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Full Length Research Paper

Effect of experimental *Schistosomiasis mansoni* infection on serum levels of iron, zinc and copper in the olive baboon (*Papio anubis*)

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Schistosoma mansoniasis **is a disease of grave concern due to its high morbidity and mortality in parts of the world. This study aimed at providing insight into the pathogenesis of *S. mansoniasis* as an aid in the development of effective control methods. Iron, zinc and copper concentrations were spectrophotometrically measured in sequential serum specimens obtained from baboons throughout the course of acute *S. mansoni* infection, following curative treatment with praziquantel and following post-treatment challenge with a second cercarial infection. The initial infection resulted in a two-fold increase in copper concentrations by Day 102 post-infection. Iron concentrations fell to almost half of pre-infection concentrations by Day 123 post-infection, while those of zinc fell to a third of pre-infection concentrations by Day 81 post-infection. These changes were seen to recover several weeks following treatment, though pre-infection concentrations were never achieved. Haptoglobin, a sensitive biomarker in the acute phase response of *S. mansoni*, was also measured at all sampling points. Haptoglobin changes were in concordance with those of the cations. The findings demonstrate that iron, zinc and copper are reactants in the acute phase response of *S. mansoni* in the nonhuman primate model, *Papio anubis*. Furthermore, these reactants are modulated in challenge infections and may be important in the immunopathology of the disease.**

Key words: Acute phase response, *Schistosomiasis mansoni*, serum iron, serum zinc, serum copper.

INTRODUCTION

During the acute phase response (APR), which takes place at the very beginning of the inflammatory process, there are changes in concentrations of a large number of serum proteins and cations (Hirvonnen, 2000). These changes are attributed to the host response to tissue injury and are therefore useful indicators for the course and severity of a disease (Alsemgeest, 1994; Mikhail and Mansour, 1982).

The APR is initiated at the site of tissue injury by mononuclear cells, which release a broad spectrum of pro-inflammatory mediators including the pro-inflammatory cytokines TNF-α, IL-1, IL-6 and IFN-γ. These mediators initiate changes in the homeostatic control of the injured animal. However, measurements of cytokines are difficult as the cytokines are very transient in the blood and prone to many interfering factors (Murata et al., 2004). It is therefore more useful to measure the end-point biomarkers, the acute phase proteins, as a gauge of the inflammatory process. Haptoglobin has been demonstrated a sensitive acute phase protein in *S. mansoniasis in* the baboon (Mungatana et al., 2007) and in other hepatosplenic schistosomiasis (El-Sahly et al., 1985).

Iron, zinc and copper ions have also been shown to vary in APRs of certain parasitic diseases (Mikhail and Mansour, 1982; Mwangi et al., 1995; Dede et al., 2008). Zinc and iron concentrations decline substantially, whereas serum copper concentrations may increase (Hayes, 1994; Dede et al., 2008). These changes reflect changes in cation binding of serum proteins (Hoppe et al., 2008), and more importantly, alterations in cellular uptake mechanisms (Anderson et al., 2000; Hirvonen, 2000).

S. mansoniasis is a disease of grave concern due to its high morbidity and mortality in certain parts of the world.

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Studies that would give insight into the pathogenesis of the disease may aid in development of effective control methods. Since controlled and effective studies of the disease in humans are not possible, the baboon provides an excellent primate model for the disease (Farah et al., 2000). To the best of our knowledge, this work is the first to profile changes that occur in serum cation levels in the APR of primate infection with *S. mansoni*. Furthermore, the changes produced in the serum levels of these cations were down modulated in challenge infection suggesting that these cations may be involved in the immunopathology of *S. mansoniasis.*

MATERIALS AND METHODS

Parasites

Biomphalaria pfeifferi snails were collected from Kangundo Division, Machakos Disrict, Kenya. They were screened by exposure to strong light to ensure that they did not have any schistosomes. The snails were housed in plastic trays at temperature between 25-28°C for 12 h of light /12 h of darkness in a snail room at the Institute for Primate Research, Nairobi. They were fed on lettuce throughout the experimental period. The snails were then infected individually with 3-6 miracidia, artificially hatched from eggs of *S. mansoni* harvested from infected baboon feaces. The infected snails were maintained in the same conditions for four weeks, after which they were put in the dark until they were required for cercarial shedding. Cercariae for infection were then obtained by exposing the infected snails to artificial light (100 watt lamp) for 1-3 h.

Hosts

Ten Kenyan olive baboons, *Papio anubis,* weighing about 6.5 kg and caught in a high altitude, non-schistosomiasis-endemic region, were used for the study. The animals were handled humanely and ethically through out the experimental period under the guidance of the Institute of Primate Research's ethics committee. The animals were quarantined for 90 days, prior to initiation of experiments, during which time they were screened for common bacterial, viral and parasitic infections. They were also tuberculin tested according to the standard procedures at the Institute of Primate Research, Nairobi, Kenya. They were tested for prior schistosomiasis infection by the Kato technique (Katz et al*.,* 1972) and miracidia-hatching test (Yole et al., 1996).

As a further safeguard against the possibility of prior exposure to schistosomiasis, serum was obtained from each animal and tested for specific *S. mansoni* soluble worm antigen preparation (SWAP) IgG using enzyme linked immunosorbent assay (ELISA), as described by Nyindo et al. (1999). Only animals with antibody concentrations below 22 µg/ml $\pm$ 3 standard deviations of the value, obtained from a laboratory colony-born baboon and negative on the Kato test were used in the study. The animals were individually caged and fed on baboon pellets twice a day. They were also fed on fruits and vegetables twice a week, while water was provided *ad libitum.*

Infection schedules comprised a large cercarial dose, of about 1000 cercariae, given once as a single-dose infection. All infections were done percutaneously following anaesthetisation by the pouch method (Farah et al., 2000). Five of the baboons were infected and the remaining five were used as uninfected controls. The controls were sampled as the infected baboons.

The baboons were all sampled for blood on Day 0 (pre-infection) and on Days 27, 54, 81 and 88 post-infection. Additionally, from Day 27 post-infection, all animals were examined weekly by miracidia hatching test for presence of viable schistosome eggs in the stool. The infected animals were then all treated with a curative dose of praziquantel on Day 88 post-infection. This treatment was repeated 14 days later and was accompanied by sampling. All the five baboons were subsequently sampled on Days 123, 136, 164, 221 and 228 post-infection. On Day 228, the baboons were infected with a post-treatment challenge of 1,000 cercariae. The infection was done as previously described. The baboons were then sampled on Days 242, 256, 270, 284 and 298 post-infection.

Blood collection

The baboons were anaesthetized with ketamine/xylaxine and 10 ml of blood was collected from the inguinal vein of each animal, allowed to clot and centrifuged (Farah et al., 2000). The serum thus separated was then stored at -70°C ready for analysis.

Gross pathology

The baboons were euthanised at the end of the experiments by intravenous administration of heparinized sodium pentobarbitol. The viscera were exposed after median incision and the extent of tissue inflammation, the density of granulomas, the extent of heaptic fibrosis and size of mesenteric lymph nodes was assessed to subjectively determine the gross pathology (Farah et al., 2000).

Determination of serum cations

Iron, zinc and copper concentration determinations were spectrophotometrically determined as described by Passey et al. (1985). The serum was digested with spectrosol grade concentrated nitric acid, diluted with de-ionized water and analyzed by aspiration into an atomic absorption spectrophotometer. Iron concentrations were measured at a wavelength of 248.3 nm and a current of 8 mAmps, within a linear range of up to 5 parts per million (ppm). Zinc concentrations were measured at wavelength of 213.9 nm, current 3 mAmps, within a linear range of up to 0.4 ppm. Copper concentrations were determined at a wavelength of 324.7 nm, current 5 mAmps, within a linear range of up to 4 ppm.

Haptoglobin determination

Haptoglobin (Hp) was measured using the method described by Makimura and Suzuki (1982) with modifications by Conner et al. (1988). The assay uses purified bovine Hp as standard. This test is based on the ability of Hp to bind to haemoglobin (Hb) and retain peroxidase activity at acidic pH, whereas free Hb loses its peroxidase activity. On addition of the substrate, peroxidase activity. Resulted in a proportionate color change, which was read off on an ELISA plate reader at 450 nm.

Statistical analysis

Statistical analysis of the data was performed using SPSS and Excel software programmes. Excel was used to compare trends in the analytes measured in infected animals and in uninfected controls. The data were considered statistically significant at $p < 0.05$ as determined by one-way ANOVA.

RESULTS

Changes in the mean serum iron concentrations (±SEM) in *S.*

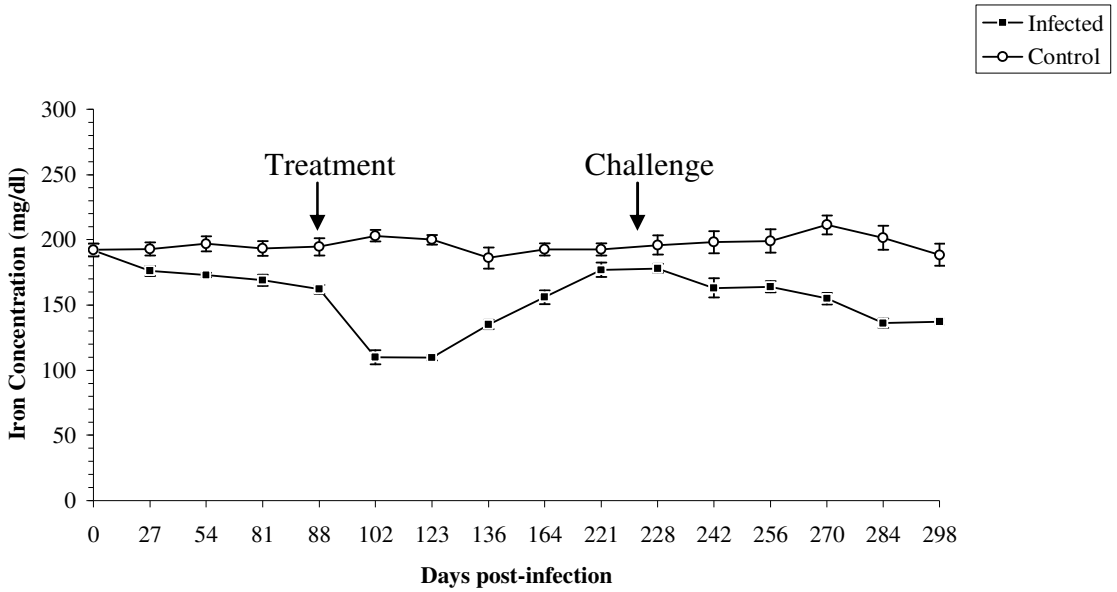

Figure 1. Mean serum iron concentrations (± S.E.M.) in µg/dl in *S. mansoni* infected baboons and in uninfected controls (n=5). Infected animals were subjected to an initial infection at Day 0, treated curatively with praziquantel at Day 88 and given a challenge infection at Day 228.

mansoni infected baboons, treated with praziquantel at Day 88 and challenged at Day 228, and their uninfected controls, are depicted in Figure 1. Following infection, mean iron concentration of the infected animals decreased gradually from pre-infection concentrations 192.0 ± 4.8 µg/dl, to reach 162.0 ±3.4 µg/dl at Day 88 post-infection, when the baboons were treated. Following treatment, the mean levels continued to decrease until Day 102, when the mean iron concentration had decreased to 109.6 ± 2.2 µg/dl. These levels were maintained until Day 123 post-infection. After this, the iron levels started to increase, reaching a peak mean concentration of 178.0 ± 3.4 µg/dl at Day 221 post-infection. At Day 228 post-infection when the baboons were challenged with *S. mansoni* cercariae, peak on concentrations were maintained. Following challenge, the serum iron levels began a gradual decrease to reach mean concentrations of 137.0 ± 2.4 µg/dl by Day 298, when the experiment was terminated. Mean serum concentrations in the infected animals showed significant differences from those of the uninfected controls at all post-infection sampling points ($p<0.05$).

Changes in the mean serum zinc concentrations (±SEM) in *S. mansoni* infected baboons, treated with praziquantel at Day 88 and challenged at Day 228, and uninfected controls, are depicted in Figure 2. Zinc concentrations of infected animals drastically fell from a mean pre-infection concentration of 230.0 ± 3.5 to 149.0 ± 3.2 µg/dl at Day 27 post-infection. The zinc levels continued to decline to reach 74.0 ± 2.7 µg/dl at Day 81 post-infection. These concentrations were maintained until Day 88 post-infection, when the baboons were treated. Following treatment, mean zinc concentrations remained at about treatment levels until Day 123 post-infection. After that, they gradually increased to182.0 ± 3.6 µg/dl by Day 228, when the animals were challenged with a second cercarial infection. Following challenge, the zinc levels showed a slight, gradual, fluctuated decrease to reach a concentration of 177.0 ± 8.4 µg/dl at Day 298 post-infection, when the experiment was terminated. Mean serum zinc concentrations of infected animals differed significantly from those of uninfected controls at all post-infection sampling points ($p<0.05$).

Changes in the mean serum copper concentrations (±SEM) in *S. mansoni* infected baboons, treated with praziquantel at Day 88 and challenged at Day 228, and uninfected controls, are depicted in Figure 3. Following infection, mean serum copper concentrations showed a gradual increase from Day 27 post-infection, with the increase taking a more drastic trend from Day 54 post-infection to reach levels of 128.0 ± 5.0 µg/dl at Day 88 post-infection, when the baboons were treated. Following treatment, the copper levels continued to increase to reach a peak of 143 ± 5.8 µg/dl at Day 102 post-infection. After this, mean copper concentrations drastically declined to reach near control levels of 83 ± 1.7 µg/dl by Day 228, when the baboons were challenged by a second cercarial dose. Following challenge, the mean copper levels started to gradually increase, reaching levels of 96.0 ± 2.8 µg/dl by Day 298 post-infection when the experiment was terminated. From Day 54 post-infection, mean copper concentrations of the infected animals were statistically different from those of the uninfected controls at $p< 0.05$, except for days 27, 228, 242, 256 and 270.

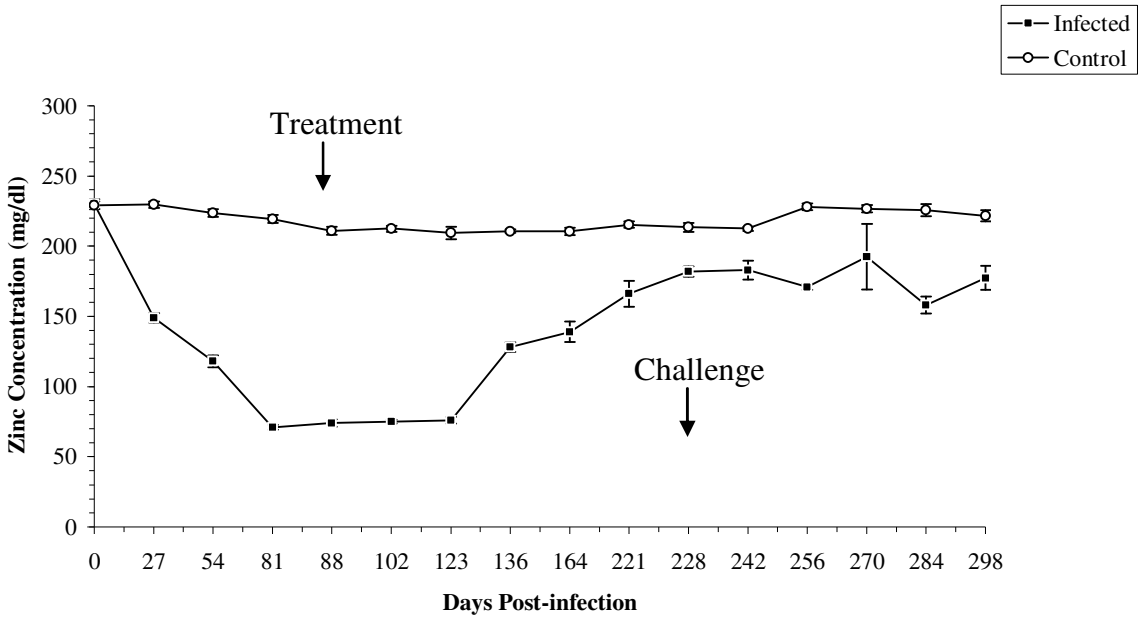

Figure 2. Mean serum zinc concentrations (± S.E.M.) in µg/dl in S. mansoni infected baboons and in uninfected controls (n=5). Infected animals were subjected to an initial infection at Day 0, treated curatively with praziquantel at Day 88 and given a challenge infection at Day 228

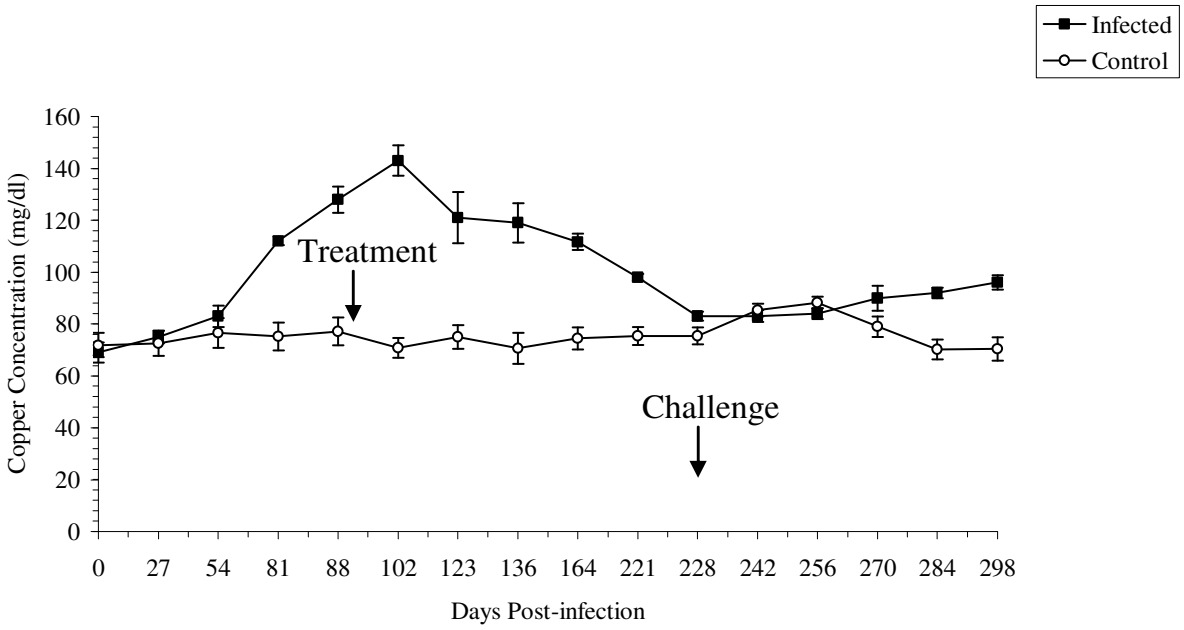

Figure 3. Mean serum copper concentrations (± S.E.M.) in µg/dl in *S. mansoni* infected baboons and in uninfected controls (n=5). Infected animals were subjected to an initial infection at Day 0, treated curatively with praziquantel at Day 88 and given a challenge infection at Day 228.

Changes in the mean serum haptoglobin concentrations (±SEM) in *S. mansoni* infected baboons, treated with praziquantel at Day 88 and challenged at Day 228, and the uninfected controls, are depicted Figure 4. The mean haptoglobin concentrations showed a four-fold increase from pre-infection concentrations of 1.68 ± 0.23 to 8.2 ± 0.81 g/l by Day 88 post-infection when treatment was instituted. Following treatment, the levels slightly increased to 8.4 g/l ± 0.86 g/l on Day 102. After this, they gradually decreased to near pre-infection levels of 2.3 ±

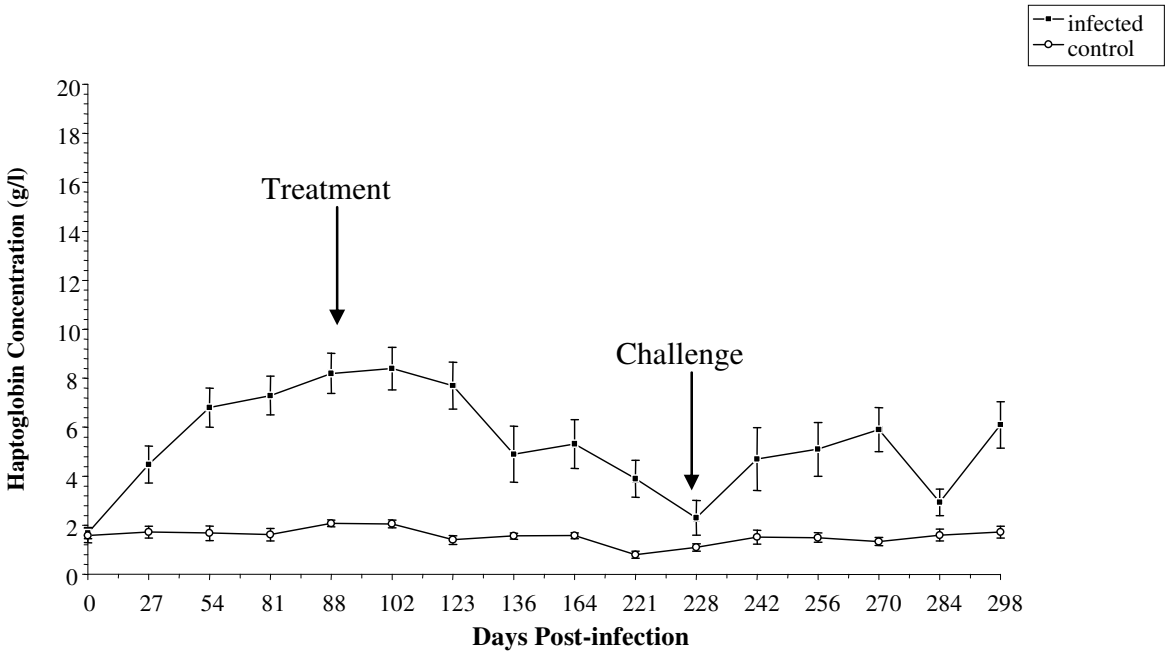

Figure 4. Mean serum haptoglobin concentrations (± S.E.M.) in g/l in *S. mansoni* infected baboons and in uninfected controls (n=5). Infected animals were subjected to an initial infection at Day 0, treated curatively with praziquantel at Day 88 and given a challenge infection at Day 228.

0.71 g/l on Day 228, when the animals were challenged with *S. mansoni* cercariae. On challenge with the parasites the haptoglobin levels showed a slow but gradual decrease to reach a concentration of 6.1 ± 0.95 g/l on Day 298, when the experiment was terminated. The haptoglobin concentrations following infection, and thereafter following challenge, were statistically different from pre-infection concentrations, until at Day 221 post-infection. Mean haptoglobin concentrations of the infected baboons were also significantly different ($P < 0.05$) from those of uninfected controls.

All infected animals tested positive for infection by the miracidia hatching assay from the sixth week post-infection. Also from the sixth week post-infection, all animals exhibited loss of appetite and passed loose stools. At termination of the experiment, the animals appeared to have lost body condition and developed ascitis, hepatomegally, splenomegally and slightly enlarged mesenteric lymph nodes. The livers of the animals demonstrated moderate density of granulomas and tissue fibrosis.

DISCUSSION

Changes in concentrations of serum cations are recognized as one of the first, and most sensitive, indicators of a metabolic change in animals as a result of parasitic infection (Beisel, 1977). In the present experiment, initial infection of the baboons resulted in decreases in serum iron and zinc concentrations, while copper concentrations were seen to increase. Following treatment and subsequent recovery of the animals, iron and zinc levels rose while those of copper fell, but pre-infection concentrations were never achieved. When the animals were challenged with a second cercarial infection, iron and zinc levels were seen to drop and copper levels increased. These changes were however more gradual and less pronounced than those following the initial infection.

The response sensitivity of iron was found to be relatively moderate with the greatest decrease of 43.2% observed on Day 102 of the experiment. These results agree with a study by Laudage and Schirp (1996), who found that schistosomiasis, was a rare cause of iron-deficiency anaemia. Moreover, Serum iron has been found to decline substantially in the APR of a number of infections (Lohuis et al., 1988a and 1988b; Mwangi et al., 1995). Decline of serum iron during the APR is a reflection of diminished brush border iron uptake and increase in iron binding proteins such as haptoglobin (Anderson et al., 2000), hepcidin (Hoppe et al., 2008), transferrin and ferritin (Sheikh et al., 2007). These proteins bind iron and preserve it, in an effort to prevent iron deficiency anaemia (Hirvonen, 2000). Indeed, upregulation of expression of genes responsible for synthesis of these iron-binding proteins has been demonstrated in acute phase response (Sheikh et al., 2007). A concurrent study by Mungatana et al. (2007) done on similarly infected baboons found evidence that haptoglobin is indeed a sensitive reactant in the APR of *S. mansoni*. Reduction of iron might be of importance in the resistance of infections which causative organisms require iron for growth (Hirvonen, 2000).

During the APR of many illnesses, there is a suddenly developing hypozincemia as a result of a sequestration of zinc within hepatocytes, thymocytes and marrow cells. Zinc sequestration then usually persists throughout the duration of the illness (Lao et al., 1987; Rhodes and Kluh, 1993). This pattern was observed in the present study when zinc dropped fairly rapidly following infection with *S. mansoni.* This drop was sustained throughout the illness before curative treatment, and a decrease as low as 69.1% was achieved on Day 81 of the experiment. The sequestration of zinc is the secondary consequence of the induced expression, caused by pro-inflammatory cytokines, of metallothionein 1 and 2 genes within these responding cells. Newly synthesized metallothionein proteins generate binding sites for zinc, and lead to the hypozincemia, which characterizes acute phase reactions (Lao et al., 1987; Rhodes and Kluh, 1993;, Taylor, 1996). In addition, zinc is a component of many enzymes, including thymulin, and other protein structures such as the zinc fingers in transcription activation factors.

Dramatic loss of zinc may therefore occur during catabolic processes, especially following severe infections. Acute zinc deficiency may then be precipitated as anabolic processes requiring zinc supervene (Vallee and Falchuk, 1993).

Copper was observed to increase up to 61% on Day 42 post- infection. In a previous similar mice experiment (Mungatana et al., 2006), copper responses were observed to be poor, with increases of only up to 16% on Day 42 post- infection. The differences in responses between mice and baboon models could be due to variations in plasma half-lives of copper between the two species. The major circulating form of copper is the blue glycoprotein, ceruloplasmin, which is synthesized in the liver. Each molecule of ceruloplasmin contains 6-8 atoms of copper. The functions of this acute phase protein are still unclear, but it is important in iron metabolism as a ferroxidase, and may have a role in regulating copper transport (Danks, 1995). It is an acute phase reactant and can increase greatly in response to infection, injury, chronic inflammatory conditions or steroid hormones (Hirvonen, 2000). Serum copper and ceruloplasmin are both increased in these circumstances as ceruloplasmin normally carries about 95% of the circulating copper (Taylor, 1996).

Depressed serum zinc and elevated serum copper levels have been demonstrated in the acute phase responses of various parasitic infections (Dede et al., 2008). A study by Mikhail and Mansour (1982) found that plasma copper in patients with active *S. mansoni* infection was significantly elevated and zinc was significantly depressed, and the degree of this correlated with the associated hepato-splenic complications of the disease. At clinical recovery, levels of copper and zinc were significantly improved in all the patients from pre-treatment values, but were within the normal range only in patients without complications. This indicated that complications associated with schistosomiasis could delay normalization of copper and zinc levels beyond the clinical cure stage. The mice study by Mungatana et al. (2006) found that some residual tissue pathology persists for some time even after curative treatment. These observations were echoed in this study when complications, including hepatomegally, splenomegally and ascites, were noted in the infected animals at perfusion. This may have hindered resolution of the acute phase response, and attainment of pre-infection concentrations of the acute phase reactants.

The pathophysiological mechanisms involved in the changes in cation concentrations are not well understood. The anorexia observed in most infected hosts could also be a contributory factor to the changes seen in serum levels of the cations. Moreover, a phenomenon termed “nutritional immunity” was proposed in which the host prevents the multiplication of bacteria in body fluids by preventing their acquisition of nutritionally important trace minerals (Weinberg, 1978). Such a mechanism has been well characterized for iron, which is tightly chelated in extracellular fluids by transferrin and lactoferrin (Weinberg, 1978; Mwangi et al., 1995). However, “nutritional immunity” involving zinc or copper has not yet been demonstrated. Adequate zinc levels are however known to be necessary for almost all manifestations of an animal’s immune response, including macrophage phagocytosis and intracellular killing, the early activation stages of antibody formation, and sequestration of zinc in the APR may then be a means of the body to conserve the vital cation. In addition, macrophage activation may also be enhanced by low serum zinc values (Taylor, 1996). Copper also is an essential trace element as copper-containing metallo-enzymes are important in iron and catecholamine metabolism, haemoglobin, elastin and collagen synthesis and free radical scavenging (Cousin, 1985; Danks, 1995).

After treatment, all the cations monitored showed positive trends back towards their normal pre-infection concentrations, in response to tissue recovery. When the baboons were challenged with a second cercarial dose 228 days after the first infection, the post-challenge serum concentrations of copper and iron attained significant differences ($p < 0.05$) from pre-infection concentrations. The post-challenge concentrations of copper, though elevated, did not significantly differ from post-infection/pre-challenge concentrations. The post-challenge changes were notably less marked than those following the initial infection. This may be explained by the fact that the liver had not fully regained its original capacity for synthesis, following fibrosis caused by the first infection, by the time of the challenge infection. Moreover, hepatic injury has been shown to be moderated by repeated exposure to *S. mansoni* cercariae (Farah et al., 2000), thus producing a moderated APR. Roth et al. (1994) also found that repeated challenge of a host results in attenuation of the release of cytokines involved in the APR, which results in downmodulation of the APR. This is an important observation as most human infections in endemic areas occurs

repeatedly.

Conclusion

The results of the study indicate that during the acute phase of *Schistosoma mansoni* infection of baboon models, there is a characteristic increase in serum copper concentrations and a decrease in serum iron and zinc concentrations. These changes were seen to reduce gradually following curative treatment. However, the concentrations did not attain normal haematological values even after curative treatment, probably because tissue trauma and inflammation had not completely ceased or due to down regulation of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IFN-γ.

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