Schistosoma mansoni cercarial elastase (SmCE): differences in immunogenic properties of native and recombinant forms

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SUMMARY

The *Schistosoma mansoni* cercarial elastase (SmCE) has previously been shown to be poorly immunogenic in mice. However, a minority of mice were able to produce antibodies against SmCE after multiple immunizations with crude preparations containing the enzyme. These mice were partially protected against challenge infections of *S. mansoni*. In the present study, we show that in contrast to the poor immunogenicity of the enzymatically active native form of SmCE derived from a crude preparation (cercarial transformation fluid), immunization of CBA/Ca mice with two enzymatically inactive forms, namely purified native SmCE or a recombinant SmCE fused to recombinant *Schistosoma japonicum* glutathione S-transferase (rSmCE-SjGST), after adsorption onto aluminum hydroxide adjuvant, induced specific anti-SmCE immunoglobulin G (IgG) in all mice within 2 weeks of the second immunization. The IgG antibody response to rSmCE-SjGST was mainly of the IgG1 subclass. These results suggest that inactive forms of the antigen could be used to obtain the optimum immunogenic effects as a vaccine candidate against schistosomiasis. Mice immunized with the rSmCE-SjGST on alum had smaller mean worm burdens and lower tissue egg counts when compared with adjuvant alone- and recombinant SjGST-injected controls. The native SmCE was antigenically cross-reactive with homologous enzymes of *Schistosoma haematobium* and *Schistosoma margrebowiei*.

Key words: cercarial elastase, *Schistosoma mansoni*, enzyme, immunogenicity, vaccine, antigen, recombinant, GST fusion protein.

INTRODUCTION

With only a single drug and no vaccine, the global elimination of schistosomiasis is a real challenge (Hotez *et al*. 2014). Only a few schistosome antigens have induced significant protection in experimental animals, and results of early experiments were often not reproducible (Bergquist and Colley, 1998). Many of these antigens were selected because they were capable of inducing strong antibody responses in infected hosts. In the 1970s, Byron Waksman proposed that such antigens may be inappropriate for vaccine development as the parasite appears to be able to survive despite the presence of high levels of antibodies against the antigens in question. Waksman's postulate was publicized by Sher (1988) and it predicates that it would be more expedient to investigate the vaccine potential of molecules that are likely to be important for parasite survival, but not so well recognized by the host's immune response (Sher, 1988; Doenhoff, 1998). One such example is the *Schistosoma mansoni* cercarial elastase (SmCE), the major proteolytic activity responsible for degradation of the diverse set of macromolecular barriers that cercariae must breach during penetration of host skin (Salter *et al*. 2002). Immune responses, which can neutralize the activity of SmCE, may thus protect the host by preventing or delaying penetration of cercariae and migration of schistosomula through the skin (Cohen *et al*. 1991). Previous reports of the likelihood that SmCE is also expressed on the surface of older skin and lung schistosomula (Marikovsky *et al*. 1990*a*,*b*; Ghendler *et al*. 1996; Fitzpatrick *et al*. 2009) may mean that the enzyme can also potentiate parasite survival during later stages of infection.

SmCE was not recognized as an antigen by antibodies in sera from schistosome-infected patients or animals (Bahgat *et al*. 2001) and its native form was found to be poorly immunogenic when injected into mice (Darani *et al*. 1997). In the latter study, a few mice repeatedly immunized with the native elastase did, however, produce anti-elastase antibodies and were found to be significantly protected against a challenge of *S. mansoni* cercariae. In this study, we present data on the immunogenicity in mice of an enzymatically inactive SmCE purified

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from cercarial transformation fluid (CTF) and a recombinant full-length, inactive SmCE fused to the *Schistosoma japonicum* glutathione S-transferase (SjGST) protein (rSmCE-SjGST) compared with the native, active SmCE present in a crude preparation [*S. mansoni* CTF (SmCTF)].

MATERIALS AND METHODS

Parasites and animals

A Puerto Rican strain of *S. mansoni* was maintained by passage through CBA/Ca inbred mice (supplied by Charles River, UK) and *Biomphalaria glabrata* snails using previously described methods (Smithers and Terry, 1965; Doenhoff *et al*. 1978*a*). Life cycles of *Schistosoma haematobium* and *Schistosoma margrebowiei* were maintained in Syrian golden hamsters and *Bulinus globosus* snails. All work with laboratory animals was conducted according to the UK Animal (Scientific Procedures) Act 1986 with personal and project licence authorities held by MICHAEL J. DOENHOFF (numbers PIL70/3255 and PPL40/3024, respectively). Animals were killed by administration of a lethal dose of pentobarbitone anaesthetic. Mice were perfused 42 days after cercarial challenge using previously described methods for enumeration of worms followed by tissue digestion for estimation of tissue egg loads (Doenhoff *et al*. 1978*a*,*b*). SmCTF and *S. haematobium* CTF (ShCTF) and cercarial homogenates from *S. mansoni* (SmCH), *S. haematobium* (ShCH) and *S. margrebowiei* (SmrgCH) were derived from their respective mechanically transformed cercariae as described elsewhere (Colley and Wikel, 1974; Smith *et al*. 2012), kept frozen at −80 °C until required for use.

Rabbit antisera reactive with SmCE, S. haematobium cercarial elastase (ShCE) or SjGST

SmCTF or ShCTF were run in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred onto nitrocellulose papers (NCPs) as previously described (Laemmli, 1970; Towbin *et al*. 1979). The respective ~27 kDa cercarial elastase bands were excised and the NCP strip was dissolved in the minimum amount of dimethyl formamide, diluted in an equal volume of isotonic saline and emulsified with Freund's adjuvants (Difco Laboratories, Detroit, Michigan, USA), Freund's complete adjuvant being used for the first injection and Freund's incomplete adjuvant for the boosting injections. New Zealand white rabbits (B&K Universal, UK) were subcutaneously immunized with 0·5 mL of the material every 2 weeks until the sera were shown by an appropriate immunoassay (immunoprecipitation or immunoblotting) to have antibody reactivity. The rabbits were then exsanguinated and their sera were collected and stored at −20 °C until required. Sera from rabbits injected with Freund's adjuvant alone were used as controls for the cross-reactivity experiment. The anti-SjGST rabbit antiserum was produced by injecting rabbits with SjGST (5 mg protein/injection) that had been purified from the *S. japonicum* worm homogenate by glutathione S-transferase (GST) affinity chromatography as previously described (Simons and Vander Jagt, 1981), emulsified with Freund's adjuvants as described above.

Plasmids and chemicals

All chemicals, antibiotics and components of bacterial growth media were purchased from Sigma (Poole, UK), unless otherwise indicated. A plasmid expressing the SmCE-GST fusion protein (pGEXCEL) was constructed from a full-length SmCE-1a cDNA clone based on the *S. mansoni* elastase HP1 gene (gi 1240057) in pBluescript II SK (designated pCEL, supplied by Jamile Khalife, Institute Pasteur, Lille, France) and the pGEXON1 vector expressing exon 1 of SmCE-1a as a SjGST fusion (Price *et al*. 1997). The plasmid pGEXON1 encodes amino-acid residues 27–78 of the SmCE-1a protein (accession number CAA94312, encoded by Genbank Z70296) cloned in-frame with the pGEX-KG GST fusion gene. Briefly, the pCEL was digested with *EcoR*I and *Sma*I to excise the SmCE cDNA fragment, minus 36 nucleotides at the 5′ end of the mature protein-coding sequence. The resulting *EcoR*I-*Sma*I excised fragment encoding residues 37–264 of mature SmCE-1a was then ligated into pGEXON1 that had been digested with *Hind*III, blunt ended, purified and digested with *EcoR*I (to remove the first exon insert except for 36 nucleotides at the insert 5′ end that were left in the correct reading frame downstream to the SjGST cDNA in the vector) using standard molecular biology methods (Sambrook *et al*. 1989). Thus, the final construct, pGEXCEL, encodes the SjGST gene fused in-frame with residues 27–264 of SmCE-1a (mature SmCE-1a).

Cloning and expression of rSmCE-SjGST and recombinant SjGST (rSjGST)

pGEXCEL vector DNA was transformed sequentially into competent JM109 *Escherichia coli* cells using standard procedures (Sambrook *et al*. 1989) in order to express the rSmCE-SjGST fusion protein (Guan and Dixon, 1991). The pGEX-KG vector was used to transform the same cells to express the rSjGST protein. General procedures for cloning, DNA manipulations and protein expression were performed as described elsewhere (Simons and Vander Jagt, 1981; Sambrook *et al*. 1989). The cells were harvested by centrifuging the culture mix at 5000 × ***g*** for 10 min (min) at 4 °C.

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The resulting cell pellet (5 g) was used to extract the recombinant protein by resuspension in 50 mL of 50 mM Tris–HCl, pH 8·0, 1 mM EDTA), 200 mM NaCl and 5% glycerol (v/v). Freshly prepared phenylmethanesulphonyl fluoride and lysozyme were added to final concentrations of 23 μg mL^{-1} and 10 mg mL^{-1}, respectively, and the suspension was stirred for 20 min at room temperature (RT) until it became viscous. Sodium deoxycholate solution (50 mg in 1 mL) was added and the mixture was stirred vigorously for a further 20 min at RT and sonicated (4 × 10 s bursts, 25% amplitude, separated by 10 s incubations on ice). The suspension was clarified by centrifugation at 42 000 × ***g*** for 30 min at 4 °C. To prepare purified soluble rSjGST, the supernatants from this step using extracts from cells carrying the unmodified vector pGEX-KG were applied to HiTrap GST columns (GE Healthcare). The pellet containing the insoluble rSmCE-SjGST protein was solubilized by re-suspending in phosphate-buffered saline (PBS), pH 7·2, containing 8 M urea at RT with shaking for 30 min, centrifuged as above, and the supernatants containing solubilized proteins were retained for purification of rSmCE-SjGST.

Antigen purification and immunization of mice

Native SmCE (giving a doublet of protein bands at ~27/28 kDa in SDS–PAGE) was purified from SmCTF by continuous-elution gel electrophoresis using the BIORAD Electro-Eluter model 422 (Bio-RAD), according to the manufacturer's instructions. An electroblot of the fraction containing the purified SmCE was stained with Protogold® (a colloidal gold solution; BioCell Research Labs, UK) according to the manufacturer's instructions. Suspensions containing rSmCE-SjGST solubilized in 8 M urea were loaded onto 10% SDS–PAGE gels, and the protein was purified by continuous-elution gel electrophoresis using the BIORAD Prep Cell model 491 (Bio-RAD) as described by the manufacturer. The proteins were eluted in 25 mM Tris-glycine, pH 8·0, with 0·3% SDS (w/v). The protein was finally dialyzed against PBS, pH 7·4. The rSjGST was purified by glutathione-Sepharose affinity chromatography using a 1 mL-GST-Trap affinity column setup in the Gradifrac system using the method described by the manufacturer (Amersham Biosciences).

Inbred male CBA/Ca mice (supplied by Charles River, UK) were injected intraperitoneally, at 2-week intervals, with 0·1 mL of protein solution in PBS (containing 10–50 μg of protein) adsorbed onto an equal volume of aluminium hydroxide gel (alum) as adjuvant. Protein concentrations were measured using the Bradford Protein assay (Bio-Rad, UK). Alum was prepared by mixing one volume of 10% aluminium potassium sulphate with two volumes of 250 mM sodium hydroxide; the mixture was stirred vigorously for 10 min and centrifuged at 1000 × ***g*** for 10 min at RT. The pellet was then resuspended in 150 mL sterile 0·9% sodium chloride solution, and the suspension was stored at 4 °C until used. Control mice received 0·2 mL alum suspension in PBS. Blood samples were collected from a superficial vein 2 weeks after each injection. Sera were separated by microcentrifugation at 13 000 × ***g*** for 5 min at RT and stored separately for each animal at −20 °C until analysis. The rabbit antiserum specific for SmCE was used as a positive control in Western blots. Mice were each challenged with 200 *S. mansoni* cercariae 2 weeks after receiving the last injection using previously described methods (Smithers and Terry, 1965; Doenhoff *et al*. 1978*a*).

Assays

SDS–PAGE analysis and Western immunoblotting. Protein samples were analysed on reducing 10 or 12% SDS–PAGE prepared as described elsewhere (Laemmli, 1970). Protein Molecular Weight Pre-Stained Standards (Amersham Pharmacia Biotech, UK) were used to determine the molecular weight of the proteins in the analysed samples. SDS–PAGE gels were run using a Bio-Rad Mini Protean II electrophoresis system and stained with Coomassie Brilliant Blue R-250 (Bio-Rad) according to the manufacturers' instructions. Proteins separated on SDS–PAGE gels were transferred electrophoretically to nitrocellulose membranes (NCP) (Amersham Pharmacia Biotech, UK) as described elsewhere (Towbin *et al*. 1979). Blots were cut into strips; one of the strips was stained with Protogold® colloidal gold solution (Agar Scientific, UK) to visualize total proteins separated in the loaded samples. The remaining strips were incubated with the primary antibody solutions for 2 h at RT, including rabbit antisera [all diluted 1/400 in PBS with Tween 20 (PBST)] or sera from immunized mice (diluted 1/200 in PBST). The NCP strips were incubated with the appropriate secondary antibody (diluted 1/1000 in PBST): alkaline phosphatase (AKP)-conjugated goat-anti-rabbit immunoglobulin G (IgG) (Scottish Antibody Production Unit) or AKP-conjugated-anti-mouse IgG (Nordic Immunological Laboratories, The Netherlands) for 2 h at RT. The membranes were developed with 0·25 mg mL^{-1} 5bromo-4chloro-3indolyl-phosphate and 0·5 mg mL^{-1} nitroblue tetrazoleum in 10 mL 0·1 M Tris–HCl, pH 9·0, 0·1 M NaCl and 0·05 M $MgCl_2$ (Sigma, UK) for 20 min at RT.

Enzyme-linked immunosorbent assays (ELISA). Indirect ELISA were performed to examine mouse sera for presence of anti-elastase antibodies and determine the endpoint titre (Voller *et al*. 1976).

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ELISA plates (96-well flat-bottomed, Thermo Life Sciences, UK) were coated with 4 μg mL^{-1} SmCTF containing the native elastase in carbonate/bicarbonate buffer, pH 9·0 (Sigma, UK) (100 μL well^{-1} and overnight incubation at 4 °C). After rinsing, the primary antibody-containing mouse sera were added to the plates diluted 1/200 in PBST and the plates incubated for 2 h at RT. Secondary antibodies [horseradish peroxidise (HRP)-conjugated goat anti-mouse IgG, IgG1, IgG2a or IgE (Nordic Immunological Laboratories, The Netherlands)] were added (100 μL well^{-1}) at 1/1000 dilution in PBST and incubated as above with gentle agitation for 2 h at RT. Plates were developed using 3, 3′, 5, 5′-tetramethyl benzidine dihydrochloride (Sigma, UK) for 5–10 min, and the reaction was stopped by addition of 50 μL of 2 M H_2SO_4. Absorbance at 450 nm was read using an ELISA plate reader.

Assay for enzymatic activity of SmCE. Gelatinase activity in samples containing ~4 μg of protein was tested on a pre-cast zymogram (gelatin) gel (10% Tris-glycine gel with 0·1% gelatin as a substrate) purchased from Invitrogen, UK. The procedure was carried out as described in the manufacturer's manual. The gels were then stained with the SimplyBlue Safe stain (Invitrogen, UK) according to the manufacturer's instructions.

Statistical procedures

All statistical calculations were performed using GraphPad Prism software version 4.0 (San Diego, California, USA). The significance of the difference between the immunized and the control groups was determined by the Mann–Whitney *U* test. Values of $P < 0{\cdot}05$ were considered to be statistically significant.

RESULTS

Production and characterization of antigens used for immunization

The native form of SmCE was purified from SmCTF by continuous-elution gel electrophoresis. Pooled fractions containing a doublet of ~27–28 kDa stained by Protogold after electrotransfer to NCPs (Fig. 1A, lane 1) was shown to be reactive in immunoblots probed with an anti-27 kDa cercarial elastase (SmCE) rabbit serum (Fig. 1A, lane 2). The plasmid pGEXEL carrying the cDNA clone of the mature SmCE-1a downstream of the SjGST was successfully constructed and transformed into *E. coli* cells that were shown to express the transformed gene following induction by 0·5 mM isopropyl-β-D-thiogalactoside at 30 °C for 60 min, the same for SjGST. The purified rSmCE-SjGST that was obtained in a soluble form in PBS (Fig. 1B, Lane 2) was antigenically reactive in Western blots probed with a rabbit anti-SjGST serum (Fig. 1B, lane 4) and an anti-SmCE serum prepared against purified native 27 kDa SmCE (Fig. 1B, lane 6). Purified SjGST (Fig. 1B, lane 1) was reactive in Western blots with the rabbit anti-SjGST serum (Fig. 1B, lane 3), but not with the rabbit anti-SmCE serum (Fig. 1B, lane 5). The native SmCE in SmCTF was enzymatically active in gelatin zymogram gels, but neither the rSmCE-SjGST nor the purified native SmCE was found to be proteolytically active in the assay (data not shown).

Antibody responses to SmCTF, purified-native SmCE and rSmCE-SjGST in mice

Four groups of six male CBA/Ca mice were immunized intraperitoneally three times at 2-week intervals. Group I (control) received only alum on PBS. Groups II, III and IV received 10 μg elastase protein per immunization in unfractionated SmCTF (containing native/active SmCE), purified native 27–28 kDa SmCE and purified rSmCE-SjGST, respectively, all adsorbed onto alum. Sera from groups III and IV immunized, respectively, with purified, native, though enzymatically inactive, SmCE and rSmCE-SjGST were shown to have anti-elastase IgG antibodies starting from bleed 2 (Fig. 2A, III and IV). Sera from the control or SmCTF-immunized mice – from all the bleeds – did not show any reactivity to SmCE when examined 2 weeks after the second (Fig. 2A, I and II) and the third immunizations (data not shown).

In a second experiment, three groups, each of 10 male CBA/Ca mice, were given four intraperitoneal injections at 2-week intervals. Group I received only alum in PBS, while groups II and III received 50 μg mouse^{-1} of, respectively, rSjGST and rSmCE-SjGST proteins on alum. In groups II and III, all mice had IgG antibodies with specificity for rSjGST (group II) or rSjGST and rSmCE (group III) detected after just two injections, while no reactivity against either rSjGST or rSmCE was detected in the control group that received alum alone. The levels of the anti-SmCE IgG produced after immunization with rSmCE-SjGST were found to be statistically significantly higher ($P < 0{\cdot}05$) when compared in ELISA with groups I and II (Fig. 2B). Most of the anti-SmCE IgG response was found to be IgG1 ($P < 0{\cdot}005$ compared with control groups I and II) (Fig. 2B) and no specific anti-SmCE IgE was detected (data not shown). Both immunized groups II and III in this experiment were found to have lower mean group worm burdens than the control group I with the greater reduction seen in group III, which had 35·3% less worms. These mice (immunized with rSmCE-SjGST) had, respectively, a mean of 42 and 45·8% fewer eggs than those received alum alone and

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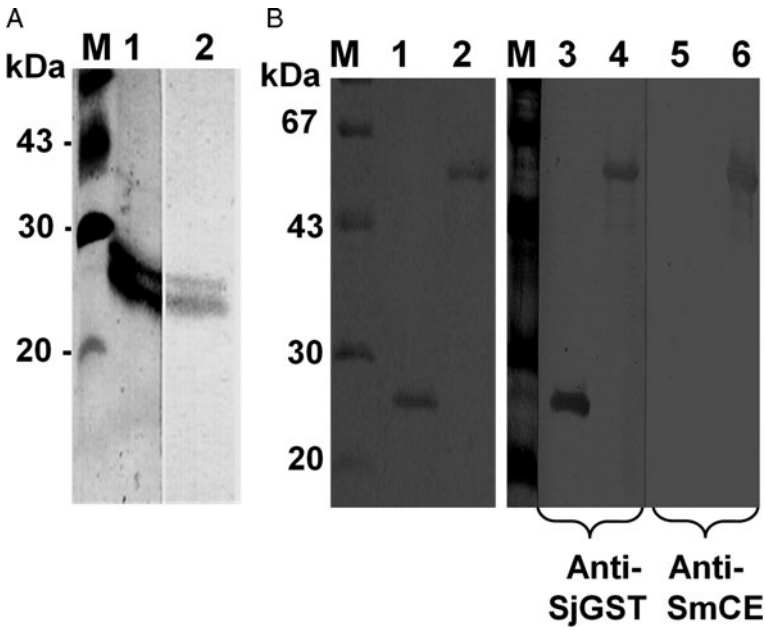

Fig. 1. Western blot of native SmCE purified from SmCTF (A) and Coomassie Brilliant Blue-stained SDS–PAGE and Western blot analysis of purified SmCE-SjGST and SjGST proteins (B). Lane M, protein molecular weight markers with sizes as indicated; (A) lane 1, Protogold-stained blot showing purified SmCE present as a doublet of 27–28 kDa in the sample; lane 2, Western blot of purified SmCE showing that the protein doublet of ~27–28 kDa reacted with antibodies in an anti-SmCE-specific rabbit antiserum. (B) Left-hand panel shows Coomassie Brilliant Blue-stained 10% SDS–PAGE gel and central- and right-hand panels show Western blots probed with anti-rSjGST or anti-SmCE rabbit serum, respectively; lanes 1, 3 and 5: purified rSjGST at ~26 kDa (~1 μg lane^{-1}); lane 2, 4 and 6: purified rSmCE-SjGST at ~53 kDa (~1 μg lane^{-1}).

the rSjGST-immunized mice in their livers (Table 1). In another independent *in vivo* protection experiment (eight mice per group), CBA/Ca mice that had received three injections of 50 μg mL^{-1} rSjGST-SmCE on alum had, respectively, a 25·1% reduction in worm burden and a 40·6% reduction in liver eggs count when compared with mice that received only rSjGST. The protection data in both experiments were, however, not statistically significant due to a large within-group variance (Table 1).

Immunological cross-reactivity of the cercarial elastases of different schistosome species

Western blots of SmCH, ShCH and SmrgCH (150 μg lane^{-1}) were probed with rabbit sera raised against the native 27 kDa SmCE or ShCE. An antigenic doublet at 27–28 kDa in SmCH and one of ~27 kDa in both ShCH and SmrgCH were found to react in the blots probed with the rabbit anti-SmCE- and anti-ShCE-specific sera (Fig. 3).

DISCUSSION

Vaccination may be the best way to obtain sustainable and long-term control of schistosomiasis (Molehin *et al*. 2016). Our own studies have been directed particularly at the SmCE. The enzymatic activity of SmCE has been shown to be important in penetration of definitive host skin (Cohen *et al*. 1991; Salter *et al*. 2002) and immune responsiveness directed against this enzyme may therefore be host-protective by directly preventing or delaying penetration of cercariae. This antigen is of interest because it seems not to be serologically recognized in infected subjects (Bahgat *et al*. 2001) and which (perhaps as a direct consequence of that property) may play an important role in immune evasion (Auriault *et al*. 1981; Marikovsky *et al*. 1988; Darani *et al*. 1997; Pleass *et al*. 2000). The present work is the first, according to our knowledge, to show that the inactive forms of the enzymes, including a purified native SmCE and rSmCE-SjGST, are more immunogenic in mice, compared with the native, after just two injections. Our Western immunoblots showed that sera from mice immunized, respectively, with enzymatically inactive, native SmCE and rSmCE-SjGST proteins had anti-elastase IgG antibody responses after two immunizations, while sera from mice immunized with the native active enzyme did not show any reactivity to SmCE till the end of the experiment at 2 weeks following the third immunization. Our finding is consistent with a previous report by Darani *et al*. (1997) who showed the native elastase to be poorly immunogenic when injected into mice. Reasons for the apparently poor immunogenicity of the native enzyme may be a consequence of the enzyme's ability to degrade host immunoglobulins and complement (Auriault *et al*. 1981; Marikovsky *et al*.

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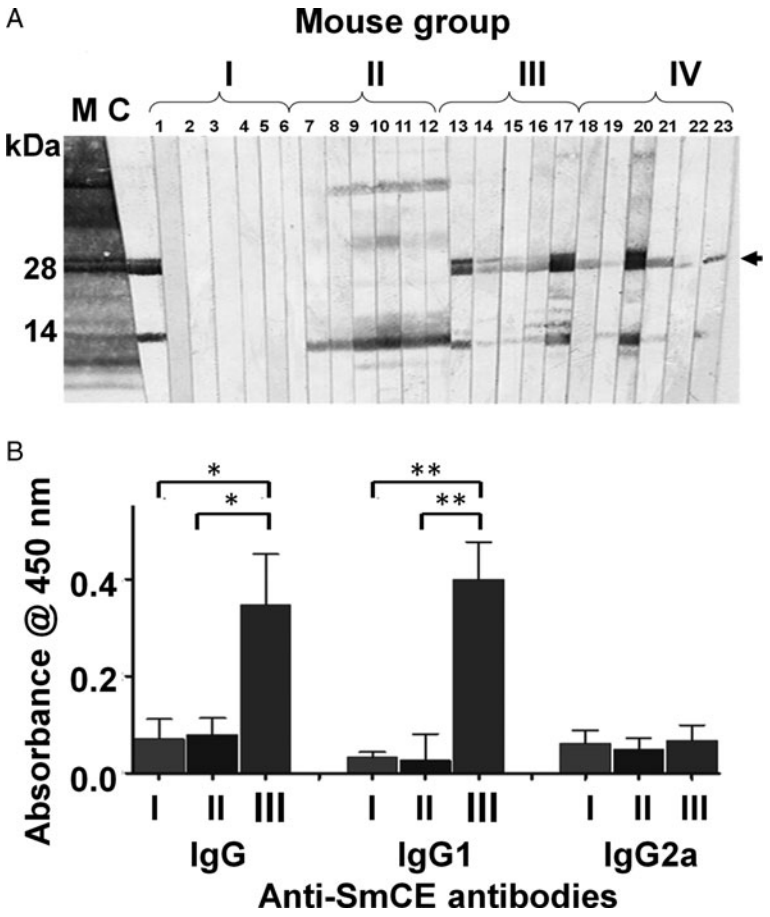

Fig. 2. Anti-SmCE antibodies in sera of mice immunized with SmCTF, purified SmCE or SmCE-SjGST after they had received two immunizations (A) and levels of anti-elastase IgG, IgG1 and IgG2a in mice immunized with rSmCE-SjGST by indirect ELISA 2 weeks following the third immunization (B). M, molecular weight markers with sizes as indicated. (A) Western blots of SmCTF (150 μg well^{-1}) were probed with the following primary antibody: C, rabbit anti-SmCE serum; I, lanes 1–6, mice sera from control mice received alum on PBS; II, lanes 7–12, sera from SmCTF-immunized mice; III, lanes 13–17, sera from mice immunized with purified native SmCE; IV, lanes 18–23, sera from mice immunized with purified recombinant SmCE-SjGST. Primary antibodies were detected with AKP-conjugated goat anti-rabbit or anti-mouse IgG, both diluted 1/1000. The position of elastase doublet at 27/28 kDa is arrowed. (B) ELISA plates were coated with SmCTF, probed with mouse sera and detected with HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2a. Columns represent mean absorbance readings for groups I (alum alone-injected mice), II (rSjGST-immunized mice) and III (rSmCE-SjGST immunized mice). Error bars represent S.D. *$P < 0{\cdot}05$, **$P < 0{\cdot}005$.

Table 1. Effect of rSmCE-SjGST immunization on *S. mansoni* worm and eggs burdens in mice

		Effect on *S. mansoni* worm and eggs counts					
		Worm			Liver eggs		
			Percentage of reduction *vs*			Percentage of reduction *vs*	
Groups		Mean ± S.D.	Control	SjGST	Mean ± S.D.	Control	SjGST
Experiment-1	Control	74·60 ± 25·70	–	–	33 998 ± 6513	–	–
	SjGST	55·60 ± 22·30	25·50	–	36 383 ± 11 318	−7·0	–
	rSmCE-SjGST	48·30 ± 28·40	35·30	13·10	19714 ± 8096	42·0	45·8
Experiment-2	Control	55·30 ± 11·8	–	–	24 643 ± 7707	–	–
	SjGST	59·40 ± 09·2	−7·4	–	31 400 ± 2251	−27·4	–
	rSmCE-SjGST	44·50 ± 18·3	19·6	25·1	18 661 ± 7533	24·3	40·6

1988; Darani *et al*. 1997; Pleass *et al*. 2000) or its interaction with serine protease inhibitors in the host (Mast *et al*. 1991; Modha and Doenhoff, 1994*a*,*b*). The latter was found to result in rapid clearance of the enzyme from the circulation preventing its normal processing by the antigen-presenting cells (Mast *et al*. 1991; Modha and Doenhoff, 1994*a*,*b*).

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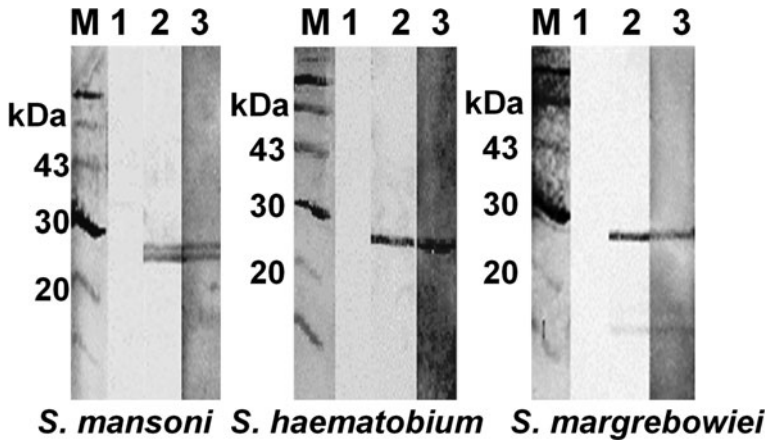

Fig. 3. Western blots of cercarial homogenates from three different schistosome species showing cross-reactivity of ~27/28 kDa cercarial elastase molecules. Blots of cercarial homogenates of *S. mansoni* (left-hand panel), *S. haematobium* (central hand panel) and *S. margrebowiei* (right-hand panel) were probed with rabbit sera raised against SmCE or ShCE. Lane M, protein molecular weight markers with sizes as indicated; lane 1, blot probed with control sera from rabbits immunized with Freund's adjuvant alone; lane 2, blot probed with rabbit anti-SmCE sera; lane 3, blot probed with rabbit anti-ShCE sera.

The SmCE-1a gene was used in the present study to produce the rSmCE-SjGST. SmCE-1a is the most highly expressed elastase isoform followed by SmCE-1b: both are responsible for 90% of the protein amount and activity (Salter *et al*. 2002; Fitzpatrick *et al*. 2009). Alignment of the SmCE-1a and SmCE-1b amino-acid sequences revealed 96·6% similarity, and the two isoforms were shown to be functionally equivalent (Salter *et al*. 2002) and immunologically cross-reactive (Darani, 1997). In this work, our detection of the SmCE doublet by a rabbit antiserum against the lower 27 kDa SmCE in immunoblots indicates, in accordance, that both isoforms may carry similar antigenic epitopes. We have shown here that the rSmCE-SjGST was antigenically reactive in Western immunoblots probed by either an anti-SmCE antiserum or anti-SjGST antibodies, indicating fusion of SjGST to elastase did not abrogate the antigenic properties of either of the fusion partners. Immunization with rSmCE-SjGST induced high levels of SmCE-specific total IgG and IgG1 in ELISA, the levels of both being statistically significantly higher when compared with alum alone and SjGST controls. High levels of IgG1 and low levels of IgG2a indicated a polarized Th2-driven response (Mountford *et al*. 1994), though elastase-specific IgE antibodies were absent in sera from the rSmCE-SjGST-vaccinated mice. The latter observation is an important consideration in vaccine development, as specific IgE results in immediate-type hypersensitivity reactions upon re-exposure to an antigen rendering vaccines that induce IgE antibodies potentially unsafe for use in humans. Such an undesirable outcome was previously observed with the hookworm Na-ASP-2 vaccine when it was tested in humans (Bethony *et al*. 2008).

The 26 kDa GST (Sj26GST) used in immunization in the present work is a fusion tag within the backbone of pGEX-KG, a commercially available bacterial expression plasmid (manufactured to produce GST-fusion proteins). Although GSTs were first identified as vaccine candidates by Smith *et al*. (1986), Sj26GST, unlike SmGST and ShGST, was shown to have 'unsatisfactory' vaccinating potential since it induced variable/inconsistent protective immune responses to *S. japonicum* in different mouse strains (Davern *et al*. 1987; Scott and McManus, 2000). In a previous research, when used as a control for immunization with another recombinant protein fused with SjGST, GST derived from *S. japonicum* was ineffective in reducing parasite burdens in a murine challenge model of *S. mansoni* (Schechtman *et al*. 2001). The latter results could possibly be a reflection of the low level of antigenic cross-reactivity between Sj26GST and the two GSTs (of, respectively, 26 and 28 kDa) of *S. mansoni* (Tiu *et al*. 1988; Henkle *et al*. 1990). Our preliminary protective data showed that mice that received rSjGST-SmCE on alum had, respectively, 25 and 40·6% more reductions in worm and liver eggs counts than mice receiving only rSjGST. In another independent experiment in the present work, mice immunized with rSmCE-SjGST had 45·8% reductions in liver eggs counts than those immunized with rSjGST alone. These mice had 35·3 and 42% fewer worms and liver eggs than unimmunized controls. Similar levels of protection (40%) with crude native SmCE have been shown in the few mice that produced antibody against the enzyme (Darani *et al*. 1997). However, the present preliminary results were not statistically significant due to large intra-group variations, especially in the rSmCE-SjGST-immunized

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groups. Inappropriate re-folding of the recombinant molecule may be the reason, even though some epitopes relevant to induction of protective immunity in the latter were retained. We suggest that in order to get consistent immunoprotective effects in all mice, it may be useful in future work to administer an inactive elastase protein that is properly folded with potentially immunoprotective B-cell epitopes being thus retained. Fusion to other smaller, relatively non-immunogenic tags may be helpful to obtain a more properly folded protein and to examine the immunoprotective capacity of the recombinant elastase immunogen independent of the SjGST.

Results of Western blots probed with 27 kDa SmCE- and ShCE-specific antisera show that the SmCE doublet is antigenically cross-reactive with a similarly sized molecule in ShCH. A 27 kDa elastase band in *S. margrebowiei*-cercarial extracts was also reacted against by the anti-27 kDa SmCE and ShCE rabbit antisera. The *S. mansoni* and *S. haematobium* elastase major isoforms were shown by Salter *et al*. (2002) to have similar protein sequences: SmCE-1a and ShCE-1a were 93·9%, SmCE-1a and ShCE-1b were 92·8%, SmCE-1b and ShCE-1a were 93·9%, and SmCE-1b and ShCE-1b were 89·9 % similar. In addition, isoforms from the same species, including SmCE-1a and SmCE-1b, and ShCE-1a and ShCE-1b had, respectively, 96·6 and 98·6% sequence similarity. SmCE-1a and SmCE-1b proteins were reported to have different molecular masses (Knudsen *et al*. 2005). In that paper, a proteomic analysis of cercarial secretions by Knudsen *et al*. (2005) revealed the presence of SmCE-1a and SmCE-1b proteins with molecular masses of 28·5 and 29·5 kDa, respectively, and alignment of the amino-acid sequences of the SmCE-1a and SmCE-1b by BLAST revealed a difference in length by an extra 10 amino acids in the latter, perhaps indicating why in the present work the SmCE protein appears as a doublet (the SmCE-1a being most probably located in the lower ~27 kDa band). No data have been reported in the literature for the relative sizes of respective elastase isoforms from *S. haematobium* and *S. margrebowiei* to indicate whether these enzymes would also appear as doublets. The lack of complete amino-acid sequence data for elastase isoforms of the latter species also prevented comparative amino-acid alignment. The present results may indicate that a vaccine based on SmCE may protect against *S. haematobium* as well. The cross-reactivity shown here with a *S. margrebowiei*, an important veterinary schistosome, may allow the vaccine to be tested for efficacy and safety in animals before trials in humans, though the reactivity of antibodies against the rSmCE protein will need to be tested against native elastases from the heterologous species.

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