

A Genetic Fusion between Sm14 and CTB does not Reduce *Schistosoma mansoni* Worm Burden on Intranasally Immunized BALB/c Mice

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Abstract

Developing a vaccine against schistosomiasis would be an important advance on the control of this chronic and debilitating disease that afflicts millions of people worldwide. Herein we describe the use of the non-toxic B subunit of cholera toxin (CTB) genetically fused to Sm14 - a fatty-acid binding protein from *Schistosoma mansoni* - as an attempt to elicit a mucosal immune response against the lung stage of this parasite by intranasal immunization. Recombinant proteins were expressed on a prokaryotic system, purified by affinity chromatography and both immunochemically and spectroscopically characterized. Intranasal immunization experiments were performed on BALB/c mice and vaccine efficacy was assessed analyzing the worm-burden after challenge infection with *S. mansoni* cercariae. The results demonstrate that Sm14 itself was not able to reduce the worm burden on intranasally vaccinated animals. The presence of CTB - either in intranasal coadministration with or genetically fused to Sm14 - did not significantly improve the protective response of Sm14 as a worm burden reduction of only 20% could be observed. In addition to that, however, CTB demonstrated a clear anti-inflammatory effect on the liver of immunized mice, which displayed hepatic granulomas around trapped eggs 15% smaller than control groups, indicating that CTB displays an immunomodulatory effect on the inflammatory responses induced by the parasite egg toxins.

Introduction

Schistosomiasis is a life threatening disease caused by blood flukes from the genus *Schistosoma* and despite effective and low cost chemotherapy is currently available, approximately 200 million people worldwide are infected with this parasite and other 600 million people are at risk of being infected [1]. Furthermore, failure in treatment with praziquantel has been reported recently indicating the development of resistant parasites [2]. As a result, developing of a vaccine would be an important advance on the control of this disease.

It is known that schistosomes are unable to replicate within the vertebrate host, this means that a vaccine, although desirable, does not need to have a sterilizing protection to provide a benefit. In fact, the World Health Organization (WHO) established that a vaccine that promotes, at least, a 40% reduction on worm burden would cause a significant impact on the global status of schistosomiasis. Sm14, a fatty-acid binding protein (FABP) from *S. mansoni* and one of the six vaccine candidates selected by the WHO for clinical trials, has been shown to reduce the worm burden in immunized mice [3]. Likewise, the elucidation of its biological and biochemical properties [4-6] led this antigen to be of particular interest of different research groups, being tested in different kinds of antischistosomiasis vaccine formulations [7-11].

Herein we describe an attempt to develop a vaccine against *S. mansoni* comprised by a genetic fusion between Sm14 and the non-toxic B subunit of cholera toxin (CTB), a molecule that has been used as mucosal adjuvant [12]. A robust mucosal immune response would be an interesting strategy to target the lung stage of *S. mansoni*. Indeed, CTB proved to elicit an adjuvant effect when used together with the 28 kDa glutathione-S-transferase (Sm28GST) antigen from *S. mansoni* [13,14]. Biochemical characterizations were performed on the rCTB-Sm14 fusion protein in order to establish its stability and biological functionality. The efficacy of this vaccine was analyzed in

terms of worm burden reduction in comparison with control groups. Furthermore, the hepatic granulomatous reactions around trapped eggs within the liver of infected mice had their areas measured and the effects of this vaccine on these inflammatory loci were also recorded.

Materials and Methods

rCTB and rSm14 expression systems

The individual cDNAs coding for the proteins rCTB [15] and Sm14 [6] were already cloned on the pAE plasmid [16], designed for high-level prokaryotic expression of recombinant proteins with a minimal N-terminal His6 tag controlled by the strong bacteriophage T7 promoter.

Cloning of CTB-Sm14 fusion construct

For the construction of the CTB-Sm14 fusion construct, the complete sm14 cDNA sequence was PCR amplified from its pAE plasmid template [6]. For this reaction, a forward primer containing

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Received December 17, 2010; Accepted December 29, 2010; Published December 30, 2010

Citation: Ramos HR, Miyasato PA, Ramos CRR, de Mattos Arêas AP, Kawano T et al. (2010) A Genetic Fusion between Sm14 and CTB does not Reduce *Schistosoma mansoni* Worm Burden on Intranasally Immunized BALB/c Mice. J Vaccines Vaccin 1: 111. doi:10.4172/2157-7560.1000111

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a *MluI* restriction site was designed and an oligonucleotide complementary to the T7 terminator region was used as reverse primer. The amplicons were then double digested with *MluI* and *HindIII* restriction enzymes and cloned into the pAE-ctb plasmid [15], at the 3' region and in frame with of the ctb DNA sequence.

Expression and purification of recombinant proteins

rSm14 and rCTB expression and purification: Expression and purification of rSm14 and rCTB were performed as previously described [6,15].

rCTB-Sm14 expression and purification: The pAE-ctb-sm14 plasmid was transformed in chemically competent *Escherichia coli* cells, which were grown on Luria-Bertani medium and induced for 3 h by the addition of 1mM isopropyl-1-thio- β -D-galactopyranoside when 0.6 of optical density at 600 nm was achieved. After the induction period, the cells were collected and mechanically lysed by French Press (Thermo Fisher Scientific Inc.) and the inclusion bodies containing the recombinant His6 tag protein were isolated from bacterial lysates and solubilized with 20 mL of solubilization buffer (8 M urea, 50 mM Tris-Cl, 5mM β -mercaptoethanol, pH 8.0). The material was then slowly diluted 200 times in refolding buffer (5 mM imidazole, 500 mM NaCl, 50 mM Tris-Cl, 100 mM D(+)-Galactose, pH 8.0).

Refolded protein solution was adsorbed on a 5 mL nickel sepharose affinity column (10 mm diameter) equilibrated with 5 column volumes (CV) of refolding buffer. The resin was washed with 5 CV of washing buffer (40 mM imidazole, 500 mM NaCl, 50 mM Tris-HCl, 100 mM D(+)-Galactose, pH 8.0) and the protein was eluted with 5 CV of elution buffer (1 M imidazole, 500 mM NaCl, 50 mM Tris-HCl, 100 mM D(+)-Galactose, pH8.0). After elution, imidazole was removed from the solution by buffer exchange using a 30 kDa cutoff centriprep system (Millipore Co.). Final buffer solution (BS) was 150 mM NaCl, 50 mM Tris-Cl, 100mM D(+)-Galactose and pH adjusted to 8.0.

rCTB-Sm14 stability improvement

For protein stability studies, proteins were kept either in BS with or without 100mM galactose for up to 3 months at 4°C, -20°C or lyophilized. Results were evaluated by non reducing SDS-PAGE [17].

Western-blot analysis

Nitrocellulose membranes, after protein transfer from 12% SDS-PAGE [17], were blocked with 5% (m/V) non-fat milk in 0.05% Tween 20/phosphate buffered saline (PBS-T). Membranes were washed three times for 10 min with PBS-T, and further incubated with a proper dilution of mouse polyclonal specific antibodies in 5% non-fat milk-PBS-T, for 1 h, followed by PBS-T washing as described above. Membranes were then incubated with a proper dilution of anti-mouse IgG peroxidase conjugate in 5% non-fat milk-PBS-T, washed with the same procedure, and revealed with ECL reagent (GE Healthcare).

SDS-PAGE of CTB-Sm14 and GM1-ELISA

The ability of the CTB-Sm14 fusion protein to fold into pentamers was analyzed by a 12% SDS-PAGE [17]. For this, samples were not boiled and a sample buffer that did not contain β -mercaptoethanol was used [15].

The GM1-ELISA protocol was adapted from a reference published elsewhere [18]. Briefly, microtiter 96-well plates were coated with 10 μ g ml⁻¹ GM1 ganglioside in PBS, pH 7.2, at 37°C for 16 h. After

three PBS-T washes, the plates were blocked by incubation with a 1% (m/V) BSA-PBS-T solution for 30 min at room temperature. The recombinant proteins were then serially diluted, from 300 mM to 300 nM, in 1% BSA-PBS-T (m/V) and incubated for 2 h at room temperature. Unbound proteins were removed from the plate by washing three times with PBS-T, and then a proper dilution of a specific polyclonal antibody was added to the plates and incubated for another hour at room temperature. After three PBS-T washes, a proper dilution of peroxidase-conjugated goat anti-mouse IgG (Sigma) was added to the plate and incubated for 1 h at room temperature. Unbound antibodies were removed by washing as described above with PBS-T and plates were revealed by the addition of 8 mg o-phenylenediamine (OPD) in 20 mL of a 0.2 M citrate-phosphate buffer, pH 5.0, in the presence of 10 μ L H₂O₂. The reaction was stopped by adding 4 M H₂SO₄. The absorbance was measured at 492 nm.

Fatty-acid binding activity of rCTB-Sm14

The fatty-acid binding ability of recombinant proteins was determined by fluorescence spectroscopy using the fluorescent fatty-acid analogue 11-(dansylamino) undecanoic acid (DAUDA) (Molecular Probes). The excitation wavelength used for DAUDA was 345 nm and emission spectra were registered from 360 nm to 670 nm. The protein and DAUDA concentrations were 2 μ M, in PBS pH 7.4 and measurements were carried out at 20°C using a SLM-AMINCO-Bowman Series II Luminescence Spectrometer (Spectronic Instruments) with 1 ml samples in a quartz cell (Ramos et al., 2003).

Circular Dichroism (CD) measurements

CD measurements were carried out at 20°C on a Jasco J-810 Spectropolarimeter equipped with a Peltier unit for temperature control. A Far-UV CD spectrum was acquired using a 1 mm path length cell at 0.5 nm intervals over the wavelength range from 190 to 260 nm. Five scans were averaged for each sample and subtracted from the blank average spectra. The protein concentration was kept at 10 μ M in 20 mM sodium-phosphate buffer pH 7.4.

Animal handling and immunization regimens

Immunization regimens and *Schistosoma mansoni* challenge: Four consecutive doses of antigen (Table 1) were given intranasally with 7 days interval to 7 weeks old females BALB/c mice, weighting around 18 to 20 g and divided in groups of 10 animals. Challenge was performed 2 weeks after the last dose. For this, animals were depilated on the abdominal region, anesthetized with 10 mg Kg⁻¹ Xylazine and 100 mg Kg⁻¹ Ketamine and had their abdominal skin exposed for 30 minutes to a 1 mL solution containing 100 *Schistosoma mansoni* cercariae (BH strain). Overall protection was calculated by the formula [(C - V) x C⁻¹] x 100, where C is the average number of worms recovered from control animals and V is the average number of worms recovered from vaccinated animals.

Animal handling protocol was approved by the Instituto Butantan's ethics committee (São Paulo, Brazil).

Animal bleeding and vaginal wash

Animals had their bloods collected by the retro-orbital plexus/sinus with a glass Pasteur pipette. Three bleedings were made, being the first one performed 7 days after the last dose; the second one performed at the moment of the challenge and the last bleeding was performed just before the perfusion. A vaginal wash, with 50 μ L 0.9% saline solution, was performed one week prior to the challenge in order to evaluate the production of secretory IgA.

ELISA

Microtiter 96-well plates were coated with 10 µg ml⁻¹ of rSm14 or rCTB in Carbonate- Bicarbonate Buffer, pH 9.6 for 16h at 4°C. After three washes with PBS-T, plates were blocked with 5% non-fat milk/ PBS-T (m/v) at 37°C for 1h. Serial dilutions of each serum in PBS-T were added to the wells and incubated for 1 h at 37°C. Bound antibodies were detected by a commercial peroxidaseconjugated anti-mouse IgG, IgG₁ or IgG_{2a} in an appropriate dilution. The addition of OPD and the following steps were performed as previously described in GM1-ELISA.

Histopathology

Livers of vaccinated animals were removed at the moment of perfusion and fixed in 4% paraformaldehyde in PBS. From each vaccinated group, three animals were selected according to their level of infection defined by the number of worms recovered (low, average and high worm burdens). Histological sections were performed using microtome at 6 µm and stained in a slide with hematoxylin–eosin (HE).

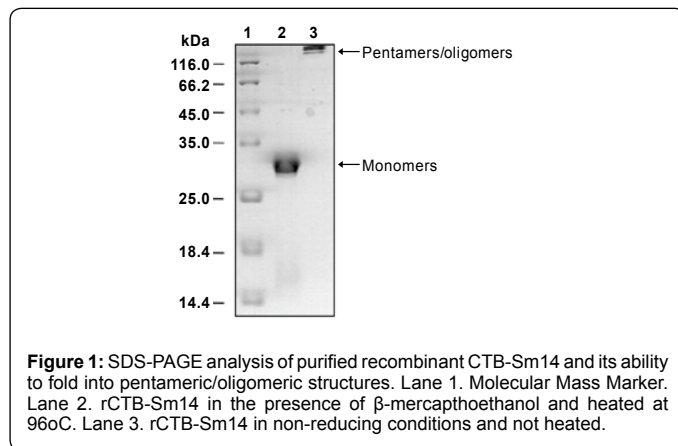


Figure 1: SDS-PAGE analysis of purified recombinant CTB-Sm14 and its ability to fold into pentameric/oligomeric structures. Lane 1. Molecular Mass Marker. Lane 2. rCTB-Sm14 in the presence of β-mercaptoethanol and heated at 96°C. Lane 3. rCTB-Sm14 in non-reducing conditions and not heated.

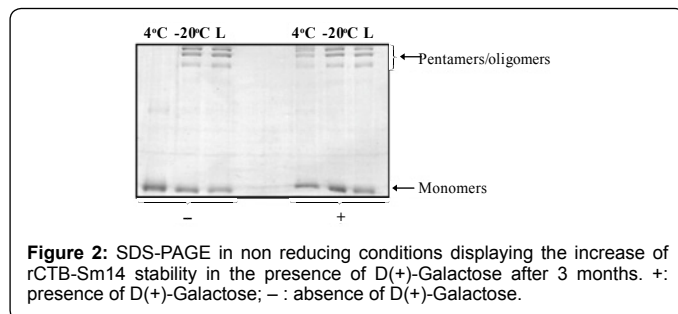


Figure 2: SDS-PAGE in non-reducing conditions displaying the increase of rCTB-Sm14 stability in the presence of D(+)-Galactose after 3 months. +: presence of D(+)-Galactose; -: absence of D(+)-Galactose.

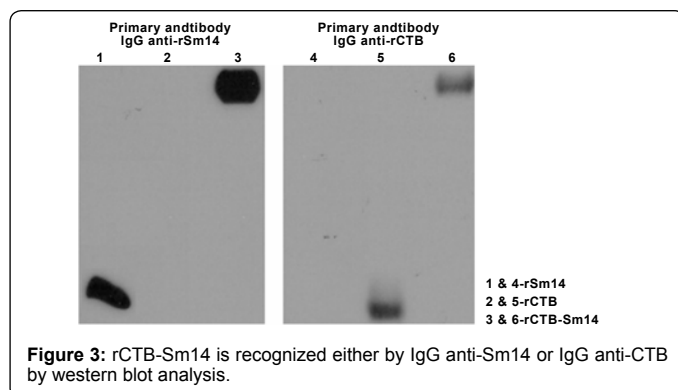


Figure 3: rCTB-Sm14 is recognized either by IgG anti-Sm14 or IgG anti-CTB by western blot analysis.

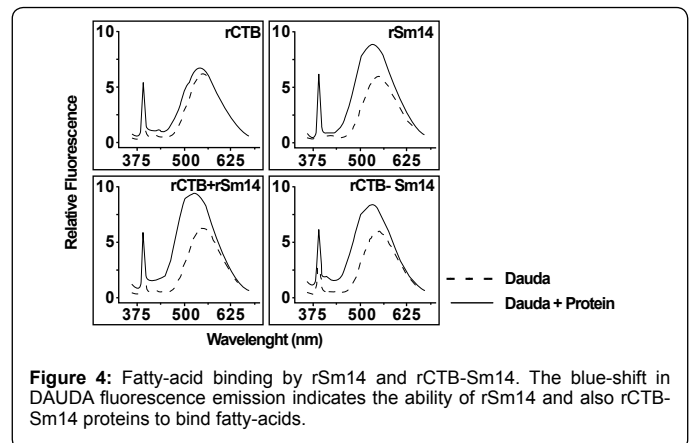


Figure 4: Fatty-acid binding by rSm14 and rCTB-Sm14. The blue-shift in DAUDA fluorescence emission indicates the ability of rSm14 and also rCTB-Sm14 proteins to bind fatty-acids.

70 granulomas with a single well-defined egg were randomly chosen and had their individual areas determined using a computer-assisted image analysis device (AxioVision Rel. 4.6, Carl Zeiss, Inc.)

Statistical analysis

Statistical analysis was performed with non-paired Student's t-test, with results with statistical significance when $p < 0.05$.

Results

Expression and purification of recombinant CTB-Sm14 fusion protein

Twenty five milligrams of purified protein per liter of induced culture were obtained and SDSPAGE analysis of eluted fractions from immobilized metal ion affinity chromatography allowed the observation of a single band of 30 kDa corresponding to the rCTB-Sm14 monomers under reduced conditions. In non-reduced and non-boiled samples, we can observe that the proteins were able to achieve quaternary pentameric/oligomeric structures (Figure 1).

Stability improvement of rCTB-Sm14 by D(+)-galactose

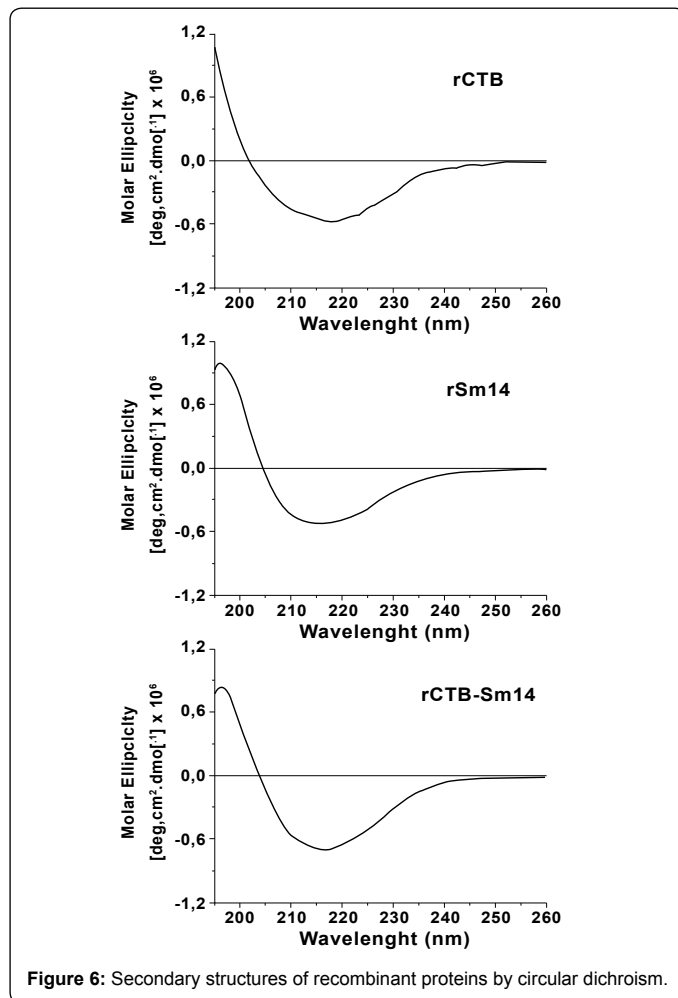
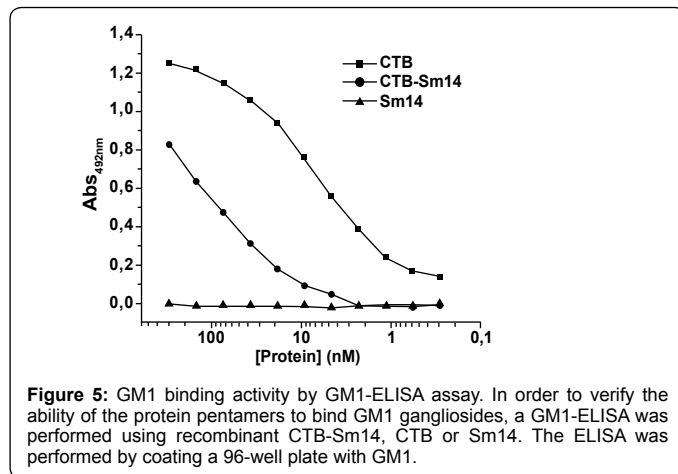
It is known that the GM1-ganglioside has on its structure two galactose residues and one Nacetyl- galactosamine residue. The terminal galactose residue displays an important function in CTB binding as determined by crystallographic studies [19]. Based on the fact that proteins should become more stable when its ligand is present, we decided to check whether the presence of galactose on CTB-Sm14 solution would improve protein stability. Indeed, this carbohydrate increased significantly the recombinant protein stability (Figure 2). Non-reducing SDS-PAGE analysis showed that the proteins, when stored in the presence of galactose, even after 3 months at 4°C, conserved their pentameric/oligomeric structure, in contrast to a sample without galactose. On the other hand, no visible difference could be observed between samples stored at -20°C and/or lyophilized. For these reasons we kept all the CTB-Sm14 at -20°C in the presence of 100 mM galactose.

The rCTB-Sm14 protein is recognized either by anti-Sm14 or anti-CTB antibodies

Western blot analysis clearly demonstrates that the recombinant fusion protein is detected by both anti-Sm14 and anti-CTB IgGs, indicating the presence of these two distinguishable fragments in a same protein (Figure 3).

Fatty-acid binding ability of Sm14 recombinant proteins

To ensure the functionality of the fatty-acid binding portion of



the rCTB-Sm14 fusion protein, we used the fluorescent fatty-acid analogue DAUDA to monitor fluorescence shifts that are caused by the internalization of this probe onto a more hydrophobic milieu. The blue shift of DAUDA fluorescence maximum from 550 nm to 532-533 nm for rSm14 and rCTB-Sm14 (Figure 4) and the concomitant increase of its fluorescence emission (both indicative of the entry of DAUDA into an apolar environment) confirmed the binding of the fluorescent fatty acid not only by the rSm14 protein but also by the recombinant fusion protein (rCTB-Sm14). As expected, figure 4 also

shows that the recombinant CTB protein alone, did not significantly bind DAUDA. However a blue-shift was observed when rSm14 was added to the rCTB containing solution (rCTB + rSm14). This demonstrates that fluorescence displacement is specifically related to the rSm14 protein and that CTB does not significantly interfere with the DAUDA binding to Sm14, showing the functionality of Sm14 portion in the fusion CTB-Sm14 protein.

GM1 ganglioside binding by rCTB-Sm14

To confirm the ability of the rCTB-Sm14 pentamers/oligomers to bind to its cellular ganglioside receptor GM1, a GM1-ELISA was performed. Recombinant CTB and CTB-Sm14 were able to bind GM1 in a dose-dependent manner. However it is clear that the presence of five Sm14 molecules in the structure imposed some interference with the binding capacity of CTB to GM1 as it showed a lower affinity than rCTB itself (Figure 5). Actually, the binding potential of CTB to GM1 has already been described elsewhere to be somewhat lower when fused to other proteins [20,21].

Secondary structure analysis of the recombinant proteins

CD analysis of recombinant proteins allowed us to infer that all molecules, as expected, displayed a regular secondary structure enriched in β sheets after the purification process (Figure 6).

Vaccination experiments

Intranasal immunization was performed as an attempt to elicit both systemic and mucosal immune response in the animals. Table 1 shows the experimental groups used in the intranasal vaccination experiments. Total IgG anti-Sm14 antibodies titers from sera of intranasally vaccinated animals could only be detected when CTB was present, being the high levels of these immunoglobulins present on those animals that received the recombinant fusion protein. Likewise, detectable levels of IgA anti-Sm14 antibodies in mucosal secretion from vaccinated animals were only observed in mice that received the recombinant fusion protein (Table 2).

However, no desirable protection levels could be observed in any vaccinated group. Furthermore, only those animals that received

Group	Dose
Saline	-
CTB	4 μ g rCTB
Sm14	4 μ g rSm14
CTB + Sm14	4 μ g rCTB + 4 μ g rSm14
CTB-Sm14	8 μ g rCTB-Sm14

Table 1: Vaccination groups with respective antigens and doses administered to animals.

Group	IgG			IgA
	1st Bleeding ^a	2nd Bleeding ^a	3rd Bleeding ^b	Vaginal Wash ^a
Saline	< 20	< 20	< 20	< 4
CTB	20	< 20	< 20	< 4
Sm14	< 20	< 20	< 20	< 4
CTB + Sm14	20	80	< 20	< 4
CTB-Sm14	640	1280	80	20

^aAt the moment of the challenge

^bAt the moment of perfusion

Table 2: Total serum IgG and mucosal IgA titers on vaccinated animals.

Group	Worm Burden \pm Standard Deviation*	Protection (%)	P value
Saline	42.53 \pm 11.86	-	-
CTB	45.69 \pm 13.77	-	-
Sm14	49.18 \pm 13.00	-	-
CTB + Sm14	35.65 \pm 9.43	21.98 ^a	0.020
CTB-Sm14	36.87 \pm 5.73	19.31 ^a	0.029

*Mean of two independent experiments

^aIn comparison with Saline + CTB group

Table 3: Worm burden and protection observed on vaccinated animals.



Group	Average (x 10 ⁴) ± S.D. (x 10 ⁴) μm ² *	Reduction (%)	P value
Saline	10.32 ± 4.12	-	-
CTB	8.89 ± 3.84	-	-
Sm14	9.99 ± 3.82	-	-
CTB + Sm14	7.44 ± 2.71	16.35 ^a	0.01072
CTB-Sm14	7.60 ± 2.79	14.51 ^a	0.04194

*Mean of two independent experiments

^aIn comparison with Saline + CTB group

Table 4: Hepatic granuloma area size around trapped eggs on vaccinated animals.

rSm14 in coadministration with rCTB and those animals that received the rCTB-Sm14 fusion protein displayed a discrete level of reduction (around 20%) on worm burden (Table 3).

Histopathology

Histological analysis of hepatic tissues from animals intranasally immunized with rSm14 in coadministration with rCTB showed a reduction of 16.35% on the area of granulomatous reaction around trapped eggs. Similarly, the recombinant fusion protein reduced in 14.51% these inflammatory areas (Table 4).

Discussion

Since the very first results from mice immunized with attenuated *Schistosoma mansoni* cercariae [22], where high levels of protection were observed, the search for an anti-schistosomiasis vaccine has turned out to be a huge challenge as those results has revealed difficult to be reproduced using individual antigens, even in the presence of strong adjuvants.

The use of the fatty-acid binding protein from *S. mansoni*, dates from 1996, when it elicited 89% protection on vaccinated rabbits and 59.6% protection on Swiss mice [3]. Later on, Sm14 would be expressed in a more feasible *Escherichia coli* system for large scale production and downstream processing and results were again promising as vaccinated animals showed a reduction of 43.9% in the worm burden [23], leading this antigen to be a subject of intense studies that resulted in its molecular and biochemical characterization [5,6], in the resolution of its three dimensional structure [4,24] and in different immunization experiments that would support Sm14's efficacy as a promising vaccine antigen candidate [8,9,11,25]. As a matter of fact, this molecule is already being tested either as a veterinary or as a human vaccine against fasciolosis and schistosomiasis, respectively [26].

Concerning the immune responses against *Schistosoma mansoni* in the attenuated cercariae model, it has been shown that optimally irradiated larvae undergo a delayed migration terminating in the lungs where the parasites die. Furthermore, this persistence in the lungs is crucial for the higher levels of resistance after challenge infection [27]. In addition to that, the recruitment of lymphocytes to the lung seems to be of great importance in generating a protective immunity [28]. These data strongly suggest the importance of the immune response elicited in the lungs and, as a result, we hypothesized that a vaccine that would induce an immune response in the mucosa-associated lymphoid tissue (MALT) might elicit a better result in vaccinated animals.

The use of CTB as a mucosal adjuvant has been widely described in diverse kinds of vaccine formulations, against different microorganisms or to induce tolerance in autoimmune diseases [20,29-37] and showed positive results when administered together with Sm28GST [13,14,38,39]. Therefore, we decided to fuse CTB to Sm14 in order to evaluate its efficacy as an intranasal mucosal Sm14 based vaccine.

Different biochemical characterizations were performed on the recombinant proteins and the results clearly demonstrated that all the proteins were at least partially folded and functional. The adjuvanticity of CTB clearly induced the secretion of anti-Sm14 IgG and IgA from vaccinated animals. The levels of antibodies, however, are barely detectable, what should explain the poor protection obtained on vaccinated animals. These data led us to think about the possibility of increasing the amount of antigen, but this would not be possible as higher doses of CTB would elicit an unspecific immune response, limiting the amount of antigen to be used (unpublished results). On the other hand, it is important to emphasize that the hepatic granuloma area size on animals which received CTB, either in coadministration with or genetically fused to Sm14, was significantly lower, indicating that the use of CTB could be a way of reducing the morbidity caused by *S. mansoni*. Indeed, this was not the first time that a reduction on hepatic granuloma was observed; the chemical conjugation of CTB with *Schistosoma mansoni* Glutathione S-Transferase (Sm28GST) had already shown a reduction on these inflammatory responses [13].

In conclusion, the data presented here showed that CTB can be used as a way of reducing the morbidity of schistosomiasis by affecting the hepatic granuloma area size, as observed and described in the previous results with Sm28GST [13,14,38,39]. Unfortunately, in our conditions, Sm14 did not induce considerable levels of protection by intranasal vaccination of the animals.

Financial Support

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Butantan.

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