Molecular and Biochemical Parasitology, 56 (1992) 103–116

 / 0166-6851/92/$05.00

MOLBIO 01831

Identification of a 33-kilodalton immunodominant antigen of *Trypanosoma congolense* as a cysteine protease

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(Received 2 January 1992; accepted 23 July 1992)

A 33-kDa protein of *Trypanosoma congolense* is a major antigen in infected cattle and the production of antibody to this antigen appeared to correlate with enhanced resistance to trypanosomiasis [4]. Immunoelectron microscopy using a monoclonal antibody (mAb 4C5) raised against the 33-kDa antigen showed a lysosomal localisation, similar to that of a previously described 32-kDa cysteine protease of *T. congolense*. Both mAb 4C5 and anti-33 kDa antibody from infected cattle bound on Western blots to the cysteine protease that had been purified by affinity chromatography on cystatin-Sepharose. Sepharose-coupled mAb 4C5 was used to affinity purify the antigen from bloodstream forms of *T. congolense*. On sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the affinity-purified antigen had a molecular mass of 33 kDa under non-reducing conditions, and 40 kDa under reducing conditions. Anti-33-kDa antibody from infected cattle bound to both non-reduced and reduced affinity-purified antigen on Western blots. Serum from a rabbit immunised with the biochemically purified enzyme also bound the affinity-purified antigen. The affinity-purified antigen displayed proteolytic activity in fibrinogen-containing SDS-PAGE and against Azocoll. It hydrolysed benzyloxycarbonyl-Phe-Arg-7-amino-methyl coumarin (Z-Phe-Arg-NHMec) with a K_m similar to that of the biochemically purified enzyme. Proteolytic and peptidolytic activities of the antigen were inhibited by the inhibitors of cysteine proteases, cystatin and *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino)butane (E-64). On two-dimensional gel electrophoresis, the antigen displayed similar characteristics to those of the biochemically purified enzyme. We conclude that the 33-kDa antigen of *T. congolense* and the cysteine protease are the same molecule.

Key words: *Trypanosoma congolense*; Antigen; Cysteine protease

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Abbreviations: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; E-64, trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane; ELISA, enzyme linked immunosorbent assay; Fbg, fibrinogen; HRP, horseradish peroxidase; Ig, immunoglobulin; kDa, kilodalton; mAb, monoclonal antibody; Mes, 4-morpholine-ethanesulphonic acid; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, Dulbecco's phosphate-buffered saline; PSG, phosphate-buffered saline containing 1% glucose; SDS, sodium dodecyl sulphate; Z-Phe-Arg-NHMec, benzyloxycarbonyl-phenylalanine-arginine-7-amino-4-methyl coumarin; 2D IEF/SDS-PAGE, two-dimensional gel electrophoresis.

Introduction

Trypanosoma congolense is a tsetse-transmitted protozoan parasite and an important pathogen in cattle. Infected animals generally experience a chronic disease in which anaemia is a principal feature. The variable surface glycoprotein (VSG) of trypanosomes is a major target for immune response and anti-VSG antibodies mediate clearance of the parasites, thus limiting the parasitaemia. However, inter- and intra-breed variations occur in the susceptibility of cattle to trypanosomiasis. Differences in the level of parasitaemia do not entirely account for such variations; there are also differences in an

individual's ability to control the development of anaemia [1–3]. The pathogenesis of trypanosomiasis is probably triggered by parasite components other than the VSG. It is possible that animals which mount an antibody response against such parasite-derived, non-VSG molecules may better resist the disease than animals which do not respond.

In a previous study, antibody responses to *T. congolense* invariant antigens were analysed during the course of an experimental rechallenge using Western blotting and ELISA [4]. Three major invariant antigens of *T. congolense* were identified. The ability to produce a detectable antibody response to one of these antigens, the 33-kDa antigen, was associated with a capacity to control the disease. Antibodies to the 33-kDa antigen were detected in those cattle which experienced moderate or no anaemia during the course of infection. In contrast, the haematocrit was severely depressed in cattle which had no anti-33-kDa antibodies prior to the challenge, and which failed to mount a detectable antibody response following reinfection. Thus, the 33-kDa antigen might be involved in the pathogenesis of *T. congolense* infections.

The present study was undertaken in order to characterize the 33-kDa antigen. We report here that this antigen is identical to a described cysteine protease of *T. congolense*.

Materials and Methods

Materials

Horseradish peroxidase (HRP)-conjugated sheep anti-mouse Ig and molecular weight markers for SDS-PAGE were obtained from Amersham International (Amersham, UK). Z-Phe-Arg-NHMec was purchased from Cambridge Research Biochemicals (Cambridge, U.K.). Proteinase K, 4-chloro-1-naphthol, Azocoll, and protease inhibitors, were obtained from Sigma Chemical Co. (Poole, U.K.). Freund's complete and incomplete adjuvants (CFA and IFA) were purchased from Gibco Laboratories, Life Technologies Inc. (Ohio, USA). DEAE-cellulose (DE-52) was obtained from Whatman Biosystem Ltd. (Maidstone, U.K.). Tween 20 was from Merck (Munich, Germany), Sepharose 4B and ampholytes were from Pharmacia (Uppsala, Sweden). Lowicryl K4M resin, goat anti-rabbit IgG 10 nm gold probe (GAR Au_{10}) and goat anti-mouse IgG 5 nm gold probe (GAM Au_5) were obtained from Agar Scientific (Stanstead, U.K.). For two-dimensional gel electrophoresis (2D IEF/SDS-PAGE), ultra-pure urea and Nonidet P-40 were purchased from ICN Biomedicals, Inc. (Cleveland, OH, USA) and Pierce (Rockford, IL, USA), respectively.

Trypanosome cultivation

Trypanosome antigens were prepared from *T. congolense* ILC-49, a stock isolated in Transmara, Kenya [5]. The trypanosomes were grown in irradiated rats (600 rads irradiation), harvested by cardiac puncture and separated from blood cells by passage over DEAE-cellulose [6]. The parasites recovered from the column effluent were washed three times in cold phosphate-buffered saline, pH 8.0, containing 1% (w/v) glucose (PSG) by centrifugation (800 × *g*, 15 min, 4°C) and stored as pellets at −70°C until required. *T. congolense* clones ILX4, IL13-E3, IL1180, and IL2281, as well as *Trypanosoma brucei brucei* IL1807 and *Trypanosoma vivax* IL2160 were grown in irradiated BALB/c mice, and prepared in a similar way. Historical information on these clones is summarised in Table I.

For immunoelectron microscopy and for cystatin affinity purification of the protease, *T. congolense* IL3000 trypanosomes (a clone from ILC-49) were grown in irradiated rats and the parasites prepared by a combination of centrifugation on isopycnic Percoll gradients [7] and chromatography on DEAE-cellulose [6]. Trypanosomes were recovered in at least 100 ml of cold isotonic PSG, pH 7.4, containing 0.1 mM hypoxanthine [8] and washed twice in the same buffer.

SDS-PAGE

The procedures used for SDS-PAGE and Western blotting have been described pre-

TABLE I

History of trypanosome clones and stocks used in the study

Species/stock	Origin	Clone	Reference
T. congolense			
ILC-49	Transmara, Kenya	ILX4	Wellde et al. [5]
		(ILNat 2.1)	Rovis et al. [37]
		IL3000	Fish et al. [38]
			Mbawa et al. [15]
LUMP69	Zaria, Nigeria	IL2281	Luckins et al. [39]
EATRO209	Busoga, Uganda	IL13-E3	Morrison et al. [40]
STIB212	Serengeti, Tanzania	IL1180	Geigy and Kauffman [41]
T. brucei brucei			
STIB247	Serengeti, Tanzania	IL1807	Geigy and Kauffman [41]
		(ILNat 3.1)	Nantulya et al. [42]
T. vivax			
Y486	Nigeria	IL2160	Leeflang et al. [43]

viously [4]. Briefly, crude lysates of ILC-49 bloodstream forms were prepared by freeze-thawing the trypanosome pellets twice in the presence of protease inhibitors (leupeptin (10 μg ml^{-1}), 1 mM phenylmethyl sulphonylfluoride (PMSF), 0.2 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 0.05 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)). Lysates were boiled in an equal volume of sample buffer (100 mM Tris-HCl/4% SDS/20% glycerol/8% 2-mercaptoethanol/0.01 bromophenol blue). Samples (5×10^6 to 10^7 trypanosomes per lane, i.e., approximately 50 μg of total protein) were loaded onto a 7.5–15% gradient gel (1.5 mm thick) and subjected to electrophoresis as described by Laemmli [9].

For electroelution, lysates from 5×10^8 trypanosomes were subjected to electrophoresis under reducing conditions on 13-cm-wide polyacrylamide gels (7.5–15%). The gels were stained with Coomassie brilliant blue, the band at 33-kDa was excised and electroeluted according to the method of Hunkapiller et al. [10]. Approximately 20 μg of the electroeluted protein was again subjected to SDS-PAGE and stained with Coomassie brilliant blue to assess the purity of the preparation.

SDS-PAGE analysis of purified cysteine protease was generally performed under non-reducing conditions to prevent the rapid autodegradation which the enzyme undergoes in presence of mercaptoethanol (unpublished observation). When reducing conditions were used, the enzyme was boiled for 2 min in non-reducing sample buffer prior to the addition of mercaptoethanol (4% final concentration) and then boiled again for a further 10 min.

Western blotting

Transfer of proteins from polyacrylamide gels to nitrocellulose membranes [11] was performed overnight at 10 V in 25 mM Tris/200 mM glycine/0.1% SDS buffer containing 20% methanol. After blocking in Tris buffer (10 mM Tris-HCl/15 mM NaCl) containing 5% skimmed milk, pH 7.4, the nitrocellulose transfers were reacted with bovine, murine, or rabbit antibodies. A 1:100 dilution of a pool of 4 post-infection bovine sera was used to detect the 33-kDa antigen in the various trypanosome lysates. The binding of specific bovine antibodies was revealed by subsequent incubation with a monoclonal antibody raised against the γ1 chain of bovine Ig [12]. Mouse monoclonal antibodies were visualised by incubation with commercial HRP anti-mouse Ig conjugate. Rabbit antibodies were detected using an HRP-conjugated goat anti-rabbit Ig (gift from V.M. Nantulya, ILRAD). The substrate used was hydrogen peroxide (0.01%, v/v) in the presence of 1-chloro-4-naphthol.

Preparation of monoclonal and polyclonal antibodies

BALB/c mice were injected intraperitoneally with 30 μg of the electroeluted 33-kDa antigen

which was emulsified in CFA. The mice were boosted 3 times at 3–4-week intervals with an equal amount of antigen in IFA. Anti-33 kDa monoclonal antibodies (mAbs) were prepared by fusing spleen cells of the immunized BALB/c mice with the myeloma line NS1 according to established methods [13]. The screening of hybridomas was done by immunoblotting using ILC-49 lysate as the antigen.

A polyclonal antibody (pAb) against a 32-kDa *T. congolense* cysteine protease [14,15] was raised in a rabbit, using the enzyme purified from bloodstream forms of IL3000 by affinity chromatography on cystatin-Sepharose [14] as described by Kos et al. [16].

Inhibition ELISA

An inhibition ELISA was used to determine if the molecule recognized by the mAb was identical to the 33-kDa antigen recognised by cattle. ELISA microplates were coated with 50 ng per well of the electroeluted 33-kDa antigen in sodium carbonate-bicarbonate buffer, pH 9.6. The plates were rinsed with PBS containing 0.1% (v/v) Tween 20 and incubated for 2 h at 37°C with serial dilutions of bovine sera in PBS. These sera were taken from 6 *T. congolense* infected animals, three which produced antibodies against the 33-kDa antigen as detected by immunoblotting ('responder' animals), and three in which specific antibodies were not detected ('non-responders'). Serum from an uninfected animal was also tested. The plates were then washed with PBS-0.1% (v/v) Tween, incubated for 1 h with mAb, followed by the same HRP anti-mouse conjugate as used for immunoblotting. ABTS (2,2'-azino bis [3-ethyl-benzthiazoline-6-sulphonic acid]) was used as the chromogen, with hydrogen peroxide as the substrate. The absorbance at 414 nm (A_{414}) was measured using a Titertek Multiskan MCC/340 microplate reader. Inhibition of binding by each serum sample was calculated from the following formula:

$$\% \text{ inhibition} = [1 - (A_{414} \text{ sample}/A_{414} \text{ mAb})] \times 100$$

Immunoelectron microscopy

Bloodstream forms of *T. congolense* ILC-49 and IL3000 were fixed in suspension in 0.1 M sodium phosphate buffer, pH 7.4, containing 8% paraformaldehyde. After fixation for 1 h at room temperature, the cells were pelleted and washed 4 times in ice-cold 0.1 M phosphate buffer/pH 7.4, containing 3.5% sucrose and 0.5 mM $CaCl_2$. The samples were processed using the enhanced membrane contrast method of Berryman and Rodewald [17] and embedded in Lowicryl K4M resin [18]. Ultrathin sections (50–70 nm thick) were incubated with mAb to the 33-kDa antigen (mAb 4C5), followed by GAM Au_5. After washing in PBS containing 3% BSA, the sections were incubated with the rabbit pAb against the *T. congolense* cysteine protease, then GAR Au_{10}. Controls consisted of reversing gold probe sizes, omitting either mAb or pAb or both, substituting non-relevant Ab, and using pre-immune rabbit serum.

Monoclonal antibody affinity purification of the 33-kDa antigen

MAb 4C5, an IgG1, was purified from ascitic fluid by precipitation with 50% ammonium sulphate and subsequent chromatography on a DEAE-cellulose column. A 10-ml affinity chromatography column was prepared by coupling 95 mg of the purified mAb 4C5 to 3 g of CNBr-activated Sepharose 4B according to the manufacturer's instructions. Frozen pellets of 4×10^9 trypanosomes were thawed and lysed by addition of 8 ml of cold distilled water. After 10 min incubation on ice, 2 ml of 10 × concentrated PBS, pH 7.4, was added. The suspension was centrifuged for 3 min at 10000 × *g*. The supernatant was loaded onto the column and the unbound antigen was eluted with cold PBS. The bound antigen was eluted with 0.1 M glycine-HCl, pH 3. Fractions (2 ml) were collected on ice and immediately neutralized by an equal volume of 0.1 M Tris-HCl, pH 8.5, containing 0.3 M NaCl. Elution fractions were coated onto microtitre plates and tested for the presence of the 33-kDa antigen in a direct ELISA. The fractions containing the 33-kDa antigen were concen-

trated in an Amicon ultrafiltration cell using a PM-10 membrane, and dialysed against PBS, pH 7.4. Protein, 20 μg as estimated by absorbance at 280 nm, was run under non-reducing conditions on a 7.5–15% SDS-PAGE gradient gel. The gels were stained with silver nitrate [19] or Coomassie blue.

Enzyme assays

Peptidolytic activity. To assess the enzymatic activity of the molecules bound by different antibodies, an assay using ELISA microtitre plates was used. Wells were coated overnight at 4°C with 10 μg per well of either mAb 4C5, the rabbit pAb against the cysteine protease, or an unrelated mAb. The coated wells were incubated for 25 min at 37°C with different amounts of biochemically purified protease, washed with PBS and then assayed for bound enzymatic activity. The peptidolytic activity was measured by the release of the fluorescent 7-amino-4-methyl coumaryl moiety from Z-Phe-Arg-NHMec [20] on a SLM 8000 spectrofluorimeter.

Z-Phe-Arg-NHMec was used to determine the peptidolytic activity of the affinity-purified antigen. The Michaelis constant, K_m was determined at 25°C in 0.1 M Mes, pH 6.0, containing 2 mM EDTA and 6 mM dithiothreitol as described previously [15] using the Enzfitter programme [21]. The robust and proportional weightings were applied. To assess the inhibition of the enzyme by E-64 or cystatin, the mAb 4C5 affinity-purified antigen was preincubated for 5 min in either E-64 (13 ng ml^{-1}) or cystatin (1.3 ng ml^{-1}) prior to assaying for residual activity against Z-Phe-Arg-NHMec (12.5 μM) in 0.1 M Mes, pH 6.0/2 mM EDTA/6 mM dithiothreitol.

Proteolytic activity. Proteolytic activity of the immunologically purified protein was assessed in fibrinogen-containing SDS-PAGE (Fbg-SDS-PAGE) as described previously [22]. The samples were not boiled or reduced prior to electrophoretic separation and analysis. For 2-dimensional analysis, 'ISO-DALT' isoelectric focusing system (pH 3.5–10) was performed in the first dimension [23]. The second dimension was SDS-PAGE in 7.5–15% gradient gels. When assaying their enzymatic activity on fibrinogen, the samples were diluted in 0.25 M Tris-HCl, pH 6.8. For silver staining of proteins, the samples were diluted in 9 M urea/5% (v/v) Nonidet P-40, and 10 μM E-64.

Proteolytic activity of the antigen was also assessed using Azocoll as the substrate. The antigen was diluted to 30 μg ml^{-1} in PBS, and the pH was adjusted to pH 6.5 by the addition of 0.2 M Mes. Azocoll and 2-mercaptoethanol were added (at final concentrations of 5 mg ml^{-1} and 10 mM, respectively) and the mixture (final volume 0.5 ml) was incubated at 37°C. Hydrolysis of the substrate was assessed by measuring the absorbance of the supernatant at 540 nm after 4 h and 20 h incubation. The assay was run in parallel in the presence of cystatin (1 μM) or E-64 (10 μM).

Results

Stock and species specificity of the 33-kDa antigen. The 33-kDa antigen was initially identified in lysates of *T. congolense* stock IL-C-49 when probing nitrocellulose transfers with sera from infected cattle [4]. A pool of positive bovine sera was used in immunoblotting on four clones of *T. congolense* derived from isolates obtained in various geographical locations within East and West Africa. The serum bound to a 33-kDa molecule in freshly prepared lysates of all four clones (Fig. 1A). The intensity of staining varied among the clones, but also varied upon the extent and mode of handling of the trypanosome lysates (i.e. freeze-thawing or extensive storage on ice, data not shown). When tested on lysates of *T. vivax* and *T. brucei*, these antibodies bound to an antigen of 69 kDa, but no reaction was observed with proteins of lower M_r (Fig. 1B). Thus all the *T. congolense* lysates tested, but not those of *T. vivax* or *T. brucei*, contained an immunologically cross-reactive 33-kDa molecule. The proteinaceous nature of this antigen was confirmed by its susceptibility to digestion by proteinase K (not shown).

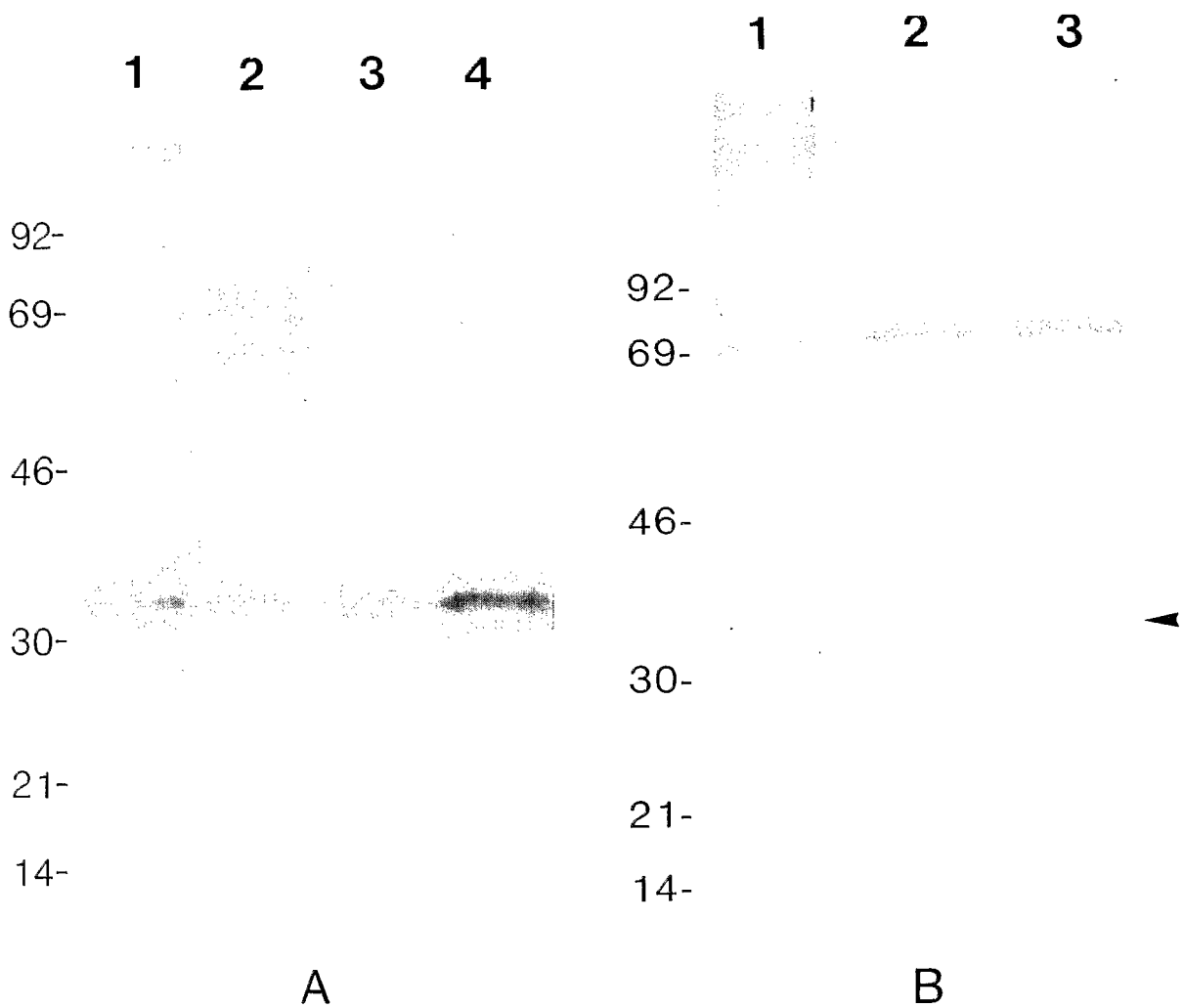

Fig. 1. Occurrence of the 33-kDa antigen in different stocks of *T. congolense* (A) and in different species of African trypanosomes (B), as determined by immunoblotting. Trypanosome lysates were run on SDS-PAGE, and transferred onto nitrocellulose membranes. The blots were probed with a pool of sera from four infected cattle (1:100 dilution). (A) Immunoblot obtained with four different clones of *T. congolense* (IL13-E3, ILX4, IL2281 and IL1180, in lanes 1, 2, 3 and 4, respectively). (B) Immunoblot obtained with lysates of *T. congolense* ILC-49 (lane 1), *T. brucei* IL1807 (lane 2) and *T. vivax* IL 2160 (lane 3). The molecular mass (in kDa) of the markers are shown on the left of each panel. The arrow indicates the position of the 33-kDa antigen of *T. congolense*.

Specificity of monoclonal antibodies for the 33-kDa trypanosome antigen. When used in immunoblotting, 3 mAbs bound to a 33-kDa molecule in whole trypanosome lysates. One of these mAbs (4C5) was tested for its ability to compete with bovine sera for binding to the electroeluted 33-kDa antigen in ELISA. The binding of the mAb 4C5 was inhibited by serum from 'responder' animals, but not by that of 'non-responders' (Fig. 2). Thus, mAb 4C5 and the serum from responder cattle reacted with the same protein.

Intracellular localisation. Immunoelectron microscopy showed that the antigen recognised by mAb 4C5 and an earlier described cysteine protease [14,15] co-localise within the same organelles of *T. congolense* (Fig. 3). These organelles have been shown to be lysosomes [14]. Reversing the size of the gold probes did

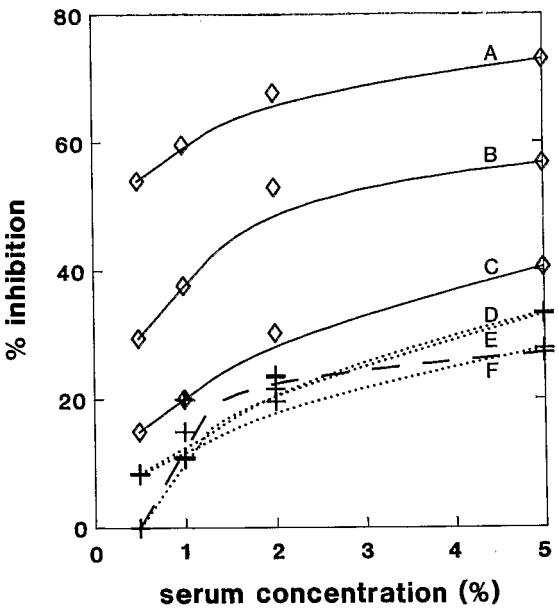

Fig. 2. Specificity of mAb 4C5 for the 33-kDa antigen of *T. congolense* in an inhibition ELISA. The graph shows the percentage of inhibition of mAb binding to the 33-kDa antigen by serially diluted bovine sera. A, B, C (—) sera from 'responder' cattle which produced detectable antibodies against the 33-kDa antigen; D, E, F (....) sera from cattle which did not respond to the 33-kDa antigen. Control serum (- - -) from an uninfected animal was also tested. Sera A, B and C inhibited the binding of mAb 4C5 to a degree that was proportional to their reactivity with the 33 kDa antigen when analysed by immunoblotting. Sera D, E and F gave a similar level of inhibition to that of the negative control.

not affect the co-localisation. However, the order in which the Ab and probes were applied was significant. A much higher signal was obtained with the mAb when used first, irrespective of the probe size. No lysosomal labelling was observed in appropriate control experiments using pre-immune sera and irrelevant antibodies.

Reactivity of mAb 4C5 with the T. congolense cysteine protease. To examine whether mAb 4C5 recognised the trypanosome cysteine protease, the biochemically purified enzyme was subjected to electrophoresis under non-reducing conditions and transferred onto nitrocellulose. MAb 4C5 bound to the non-reduced protease on a Western blot (Fig. 4,

Fig. 3. Immunoelectron micrograph showing co-localisation of the 33-kDa antigen and the cysteine protease in a lysosome of *T. congolense* ILC-49. The section was probed with anti-33-kDa mouse mAb 4C5 (1:20 dilution of ascites), followed by 5 nm gold probe, then with a rabbit pAb (1:200 dilution) raised against the biochemically purified cysteine protease, followed by 10 nm gold probe (arrows). The small number of gold particles outside the lysosome is equivalent to the background labelling obtained using pre-immune serum. No labelling of lysosomes was observed with the pre-immune serum. Scale bar: 0.2 μm.

strip 1). Bovine sera which contained anti-33 kDa antibodies, also reacted with the purified enzyme (Fig. 4, strips 3 and 5) whereas the serum from an infected animal which did not respond to this antigen did not bind to the enzyme (Fig. 4, strip 4). Weak labelling was also observed at 31 kDa, which is probably an autodegradation product of the protease.

Peptidolytic activity of the molecules bound by mAb 4C5. To exclude the possibility that

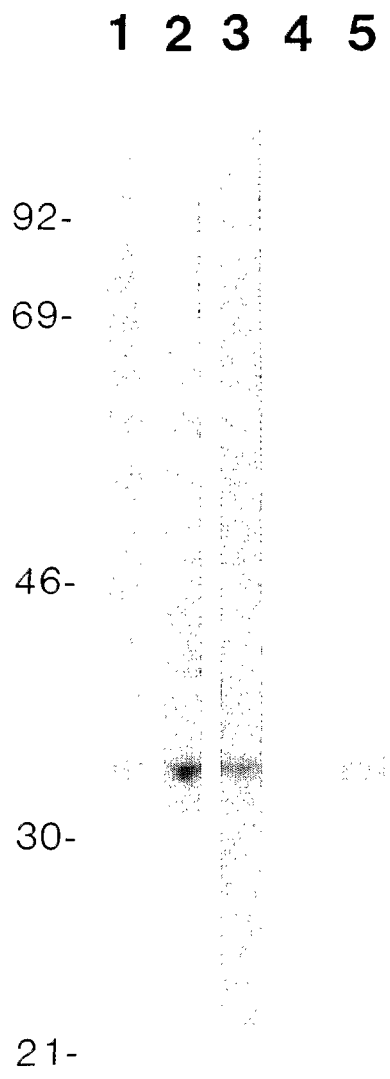

Fig. 4. Detection of the biochemically-purified cysteine protease by bovine infection sera and mAb 4C5 on a Western blot. The protease, purified by cystatin affinity chromatography, was run under non-reducing conditions on SDS-PAGE and transferred onto nitrocellulose. The nitrocellulose membrane was cut into strips and probed with the following antibodies. Strip 1, mAb 4C5 (culture supernatant); strip 2, rabbit polyclonal antibody raised against the purified protease (1:100 dilution); strips 3 and 5, sera from two 'responder' cattle (1:50 dilution); strip 4, serum from a 'non-responder' steer (1:50 dilution). The position of molecular mass markers is indicated on the left (in kDa).

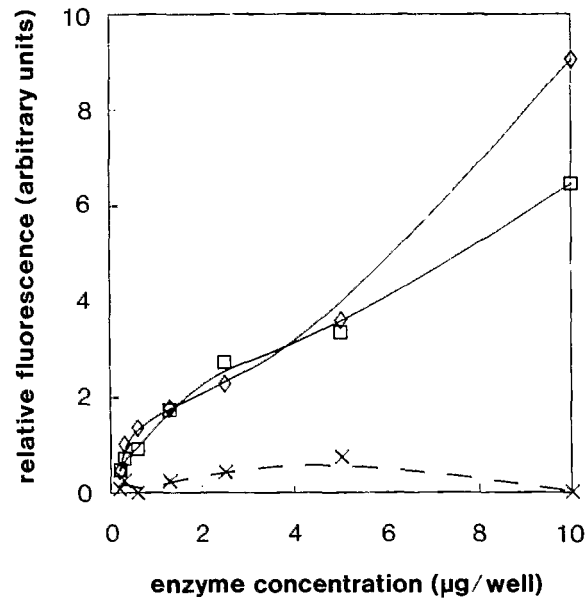

Fig. 5. Peptidolytic activity of the molecules bound by different antibodies. The mAb 4C5 (◇), the rabbit polyclonal anti-protease (□) and an unrelated mAb (×) were used to coat different wells of an ELISA plate. After incubation with different amounts of purified enzyme, the substrate Z-Phe-Arg-NHMec was added, and the fluorescence released by hydrolysis of the substrate was measured. The experimental variation for each point was less than 5%.

mAb 4C5 reacted with a contaminant in the protease preparation, ELISA microplates were coated with the mAb and then incubated with the purified enzyme. In contrast to control wells that were pre-coated with non-specific antibody, the mAb 4C5-coated wells bound a molecule which hydrolysed Z-Phe-Arg-NHMec, a substrate for lysosomal proteases of African trypanosomes [20] (Fig. 5).

Monoclonal antibody affinity purification of the 33-kDa antigen. An affinity column of Sepharose conjugated to mAb 4C5 was used to purify the 33-kDa antigen. When the lysate from 4×10^9 trypanosomes (approximately 20 mg total protein) was loaded onto the column, approximately 200 µg of protein was eluted by the low pH buffer. After subjecting 20 µg of the eluate to SDS-PAGE, Coomassie blue or silver staining revealed a single band corresponding to 33 kDa (Fig. 6A, lane 2). A weak

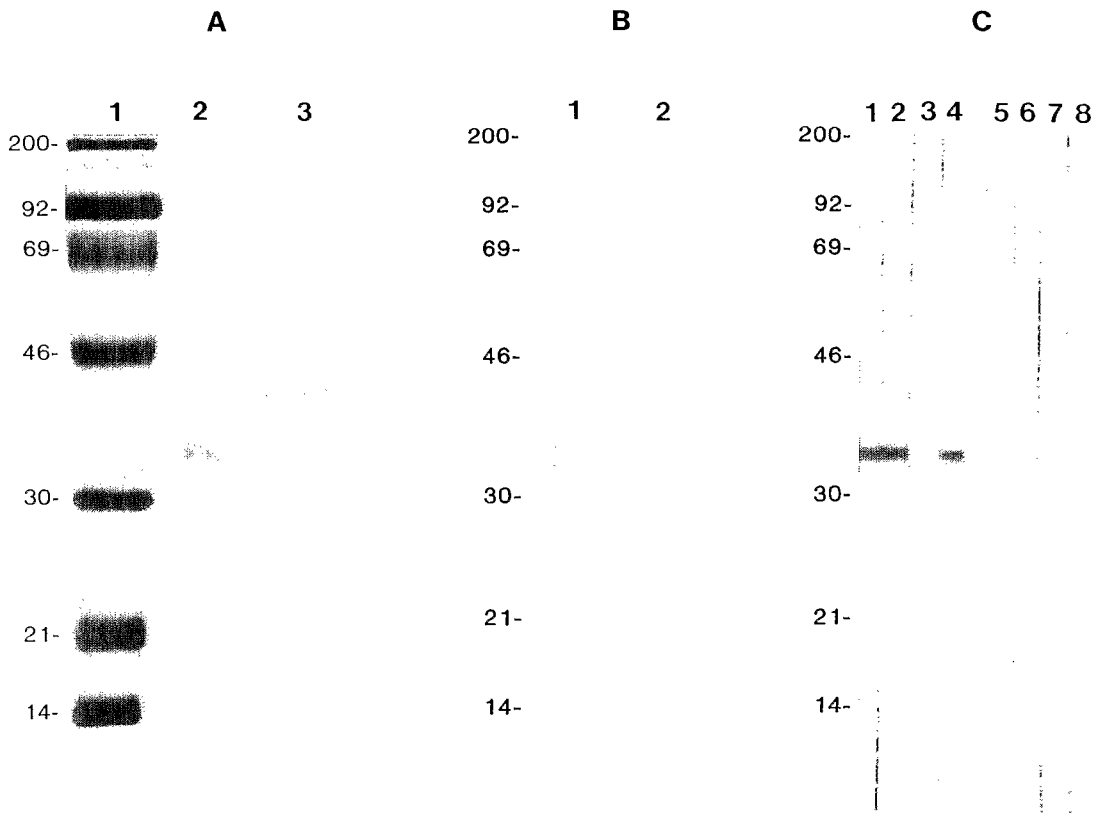

Fig. 6. Monoclonal antibody-affinity purification of the 33-kDa antigen. (A) Coomassie stain of the non-reduced (lane 2), and reduced (lane 3) affinity-purified antigen after SDS-PAGE. The positions of the markers (in kDa, lane 1) are indicated on the left of the panel. (B) Western blot of the non-reduced antigen (lane 1) and reduced antigen (lane 2) probed with mAb 4C5. (C) Western blot of the non-reduced antigen (lanes 1,2,3,4) and reduced antigen (lanes 5,6,7,8) probed with sera from two 'responder' cattle (lanes 1,2 and 5,6), a 'non-responder' steer (lanes 3 and 7) and the rabbit anti-protease antibody (lanes 4 and 8).

additional band was sometimes seen at approximately 31 kDa, as was observed with the biochemically purified cysteine protease (Fig. 4 and 7A). Under appropriate reducing conditions (see Materials and Methods) this molecule had an apparent molecular mass of approx. 40 kDa (Fig. 6A, lane 3). MAb 4C5 bound to the unreduced molecule, but did not bind to the reduced molecule on Western blots (Fig. 6B). The serum from two 'responder' animals reacted with both reduced and non-reduced antigen, although the reaction with the reduced antigen was weak (Fig. 6C, lanes 1, 2 and 5, 6, respectively). The rabbit antibody raised against the biochemically purified cysteine protease reacted strongly with the non-reduced antigen (Fig. 6C, lane 4). Like bovine antibodies, the rabbit antiserum reacted weakly with the reduced molecule (Fig. 6C, lane 8).

When analysed by Fbg-SDS-PAGE gels, the purified antigen exhibited proteolytic activity at 33 kDa, like the cysteine protease purified by cystatin affinity chromatography (Fig. 7A). Its proteolytic activity against Azocoll was inhibited by 1 μM and 10 μM of the cysteine protease inhibitors, cystatin and E-64 respectively (Fig. 7B). The antigen hydrolysed the fluorogenic substrate Z-Phe-Arg-NHMec with a K_m of 7.3 μM ($\pm$ 0.8 μM) and this hydrolysis

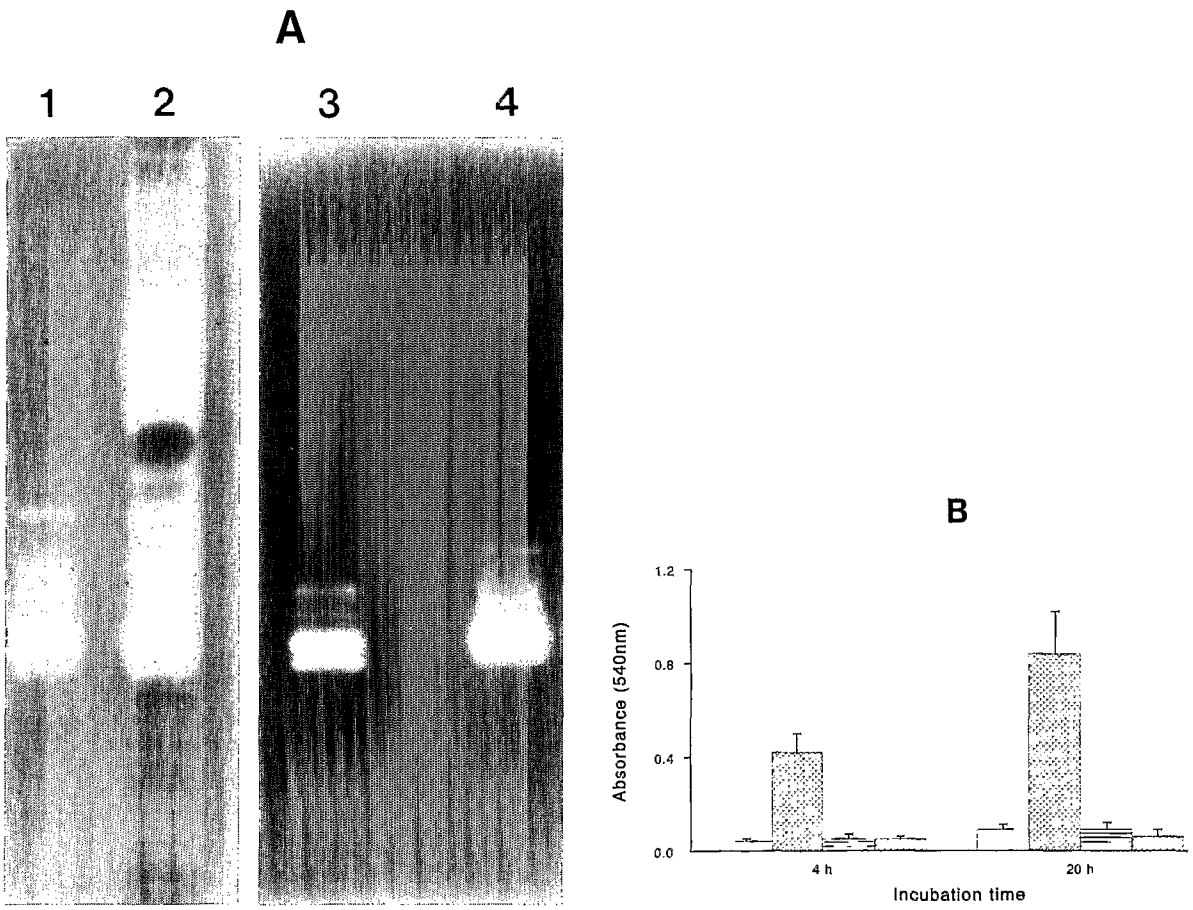

Fig. 7. Enzymatic activity of the 33-kDa antigen. (A) Fibrinolytic activity in Fbg-SDS-PAGE after 24 h incubation at 37 °C. Lanes 1 and 4, mAb affinity-purified antigen (5 μg per lane); lane 2, crude lysate of *T. congolense* ILC-49 (5×10^7 trypanosomes); lane 3, cystatin affinity-purified enzyme (5 μg). The band seen at lower M_r corresponds to the 30-kDa band occasionally seen on Western blots and is probably due to partial degradation. (B) Proteolytic activity on Azocoll. (Open columns) spontaneous hydrolysis of Azocoll in absence of antigen; (cross-hatched) hydrolysis of Azocoll induced by the purified antigen (30 μg ml^{-1}); (horizontally hatched) hydrolysis induced by the antigen (30 μg ml^{-1}) in presence of cystatin (1 μM); (diagonally hatched) hydrolysis of Azocoll in presence of E-64 (10 μM).

was inhibited completely by preincubating the antigen for 5 min with cystatin (1.3 ng ml^{-1}) or E-64 (13 ng ml^{-1}). Pronounced substrate inhibition was observed at Z-Phe-Arg-NHMec concentrations equal to or greater than 25 μM. The enzyme was unstable when stored on ice, where a progressive loss of activity with time was observed against Z-Phe-Arg-NHMec ($t_{1/2}$ = 1–2 h).

Silver-stained 2D gels (IEF/SDS-PAGE) reproducibly showed a similar protein pattern, a complex of two adjacent spots, whether they were loaded with the biochemically purified enzyme, the 33-kDa antigen, or the two proteins simultaneously (Fig. 8A, B, C). The complex may be due to isoforms of the enzyme or may result from carbamylation. Under the conditions used for Fbg-2D gels, both the antigen and the enzyme resolved into multiple spots of proteolytic activity, within the same range of pI and M_r (Fig. 8D, E). When the antigen and the enzyme were mixed, the patterns of fibrinolytic activity of both preparations were super-imposed in the gel (Fig. 8F).

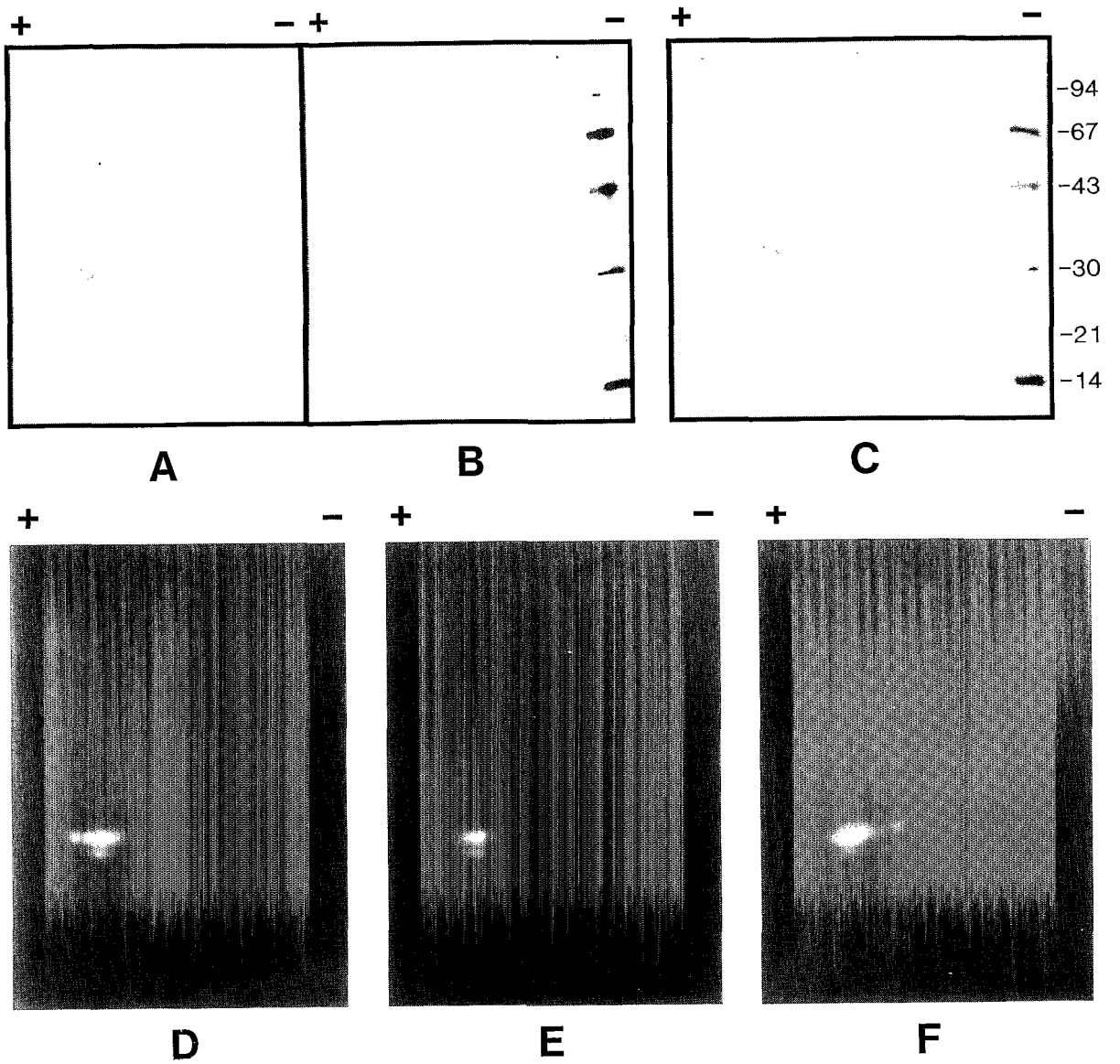

Fig. 8. Comparison of mAb affinity-purified antigen and cystatin affinity-purified protease, by 2D IEF/SDS-PAGE. The first (horizontal) dimension was IEF (pH 3.5–10) and the second dimension (vertical) was SDS-PAGE in 7.5–16.5% gradient gels. (A, B, C) Protein profile after silver staining. Approximately 10 μg or total protein were loaded on each gel. Samples (A) and (B) were run on the same SDS-PAGE gel. The molecular mass markers (indicated in kDa) were run on the right side of each gel. (D, E, F) Enzymatic activity in 2D IEF/Fbg-SDS-PAGE, after 48 h incubation at 37°C. Approximately 2 μg of total protein were loaded on each gel. (A, D) purified antigen; (B, E) cystatin affinity-purified enzyme; (C,F) mixture of antigen and enzyme.

Discussion

We have demonstrated that a major antigen of *T. congolense* is a cysteine protease. The trypanosome antigen shares similar molecular mass (33 kDa) and intracellular localisation

(lysosomal) with a cysteine protease purified employing a cystatin affinity resin [14,15]. The identity between the two proteins was shown using a mAb raised against the 33-kDa antigen. The anti-33-kDa mAb bound to purified cysteine protease on Western blots. Using immobilised mAb as an affinity resin, a 33-kDa protein was purified from bloodstream forms of *T. congolense*. This protein was bound by anti-33-kDa antibody from infected cattle and was also recognised by a rabbit serum raised against the biochemically purified cysteine protease. The affinity-purified antigen displayed proteolytic activity characteristic of cysteine proteases, confirming that the antigenic and enzymatic activities are associated with the same molecule.

Rautenberg et al. [24] initially described a 31-kDa thiol protease which was purified from bloodstream forms of *T. congolense* by affinity chromatography on thiopropyl-Sepharose. Lonsdale-Eccles and Grab [20] subsequently demonstrated that the lysosomal system of African trypanosomes contains a cysteine protease. This protease has a size of 27 kDa in *T. brucei* and 31 kDa in *T. congolense* [20,22]. It is found only in the VSG-coated forms of the parasite [25], from which it can be purified by cystatin affinity chromatography [14].

In the work reported here, the enzyme bound by the immobilised mAb was successfully eluted from affinity beads using acidic conditions and retained antigenicity and enzymatic activity. This enzyme represented approximately 1% of the total cellular protein in lysates of *T. congolense* ILC-49. The K_m for the hydrolysis of Z-Phe-Arg-NHMec (7.3 μM) by the mAb affinity-purified antigen is similar, but not identical, to that reported (4.4 μM) for a *T. congolense* cysteine protease purified by affinity chromatography on thiopropyl-Sepharose [15]. The differences in K_m may be the result of an inaccurate determination as a consequence of the instability of the enzyme and its inhibition by substrate at a concentration of 25 μM and above. However, it is also possible that the two purification procedures produce distinct subpopulations of *T. congolense* cysteine proteases with different K_m values.

Under reducing conditions, the *T. congolense* cysteine protease, like the *T. brucei* homologous enzyme [26], undergoes rapid autodegradation. However, heat inactivation followed by prolonged reaction under strong reducing conditions resulted in the successful reduction of the purified *T. congolense* enzyme. The reduced protein exhibited an apparent molecular mass in SDS-PAGE of 40 kDa. In trypanosome lysates subjected to electrophoresis under standard reducing conditions, the antigen was found at 33 kDa. This suggests that only partial reduction of the enzyme was achieved in the lysates.

The data shown here indicate that the protease is antigenically conserved in different isolates of *T. congolense*. The amount of enzyme appeared to vary between different isolates. However, because of the lability of the enzyme, the amount also varied between different preparations of the same stock. The post-infection bovine serum did not cross-react with proteins of similar molecular mass in *T. vivax* or *T. brucei*, suggesting that the antigen is specific for *T. congolense*. Thus, the 33-kDa protease is potentially a serodiagnostic reagent.

Proteases of parasites are increasingly regarded as potential targets for chemotherapeutic attack [27]. In contrast, only a few reports on the antigenicity of these molecules are available. Proteases from *Schistosoma mansoni* [28] and from *Leishmania* sp. [29,30] have been shown to elicit antibodies in infected hosts and a major antigen of *T. cruzi* was shown recently to be a cysteine protease [31,32]. The high immunogenicity of the *T. congolense* cysteine protease in cattle suggests that this may be a common characteristic of this class of enzymes. The cysteine proteases of protozoan parasites share large regions of homology with mammalian cathepsin L, mainly in the vicinity of the active site [31,33–35]. The sequence of the gene encoding the *T. congolense* enzyme has not been reported. However, in *T. brucei*, the sequence

of the gene encoding the cysteine protease predicts an unusual C-terminal extension, which distinguishes the trypanosome enzyme from human cathepsin L [35]. Thus the C-terminal portion of the molecule is likely to be the major target for the immune response. Another consequence of the homology between trypanosome and mammalian cysteine proteases, is the likelihood that these proteins carry few T-cell epitopes. Consequently, specific T-cell responses, which are dependent on antigen presentation in the context of the major histocompatibility complex antigens, may be very sensitive to genetic restriction. The efficiency of specific T-cell help might determine the isotypic and quantitative characteristics of antibody responses to the 33-kDa protease. This is supported by our observations that there are breed and individual differences in the humoral response to this antigen [4].

The 33-kDa antigen may play a role in the disease process by virtue of its proteolytic activity. By their ability to degrade host proteins (e.g., complement components, cell surface proteins, immunoglobulins and haemoglobin), proteases have been implicated in the pathogenesis of several parasitic diseases (reviewed in ref. 36). We are currently studying the interactions between the parasite enzyme and protease inhibitors of normal plasma as well as investigating the possibility that the circulating antigen expresses enzymatic activity. Considering the potential deleterious effects of the cysteine protease on host proteins and the apparent association between antibody response and resistance to the disease, we speculate that specific antibodies may block some functions of the enzyme, thus mitigating the pathogenic effects of trypanosome infection.

Acknowledgements

We thank G.W.N. Mpimbaza for performing fibrinogen SDS-PAGE, Dr W.R. Fish for providing *T. congolense* clone IL3000, Dr O.K. ole-MoiYoi and M. Shaw for critically reviewing this manuscript and Mrs L. Agina for secretarial assistance. This is ILRAD publication number 917.

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