately after removal.

RESULTS

Correlation between a cationic serine peptidase/protease and a 27 kDa antigen

A cationic antigen that was precipitated out from *S. mansoni* CH by a polyspecific rabbit anti-CTF serum in immunoelectrophoresis was shown to have chymotrypsin-like peptidolytic activity through its ability to hydrolyse NAPBNE (Fig. 1A and B).

The antiserum used in Fig. 1 was polyspecific, but only 1 precipitin line resulting after IEP was found to hydrolyse the peptidase substrate, and in combination with chromogenic substrate the serum could therefore be used for the specific display of the cationic peptidolytic activity. When aliquots of CH were incubated with a range of protease inhibitors and subjected to radial immunodiffusion the immunoprecipitated peptidolytic activity was inhibited by PMSF and DFP, indicating that the cationic antigen is a serine protease (Fig. 1C). No inhibitory effect was found with 2 other serine protease inhibitors TLCK and TPCK, nor with bestatin, phenanthroline, pHMB, EDTA and SBTI.

Radio-isotope labelling with DFP confirmed that the cathodally migrating antigen in *S. mansoni* larval extracts is a serine protease, and was used to determine its relative molecular size. Thus, CH was incubated with [1,3-^{3}H]DFP and subjected to immunoelectrophoresis with rabbit anti-CTF serum, followed by autoradiography. Fig. 2A and B show that the antigen present in a cathodal immunoelectrophoresis precipitin arc had become labelled by the DFP, and furthermore, the labelled arc was in an electrophoretically similar position to the cationic antigen in CH which hydrolysed NAPBNE in Fig. 1A and B.

Radio-isotope labelled material in the IEP precipitin arcs of Fig. 2A and B gave an autoradiographically active band at approximately 27 kDa in SDS–PAGE plus autoradiography (Fig. 3, lane 3), which corresponded to the lower band of a radioactive 27/28 kDa doublet present in both the DFP-labelled CH (Fig 3, lane 2) and in immune complexes co-precipitated out by polyspecific anti-CTF serum and protein A (Fig. 3, lane 4). In DFP-labelled CH there was radio-isotope labelling of further high molecular weight material that had failed to enter the gel, and labelled material of approximately 17 kDa was also present.

Attempts to purify the proteolytic activity in CH by column chromatography (gel filtration or ion exchange) were hampered by a failure of the activity to emerge from a variety of fractionation matrices that were tested. Partial purification was, however, achieved by preparative electrophoresis (PEP) in a bed of inert vinyl chloride copolymer beads (Dunne *et al*. 1981), a procedure which took particular advantage of the cationic nature of the proteolytic activity under study (as indicated by immunoelectrophoresis, Fig. 1). Aliquots from all the fractions were tested for azocollagenolytic activity and for peptidolytic activity in RID with a rabbit anti-CTF serum followed by 'staining' with NAPBNE and it was confirmed that the enzymatic and antigenic activities were found in the most cathodal fractions (results not shown).

Fractions containing antigenic material which hydrolysed NAPBNE after immunoprecipitation in RID were pooled and subjected to immunodiffusion, SDS–PAGE and immunoblotting. After concen-

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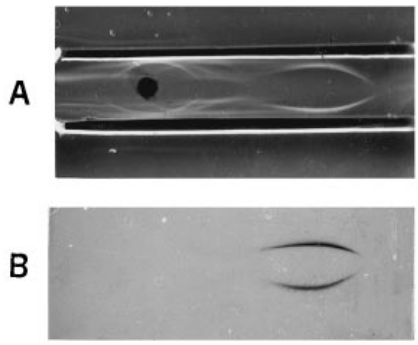

Fig. 2. Immunoelectrophoresis and autoradiography of CH labelled with tritiated-DFP. IEP of [1,3-^{3}H]DFP-labelled CH with anti-CTF serum photographed over (A) indirect light and (B) direct light after washing, drying and autoradiography.

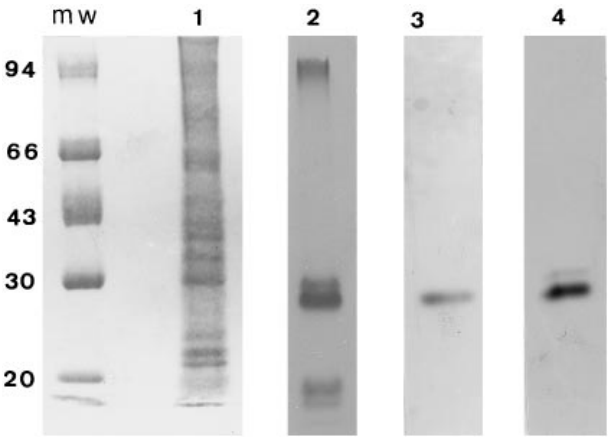

Fig. 3. SDS–PAGE analysis of CH after radio-isotope labelling with tritiated DFP. MW, Coomassie blue-stained molecular weight standards; Lane 1: Coomassie blue-stained, unfractionated CH; Lane 2: autoradiograph of unfractionated CH radio-isotope labelled with [1,3-^{3}H]DFP; Lane 3: autoradiograph of cationic [1,3-^{3}H]DFP-labelled precipitin arcs excised from IEP plates prepared as for Fig. 2; Lane 4: autoradiograph of material in CH labelled with [1,3-^{3}H]DFP and co-precipitated with rabbit anti-CTF serum and protein A-Sepharose (as described by Simpson, James & Sher, 1983).

tration the pooled fractions contained material which yielded a single line stainable by NAPBNE in immunodiffusion with polyspecific rabbit anti-CTF serum (Fig. 4A and B). In SDS–PAGE of the pooled fractions a single band of 27 kDa was detectable by Coomassie blue staining (Fig. 4C, lane 2). In an immunoblot of the partially purified material with a rabbit anti-CTF serum a single band of antigenic activity at 27 kDa was apparent (Fig. 4D, lane 2), and in gelatin substrate/PAGE zymography the pooled fractions gave a band of hydrolysis at 27 kDa (Fig. 4E).

When an antiserum raised by immunizing rabbits with replicate NAPBNE-stained precipitin arcs excised from IEP plates prepared and stained as in Fig. 1 was applied in Western immunoblots of unfractionated CH, antigenic activity was found principally at 27 kDa with additional activities at 28 and 60 kDa (Fig. 4F).

Hydrolysis of specific rabbit antibodies and human immunoglobulin by S. mansoni *larval protease*

Several rabbits were immunized with the cationic antigen isolated by precipitation in IEP (Dunne *et al.* 1986) and, contrary to our experience with most other schistosome antigens, when these sera were tested in IEP none of them distinctively precipitated out any antigens from otherwise untreated CH. However, it was fortuitously observed that these antisera gave a readily visible cathodal precipitation reaction in IEP if the CH had been pre-incubated with PMSF (Fig. 5). The precipitin arc formed in this instance was in a similar cathodal position to those which hydrolysed NAPBNE (Fig. 1B) or reacted with DFP (Fig. 2B). Pre-incubation of CH with the serine protease inhibitors TPCK or TLCK failed to improve the immunoprecipitability of the cationic antigen by the monospecific sera (Fig. 5).

It was postulated that the absence of precipitin arcs after immunoelectrophoresis of untreated CH was due to degradation of specific immunoprecipitating antibody by the enzyme. The ability of both crude CH, and a preparation containing the 27 kDa CH protease partly purified by PEP, to hydrolyse non-reduced ^{125}I-labelled human immunoglobulin was confirmed by a combination of SDS–PAGE and autoradiography (Fig. 6). The iodinated IgG suffered progressive reduction in relative molecular size during 18 h incubation with either crude CH (Fig. 6A) or with the pooled and concentrated PEP fractions containing purified enzyme (Fig. 6B). The CH-induced hydrolysis of IgG was inhibited by pre-treatment of the larval extract with 3×10^{-5} M DFP (Fig. 6C), boiling the CH for 30 min (Fig. 6D), or immunoabsorption of the CH with antiserum from a rabbit immunized with the cationic IEP arcs with peptidolytic activity (Fig. 6E).

Anomalous immunological properties of the 27 kDa S. mansoni *larval protease*

Figure 7 shows the antibody responses of rabbits that had been immunized with different preparations containing the 27 kDa *S. mansoni* larval protease. CTF was chosen for the antigen preparation for immunoblots because, as comparison of lanes 1 and 2, Fig. 7A shows, the 27 kDa band is as prominent in CTF as in CH and, as might be expected, the former extract contains fewer contaminant protein bands.

The antibody responses of 2 rabbits hyperimmunized with CTF is shown in Fig. 7B, lanes 1 and 2. There is a marked difference between the 2 rabbits with respect to the intensity of antibody reactivity against the 27 kDa band.

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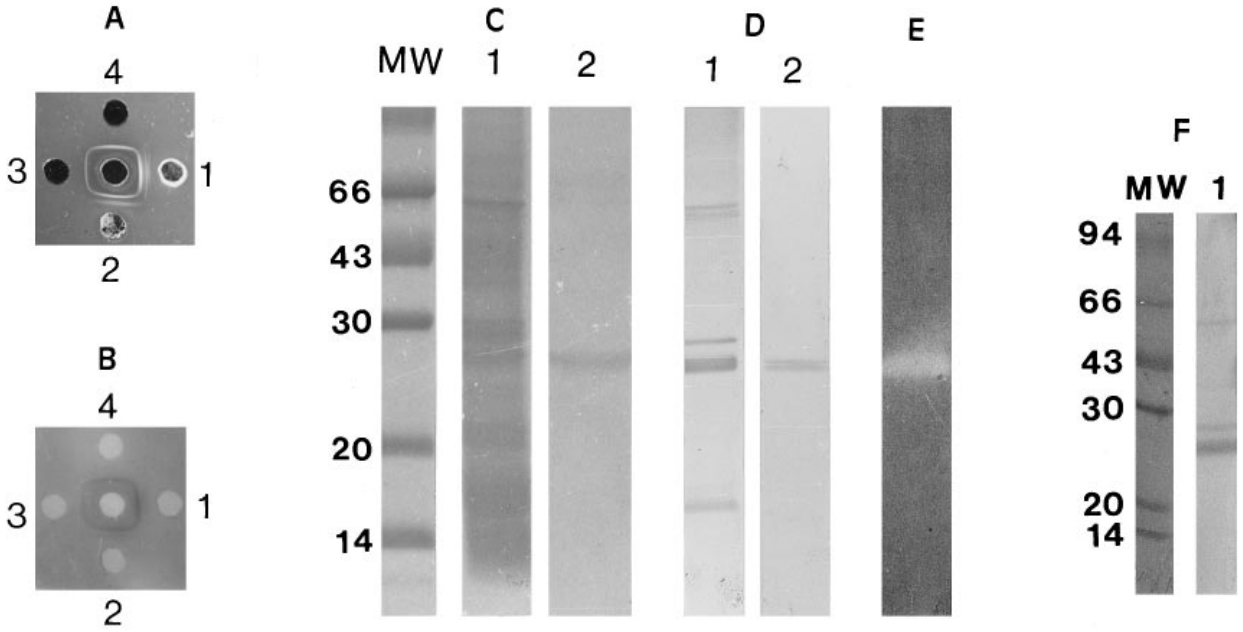

Fig. 4. SDS–PAGE, immunoblotting and substrate gel electrophoresis of fractions purified by preparative electrophoresis (PEP). (A and B) Immunodiffusion reaction between anti-CTF serum (centre well) and either unfractionated CH (wells 1 and 2), or pooled and concentrated PEP fractions of CH containing cathodally migrating peptidolytic activity (wells 3 and 4). Photographed (A) over indirect light or (B) over direct light after washing and immersion in NAPBNE and FBB. (C) Coomassie blue-stained PAGE of: MW, molecular weight standards; Lane 1, unfractioned CH; Lane 2, pooled and concentrated PEP fractions of CH containing partly purified peptidolytic activity. (D) Western blot with rabbit anti-CTF serum of: Lane 1, unfractionated CH; Lane 2, pooled and concentrated PEP fractions containing partly purified peptidolytic activity. (E) Gelatin substrate PAGE of PEP fractions containing pooled and concentrated enzymatic activity. (F) MW, molecular weight standards; Lane 1, immunoblot reaction between unfractionated CH and rabbit antiserum raised by immunization with replicates of the cathodal NAPBNE-stained precipitin line in Fig. 1.

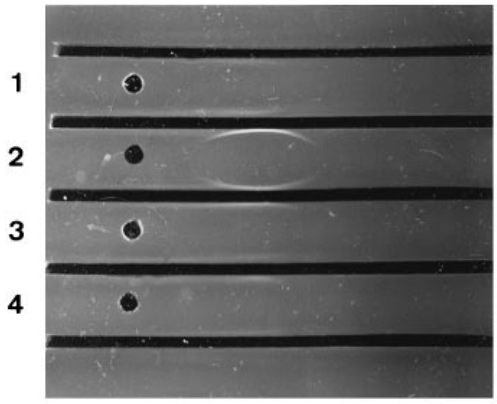

Fig. 5. IEP of anti-CTF serum with CH pre-incubated with serine protease inhibitors. Antigen wells contained; (1) control CH without inhibitor; (2) CH + PMSF; (3) CH + TLCK; (4) CH + TPCK. The CH was incubated for 30 min at 37 °C with inhibitors at a concentration of 1 mM.

The results in Fig. 4F indicated immunological cross-reactivity between the 27 kDa antigen and a less intensely staining 28 kDa molecule. Further evidence for this cross-reactivity is given in Fig. 7C. Thus, each of 2 rabbits immunized respectively with either of the 2 antigens alone, after separation of the molecules by SDS–PAGE and electroblotting, produced antibodies which reacted with both antigens. However, the cross-reactivity was not fully reciprocal in so far as the rabbit immunized with NCP carrying the 28 kDa molecule (Fig. 7C, lane 1) reacted less intensely against the 27 kDa molecule than serum from the rabbit immunized with the 27 kDa molecule (Fig. 7C, lane 2).

Three rabbits that had been repeatedly infected with large numbers of unattenuated *S. mansoni* cercariae percutaneously via the ear produced a variety of antibodies reactive against the constituents of CTF (Fig. 7D, lanes 1–3), but reactivity against the 27/28 kDa complex was strikingly absent when compared with the reactivity of, for example, the serum raised by specific immunization with electrotransferred 27 kDa protein (Fig. 7C, lane 2).

Four rabbits that had been immunized with immunoelectrophoresis precipitin arcs containing the cathodally migrating peptidase (Fig. 1) gave reactivities of differing intensity in immunoblots (Fig. 7F, lanes 2–5), but precipitin lines indicating identical reactivity in immunodiffusion (Fig. 7G). The rabbit immunized with the 27 kDa line on NCP, and which reacted well with the homologous antigen in immunoblots (Fig. 7C, lane 2 and 7F, lane 1), did not immunoprecipitate any antigen in immunodiffusion (Fig. 7G, wells numbered 1).

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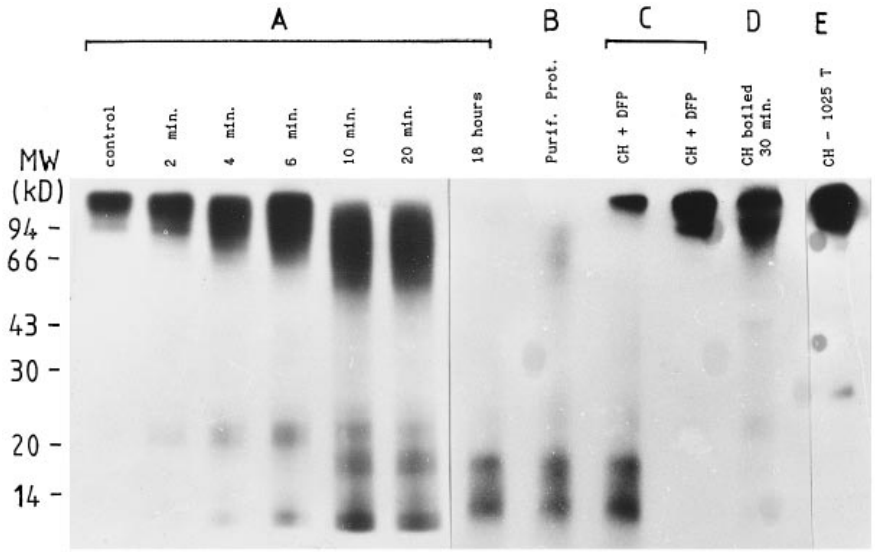

Fig. 6. Hydrolysis of ^{125}I-labelled human immunoglobulin by CH and purified larval protease. (A) An incubation mixture which consisted of 1 cm square polyacrylamide containing radio-isotope labelled immunoglobulin was macerated in 100 μl of PBS containing 1 mg (protein equivalent) CH. Aliquots of supernatant (10 μl) were removed at the different times indicated and subjected to PAGE and autoradiography. Control = sample removed at start of incubation period. (B) As in (A) but CH replaced by 100 μl containing 25 μg (protein equivalent) of pooled and concentrated PEP fractions containing partially purified peptidolytic activity (see Fig. 4). (C) Mixture as in (A) 18 h, but with DFP added at the start of incubation; $1{\cdot}5 \times 10^{-5}$ M DFP (left lane), 3×10^{-5} M DFP (right lane). (D) As in (A) 18 h, but CH subjected to 100 °C for 30 min before incubation. (E) As in (A) 18 h, but CH absorbed with rabbit antiserum 1025T raised by immunization with the cathodal NAPBNE-stained precipitin line in Fig. 1.

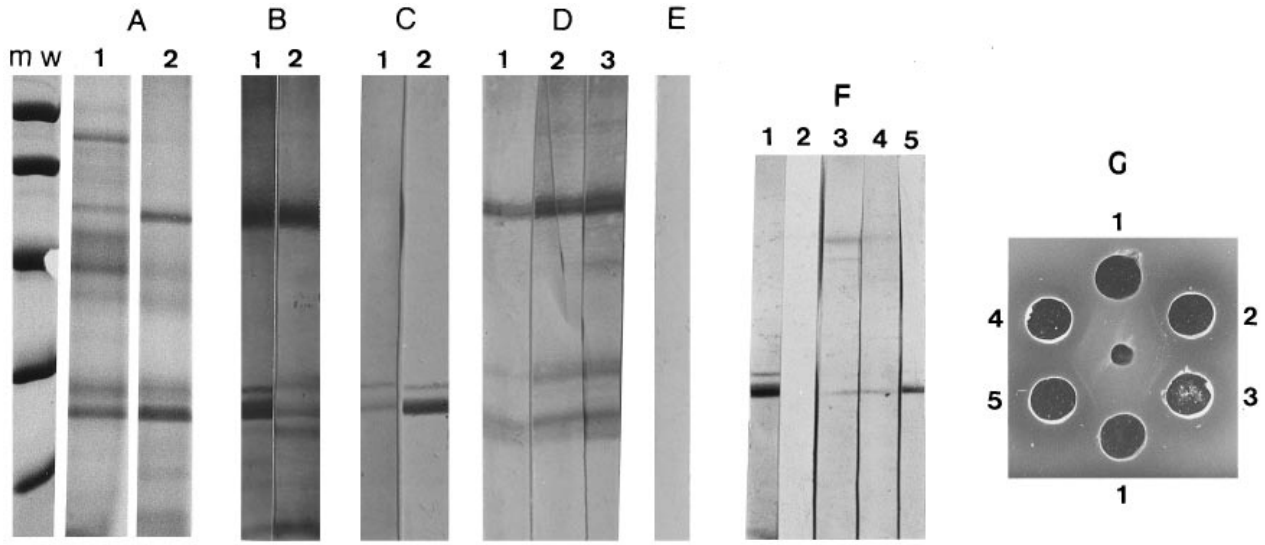

Fig. 7. Western immunoblot reactivity of rabbit antisera. MW, molecular weight standards; (A) Coomassie blue-stained PAGE of (1) unfractionated CH, and (2) unfractionated CTF. (B) Sera from 2 rabbits hyperimmunized with CTF. Both rabbits were given the same number of injections containing the equivalent amounts of protein. (C) Sera from 2 rabbits immunized with NCP carrying either (1) the 28 kDa band, or (2) the 27 kDa band of protein present in CTF (see (A), lane 2). (D) Sera from 3 rabbits given equivalent multiple infections of *Schistosoma mansoni* cercariae via the ear pinna at fortnightly intervals. (E) Normal rabbit serum. (F) Lane 1, serum from the same rabbit as in lane C2. Lanes 2–5, sera from 4 different rabbits immunized with the cathodal NAPBNE-stained immunoelectrophoretic precipitin arc in Fig. 1. (G) Immunodiffusion of the 5 rabbit sera in (F) (outer wells, with numbers the same as lanes in F) against PMSF-treated CTF (centre well).

Figure 8 shows the anti-CTF reactivities in individual sera from a group of 16 inbred CBA/Ca mice that had been injected 5 times at approximately 2 week intervals with CTF on alum adjuvant. Only 5 mice (Fig. 8, upward pointing arrows) had produced clear antibody responses against the 27 kDa antigen, while reactivity against some other antigens in CTF appeared more consistent through-

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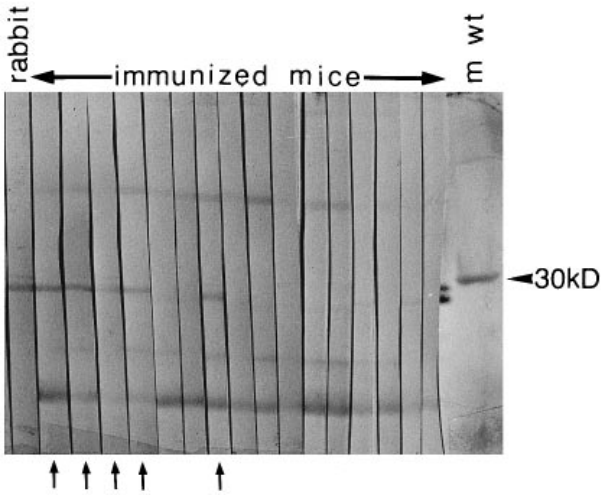

Fig. 8. Reactivity of CTF-immunized mice in Western blots of CTF. Immunized mice = sera from 16 CTF-immunized mice tested individually on electroblotted CTF. Rabbit = serum from same rabbit as in Fig. 7(C), lane 2. m wt = 30 kDa marker protein detected by staining NCP paper for protein after electrotransfer from polyacrylamide gel. Note the ends of 2 intensely stained CTF-derived bands of protein to the left and slightly below the 30 kDa marker, presumed to be the 27/28 kDa protease complex (as evident in Fig. 7A, lane 2).

out the group. The results in Table 1 show that in ELISA, using cercarial protease that had been partly purified by preparative electrophoresis as antigen, the group of 5 antibody 'responder' mice had significantly greater specific antibody titres than control unimmunized animals.

The 5 mice that had responded to the 27 kDa antigen (Fig. 8) were challenged with 200 *S. mansoni* cercariae, together with a group of 6 'non-responder' mice selected at random from the remaining 11 CTF-immunized animals, and a naive group of untreated CBA/Ca mice. Table 1 shows that after perfusion and worm enumeration 6 weeks after infection, the mice that had responded to the 27 kDa antigen had a 40% reduction in worm burden compared with the unimmunized control group ($P < 0{\cdot}001$), while the worm count in the group which had not produced antibody was not significantly different from that of the untreated challenge control group. A difference between antibody 'responder' and 'non-responder' mice was also reflected in the liver egg counts (Table 1).

DISCUSSION

A previously identified *S. mansoni* cationic cercarial antigen with peptidolytic activity (Doenhoff *et al.* 1988) has been further characterized, particularly with respect to its immunological properties. It was considered important first to establish whether it was related to other *S. mansoni* proteolytic enzymes that have so far been described.

Inhibition of the immunoprecipitable enzyme by DFP and PMSF indicates it is a member of the serine protease family, though TLCK, TPCK and SBTI – 3 further inhibitors of serine proteases – had no effect on it. Bestatin, pHMB, 1–10 phenanthroline and EDTA, inhibitors respectively of exopeptidases, cysteine proteases and metalloenzymes, had no effect on the enzyme. The inhibition profiles were, however, determined after the enzyme had been isolated in immune complexes and this may have affected inhibitor and substrate specificity.

Hydrolysis of NAPBNE and inhibition by PMSF are suggestive of chymotrypsin-like activity. Although NAPBNE can also be hydrolysed to some extent by trypsin and pancreatic elastase type II (Ohlsson, Westron & Karlsson, 1987), it was found that the compound succinyl-Ala-Ala-Pro-Phe-nitroanilide, which is more specific for chymotrypsin (Delmar *et al.* 1979), was hydrolysed by the enzyme after it had been partly purified (data not shown). Paradoxically, however, TPCK was not inhibitory to the enzyme in CH prior to quantification in radial immunodiffusion, nor did it act as effectively as PMSF for the result in Fig. 5, despite its being selectively reactive with histidine residues of chymotrypsin.

The reactivity of the larval enzyme with DFP was exploited to give an estimated molecular size of 27 kDa by SDS–PAGE and autoradiography. Further evidence that at least part of the proteo/peptidolytic activity being studied here was approximately 27 kDa is provided by the following observations. (i) The pooled fractions containing peptidolytic enzyme activity isolated by preparative electrophoresis gave bands of this size in Coomasie blue-stained PAGE gels, and in Western immunoblots with polyspecific rabbit antisera. (ii) The partly purified enzyme gave a band of proteolytic activity at approximately 27 kDa in a gelatin substrate/PAGE assay. (iii) Antisera raised by immunizing rabbits with the cathodal NAPBNE-stained precipitin arcs reacted against a 27–28 kDa doublet in immunoblots of unfractionated CH.

On the basis of molecular size, inhibition profile and substrate preference the enzyme identified and isolated here therefore has similar properties to the enzymes that have already been respectively identified and studied by several laboratories (Landsperger *et al.* 1982; McKerrow *et al.* 1985; Newport *et al.* 1988; Marikovsky *et al.* 1988*a*, *b*; Pierrot *et al.* 1996). The relative cationicity of the enzyme described here is also consistent with previous work indicating that the major larval serine protease has a higher than neutral pI (McKerrow *et al.* 1985; Marikovsky *et al.* 1988*a*). There are some discrepancies in properties of the enzyme(s) characterized in different laboratories: for example, Marikovsky *et al.* (1988*a*) found that antibodies reactive against their 28 kDa protease did not cross-react

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Table 1. Antibody responses, worm burdens and tissue egg counts in CTF-immunized mice which responded or failed to respond with antibody production to the 27/28 kDa protease, compared with non-immunized control mice

Group	ELISA (OD_{414})	*P*	Mean no. of worms	Reduction (%)	*P*	Liver egg count ($\times 10^{-3}$)	Reduction (%)	*P*
Control	$0{\cdot}12 \pm 0{\cdot}04$		$114{\cdot}7 \pm 7{\cdot}4$			$67{\cdot}3 \pm 1{\cdot}1$		
'Responder'	$0{\cdot}39 \pm 0{\cdot}16$	$< 0{\cdot}01$	$60{\cdot}6 \pm 16{\cdot}7$	47	$< 0{\cdot}001$	$25{\cdot}9 \pm 10{\cdot}5$	62	$< 0{\cdot}001$
'Non-responder'	$0{\cdot}28 \pm 0{\cdot}09$	N.S.	$100{\cdot}2 \pm 20{\cdot}5$	13	N.S.	$54{\cdot}2 \pm 7{\cdot}1$	20	N.S.

N.S., $P > 0{\cdot}05$.

with a 60 kDa molecule with proteolytic activity, but rabbit antisera produced here against isolated immunoprecipitin arcs reacted against both antigens. Furthermore, immunoblotting suggested that the 27 kDa moiety exists as a doublet, a notion that is consistent with the results of SDS–PAGE and autoradiography of DFP-labelled CH. The rabbit antisera raised, respectively, by Pierrot *et al*. (1996) against a recombinant expression product of their cloned cercarial elastase gene, and by Ghendler *et al*. (1996) against soluble enzyme, also gave 2 bands of reactivity of differing intensity at 27/28 kDa and very similar to the patterns observed here.

Although it has been suggested that there is only 1 serine protease gene in schistosomes (McKerrow, Newport & Fishelson, 1991), 4 sequences for this gene are currently registered, and 3 of these are different from each other. Thus, 2 sequences with respective PID accession numbers g160948 (Newport *et al*. 1988) and g1240058 (Price, Doenhoff & Sayers, 1997) are identical and the estimated molecular weight for the mature protein is 22485 Da. Two sequences with PID numbers g1103831 and g1103829 (Pierrot, Capron & Khalife, 1995) are respectively 81% and 97% identical with the Newport/Price sequence and would give mature proteins with estimated molecular weights of 21938 Da and 23552 Da respectively. Explanations for these discrepancies could thus be sought in terms of expression of different genes (or differential splicing) and/or post-translational modifications, including glycosylation, or in strain differences between isolates of the parasite.

It might be expected that an enzyme which has a pivotal role in larval infectivity (McKerrow *et al*. 1991) would have paradoxical immunological properties. That host immune responses against the protease may be modulated is indicated by the following observations. (i) Production of antibody to the enzyme in infected animals is relatively transient and/or weak (Toy *et al*. 1987). (ii) The majority of rabbits we have immunized with CTF have produced antibodies which precipitated the enzyme in immunoelectrophoresis, but we have found no such precipitating antibody in serum from rabbits that have been either immunized with unfractionated CH (although the latter extract contains the enzyme in a form antigenically reactive with anti-CTF sera), or subjected to repeated percutaneous infections with large numbers of live unattenuated *S. mansoni* cercariae. (iii) The 27 kDa and 60 kDa moieties which the results in Fig. 4 indicate may be immunologically cross-reactive are differentially glycosylated (Marikovsky *et al*. 1988*a*) and there is evidence that glycosylation profiles may be important in the modulation of anti-schistosome immunity (Yi *et al*. 1986; Butterworth *et al*. 1988). (iv) Immunoglobulin G and specific antibody are degraded by the enzyme (Auriault *et al*. 1981), and the products of IgG cleaved by schistosome larval proteases have been shown to inactivate macrophages (Auriault *et al*. 1980). Proteolytic activity in products released by schistosomula during *in vitro* culture has also been shown to modulate IgE metabolism (Verwaerde *et al*. 1986).

Results indicate that in addition to its antigenicity (i.e. ability to react with specific antibody) the immunogenicity of the 27 kDa enzyme (i.e. its capacity to induce specific antibody responses) is compromised. We do not know of any other example in which the antibody response of inbred mice to an antigen can be characterized as 'responder' or 'non-responder' to the extent apparent in Fig. 8. Down-regulation of the immunogenicity of the 27 kDa antigen, such that only a minority of animals produce antibody responses, may be a consequence of it being a schistosome product, the immunological inactivation of which would have negative consequences for parasite survival. Alternatively, the intrinsic proteolytic activity of the antigen may interfere with the normal activities of the immune system. For example, the immunoglobulin receptors on B-cells with which the enzyme came into contact in their capacity as antigen presenting cells might be degraded in the same way as soluble immunoglobulins, resulting in abnormal antigen processing and presentation of derivative peptides by MHC when compared with processing and presentation of antigens without such enzymatic activity. Interaction with host protease inhibitors (Modha & Doenhoff, 1994) may also result in a heterologous protease being deviated from normal antigen processing

pathways. In this context we have some preliminary unpublished evidence to suggest that other serine proteases are also poor immunogens.

The results do, however, indicate that animals which have generated antibody responses against this schistosome antigen are partly protected against infection. The membrane-bound form of the protease (Ghendler *et al*. 1996) present on larvae and adult worms may be the actual target of protective immune responses (i.e. rather than soluble enzyme excreted during penetration). Our attention is now focused on developing means by which the immunogenicity of this protease can be enhanced so that it will induce protective immune responses in a more predictable manner. We have recently cloned and expressed the 3 exons of which the larval protease is comprised (Price *et al*. 1997), and have shown the respective expressed fusion proteins to be antigenically reactive in immunoblots with the antiserum raised by immunizing a rabbit with the 27 kDa native protease on NCP. Studies are underway to compare the immunogenicity of these fusion proteins, as well as that of an expression product of the full length gene, with that of the native protease.

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