Original Article

Potency of detergents in enhancing Schistosoma mansoni tegumental antigens

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Abstract

Introduction: Vaccine strategies represent an essential component for the future control of schistosomiasis. This work aimed to evaluate the role of detergents for potentiating the effect of *Schistosoma mansoni* tegumental antigens (TA) against challenge infection.

Methodology: Two detergents; Triton X-100 (TX-100) and sodium dodecyl sulfate (SDS), were selected for extraction of tegumental proteins. Mice were vaccinated with two doses of each preparation (100 µg protein) at time intervals 15 days before infection. Evaluation was performed by estimating particular metabolic pathways, including the Krebs cycle (via succinate dehydrogenase); glycolysis (lactate dehydrogenase); gluconeogenesis (glucose-6-phosphatase); hydrolytic enzymes (acid phosphatase); and nucleic acid catabolic enzymes (5`-nucleotidase). Serum protein profiles, worm burden, ova counts, spleen and relative liver weights were also investigated. The work was extended to study the histopathological picture of the liver including granuloma count, and total infection area. Vaccinated post-challenged mice showed amelioration of the selected biochemical parameters and reduction in worm and ova count ($P \leq 0.0001$) as well as improvement in the histological features of the liver.

Results: Triton X-100 potentiated the protective effect of *S. mansoni* tegumental antigen and extracted effective proteins better than SDS, while TA alone recorded the lowest level of protection.

Conclusion: TX-100 will be more efficient for future studies in molecular identification of novel candidate tegumental proteins.

Key words: *Schistosoma*; detergent; enzymes; vaccine; protein; liver; histopathology

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Introduction

The major goal of research on the immune response to schistosomiasis is to develop a vaccine. Vaccines that can reduce schistosomiasis morbidity and mortality by lowering the intensity of infection or by modifying the immune response to parasite-derived antigens should be adopted for practical use even if they are not effective in complete elimination of the parasites [1].

Schistosomes are covered by a living syncytium, called the tegument, which plays an important role in nutrient uptake and immune evasion [2]. Surface proteins of schistosomes are exposed to host tissues and thus present potential candidate molecules for the development of new intervention strategies [3]. Recently, tegumental proteomic research identified many proteins that may be potential targets for diagnosis, drugs and vaccines [4]. Fallon and Doenhoff [5] observed that the efficacy of praziquantel treatment was significantly enhanced in CBA/Ca mice that had been immunized prior to *Schistosoma mansoni* infection with a crude extract of worm membrane antigens.

Detergents are chemical compounds that cause proteins to stop binding or prevent them from binding after their bonds have been broken. Because the structure of detergents is similar to the structure of cell membranes, the two can interact. The detergent can break through the protective cell membrane to enter the cell, surround the protein and pull it out from the membrane. Then the protein is denatured to form a single line, revealing its primary structure, which can be analyzed either to determine its amino acid components or for molecular identification of a new candidate protein fraction [6,7].

The aim of the present work was to evaluate the potency of two detergents, non-ionic Triton X-100 (TX-100) and ionic sodium dodecyl sulfate (SDS), in extracting *S. mansoni* tegumental proteins for protection against schistosomiasis. Three tegumental preparations were tested: tegumental antigens (TA) without detergent, TA with TX-100, and TA with SDS. The evaluation process was mediated through

the estimation of certain enzymes representing different metabolic pathways in the main host and their serum protein profiles. Diminution in worm burden, ova count, granuloma diameter and frequency were also examined. The evaluation was confirmed by a liver histopathology study.

Methodology

Chemicals

All chemicals used were of high analytical grade, products of Sigma (Saint Louis, United States), Merck (Munich, Germany) and BDH (Dorset, England).

Animals

Male Swiss albino CDI mice (20-25g) were obtained from the Theodor Bilharz Research Institute, Cairo, Egypt, and maintained on excess water and a stock commercial pellet diet (El-Kahira Company for Oil and Soap, Cairo, Egypt).

Extraction buffer

Three preparations of 0.01 M phosphate buffered saline (PBS) were prepared for tegumental antigen (TA) extraction. The first contained no detergent and is henceforth referred to as PBS-TA; the second contained 2% Triton X-100 (TX-TA); and the third preparation contained 0.02% SDS (SDS-TA).

Antigen preparation

The tegumental antigen of adult worms was prepared according to the method of Hillyer [8] with slight modifications. Worms were collected by liver perfusion of ten *S. mansoni* infected mice, washed in 0.01 M PBS, and divided into three groups. Each group was suspended in a different extract buffer at 4°C for two hours, centrifuged at 10,000 rpm for one hour at 4°C, and the supernatants were then stored at -20°C until used.

Antigen administration regimens

The protein content of each extraction was determined by the method of Bradford [9]. The antigen administration protocol was performed according to Maghraby *et al*. [10]. Each mouse was sensitized with a single subcutaneous injection of the selected antigen in a 50 µg protein dose. After 15 days, a second inoculation with the same antigen concentration was administered.

Schistosoma mansoni *post challenge*

Fifteen days after the last antigen injection, all vaccinated mice were infected with 80 cercariae of *S. mansoni* (Egyptian strain) by the tail-immersion technique [11] and observed for two months.

Experimental design

The mice were divided into five groups containing six mice each. Group 1 was comprised of normal healthy control mice, each of which received two doses of 50 µl 0.01 M PBS/week for two weeks. Group 2 was comprised of *S. mansoni* infected mice. Groups 3 to 5 were made up of *S. mansoni* infected mice pre-vaccinated with 100 µg of each antigen preparation following the same immunization schedule described above. All mice were sacrificed two months after the last injection.

Ethics

Anesthetic procedures and handling of the animals complied with the guidelines of the Ethical Committee of the Federal Legislation and National Institutes of Health Guidelines in the United States, and were approved by the Medical Ethical Committee of the National Research Centre in Egypt.

Spleen and relative liver weight

Body, liver and spleen weight (in grams) of each group were measured. Relative liver weight (%) was calculated as liver weight/body weight x 100.

Preparation of tissue homogenate

Liver tissue was homogenized in 0.9 M NaCl (1:9 w/v). The homogenate (4oC) was centrifuged at 3,000 r.p.m. for 5 minutes and the supernatant was stored at -80°C for different enzyme assays.

Preparation of serum sample

The sub-tongual vein was punctured in each animal, and blood was collected in a clean, dry test tube, left 10 minutes to clot, and then centrifuged at 3,000 rpm for serum separation. The separated serum was stored at -80oC for later analyses of serum total protein and protein profiles.

Parameter assays

Enzyme activities were evaluated using the end point assay method.

Succinate dehydrogenase: reduction of flavin adenine dinucleotide (FAD) was coupled with a reduction of tetrazolium salt as 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT), and the resulting formation of INT was measured colorimetrically at 490 nm [12].

Lactate dehydrogenase: the reduction of nucleoside derived amino acids (NAD) was coupled with the reduction of tetrazolium salt with phenazine methosulfate (PMS), serving as an intermediate election carrier, and the resulting formation of INT was measured colorimetrically at 503 nm [13].

The remaining three enzymes, glucose-6-phosphatase (G-6-Pase), acid phosphatase and 5`-nucleotidase, were measured colorimetrically as liberated inorganic phosphorus at 660 nm [14-16].

Serum protein electrophoretic separation was performed by the method of Hames and Rickwood [17] using 0.3% polyacrylamide gel and a 0.7% amido black stain. Each protein fraction was expressed as a percentage of the total area. The total area was related to the total protein concentration; therefore, each fraction was calculated and expressed as mg/ml.

Worm count

Worms were recovered by liver perfusion as described by Smithers and Terry [18]. The difference in the percentage of worms recovered from challenged mice relative to vaccinated mice was calculated by the method of Tendler *et al*. [19] as follows:

$$P = (C - V)/C * 100$$

Where, P = the % of protection, C = the mean number of parasites recovered from infected animals, and V = the mean number of parasites recovered from vaccinated animals.

Ova count

The number of ova per gram of tissue was counted by the method of Cheever *et al*. [20].

Oogram determination

All viable and dead eggs were counted microscopically in the liver and classified according to the method of Pellegrino *et al*. [21].

Histopathology

Representative slices from liver tissue were taken from the eviscerated animals and fixed in 10% formalin buffer. Paraffin-embedded sections (4 µm thick) were taken after fixation and the slides were stained using haematoxylin and eosin (H&E) following the protocol of Hirsch *et al*. [22].

Granuloma diameters and count

The granuloma count was conducted in five successive fields of view (magnification 100) from serial tissue sections more than 25 µm apart. Granuoloma diameters were measured using an ocular micrometer for the lobular granuloma with central ova.

Statistical analysis

Data was analysed by one-way analysis of variance (ANOVA) using the Costat Computer Program (CoHort Software, version 6.303, Monterey, United States). The significance level between groups was set at P ≤ 0.0001.

Results and discussion

The metabolic relationship between parasites and their hosts, and metabolic changes developed in the host as a consequence to infection or vaccination, are of great importance. Succinate and lactate dehydrogenase enzyme activities decreased significantly in *S. mansoni* infected mice by 57.7% and 31.3% respectively, while glucose-6-

Table 1. Effect of vaccination of *S. mansoni* teugmental antigens on liver enzymes of certain metabolic pathways.

Parameters	Control	Infected	Antigen preparations		
			PBS-TA	**TX100-TA**	**SDS-TA**
Succinate dehydrogenase	0.78 ± 0.03^a	0.33 ± 0.02^e (-57.7)	0.43 ± 0.02^d [12.8]	0.65 ± 0.02^b [41.0]	0.61 ± 0.02^c [35.8]
Lactate dehydrogenase	110.99 ± 2.09^a	76.29 ± 4.04^e (-31.3)	82.53 ± 2.13^d [5.6]	97.56 ± 3.15^b [19.2]	91.49 ± 1.24^c [13.7]
Glucose-6-phosphatase	57.94 ± 3.11^d	88.86 ± 1.78^a (+53.4)	71.26 ± 2.45^b [30.4]	66.48 ± 5.22^c [38.6]	67.58 ± 2.95^{bc} [36.7]
Acid phosphatase	15.25 ± 1.24^d	24.29 ± 1.78^a (+59.3)	20.59 ± 1.10^b [24.3]	17.41 ± 1.04^c [45.1]	19.58 ± 1.00^b [30.9]
5`- nucleotidase	186.14 ± 4.12^d	293.21 ± 4.42^a (+57.5)	255.63 ± 3.45^b [20.2]	195.91 ± 2.28^c [52.3]	200.56 ± 3.57^c [49.8]

Enzyme units: µmol/min/mg of protein. Data are means ± S.D. of six mice in each group. Analysis of data is carried out by one-way ANOVA. Unshared superscript letters between all groups are significant values at p ≤ 0.0001. Numbers between round brackets are percentage changes over control group. Values between square brackets are improvement percentages (i.e. mean vaccinated-mean infected/ mean control x 100).

phosphatase, acid phosphatase and 5`-nucleotidase showed significant increases by 53.4%, 59.3%, and 57.5%, respectively (Table 1). This observation is in accordance with the results of Hamed and Hetta [23], who detected the same variation in enzyme activities after *S. mansoni* infection. The same authors attributed the diminution in succinate and lactate dehydrogenases to *Schistosoma* toxins, which could have affected both mitochondrial and plasma membranes, and led to enzyme leakage. The mitochondrial changes could also have originated from the limited amount of oxygen present as a result of inflammation; hence mitochondrial oxidation, Krebs cycle intermediates, and enzyme activities are repressed [23,25]. Lactate dehydrogenase (LDH) inhibition was found to be in the direction of lactate oxidation; therefore, lactate was accumulated and glycogen depleted, which confirms the inhibition of aerobic respiration and the stimulation of anaerobic glycolysis [26].

The observed increase in glucose-6-phosphatase activity could have been a result of the adverse effect of *Schistosoma* toxins on the endoplasmic reticulum, where the enzyme is usually located, or caused by the elevation of cytosolic calcium, which can trigger the conversion of the inactive enzyme phosphorylase-b to active phosphorylase-a, consequently degrading glycogen into glucose [24,27].

All lysosomal enzymes are activated in conditions characterized by increased tissue catabolism; therefore, acid phosphatase enhancement was related to the increase in tissue catabolism as a result of the increase in metabolic worm products [24]. In addition, purine and pyrimidine nucleotides catabolism were also increased, leading to the activation of 5`-nucleotidase, which plays a major role in nucleic acid catabolism. The increased catabolism stimulated the active transport and phagocytosis processes, leading to further activation of 5`-nucleotidase through the plasma membrane where the enzyme is usually localized [23].

Vaccinated mice with different tegumental antigens showed a decrease in succinate dehydrogenase by 12.8%, 41%, and 35.8% for PBS-TA, TX100-TA and SDS-TA, respectively, while lactate dehydrogenase values increased by 5.6%, 19.2%, and 13.7%. We also observed improvement in the levels for glucose-6-phosphatase (30.4, 38.6, 36.7%), acid phosphatase (24.3, 45.1, 30.9%) and 5`-nucleotidase (20.2, 52.3, 49.8%) in vaccinated animals (Table 1). Gene repression or activation may explain the molecular basis for metabolic changes. Transcriptional regulation is an effective way of changing enzyme activity [28].

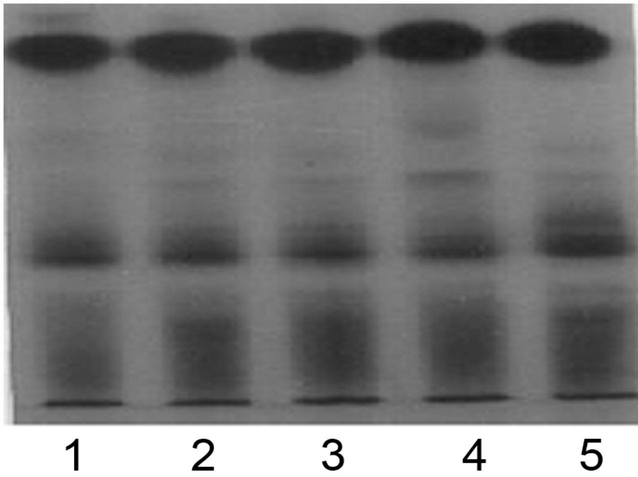
The electrophoretic protein separation patterns of infected mice revealed significant increases in total protein levels and α, β and γ globulin fractions (56.1%, 62.8%, 21.7%, and 98%), while the serum albumin level showed a significant decrease by 25.7% (Table 2 and Figure 1). These results agree with those of El Shenawy *et al*. [29] and Barros *et al*. [30], who also observed that, after *S. mansoni* infection, elevation in protein and globulin (antibody) levels are associated with infection and liver dysfunction; hence globulin elevation represents a responsive mechanism enhancing the immunity of

Table 2. Effect of vaccination of *S. mansoni* tegumental antigens on serum protein profile, liver and spleen weight.

Parameters	Control	Infected	Antigen preparations		
			PBS-TA	TX100-TA	SDS-TA
Serum protein	179.93 ± 9.03^{e}	280.87 ± 9.55^{a} (+56.1)	240.74 ± 7.91^{b} [11.2]	200.02 ± 5.49^{d} [44.9]	212.12 ± 6.55^{c} [38.2]
Albumin	140.48 ± 4.09^{a}	104.32 ± 3.04^{e} (-25.7)	114.74 ± 3.93^{d} [7.4]	134.19 ± 2.98^{b} [21.3]	124.76 ± 3.54^{c} [14.6]
α-globulin	15.31 ± 0.63^{d}	24.93 ± 0.63^{a} (+62.8)	21.65 ± 1.82^{b} [21.7]	17.47 ± 1.12^{c} [48.7]	19.23 ± 0.92^{c} [37.2]
β-globulin	20.41 ± 1.65^{bc}	24.89 ± 1.36^{a} (+21.7)	21.89 ± 1.69^{b} [14.7]	18.92 ± 1.04^{c} [29.2]	20.47 ± 0.63^{bc} [21.6]
γ-globulin	16.11 ± 0.72^{e}	31.90 ± 1.23^{a} (+98.0)	28.19 ± 1.96^{b} [23.0]	17.69 ± 1.07^{d} [88.2]	19.36 ± 1.11^{c} [77.8]
Liver weight	5.01 ± 0.62^{c}	6.00 ± 0.21^{a} (+19.8)	5.51 ± 0.38^{b} [9.8]	5.06 ± 0.26^{bc} [18.8]	5.51 ± 0.35^{b} [11.8]
Spleen weight	0.49 ± 0.03^{d}	0.74 ± 0.04^{a} (+51.0)	0.67 ± 0.02^{b} [14.3]	0.52 ± 0.03^{d} [44.9]	0.57 ± 0.02^{c} [34.7]

Data are means ± S.D. of six mice in each group. Serum protein profile units: mg/ml serum. Liver and spleen weight units: g. Analysis of data is carried out by one-way ANOVA. Unshared superscript letters between groups are significant values at $p \leq 0.0001$. Numbers between round brackets are percentage changes over control group. Values between square brackets are improvement percentages (i.e. mean vaccinated-mean infected/ mean control x 100).

Figure 1. Electrophoretic separation pattern of serum protein in normal, infected, and vaccinated mice. 1- Normal healthy serum. 2- *S. mansoni* infection. 3- Vaccination with PBS-TA. 4- TX100-TA. 5- SDS-TA.



the host. The protein profile in *S. mansoni* infected mice pre-immunized by the three selected tegumental antigens recorded improvement in all fractions, where TX100-TA showed the highest enhanced level (Table 2 and Figure 1).

Relative liver weight and spleen weight recorded significant increases in infected mice by 19.8% and 51%, respectively. The increase in the relative liver weight may be attributed to both egg deposition by worms and several metabolites released by *S. mansoni* which affect the host hepatic tissue.

Moreover, *Schistosoma* eggs are known to produce several tissue reactive substances including lipids, antigens, enzymes, reticuloendothelial cells infiltration and fibrogranulomatous tissue that lead to liver enlargement [31]. Spleen enlargement was likely caused by the increase in its matrix components, especially type IV collagen and fibrous deposition [32]. Vaccination with TX100-TA enhanced relative liver and spleen weights by 18.8% and 44.9%, respectively (Table 2).

Chronic morbidity during infection with *S. mansoni* develops as a result of schistosome worms and eggs lodging in the liver and causing granulomatous inflammation that ends with fibrosis [33]. This result is in supported by the worm and ova counts observed in *S. mansoni* infected mice (Table 3). All selected vaccines showed a reduction in worm burden, as well as in total live and dead ova by variable degrees; however, TX100-TA recorded a higher level of protection than the other preparations (Table 3). The same observations were noted in the determination of granuloma diameter, count, and total infection area, where TX100-TA recorded diminution by 57.9%, 56.3% and 80.9%, respectively. It was observed that there was no relationship between egg count and worm pairs, which may be due to the death of some worms or their loss from the portal system; hence the number of eggs found might include eggs laid by worms not recovered by perfusion. Therefore, egg count is not dependent upon worm burden but upon worm

Figure 2. Haematoxylin and eosin (H&E) stained liver sections of control, infected and vaccinated mice. Normal healthy liver (A), *S. mansoni* infection (B), vaccination with PBS-TA (C), TX100-TA (D) and SDS-TA (E). Magnification 200 x.

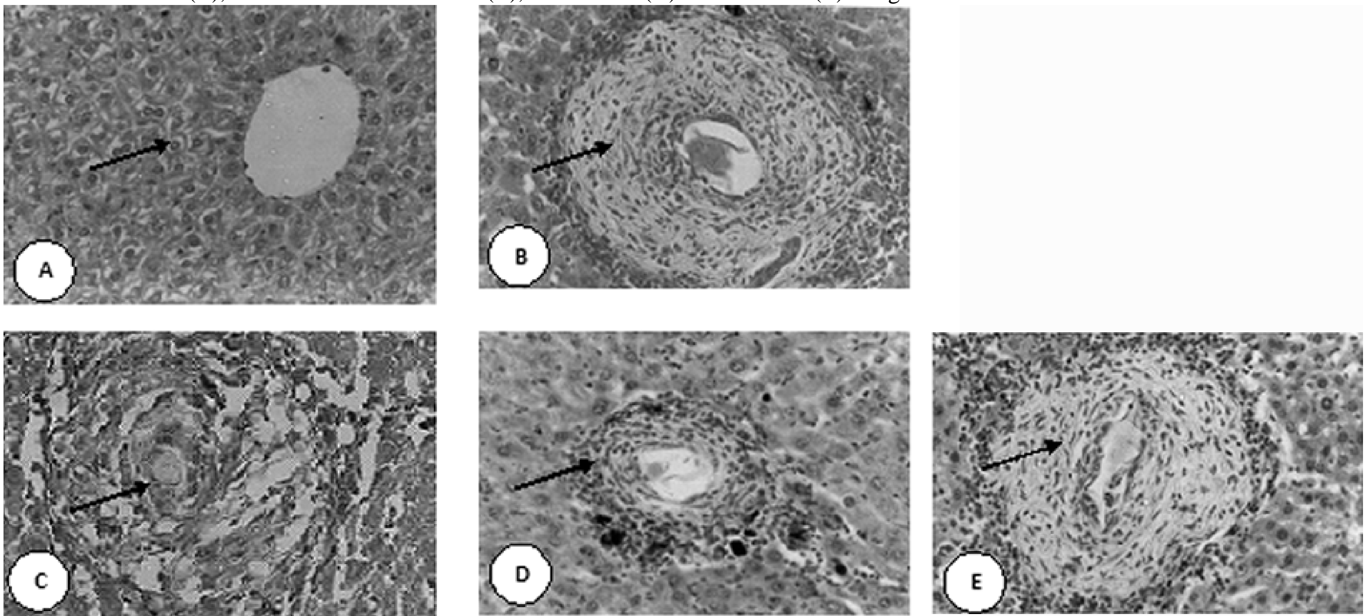


Table 3. Effect of vaccination of *S. mansoni* teugmental antigens on worm burden, ova count, granuloma count and diameter.

Parameters	Infected	Antigen preparations		
		PBS-TA	TX100-TA	SDS-TA
Total worm	26.50 ± 1.87[a]	16.38 ± 1.47[b] (-38.2)	8.50 ± 1.05[d] (-67.2)	10.33 ± 1.03[c] (-61.0)
Living egg	33.33 ± 2.16[a]	26.50 ± 1.88[b] (-20.5)	8.67 ± 1.05[d] (-77.5)	10.50 ± 1.16[c] (-69.5)
Dead egg	45.66 ± 1.63[a]	37.50 ± 1.87 [b] (-17.9)	25.50 ± 1.78[d] (-44.2)	27.00 ± 1.41[c] (-38.7)
Total egg	78.99 ± 1.75[a]	64.00 ± 3.63[b] (-18.6)	34.17 ± 2.64[d] (-56.6)	37.50 ± 1.87[c] (-52.3)
Granuloma diameter (μm)	133.83 ± 2.63[a]	110.66 ± 5.72[b] (-17.3)	56.33 ± 2.16[d] (-57.9)	66.33 ± 2.16[c] (-50.4)
Granuloma count	12.16 ± 1.47[a]	8.83 ± 1.47[b] (-17.8)	5.32 ± 0.52[c] (-56.3)	6.22 ± 0.42[c] (-48.8)
Total infection area (μm)/LPF	1628.83 ± 202.18[a]	1002.66 ± 127.36[b] (-38.4)	310.66 ± 41.35[c] (-80.9)	416.17 ± 32.83[c] (-74.4)

Data are means ± S.D. of six mice in each group. LPF - Low power field of the microscope. Total area of infection = number of granuloma x granuloma size. Analysis of data is carried out by one-way ANOVA. Unshared superscript letters between groups are significant values at $p \leq 0.0001$. Numbers between parentheses are percentage changes over infected group

fecundity status [34].

The observed decrease in worm and egg counts attenuated the oxidative stress and reduced the reactive oxygen species (ROS) that evolved as a result of infection, which gives additional support to the improvement observed in certain biochemical parameters, as ROS played a deleterious effect on cell organelles where most enzymes are usually located [35].

The histopathological findings of normal liver showed hepatic lobules formed of cords of normal liver cells radiating from the central vein to the periphery of the lobule. The cell cords were separated by narrow blood sinusoids lined by endothelial cells (Figure 2A). The infected liver showed multiple granulomatous lesions and focal areas of necrosis. The granulomatous reaction resulted from periportal cellular infiltration around mature ova and extended toward similar lesions neighbouring the portal tract. The brownish black bilharzial pigmentation was also observed in Kupffer cells of the infected liver sections (Figure 2B). These observations were clearly marked by comparing infected and uninfected livers.

Vaccinated livers showed a noticeable degree of improvement represented by fewer granuolomas of reduced size, and minimal degenerative changes in the ova (Figures 2C, D and E), as compared with liver sections of *S. mansoni* infected mice (Figure 2B). Antigen administration, however, did not appreciably change the type of the granulomatous reaction.

In conclusion, vaccination with *Schistosoma* tegumental antigens successfully protected against challenge infections by variable degrees. This protection was observed in the reduction in worm count, egg load, granuloma size, and frequency, as well as in certain metabolic parameters that revealed the tendency of the cells to restore their physiological function and assist in the immune-mediated destruction of eggs, hence ameliorating the liver's histopathological response. Detergents played a significant role in improving the protective effect of the selected antigens, and TX-100 was more effective than the others tested. Further work is still needed in the molecular identification of proteins extracted by TX-100 for possible protection against schistosomiasis.

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