EFFECT OF REACTIVE OXYGEN TREATMENT ON THE POTENCY OF SCHISTOSOMA MANSONI WORM ANTIGENS IN INDUCTION OF RESISTANCE TO CHALLENGE INFECTION IN MICE

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SUMMARY: The present study was conducted to investigate the effect of reactive oxygen (as NaOCI) treatment on the potency of S. mansoni whole worm antigens in induction of protective resistance to challenge homologous infection.

Five antigen preparations each treated with specific reactive oxygen concentrations were used. For each antigen preparation, mice were individually immunized, twice a week for 5 weeks, with 0.5 mg protein. Two weeks post immunization, mice were individually exposed by tail immersion to a challenge single dose of 100 S. mansoni cercariae. Two months later, animals were sacrificed and the degree of resistance was evaluated using worm burden, egg count, and liver pathophysiology and histopathology as indicative criteria.

The results obtained show that, immunization of mice with untreated antigens caused 26.3% reduction of the worm burden. Immunization of mice with treated antigens furthered the degree of resistance to a maximum of 62.9% by inoculation with antigen preparation treated with 100 ppm of NaOCI. Treatment with higher concentrations of hypochlorite did further the immunogenicity of worm antigens. In view of the relevant studies, however, the increase in the immunogenicity of the worm antigens by reactive oxygen treatment could be attributed to its disruptive effect on the carbohydrate coat thereby making some antigenic epitopes more accessible to the immune response.

Key words: S. mansoni, immunization, hypochlorite, adult worm antigens.

INTRODUCTION

Antigenic preparations from *S. mansoni* adult worms have been tested for their efficacy in stimulating resistance to subsequent infection of experimental animals. Immunization of mice with adult worm extract resulted in 54% reduction of the parasite load while adult worm-immunized rabbits displayed 88% reduction and developed higher levels (91 - 100%) of cytotoxic antibodies (31,32). Actually, although these trials resulted in a marked increase in the immunogenicity of the adult whole worm preparations, complete degree of resistance was not attained.

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It is well known that, cercaria is covered with complex carbohydrate network which constitutes the glycocalyx surrounding the cercarial tegument. This carbohydrate network interferes with the development of higher degree of resistance (16,34). However, some trials have been carried out to stimulate higher levels of resistance to challenge infection in experimental animals. A range of studies on the induction of resistance to *S. mansoni* infection of mice with cercariae attenuated by irradiation has been conducted (10,13,22, 24,25,37). Substantial levels of protection against challenge infection can be produced when cercariae are attenuated by UV or gamma-irradiation (6).

Wales *et. al.* (34) and Mountford *et. al.* (25) suggested that radiation induce a pronounced modification of the complex carbohydrate network, which constitutes the glycocalyx, expressed at the surface of cercariae and schistosomula. Radiolysis, therefore, seems to induce degrading changes in carbohydrate polymers (23,28,33) that could make antigenic determinants in the glycocalyx itself more accessible to antibody binding, or may expose epitopes normally hidden in the underlying membrane. As a result, new antigenic determinants could be presented to helper T-lymphocytes, and therefore stimulating potent protective immunity (34,25).

In the view of the forgoing concept El-Shaikh *et. al.* (14) carried out a trial to investigate whether utilization of reactive oxygen would produce effects similar to that induced by irradiation in promoting the antigenicity of the parasite antigens. The results of the trial showed that utilization of reactive oxygen resulted in a significant but limited potentiation of the antigenicity of the cercarial antigens. Therefore, the present study was conducted to investigate the effect of such treatment on the antigenicity of the whole worm antigen preparation.

MATERIALS AND METHODS
Whole antigen preparations (WAP)
Adult worms were obtained from *S. mansoni*-infected mice according to the technique described by Christensen *et. al.* (8). The perfused worms were freed of extraneous host tissue and blood clots by washing them in saline solution.

Worms were then repeatedly washed with 0.01 M phosphate-buffered saline (PBS, pH: 7.4) and were divided into 6 patches; each of 30 adult worms. The first patch was saved untreated one while the other five patches were treated with different concentrations of NaOCl solution (Winlab, UK). Five concentrations of hypochlorite ranged from 10-200 ppm were chosen for treatment of the worm patches. For each patch, a whole worm homogenate was prepared by grinding the *S. mansoni* adult worms with Teflon/glass tissue homogenizer (797S B. Braun Melsungen AG, Germany) with the homogenizing tube immersed in an ice bath. The protein content of these preparations was determined colorimetrically as described by Henry (19). The homogenate was adjusted with PBS to contain 10 mg protein / ml then stored at -20°C until used.

Experimental animals and groups
Adult male albino mice *Mus musculus*, weighing between 16 and 22 g at the beginning of the experiments were used in the study. For evaluation of the effect of treatment of the whole worms antigens with reactive oxygen on their potency in induction of resistance to challenge *S. mansoni* infection, six groups each of 12 mice were used. The first group was immunized with untreated worm antigen preparation (WAP) as a control. The other five groups were immunized with hypochlorite-treated WAP. Each group was immunized with an antigen preparation treated with one of the NaOCl concentration adopted. At first, one ml of the whole worm homogenate was emulsified with an equal volume of Freund's complete adjuvant (FCA, F-5881, Sigma Immunochemicals). Each ml of the adjuvant contained 1 mg of *Mycobacterium tuberculosis*; heat killed and dried 0.85 ml paraffin and 0.15 ml mannide monooleate. The immunization schedule was performed according to methods of Hillyer (20) and Tendler *et. al.* (32). Each mouse was sensitized with an initial injection of 0.1 ml of the emulsified antigen in 5 subcutaneous sites with a total dose of 0.5 mg protein followed by 9 intraperitoneal inoculations with the same antigen concentration without adjuvant. Two doses a week for five weeks; i.e. each mouse received a total dose of 5 mg of the appropriate antigen.

Two weeks after the last inoculation, all mice were individually exposed by the body immersion method to a challenge single dose of 100 *S. mansoni* cercariae. For comparison purposes, one additional group was considered as a non-infected control group received adjuvant only. The techniques used for animal infection were as those described by Liang *et. al.* (21).

Evaluation of the degree of resistance

Two months post the challenge infection; mice of each group were individually weighed and sacrificed by decapitation. Non-heparinized samples were saved and sera were collected for serologic analysis. Serum albumin was determined according to the method described by Doumas *et. al.* (11). Livers of mice were perfused for worm recovery according to the method described by Christensen *et. al.* (8). All worms whether male or female or couple from perfusion fluid, intestine, mesentry and liver were counted. The percentage worm reduction (% protection) was calculated according to the following formula:

$$\% \text{ Protection} = \frac{(C - I)}{C} \times 100$$

where;

C : is the mean worm recovery of non-immunized group,

I : is the mean worm recovery of immunized group.

Liver was excised, and accurately weighed then selected fragments of the liver were used for oogram and histological preparations. Another part of the liver tissue was weighed and 10% homogenate was prepared for determination of total lipids (17) while 2.5% homogenate was used for determination of enzymatic activities of aspartate (AST), and alanine aminotransferases (ALT) according to the method of Reitman and Frankel (29).

Enzyme-linked immunosorbent assay (ELISA) antischistosomal antibody level

Five µg of WAP protein in 100 µl 0.06 M carbonate buffer (pH 9.6) were placed into each of polystyrene 96 well of the flat bottom microtitrate plates (greiner F ELISA). The plates were incubated overnight at room temperature. The plates were washed 3-times with PBS/T-20 (0.05% Tween-20 in 0.01 M PBS, pH: 7.2). Free sites were blocked with 200 µl/well of 0.25% bovine serum albumin (BSA fraction V, Sigma) in carbonate buffer at 37°C. Sera were diluted to 1/28 in 0.25% BSA in PBS/T and applied as 100 µl/lwell and incubated for 2 hours at 37°C. A hundred µl of anti-mouse IgG (peroxidase conjugate, Sigma) was diluted to 1/5000 in 0.25% BSA, PBS/T-20 and were incubated at 37°C for 1h. The plates were washed 5 times with PBS/T-20 to develop the reaction. Plates were incubated at room temperature in complete darkness for 30 min with 100 µl/well of peroxidase substrate (o-phenylene diamine dihydrochloride, OPD, Sigma). OPD was dissolved in 25 ml of 0.05 M phosphate-citrate buffer at pH 5 and containing 0.03% sodium perborate (Sigma). The enzyme reaction was stopped by the addition of 50 µl/well of 8 NH_2SO_4. The absorbance was measured as optical density (OD) values at 492 nm using ELISA-reader (Bio-Rad Microplate Reader Richmond, CA).

Statistical analysis

Student's 't' test was used to calculate the significance of the differences observed between mean values of experimental and control groups in each experiment at a level of significance of $p < 0.05$.

Figure 1: Worm burden of mice immunized with hypochlorite-treated worm antigen preparation and challenged with *S. mansoni* infection.
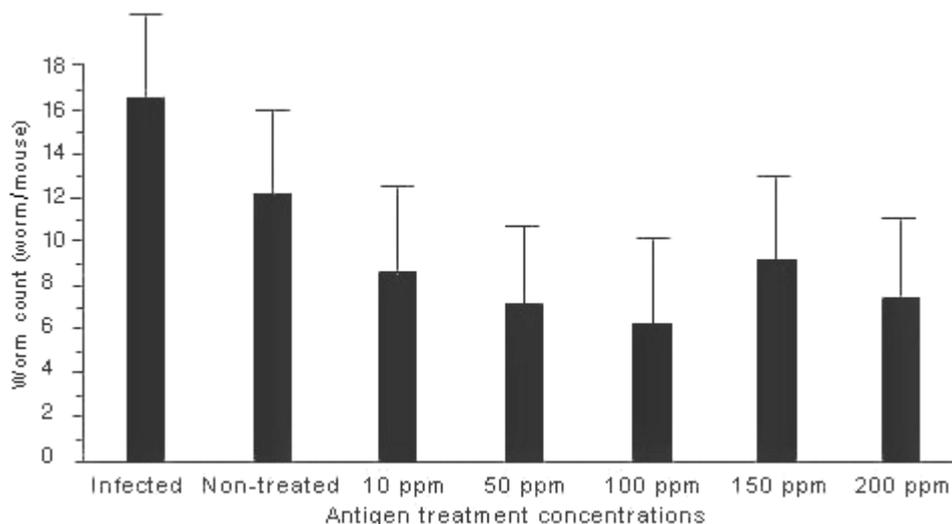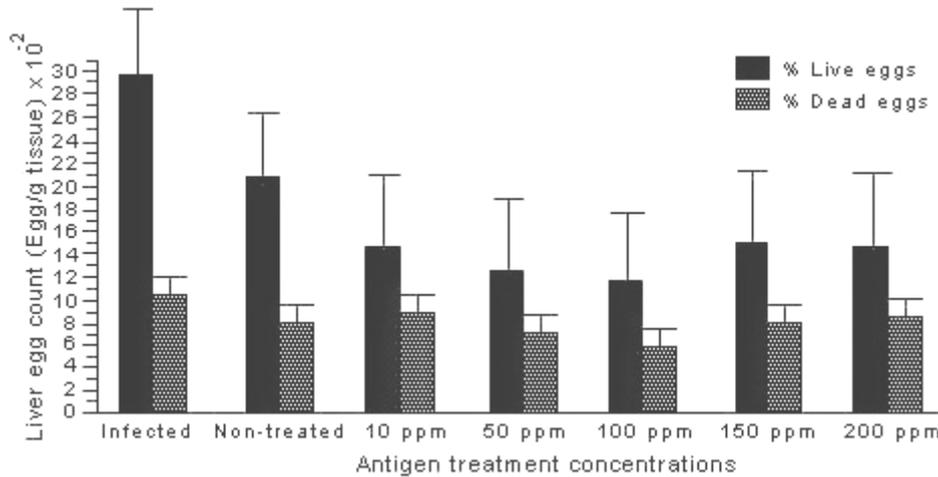
Figure 2: Liver egg load mice immunized with hypochlorite-treated worm antigen preparation and challenged with *S. mansoni* infection.

RESULTS

Effect of immunization on the worm burden and egg count

Infection of mice with *S. mansoni* cercariae (100 cercariae/mouse) resulted in a worm recovery of 16.7 ± 2.2 worm/mouse. Immunization of mice with untreated whole worm antigens and challenged with the same dose of cercariae resulted in a percentage reduction of 26.3% as compared with infected control mice. Immunization of mice with hypochlorite-treated whole worm antigens displayed further reduction in the worm burden. A maximum percentage reduction of 62.9% was obtained by immunization of mice with antigen-treated with hypochlorite at a concentration level of 100 ppm as compared with infected mice (Figure 1).

The total liver egg count of infected control mice was found to be 39.8 ± 6.9 x 10² egg per gram liver tissue, of which 74.1% were found to be live and 25.9% were dead. Immunization of mice with untreated whole worm antigens and challenged with *S. mansoni* cercariae showed a moderate decrease of 29.1% in the total liver eggs. Of that eggs load, 71.6% were found to be live and 28.4% were dead. On the other hand, immunization of mice with hypochlorite treated-whole worm antigens showed a moderate decrease in the

total liver egg load particularly in mice immunized with antigens treated with higher concentrations of hypochlorite (Figure 2).

Effect of immunization on serum anti-WAP IgG antibody level

Infection of naive normal mice with *S. mansoni* cercariae resulted in a highly marked elevation in serum anti-SAWP IgG antibody level as compared with normal uninfected mice. Immunization of mice with untreated whole worm antigens moderately increased the specific IgG antibody level by 18.0% as compared with infected control mice. Immunization of mice with hypochlorite treated-whole worm antigens displayed further increase of the specific IgG antibody level. Maximum increases of 25.8% and 28.1% were observed in mice immunized with antigens treated with 100 - 150 ppm hypochlorite as compared with mice immunized with untreated antigens (Figure 3).

**Effect of immunization on the liver function**

S. mansoni infection caused a moderate decrease of serum albumin by 21.8% as compared with normal non-infected mice. Immunization of mice with untreated whole worm antigens did not display any marked

Table 1: Liver function of mice immunized with hypochlorite treated adult worm antigens and challenged with *S.Mansoni* infection.

Animal groups	Albumin		Total lipids		ALT		AST	
(Antigen treatment concentration)	Mean ±SD	% change	Mean ±SD	% change	Mean ± SD	% change	Mean ±SD	% change
Non - immunized and non challenged (a)	31.2 ±2.96	-	15.9 ±3.2	-	94.0 ±6.6	-	95.2 ±8.9	-
Non - immunized and challenged	24.4 ± 4.9 *	- 21.8	35.5 ±10.6 *	123.3	83.5 ±5.2 *	-11.2	65.2 ±3.1 *	- 31.5
Immunized untreated and challenged (b)	24.5 ± 2.8 *	- 21.5	54.1 ±10.0 *	240.3	70.4 ±3.4 *	- 25.1	64.1 ±17.5 *	- 32.7
Immunized (10ppm) and challenged	27.0 ± 3.5	10.2	32.0 ±7.0 *	- 40.9	59.3 ±17.4	-15.8	73.3 ±26.7	14.4
Immunized (50ppm) and challenged	30.1 ±1.6 *	22.9	32.9 ±10.6 *	- 39.2	55.5 ±5.5 *	- 21.2	61.2 ±11.4	- 4.5
Immunized (100ppm) and challenged	26.3 ± 4.8	7.3	52.4 ±9.2	- 3.1	55.8 ± 4.5 *	- 20.7	53.8 ±21.9	-16.1
Immunized (150ppm) and challenged	28.6 ±3.6 *	16.7	51.5 ±7.8	- 4.8	63.2 ±5.1 *	-10.2	59.1 ±14.6	- 7.8
Immunized (200ppm) and challenged	22.5 ±1.9	- 8.2	49.8 ±9.3	- 7.9	78.5 ±2.3	11.5	56.2 ±7.9	-12.3

Mice were exposed to 100 cercariae/animal two weeks post the last antigen inoculation. Antigen total dose was 5 mg protein / mouse.* indicates level of significance, which was calculated as P<0.05. The percentage changes (%) are calculated relative to 8b) immunized untreated mice except (a) relative to normal mice. Albumin unit is expressed as g/l and for total lipids is expressed as mg lipids/g liver tissue. Enzyme activity is expressed as Reitman and Frankel U/g liver tissue for each of AST and ALT.

Figure 3: Antischistosomal IgG serum level of mice immunized with hypochlorite-treated worm antigen preparation and challenged with *S. mansoni* infection.
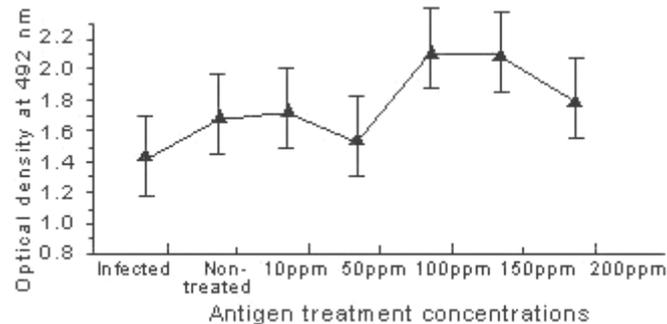
changes in serum albumin compared with infected control mice. On the other hand, immunization of mice with hypochlorite treated whole worm antigens moderately increased the serum albumin by 22.9% and 16.7% for mice immunized with antigens treated at levels of 50 ppm and 150 ppm respectively (Table 1).

The total lipids content of the liver homogenate of *S. mansoni*-infected mice showed a highly markedly increase of 123.3% as compared with normal uninfected mice. A slight increase in the total lipids of (52.4%) was observed in mice immunized with untreated whole worm antigens as compared with infected control mice. As compared with mice immunized with untreated antigens, mice immunized with hypochlorite treated antigens particularly 10-50 ppm resulted in a moderate decrease (40.9% and 39.2%) in the total lipids content of the liver (Table 1).

The enzymatic activities of AST and ALT in the liver homogenate of *S. mansoni*-infected mice showed moderate decreases of 31.5% and 11.2% respectively as compared with normal mice. Mice immunized with untreated whole worm antigens resulted in slight decreases in the activity of aspartate and alanine aminotransferases compared with infected control mice. However, immunization of mice with hypochlorite-treated whole worm antigens did not display further decrease of aspartate or alanine aminotransferase activity as compared with mice immunized with untreated antigens (Table 1).

Histopathologic investigation showed that the liver sections of *S. mansoni*-infected mice showed numerous granulomatous reactions containing viable eggs. Also, the infected liver sections showed pronounced periportal inflammatory cellular infiltration around mature eggs as well as hepatic veins (Figure 4a). The liver sections of mice immunized with untreated adult whole worm antigens slightly decrease the granuloma frequency (Figure 4b) as compared with infected liver sections. Moreover, the liver sections of mice immunized with hypochlorite-treated whole worm antigens at a concentration level of 100 ppm showed appreciable suppressive effect on the granuloma frequency (Figure 4c) as compared with infected liver sections. The ability of hypochlorite treated-antigens to lighten the severity of hepatic egg-induced granulomatous responses could be attributed to its high immunogenicity that resulted in a marked reduction in the worm burden and consequently decreases the total egg number deposited in the liver tissue.

DISCUSSION

Previous studies on experimental animals showed that immunization of mice with *S. mansoni* antigens prepared from adult worms resulted in a statistically significant degree of protection after the challenge infection (35) and animals (mice) immunized with either whole worm homogenates, freeze-thawed extract of adult worms or adult worm membrane antigens

Figure 4: Augmented granulomatous reaction size in S. mansoni-infected mice (a), moderate reaction in mice immunized with untreated worm antigen preparation (b), and diminished reaction in mice immunized with hypochlorite-treated worm antigen preparation at a concentration level of 100 ppm (c). Haematoxylin and Eosin, X 100.
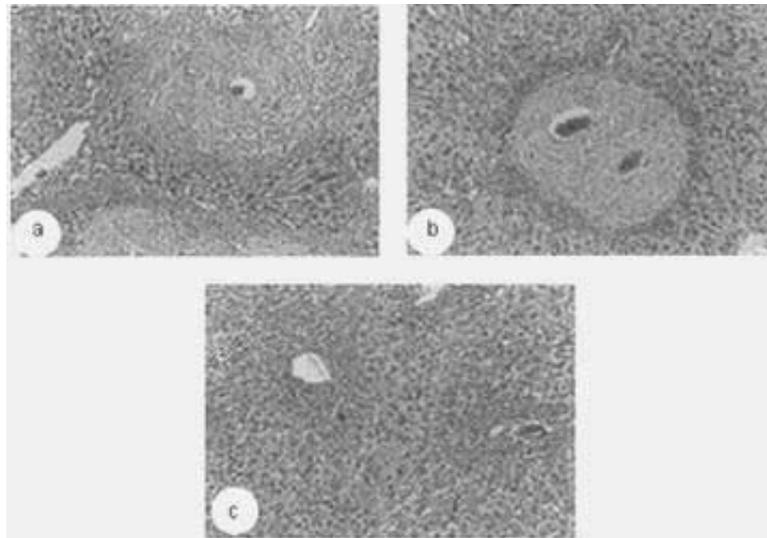
extracted with hypertonic 3M KCl produced moderate to high levels of cytotoxic antibodies. But a firm correlation between such antibody level and resistance to infection could be established (27). However, immunization of mice with adult worm extract resulted in a reduction of 54% in parasite load (31). Lower percentages were obtained in mice either vaccinated with recombinant Sm-14, 48% (30) or mice vaccinated with glutathione S-transferase, 45% immunity (7). Unexpectedly, immunization of mice with untreated whole worm homogenate caused much lower percentage of reduction (26.3%).

In the view of previous studies, the higher degree of resistance obtained from vaccination of mice with adult worm extract (31), recombinant Sm-14 (30) or glutathione S-transferase (7) may attribute the considerably higher resistance level to the fact that saline extract or purified antigens appeared to be more immunogenic than whole homogenates. It is possible that crude antigens may dissipate the antibody response or result in competition of antigens (2). Thus, immunogenic antigens, if present, may be unable to elicit effective antibody production. Therefore, the immunization of mice with whole worm antigens resulted in a less degree of resistance than previous purified antigens.

In the present study, however, showed that treatment of whole worm homogenate with NaOCl promoted their potency to induce higher level of protection. A maximum level of protection of 62.9% was obtained at a level of antigen treatment of 100 ppm compared with infected control mice comparable results were obtained by El-Shaikh *et. al.* (14) using NaOCl treated cercariae. The increase of percentage resistance due to hypochlorite treatment could be explained in the view of the study of El-Shaikh *et. al.* (14) and it may indicate that hypochlorite could induce some modification of surface antigens of the worm tegument. These modifications, therefore, seem to contribute in promoting the immunogenicity of some worm antigens. However, the decrease in the total liver egg count of mice immunized with hypochlorite treated antigens seems to be a direct response to the reduction in the number of the worm as a result of immunization.

Our results showed also that treatment of worm antigens with reactive oxygen caused quite an increase in the level of antischistosomal IgG level as compared with its level in mice immunized with untreated antigens. El-Shaikh *et. al.* (14) observed a higher level of IgG in the sera of mice immunized with cercariae treated with NaOCl. However, Dunne *et. al.* (12) using periodate as oxidizing agent for treatment of worm antigen preparations and he found that there was a correlation between the levels of antibody to carbohydrate epitopes extracted from the schistosomulum and the egg, and a correlation between antibodies to non-carbohydrate epitopes extracted from the schiotosomulum and the adult worm. They also suggested that similar surface epitopes might be recognized human and murine antibodies.

The present study showed also that, *S. mansoni* infection caused marked liver dysfunction as manifested by a decrease in serum albumin content. These findings are in parallel with those previously reported by El-Sharkawy *et. al.* (15), Goodgame *et. al.* (18), Zakaria *et. al.* (38) and Al-Okdah (3). They found that, the level of serum albumin was decreased in patients-infected with *S. mansoni* and they attributed this hypoalbuminaemia to a defect in the synthetic ability of the hepatocytes. However, although immunization of mice with untreated antigens resulted in marked decrease in the worm burden and the liver egg count, concomitant alleviation in the serum albumin level was not observed.

It is worth mentioning that total lipid of the liver was increased after *S. mansoni* infection. However, mice immunized with untreated whole worm antigens showed a further elevated total lipid content in liver homogenate of mice. But, immunization of mice with treated whole worm antigens resulted in a slighter increase of total lipids when means that the severity of the liver derangement is slighter and this could be attributed to the fewer worm burden brought about by immunization. However, patients with hepatosplenic disease had a decreased level of total serum lipids,

phospholipids and cholesterol esters (9). Malabsorption may also be contributing factor in the decrease of protein biosynthesis, through the defect in the absorption of amino acids, which are considered as the building blocks of any protein molecules. Thus a defect in protein metabolism may lead to defects in enzyme synthesis and hence derangement of many anabolic pathways including those involved in lipid biosynthesis in the liver (26).

Our data showed that, *S. mansoni* infection decreased transaminase activity in the liver tissue homogenate. The activity of AST in the liver tissue homogenate of mice immunized with either untreated or treated whole worm antigens did not induce any change while the activity of ALT was slightly decreased in mice immunized with either untreated or treated whole worm antigens. The decrease of transaminases activity has been reported in the liver tissue homogenate of *S. mansoni*- infected mice (1,4,5). Because ALT is more sensitive in both acute and obstructive liver disease while AST is more sensitive in most chronic and infiltrative lesions (36), the reduced activity of the transaminases in the liver tissue homogenate reported herein showed that transaminases recovery to the normal level was not reached. This may indicate that complete elimination of the worms in immunized mice was not reached. Histopathological findings suggested the results of immunization, where the fewer worm burden of immunized mice result in less severe pathological changes in the liver, particularly the frequency of the inflammatory reactions.

On the whole, present study showed that treatment of adult worm antigens with reactive oxygen released from hypochlorite caused significant but limited increase in their immunogenicity and that increase in the immunogenicity of the adult worm antigens by such way of oxidation may be ascribed to the disruptive effect on the carbohydrate coat that hides the antigenic epitopes, thereby making them more accessible to the immune system.

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