

A mucosally administered recombinant fusion protein vaccine against schistosomiasis protecting against immunopathology and infection

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Abstract

We have constructed and efficiently produced and purified a candidate vaccine against schistosomiasis consisting of a novel hybrid protein in which two dominant T- and B-cell epitopes from *Schistosoma mansoni* 28 kDa glutathione-S-transferase (Sm28GST) antigen (a.a 24–43 and 191–212) are fused to cholera toxin B subunit (CTB). Intranasal treatment of *S. mansoni*-infected mice with the hybrid protein, which similar to native CTB was assembled into receptor binding pentamers, significantly reduced total worm burden and liver egg counts due to the induction of Sm28GST-specific antibodies. Immunopathologic granuloma formation in the liver was also significantly suppressed and there was an almost complete suppression of delayed-type hypersensitivity reactions to both Sm28GST and to total soluble egg antigen in infected animals. The results suggest that this type of hybrid protein could be used as a combined anti-immunopathology and anti-infection vaccine against schistosomiasis.

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1. Introduction

The use of the non-toxic B subunit of cholera toxin (CTB) as a carrier for mucosal administration of proteins and peptides to achieve either immunity or tolerance is well documented [1]. Whereas chemical conjugation of antigenic proteins to CTB has elicited strong mucosal immune responses to linked antigens, the addition of B-cell epitopes to CTB by the generation of gene fusion proteins has been less successful and antibody responses to the fused peptides have been unimpressive [2–8]. Recently, we have reported that mucosal administration of even minute amounts of specific antigens chemically linked to CTB could induce peripheral tolerance [9] and could thereby prevent and treat various experimental auto-immune diseases [10–12]. It is clear that CTB in these studies served, not only as an ef-

ficient carrier to present T-cell epitopes but also exhibited direct immunomodulating properties and in light of these findings the use of peptide epitopes genetically fused to CTB appears to be more promising [13,14].

In the context of several chronic parasite infections, the possibility of using well-defined peptide epitopes fused to CTB both to modulate immunopathological responses and to give rise to protective antibodies interfering with the infection process is particularly interesting. Indeed pathology resulting from a parasite infection is often related more to inflammatory immune responses to parasite antigens than to the infection itself. We therefore used this approach to construct an anti-inflammatory and potentially also an anti-infectious vaccine against a major parasitic disease, schistosomiasis. The major pathological feature of this chronic helminth infection is the development of T-cell dependent granulomatous reactions around parasite eggs trapped in the liver, intestines and various other tissues of the host [15]. Among various candidate vaccines recently developed against human and veterinary schistosomiasis, Sm28GST has been recognized as perhaps the most promising molecule [16]. Previous studies have indicated that

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immunization with Sm28GST leads not only to a significant reduction in worm burden but also to a pronounced decrease in parasite fecundity in various animal models [17].

We have recently demonstrated that intranasal administration of full length Sm28GST chemically linked to CTB can both partly limit parasite development and suppress liver granuloma formation after *S. mansoni* infection [18]. Peptide mapping studies of the Sm28GST molecule show that the dominant T- and B-cell epitopes are carried by the N-terminal peptide 24–43, the C-terminal peptide 190–211, and the peptide 115–131 and immunization with each of these peptides gives rise to protective antibodies active against both worms and eggs [17,19]. The present study demonstrates that mucosal administration of dominant T- and B-cell epitopes of Sm28GST genetically linked to CTB is sufficient to protect against parasite infection as well as to suppress systemic T-cell-mediated granulomatous reactions. The results show that i.n. administration of microgram amounts of a CTB-based hybrid protein to mice during an ongoing infection can inhibit both parasite development and granuloma formation. This could provide the basis of a well-defined recombinant vaccine with combined anti-infectious and anti-immunopathological effects. The findings clearly support the use of immunoregulating treatment to control pathology during ongoing schistosomiasis, and hold out the possibility of extending the application of CTB–peptide hybrid proteins to other parasitic diseases based on well-defined specific protective or inflammatory epitopes.

2. Materials and methods

2.1. Animals and *S. mansoni* infection

Female C57BL/6, CBA and BALB/c mice (ALAB, Solentuna, Sweden), aged 6–8 weeks, were infected percutaneously (p.c.) with 60 *S. mansoni* (Puerto Rico strain) cercariae obtained from *Biomphalaria glabrata* snails [20]. After 7 or 8 weeks of infection, total worm counts were determined after whole-body perfusion with heparinized saline. Liver egg counts were determined after digestion of pre-weighed tissues with 4% potassium hydroxide [17].

2.2. Construction of CTB::GST1,3 hybrid protein carrying peptides 24–43, and 191–212

The complete Sm28GST gene was amplified from the cloned cDNA gene contained in plasmid pTG54 [21] such that it carried an in-frame NheI site at the 5'-terminus and a HindIII site incorporated into a TAA stop codon at the 3'-terminus, and was then cloned into a general-purpose expression vector pMT-tac1. DNA encoding a.a. 49–191 was removed from the gene by reverse PCR in which the entire plasmid excluding the region to be deleted was amplified. The primers introduced a unique KpnI site and amplified

DNA was digested with KpnI, ligated and electroporated into *E. coli* strain XL-1 Blue (Stratagene, San Diego, CA). The 5' end of Sm28GST gene was then added to the 3' end of a CTB gene to which a linker with a unique in-frame NheI site had been added. The nucleotide sequence encoding the residues 1–23 of Sm28GST was removed from the resulting plasmid by digestion with NheI and PvuII and insertion of a short synthetic linker that added back 4 residues from a.a. 24 of Sm28GST removed by digestion with PvuII and brought the resulting dual peptide into frame with the mature CTB gene. The sequence of the resulting dual peptide in the final plasmid (pML-CTB::Sm28 (24–43, 190–212) was confirmed by DNA sequencing. The CTB gene used lacked the signal peptide directing transport of the synthesized protein into the periplasmic space. This resulted in the cytoplasmic accumulation of the product (CTB::GST1,3) as monomers which formed insoluble inclusion bodies [22]. The hybrid protein is expressed from the tac promoter under the control of the lacI^q repressor carried on the same plasmid, and expression was induced by addition of IPTG to the medium. For production of the hybrid protein the plasmid pML-CTB::Sm28GST (24–43, 191–212) was transferred into *E. coli* BL21 [23].

2.3. Expression and assembly of CTB::GST1,3 hybrid protein

The growth of cultures and procedures used for the isolation of inclusion bodies and assembly, and purification of CTB::Sm28GST (24–43, 191–212) are same as those described previously [14]. The purity and degree of assembly of the protein was checked by SDS-PAGE and GM1 binding ability by GM1 ELISA [24]. The protein was further purified by ion exchange followed by gel filtration [25]. Expression of the Sm28GST peptide was confirmed by reactivity with a polyclonal antibody raised against the 191–212 peptide in GM1 ELISA. CTB::OVA is a CTB fusion protein carrying an irrelevant peptide epitope derived from chicken ovalbumin (a.a. 324–340) [26].

2.4. Additional antigens

rSm28GST was expressed in *E. coli* (strain TFE901) containing the plasmid pTG54 and purified as described earlier by affinity chromatography on a glutathione column [17]. Soluble egg antigen (SEA) from *S. mansoni* eggs were prepared as previously described [27].

2.5. Mucosal immunization regimens

Unless otherwise mentioned, anaesthetized mice received five consecutive doses of CTB::GST1,3 at 1 week interval starting 1 week after *S. mansoni* infection. Each dose consisted of 20 or 100 µg of hybrid protein given by i.n. instillation. Control groups received concomitant treatments with 20 µg CTB::OVA in 25 µl PBS or with 25 µl PBS alone.

2.6. Induction and measurement of delayed-type hypersensitivity reactions

Delayed-type hypersensitivity (DTH) reactivity to Sm28GST and SEA was evaluated in infected BALB/c mice as described before [18]. Infected animals were challenged with 10 μ g of Sm28GST, or SEA injected into the left and right foot-pads, 6 week after p.c. infection with 60 cercariae. Foot-pad thickness was measured immediately before and then 3, 24 and 48 h after foot-pad challenge, using a dial gauge caliper (Oditest, Essen, Germany). The resulting values referred to as early (3 h) and delayed (24 and 48 h) specific DTH foot-pad thickness increment, were expressed in units of $\text{cm} \times 10^{-3}$.

2.7. Histopathology

After 7 weeks of infection (i.e. 1 week after the final treatment), animals were sacrificed and the livers excised. The ventral median lobe of the liver was fixed in 4% phosphate-buffered paraformaldehyde, pH 7.4, dehydrated and embedded. Serial 8 μ m thick sections were prepared and stained with haematoxylin-eosin for measurement of granulomas.

The surface area of individual granulomas (a granuloma being defined as containing a single egg) was determined using a computer-assisted image analysis device (Leitz, Dresden, Germany). All granulomas were measured in two liver sections selected to be sufficiently distant from each other (ca. 200 μ m) to ensure that a granuloma was not measured twice. Data were expressed as mean granuloma size ($\mu\text{m}^2 \times 10^{-3}$), and mean percentage of the total eosinophils in the area of the liver sections that occurred within the granulomas.

2.8. Antibody determination

Sera were prepared from blood samples collected immediately before perfusion. Lung and tissue extracts were prepared by cycles of freezing and thawing in the presence of 2% saponin as described previously [28]. Although the saponin extraction procedure has only been described for determination of IgG and IgA in a limited number of tissues [28], the method has been extensively validated for a wide range of tissues and antibody classes and isotypes and has furthermore been extended to allow the extraction and determination of cytokines in a range of tissues (J. Holmgren, unpublished data). Antibody titers against Sm28GST were estimated by ELISA [29]. Polystyrene 96 well plates (Dynatech Lab Inc., Chantilly, VA, US) were coated with Sm28GST (0.5 μ g in 50 μ l PBS per well) for 3 h at 37 °C. After three washes with PBS-Tween (0.1%), sera or tissue extracts were serially diluted in PBS-Tween with 0.5% (w/v) gelatin and incubated overnight at 4 °C. Following washes with PBS-Tween, plates were incubated for 4 h at 37 °C with HRP-labeled goat anti-mouse IgG1, IgG2a, IgA,

or IgE (Southern Biotechnology Association, Birmingham, AL) diluted in PBS-Tween. Following washing and addition of enzyme substrate, solid phase-bound HRP activity was monitored spectrophotometrically. The titer of a serum or tissue extract was defined as the reverse of the highest dilution yielding an absorbance value of 0.4 above the background level.

3. Results

3.1. Anti-infectious effect of CTB::GST1,3 treatment in *S.mansoni*-infected mice

Previous work has shown that i.n. administration of Sm28GST alone either before or a few weeks after infection with *S. mansoni* neither affected the worm burden, nor the liver egg counts in infected animals. The extent of liver granuloma formation was also unaffected [18]. Recently, we have shown that i.n. administration of whole Sm28GST conjugated to CTB was more effective [18]. Here, we wanted to determine whether immunodominant peptides (24–43 and 191–212) of Sm28GST genetically linked to CTB would be able to modulate an ongoing *S. mansoni* infection.

C57BL/6 and CBA animals were treated with CTB::GST1,3 hybrid protein and sacrificed 7 and 8 weeks, respectively, after the initial infection. A significant reduction in worm burden and an even more pronounced decrease in liver egg counts were obtained compared to untreated or CTB::OVA treated animals (Table 1).

3.2. Increased the levels of Sm28GST-specific antibodies in protected *S.mansoni*-infected animals

Animals infected with *S. mansoni* or immunized i.n. with Sm28GST alone, give low serum antibody responses against the parasite antigen in the first 7 weeks post-infection [18]. Treatment with the CTB::GST1,3 hybrid protein dramatically increased serum IgG1 and IgG2a antibody responses to Sm28GST in C57BL/6 mice as compared to the control groups that were either untreated or treated with CTB::OVA (Table 2).

Higher IgA titers were found both in serum and lung of infected C57BL/6 mice after i.n. treatment with CTB::GST1,3 as compared to control animals. Interestingly, anti-Sm28GST IgE antibodies were produced both in the lungs and the liver of infected C57BL/6 mice treated with CTB::GST1,3, but were absent from the same tissues in untreated mice or those treated with CTB::OVA (Table 2). Serum Sm28GST-specific IgE was not found in any of the treated groups. In CBA mice where the effects of CTB::GST1,3 treatment on infection were similar to those seen in C57BL/6 mice, antibody responses including IgE, were also found to be elevated in the same way (results not shown).

Table 1

Effects of nasal administration of CTB::GST1,3 protein on *S. mansoni* infection and on liver granuloma formation

Treatment i.n. ^a	Mean $\pm$ S.D. (% reduction)		
	Worm burden	Egg counts ($\times 10^{-3}$)	Granuloma size (μm^2) ($\times 10^{-3}$)
Exp I (C57BL/6)			
Nil	16 $\pm$ 2.5	10.8 $\pm$ 3.0	130.9 $\pm$ 25.6
CTB::OVA (20 μg)	19.3 $\pm$ 4.5	13.6 $\pm$ 3.45	118.8 $\pm$ 11.2
CTB::GST (20 μg)	11.4 $\pm$ 4.0 (29%)	5.82 $\pm$ 2.25 (46%)	60.9 $\pm$ 14.4 (53%)
CTB::GST (100 μg)	11.8 $\pm$ 2.0 (26%)	6.45 $\pm$ 2.98 (40%)	66.3 $\pm$ 18.7 (49%)
P-value (vs. PBS)	<0.01; 0.05	<0.01; <0.01	<0.01; <0.01
Exp II (CBA)			
Nil	12.0 $\pm$ 2.4	6.70 $\pm$ 2.82	138.6 $\pm$ 19.5
CTB::OVA (20 μg)	11.8 $\pm$ 1.5	7.42 $\pm$ 0.87	140.3 $\pm$ 29.2
CTB::GST (10 μg)	6.20 $\pm$ 1.7 (48%)	1.20 $\pm$ 0.56 (82%)	39.3 $\pm$ 34.3 (72%)
CTB::GST (20 μg)	8.40 $\pm$ 2.0 (30%)	3.18 $\pm$ 0.36 (53%)	86.2 $\pm$ 21.5 (38%)
P-value (vs. PBS)	<0.01; <0.05	<0.001; <0.01	<0.001; <0.05

^a Worm burden, egg counts and liver pathology were examined from groups of six–eight mice animals sacrificed 7 weeks after infection. Reduction in percentage of control value from the untreated group was used as control for the calculations. Statistical significance was performed by Student's *t*-test; NS: not significant.

3.3. Anti-immunopathological effect of intranasal CTB::GST1,3 treatment in *S. mansoni*-infected mice

Histological examination of liver specimens from C57BL/6 and CBA mice collected 7 weeks after infection with *S. mansoni* showed a significant reduction in both the overall liver pathology (the total inflamed liver volume, not shown) and in the mean hepatic granuloma size in animals treated with CTB::GST1,3 as compared to untreated mice (Table 1). Whereas a tendency could be seen for CTB::OVA treatment to exacerbate worm burden and

liver egg counts, there was no discernible effect on liver pathology or granuloma size in either of the mouse strains used when compared with the untreated controls.

No correlation could be observed between worm burden or egg counts and liver granuloma sizes in these mice, supporting the view that suppression of granulomatous reactions was not the mere result of a reduction in worms and/or eggs ($r = 0.03$, $P > 0.2$ for regression analysis of worm burden versus granuloma size, and $r = 0.15$, $P > 0.2$ for liver egg counts versus granuloma size).

We have shown previously that inhibition of egg-associated granuloma formation in livers of BALB/c mice is correlated with suppression of DTH responses induced by i.n. treatment with protein Sm28GST chemically linked to CTB [18]. Thus, in order to compare the effects of i.n. treatment with the CTB::GST1,3 protein with our previous results, we selected BALB/c mice and examined the effects of i.n. treatment with DTH responses. The results indicated that the reduction in granuloma size was associated with a suppression of DTH against not only Sm28GST itself, but also against a soluble egg antigen preparation (Fig. 1).

4. Discussion

In this study, we have constructed and efficiently produced a candidate vaccine against schistosomiasis consisting exclusively of a novel hybrid protein in which dominant T- and B-cell epitopes of Sm28GST (a.a. 24–43 and 191–212) [17] are fused to CTB. Two out of the three immunodominant epitopes were initially chosen for technical reasons in order to reduce the length of the peptide added to the CTB. This was done in order to reduce the possibility of inefficient assembly of CTB pentamers in vitro due to interference from the added peptide. Since immune responses to each of the epitopes appears to give some level of protection those that

Table 2

CTB::GST1,3 hybrid protein treatment induces serum, mucosal and target organ antigen-specific antibodies in infected mice

	Titer of anti-Sm28GST-specific antibodies C57BL/6			
	IgG1	IgG2a	IgA	IgE
In serum				
PBS	<40	<40	<40	<40
CTB::OVA (20 μg)	<40	<40	<40	<40
CTB::GST (20 μg)	14,398	562	183	<40
CTB::GST (100 μg)	163,682	6252	622	<40
In lung				
PBS	<10	<10	<10	<10
CTB::OVA (20 μg)	<10	<10	<10	<10
CTB::GST (20 μg)	89	183	140	1212
CTB::GST (100 μg)	1,159	242	627	584
In liver				
PBS	<10	<10	<10	<10
CTB::OVA (20 μg)	<10	<10	<10	<10
CTB::GST (20 μg)	44	40	36	5227
CTB::GST (100 μg)	256	165	58	3521

Groups of six–eight mice were weekly i.n. treated with five doses of 20 or 100 μg of CTB::GST, or control regimens initiating 1 week after infection. Sera, weighted lungs and livers were collected 7 weeks after infection. Geometric mean is presented in the table.

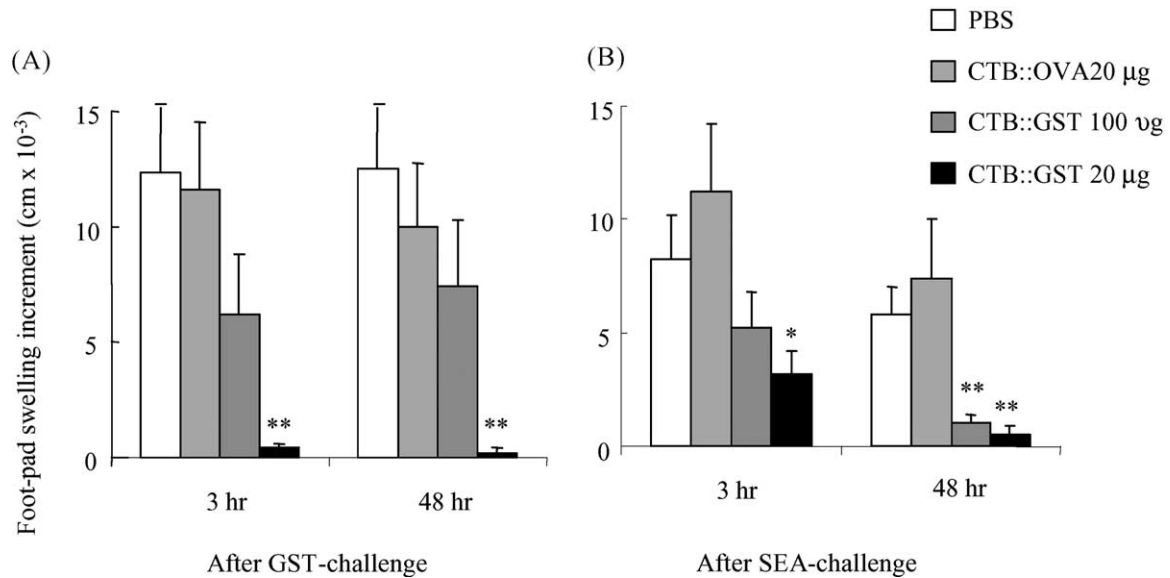


Fig. 1. Delayed-type hypersensitivity to Sm28GST (A) and soluble egg antigen (SEA) (B) with and without intranasal treatment CTB::Sm28GST1,3 after 3 and 48 h.

covered regions of the catalytic site were chosen preferentially.

We show that mucosal (i.n.) administrations of the resulting hybrid protein to mice infected with *S. mansoni* strikingly and consistently suppressed immunopathological liver granuloma formation and could also decrease worm burden and egg production. These effects were associated with induction of peripheral suppression of specific DTH in treated animals, and with Sm28GST-specific antibody production, respectively. To our knowledge, this is the first time that it has been shown that peptide epitopes genetically fused to CTB can give rise to both these effects simultaneously. The results suggest that this type of hybrid protein could be used as a combined anti-immunopathology and anti-infection vaccine against schistosomiasis.

Previous studies indicate that mucosal routes of immunization that give rise to IgA and IgE antibodies against Sm28GST especially at the induction and target sites evoke partial protection against infection [16–30]. Serum IgA from humans infected with *S. mansoni* was capable of neutralizing Sm28GST enzymatic activity as well as reducing female worm fecundity in vitro [31]. Our results are consistent with these findings and thus the decrease in worm burden and tissue egg counts observed after treatment with the hybrid protein may be due to increased mucosal and systemic Sm28GST-specific antibodies. Successful treatment gave rise to antigen-specific IgE in the liver and lungs of infected animals and to elevated levels of Sm28GST-specific IgA in serum and in lung tissue. Serum antibody responses to Sm28GST, mainly IgG1 and IgG2a were also dramatically increased.

The reductions in worm burden and egg counts were obtained even when i.n. treatment with CTB::GST1,3 was initiated 1 week post-infection. During the 5-week treatment

period most schistosomulae leave the lung vasculature and reach maturity. This suggests that the treatment affects both immature and mature parasites and is consistent with earlier reports indicating that young worms appear to be vulnerable to immune attack [32] and that mucosal vaccination of mice with recombinant GST can induce damage to adult worms after a challenge infection with *Schistosoma japonicum* [33].

The marked reduction in liver inflammation observed after i.n. treatment with CTB::GST1,3 appears not merely to be the result of a decrease in parasite burden, female worm maturation, or tissue egg deposition and is an important distinguishing feature of treatment with CTB-linked epitopes as opposed to immunization with the antigen alone. Indeed, whereas a reduction in worm burden can lead to a reduction in pathology, the underlying inflammation surrounding each egg embedded in the liver may well be undiminished. Our results show that in both C57BL/6 and CBA mice the worm burden and egg counts as well as the local inflammation surrounding the eggs in the liver are significantly decreased.

In conclusion, this work describes for the first time the construction and use of a genetically constructed parasite epitope-CTB candidate vaccine against schistosomiasis which when administered to infected animals by a mucosal route, consistently inhibits egg-induced pathology and can also limit the worm burden and egg counts in responding individuals. Such profound therapeutic and protective effects had only previously been achieved with whole protein antigens chemically conjugated to CTB. The potential of this novel antigen for immunoregulating intervention in severe forms of schistosomiasis not reversible by the usual praziquantel treatment will now be investigated. In more general terms the results of this study demonstrate the potential of CTB-peptide fusion proteins as the basis of non-invasive immunization strategies to block T-cell-mediated pathology

without compromising the ability to mount effective mucosal and humoral immune responses. The presented genetically engineered hybrid protein has clearly potential in the development of a new effective treatment for a parasitic disease that affects more than 200 million people world-wide.

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