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The effects of immunization with recombinant Sm14 (rSm14) in reducing worm burden and mortality of mice infected with *Schistosoma mansoni*

Os efeitos da imunização com Sm14 recombinante (Sm14r) na redução da carga de vermes e na mortalidade de camundongos infectados com *Schistosoma mansoni*

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Abstract *To investigate whether mice immunization with the recombinant form of a 14.7 KDa* Schistosoma mansoni *protein (rSm14) confers protection against a* S. mansoni *lethal challenge infection, rSm14-immunized mice were challenged with different cercarial burdens. A significant protection was detected in immunized mice challenged with 100 or 1,000* S. mansoni *cercariae when compared with their controls (p< 0.004 and p< 0.01 respectively). Differently from previous report, none of the mice from the control group (not immunized and infected with 1000 cercariae) died before the 30th day post-infection. A direct correlation between the number of challenge cercariae and the precocity of mice death was found. IgM anti-rSm14 antibodies were significantly produced (p< 0.05) mainly in the groups of immunized mice infected with 500 or 1000 cercariae. IgG and IgA anti-rSm14 antibodies were not significantly detected. In Western immunoblots, all mice sera showed a specific antibody response with a 14.7 KDa antigen being reacted with particular intensity in sera from immunized mice. The results show that immunization with rSm14 reduced mice worm burden independently of the cercariae load of challenge infection. No correlation was found between serum antibodies and worm burden reduction. In relation to cercarial load and the rate and precocity of mice mortality a direct correlation was found.*

Key-words*:* Schistosoma mansoni. *recombinant Sm14. Immunization. Fatty acid binding protein.*

Resumo *A fim de investigar se a imunização de camundongos com a proteína recombinante de14,7 KDa (Sm14r) de* Schistosoma mansoni *confere proteção contra uma infecção letal por* S. mansoni*, camundongos imunizados com Sm14r foram infectados com diferentes cargas de vermes.Uma proteção significativa foi demonstrada nos camundongos imunizados e infectados com 100 ou 1.000 cercárias de S. mansoni quando comparados com os controles (p< 0,004 e p< 0,01 respectivamente). Diferentemente de resultados anteriores, nenhum camundongo do grupo controle (não imunizado e infectado com 1.000 cercárias) morreu antes do 30º dia após infecção. Uma correlação direta entre o número de cercárias e o tempo de morte dos camundongos foi detectada. Anticorpos IgM anti-Sm14r foram produzidos significativamente (p< 0,05), principalmente, nos grupos de camundongos imunizados e infectados com 500 ou 1.000 cercárias. Anticorpos IgG e IgA anti-Sm14r não foram produzidos em quantidades significativas. Os testes de immunoblots demonstraram que todos os soros de camundongos revelaram uma banda específica de aproximadamente 14,7 KDa, e com maior intensidade nos soros dos camundongos imunizados. A maior reatividade foi encontrada no soro no período da terceira imunização.Os resultados mostram que imunização com Sm14r reduz a carga de vermes independentemente da carga de cercárias da infecção. Nenhuma correlação foi encontrada entre tipos de anticorpos e redução da carga de vermes. Uma correlação direta foi encontrada em relação a carga de cercárias e a taxa e precocidade da mortalidade dos camundongos.*

Palavras-chaves: Schistosoma mansoni. *Imunização. Sm14 recombinante. Proteínas carreadoras de ácidos graxos.*

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The study of candidate antigens for a vaccine against schistosomiasis is a subject that has aroused great interest, mainly in the last two decades. Immunization with secreted and excreted antigens (SE) derived from *Schistosoma mansoni* adult worms conferred protection against lethal infection when mice were challenged with 1,000 *S. mansoni* cercariae[13]. The complete sequence of nucleotides from a Sm14 expressed in *Escherichia coli* has already been determined. This protein contains a sequence of aminoacids with significant homology to proteins of fatty acids binding proteins (FABPs) family[7]. The protective activity of recombinant Sm14 (rSm14) in immunized mice challenged with *S. mansoni* cercariae has been shown[12]. The authors reported that similar protection levels were detected in immunized mice with rSm14 in the presence or absence of Freund's complete adjuvant.

As Sm14 is one of the component antigens of SE, it would be important to evaluate whether this protein has the same prophylactic potential of SE against lethal infection by *S. mansoni*. Thus, an experimental protocol was carried out in which mice were previously immunized with the recombinant protein and challenged with different *S. mansoni* cercarial burdens, including those expected to be lethal.

MATERIAL AND METHODS

Parasite and hosts. Female Swiss mice were used for immunization with rSm14 and posterior challenge infection with *S. mansoni* cercariae (LE strain) kept routinely on *Biomphalaria glabrata* snails, at our laboratory.

Obtention and concentration of cercariae. *B. glabrata* snails infected with *S. mansoni* were put into a beaker with dechlorinated water and exposed to artificial light for 2 hours. The discharged cercariae were concentrated according to the technique previously described[8].

Infection and recovery of parasites. Mice were infected with different cercarial burdens by subcutaneous route[9]. Perfusion of mice for worm recovery was carried out as prescribed[9]. In summary, after sacrifice of the animals by cervical fracture, the viscera were exposed, the portal vein was torn and the terminal portion of the large intestine was tied up. Mice were then perfused through the thoracic aorta artery and also through the hepatic hilum.

Recombinant Sm14 (rSm14). Expression and purification of rSm14 has been carried out at the Instituto Butantan, São Paulo, Brazil. Briefly, a BL21-DE3 *Escherichia coli* colony transformed with plasmid pRSET6xHis was induced in culture medium in presence of ampicillin 100µg/ml plus 1mM Isopropyl β-D-thiogalactopyranoside (IPTG) for 3 hours at 37°C. Cell recovery was carried out by centrifugation at 5,000rpm for 15 minutes. Cells were suspended, and submitted to French pressure at 2,000 GAGE 3X. The lysate was centrifuged at 5,000rpm for 15 minutes at 4°C. The soluble cell-free extract was centrifuged at 5,000rpm for 15 minutes, filtered and used for purification of rSm14 using a 5ml Ni-Sepharose column (Armershan Pharmacia Biotech, England, U.K).

Experimental protocol. Mice were divided into seven groups of 12 animals each. Mice were immunized (footpad injection) with 3 doses of rSm14 (20µg) on days 0, 7 and 21. Forty-five days after the last immunization, the animals were infected with 100, 500 or 1,000 cercariae. Control groups received phosphate buffer saline (PBS) on the same days of the above immunization protocol, and were infected with 100, 500 or 1,000 cercariae, respectively, on the same date for infection of immunized groups (Table 1). One group of mice was immunized with rSm14 (10µg), on the same date of the other groups. Forty-five days after challenge infection, half of each group was sacrificed for worm burden evaluation. The other half was kept under observation to evaluate the kinetic of mortality in rSm14-immunized mice and respective controls bearing a heavy infection with *S. mansoni*.

Table 1 - Protective activity of rSm14 in mice previously immunized and challenged with different S. mansoni *cercarial burdens.*

Immunization Protocol	Number of cercariae	Worm recovery Mean ±SD	Protection %	Significance level
rSm14 (3 x 10µg)	100	17.6 ± 8.6	36.9	0.05
rSm14 (3 x 20µg)	100	14.1 ± 4.9	49.5	0.004
PBS (3 x)	100	27.9 ± 5.3	-	
rSm14 (3 x 20µg)	500	93.7 ± 19.8	7.8	NS*
PBS (3 x)	500	101.7 ± 18.0	-	
rSm14 (3 x 20µg)	1.000	181.0 ± 65.2	41.2	0.01
PBS (3 x)	1.000	335.0 ± 94.5	-	

*NS = Not significant

Enzyme-linked immunosorbent assay (ELISA). Mice were anesthetized with ether for blood collection, through the orbital plexus throughout the experiments, or through the brachial vein, before perfusions. Blood collected was kept on ice for 30 minutes and later for 30 minutes at room temperature. Sera were centrifuged, aliquoted and stored at -20°C until use.

Flat-bottomed microtiter plates (Maxsorp, Nunc) were coated with rSm14 (1µg/well) in carbonate buffer pH 9.6 overnight. Microtiter plates were then washed

3X in phosphate buffer saline containing 0.05% Tween 20 (PBST20) and non-specific binding sites were blocked with 2.5% non-fat milk in PBST20 for 1 hour at 37°C. Plates were washed 3X with PBST 20. Sera were diluted 1/100 in PBST20 and incubated for 1 hour at 37°C. After incubation, plates were washed 3 X with PBST20 and then a second goat anti-mouse IgG, IgM or IgA antibody conjugated with peroxidase (Sigma, St. Louis, Mo, USA) was added at dilution 1/3,000. After a new incubation at 37°C for 1 hour, the plates were once more washed 3 X, and substrate 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS - Sigma, St. Louis, Mo, USA) was added. Readings were taken using ELISA reader (BIORAD Mod. 3550) at 405nm.

Western-blotting. rSm14 was applied into 15% polyacrylamide gel containing sodium dodecylsulphate (SDS-PAGE), as described[4]. SDS-PAGE gels were electrotransferred onto nitrocellulose membrane (Armershan Pharmacia Biotech, England, UK). After transferring, membrane was stained with Ponceau's solution and then sliced into strips. Membrane strips were blocked with 5% skimmed milk in transblotting solution containing 0.05% Tween 20 (TBST20) for 2 hours. Strips were then washed 3X with TBST20 for 10 minutes. Mice sera were added at 1/100 dilution at room temperature for 1 hour. After washing in TBST20, a secondary alkaline phosphatase-labeled goat anti-mouse IgG (Promega, Madison, WI, USA) was added at dilution 1/5,000 for 1 hour at room temperature. Development was carried out by addition of p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBTBCIP - Bio-Rad Lab., CA, USA) as substrate. Reaction was interrupted with milli-q water.

Statistical analysis. Percentage of protection conferred in immunized groups was assessed according to the following formula: P = [(C-V)\ C] x 100 where C represents the mean number of parasites in control animals, V represents the mean number of parasites in vaccinated animals. Analysis of variance test with statistical level of significance set at $P < 0.05$ was used for the studied groups.

RESULTS

Protection against infection. Mice immunized with rSm14 (10 or 20µg) and infected with 100 cercariae showed 36.9% and 49.5% protection respectively in relation to the non-immunized control group. rSm14-immunized group and challenged with 500 cercariae did not show significant worm burden reduction. However, those infected with 1,000 cercariae presented 41.2% of worm burden reduction ($p<0.01$) (Table 1).

Mortality rate. The group of mice immunized with 20µg/rSm14 and challenged with 100 *S. mansoni* cercariae presented on day 66 after infection 14% of dead animals, whereas the control group reached 50% mortality (Figure 1). Immunized group and infected with 500 cercariae (which did not show significant worm burden reduction) presented, on day 59 after infection, a mortality rate of 42.8%, whereas the control group

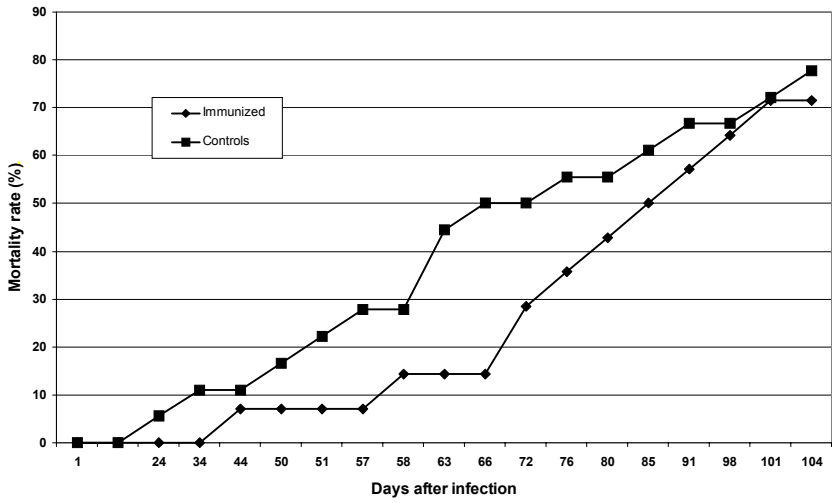

Figure 1 - Mortality rate of mice immunized with rSm14 (20µg) and infected with 100 S. mansoni *cercariae.*

showed 76.9% (Figure 2). rSm14-immunized mice and challenged with 1000 cercariae demonstrated that 48 days after infection the mortality rate was 38% against 82% mortality for the control group (Figure 3). Statistical analysis of the mortality rate among the immunized and control groups showed no significant differences probably due to the reduced number of surviving mice along the experiment. Days that represent 50 or 90% mortality of immunized groups and respective controls showed that a direct correlation has been found between the number

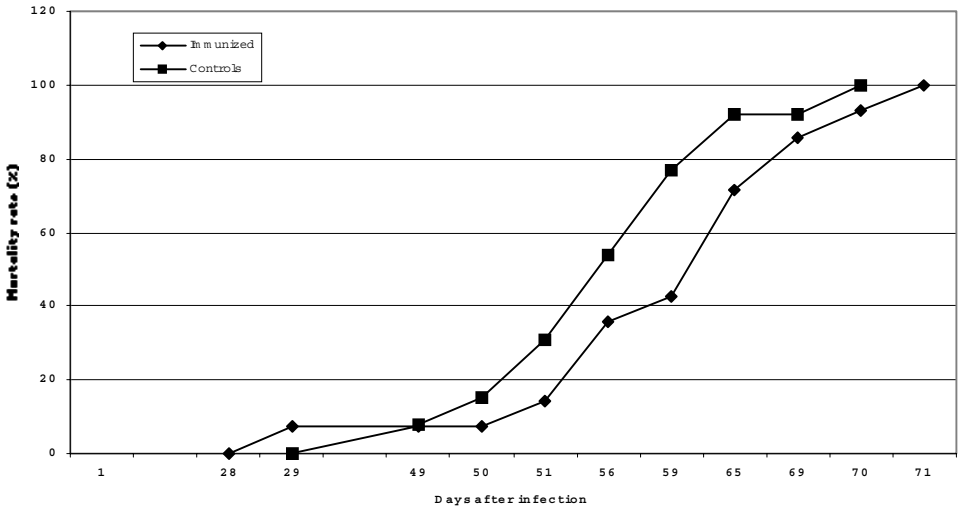

Figure 2 - Mortality rate of mice immunized with rSm14 (20µg) and infected with 500 S. mansoni *cercariae.*

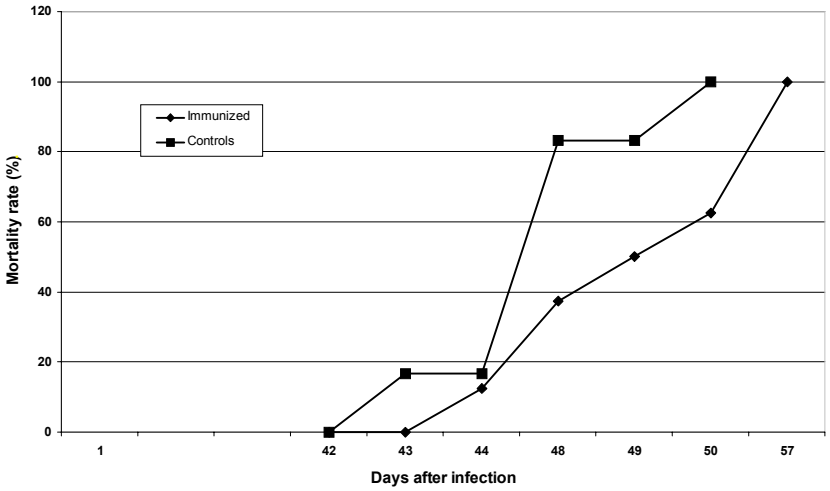

Figure 3 - Mortality rate of mice immunized with rSm14 (20µg) and infected with 1.000 S. mansoni *cercariae.*

of cercariae of the challenge infection and the precocity of the animals death. It is interesting to remark, this correlation has been found not only in the two immunized and protected groups, but also in the non-protected one (Tabela 2).

Evaluation of the immune response – ELISA: it was not detected any significant difference in IgA and IgG anti-rSm14 (20µg) antibody levels in sera from immunized mice collected 45 days after infection in relation to the control groups. There was no difference in IgG anti-rSm14 antibody levels between 10 or 20µg-immunized groups (data not shown). However, IgM anti-rSm14 antibody levels in sera from mice immunized with rSm14 (20µg), collected 45 days after infection with 500 or 1,000 cercariae, were statistically significant when compared with non-immunized mice groups (Figure 4). *Western blotting*: as can be seen in (Figura 5), immunoblotting demonstrated that sera of mice collected at the same date of the third immunization showed the highest reactivity against rSm14 (lane 7). Sera from immunized mice (rSm14 / 20µg) which were infected with 100 cercariae as well as their respective controls presented only a slight reactivity. However, sera proceeding from mice immunized with rSm14 (20µg) and

Table 2 - Effect of immunization with rSm14 on mortality time after infection with different S. mansoni *cercarial burdens.*

Mortality		Group infected with 100 cercariae	Group infected with 500 cercariae	Group infected with 1000 cercariae
M50	C	66	55	45
	I	85	60	49
M90	C	>104	64	49
	I	>104	70	57
Protection %		49.5	7.8	41.2

M50 = mortality of 50% of the animals expressed in days. M90 = mortality of 90% of the animals expressed in days. C = controls. I = immunized mice

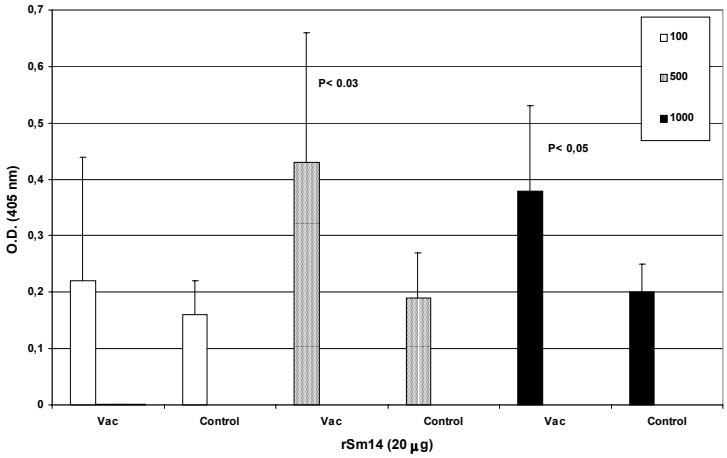

Figure 4 - IgM anti-rSm14 antibody levels in sera from immunized mice.

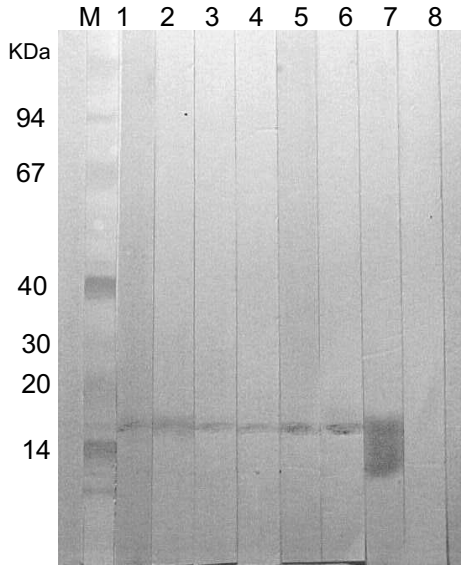

Figure 5 - Western Immunoblotting of rSm14 recognition by immune or normal mice sera.
M = Molecular weight. 1) 20µg + Inf. 100 cercariae. 2) 20µg + Inf. 500 cercariae. 3) 20µg + Inf. 1000 cercariae. 4) Inf. 100

infected with 500 cercariae (lane 5) reacted against the rSm14 antigen showing a reactive band with approximately 14 KDa. Conversely, the respective control group demonstrated light reactivity. In relation to the group infected with 1,000 cercariae, the non-immunized group (lane 6) presented higher activity when compared with the rSm14-immunized group (lane 3).

DISCUSSION

In the present report we have studied the humoral response induced in rSm14-immunized mice as well as the capacity of the recombinant protein in protecting mice from different challenge infections (100, 500 or 1,000 cercariae) including that supposed to be lethal for the animals up to 30th day after infection. On the contrary to findings previously reported in the literature[13], our results have demonstrated that *S. mansoni* infection of mice with 1,000 cercariae did not lead the animals to death up to the 30th day of infection. The diverging results between two similar infection protocols could be perhaps explained by the use of a *S. mansoni* strain, which has been maintained at the laboratory of the Centro de Pesquisas René Rachou, Belo Horizonte, Brazil, routinely, for more than 30 years. The strain used in our experiments may have had its virulence altered after maintenance for all these years in genetically different hosts from that used by other authors. This diminished virulence could develop a less drastic pathogenesis in mice. The immunization schedule used was able to attain a significant protection (about 50%) in the group of animals immunized with rSm14 (20µg) and infected with 100 or 1,000 cercariae, thus suggesting that there was no correlation between the infectious burden and worm burden reduction conferred by the immunization. For unknown reasons, the immunized group and that infected with 500 cercariae did not show significant protection when compared to the control group. As far as the mortality rate is concerned, a notable delay on M50 was detected in the immunized animal groups infected with 100, 500 or 1,000 cercariae. It is worthwhile to note that when a higher number of cercariae were inoculated the mortality rate of mice not only was more precocious, but also higher. The same trend was observed in M90 to both immunized and control group. In regard to mortality rate the results showed that in spite of the groups of immunized mice challenged with 100, 500 or 1.000 *S. mansoni* cercariae had presented on day 66 (14% of dead animals whereas the control group reached 50% of mortality), 59 (42.8%, whereas the control group showed 76.9% mortality) and 48 (38% against 82% mortality) respectively, statistical analysis of the mortality rate among the immunized and control groups showed significant differences probably due to the reduced number of surviving mice during the experiment. ANOVA simulation tests using groups formed by 15 mice demonstrated significant differences among immunized and infected groups and their respective controls.

Previous studies have demonstrated the role of the host's humoral response in the elimination mechanisms of *S. mansoni* from mice submitted to different immunization protocols [11 5 6 2]. Immunization of mice with rSm14 did not produce significant IgG and IgA anti-rSm14 antibody levels. However, significant IgM anti-rSm14 antibody levels could be detected, mainly in the groups of immunized mice and infected with 500 or 1,000 cercariae.

Other studies using human models, have detected low but significant IgM anti-rSm14 antibody levels in human serum from individuals in different clinical forms (compensated hepatosplenic and individuals termed *normal endemic* who were stool-negative in repeated examinations but living in continuous contact with contaminated water) but IgM anti-rSm14 antibody levels were not significant in human serum from patients at intestinal, acute or decompensated hepatosplenic[1]. Immunoblotting showed that sera of mice collected at the same date of the third immunization presented the highest reactivity against rSm14. Sera from immunized mice that were infected with 100 cercariae presented a slight reactivity whereas its control group was negative. Sera from mice immunized with rSm14 (20µg) and infected with 500 cercariae reacted against rSm14 showing a reactive band. Its respective control group demonstrated a light reactivity. The lack of correlation between protection and IgG, IgM or IgA antibody levels could be due to immunization of mice with rSm14 leads rather to a cellular immune response than a humoral immune response. The study on cytokine profile to detect IFN-γ or TNF-α and lymphoproliferative assays would lead to a better understanding of the effector mechanisms involved in worm burden reduction of rSm14-immunized mice. According to some researchers, optimal vaccination against *S. mansoni* requires the induction of both humoral and cell-mediated immune mechanisms[3 14]. In summary, although it has been observed up to 50% of protection in rSm14-immunized mice challenged with 100 or 1,000 cercariae, no correlation was found between mortality rates and protection, but in all three groups immunization apparently induced a delay in mortality rates. It was not found either a correlation between protection and IgG, IgM or IgA antibody levels. However, a direct correlation was achieved between cercarial burden and the rate and precocity of mice mortality. Further studies must be carried out in order to clarify whether immunization of mice with rSm14 leads to production of IgE anti-rSm14 antibodies as well as a rSm14-derived cellular immune response in mice.

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