The Effect Of Immunizing Swiss White Mice With Snail Soluble Proteins And Challenging Them With Schistosoma Mansoni

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Abstract

Schistosomiasis infects approximately 700 million people in 74 endemic countries. It causes high morbidity. Although the drug of choice, Praziquantel is effective against Schistosoma mansoni, there is a problem of rapid re-infection, high cost and risk of drug resistance. A vaccine would have a long term effect and would complement chemotherapy, but, there is no Schistosomiasis vaccine in the market, although many candidate vaccines have been developed and tested. The intermediate host of S. mansoni, the snail, has been found to share proteins with the parasite. The present study tested the effect of immunizing Swiss white mice with soluble proteins prepared from the snail intermediate host, Biomphalaria pfeiferri. DG was prepared from the digestive gland and RT from the rest of the snail body. The immunized mice were challenged with Schistosoma mansoni, and worm reduction, cytokine responses, IgG responses and gross pathology analyzed. Worm reduction in RT was 60.5% while that of DG was 43.3%. RT stimulated significantly higher responses of interferon gamma and interleukin-5 compared to DG. RT produced higher IgG responses than DG. Also, RT had more reduced pathology compared to DG. These results imply that both cellular and humoral responses were involved in the protection against S. mansoni, as shown by high production of Interferon gamma, Interleukin 5 and IgG. This resulted to reduced worm counts and reduced pathology. Although the two soluble proteins were protective, RT was a better in terms of higher worm reduction, higher cellular and humoral protective responses and least pathology.

INTRODUCTION

Schistosomiasis infects approximately 700 million people in 74 endemic countries. Those infected are 207 million, with 85% of the cases occurring in Africa (WHO, 2010). The total number of Disability Adjusted Life Years (DALY) lost to schistosomiasis is estimated at 1.532 million per year, of which 77% are in sub-Saharan Africa (Gryseels et al., 2006).

Chemotherapy remains the cornerstone of intervention but rapid re-infection demands frequent re-treatment and emphasizes the need for a more long-term approach. In addition, the drug of choice, Praziquantel, is expensive, and there is possibility of drug resistance (Ross et al., 2002, WHO, 2010).

Host immune responses are central to strategies for controlling both infection and pathology in schistosomiasis (Capron, 1992). Parasite egg productions, granuloma formation, disease severity, resistance to re-infection and drug efficacy all depend on immunological factors (McCarthy & Nutman, 1996). Therefore vaccine strategies represent an essential component of the control of this chronic debilitating disease where the deposition of millions of eggs in tissues is the main cause of pathology.

The optimism towards the development of Schistosomiasis vaccine stems from the findings that continual exposure to Schistosomiasis elicits partial immunity (Butterworth et al., 1996; James et al., 2001) and that complete, sterilizing immunity is not required since the parasite does not replicate within the human host.

Various antigens have been researched on as possible vaccine candidates for schistosomiasis. These include: the 63 kD parasite myosin, the 97 kD paramyosin, the 28 kD triose phosphate isomerase (TPI), a 23 kD integral membrane protein (Sm23), and the 26 and 28 kD glutathione-S-transferases (GSTs). By 2006, S. haematobium 28kD GST subunit vaccine (Lille Pasteur Institute) was at phase II clinical testing; S. mansoni paramyosin + TPI multiepitope (Bachem/USAID/SVDP) was at preclinical stage and also S. mansoni SM14 (Fiocruz) was at preclinical stage (WHO

2009). There is no schistosomiasis vaccine in the market.

S. mansoni intermediate host are aquatic hermaphroditic snail species of family Planorbidae. Common antigens between different species of Schistosoma and their intermediate hosts have been reported (Chacón et al., 2000; Dissous et al., 1990; Iwanaga et al., 1994; Jackson, 1976; Yoshino & Cheng, 1978;). Chacón et al., 2000 demonstrated that sera from schistosome-infected persons reacted against soluble crude Biomphalaria glabrata antigen (SBgA) by ELISA (100% of sensitivity) and that sera from mice immunized with SBgA recognized several homologous snail molecules by Western-blot.

The objective of the present study was to determine the effect of immunizing mice with soluble proteins, RT and DG prepared from Biomphalaria pfeifferi, the intermediate host of S. mansoni in Kenya. The mice were challenged with S. mansoni, and the worm counts, immunolological responses and pathology analyzed.

MATERIALS AND METHODS

Swiss white mice were divided into 2 experimental groups, DG (vaccinated with DG), RT (vaccinated with RT), and an infected control, IC. DG and RT were immunized and given two boosters of their specified proteins. All the three groups were challenged with 150 S. mansoni cercariae after the final boost. Blood was collected (wk 2, 4 & 6 post-infection, pi) and serum prepared for IgG ELISA. Lymph nodes and spleen cells were cultured (wk 2 & 4 pi) for supernatants for Cytokine ELISA. Perfusion and gross pathology was carried out at wk 6 pi. Six mice were sampled at each specified time points. The study was repeated.

HOSTS AND PARASITES

Swiss white mice used in this study, were maintained at the Rodent House, Institute of Primate Research (IPR). They were fed with nutrient pellets (Laboratory Cho from Unga Feeds® CO.) and supplemented with carrots and kale leaves. Water was supplied ad libitum. S. mansoni intermediate host, Biomphalaria pfeifferi were obtained from Kakuyuni, Kangundo and maintained at the Snail room at IPR, at 22-25°C, 12 h of light and 12 h of darkness, and fed on dried lettuce.

VACCINATION AND CHALLENGE

The digestive gland of colony bred snails was dissected out from the rest of the snail body. The two tissues were homogenized separately in a glass mortar and pestle. The homogenates were centrifuged for 1 h at 10,000 g at 4°C to obtain the soluble proteins. The concentration of the proteins was assayed using Bradford method (1976). Two groups of mice, DG receiving soluble protein from the snail digestive gland, and RT receiving soluble protein from the rest of the body, were immunized with 50 µg of the specified soluble protein in 100 µl of complete Freund's adjuvant intra-peritoneally. Each of the groups received 2 boosters, at weeks 2 and 4 of 25 µg of specified protein in 100 µl of incomplete Freund's adjuvant intraperitoneally. One week after the final vaccination, the two groups of immunized mice, DG & RT, and a control IC, were challenged abdominally with 150 S. mansoni cercariae, using the method of Smithers and Terry (1965).

PREPARATION OF ANTIGENS

0-3 hour release protein was prepared by artificially transforming cercariae (Ramalho-Pinto et al. 1974) and separating the heads from tails on a discontinuous Percoll gradient (Ladzins et al. 1982). The heads were cultured in Complete medium [RPMI 1640 (Sigma), containing 10% foetal calf serum (Gibco BRL, Germany), Gentamycine 2mM (Sigma) and 1% -mercaptoethanol] for 3 h. The schistosomules were pelleted, and the supernatant containing the proteins released during penetration obtained. Protein concentration was determined using Bradford (1976) method. Schistosoma mansoni Soluble worm antigen (SWAP) was prepared as described by Yole et al. 1996.

PREPARATION OF SERUM

At weeks 2, 4 and 6 post-challenge, blood was obtained from anaesthetized (mixture of Rompun and Ketamine in ratio of 20:1) DG, RT, IC and naïve mice by heart puncture. Serum was prepared from the blood and stored at -20°C before use in IgG Enzyme linked immunosorbent assay (ELISA).

PREPARATION OF LYMPH NODES AND SPLEEN CELLS AND CELL CULTURE

The inguinal and auxillary lymph nodes, which drain the abdominal area and the spleen, were obtained from the mice which had been bled. Lymph node cells and spleen cells were prepared as described by Yole et al. 2006. Flat-bottomed 48-well culture plates (costar®, Corning Incorporated, NY, USA) were used for culture and 6 x 105 cells were dispensed in each well. Duplicate wells were set for each regime. Negative control had only complete medium and cells. Positive control had 1g/ml of Concanavalin A, while test wells had 10g/ml of either

SWAP or 0-3 hr release protein. The total volume of culture medium per well was 400µl. The plates were incubated at 37ºC, 5% CO2 for 48 h for Con A and 72 h for the other set-ups. The supernatants were collected from each well and were stored at -20°C before use in Cytokine ELISA.

CYTOKINE ELISA FOR INTEFERON GAMMA (IFN) AND INTERLEUKIN-5 (IL-5)

MABTECH Inteferon gamma and IL-5 kits were used for the assay. Nunc-Immulon ™ (Maxisorp™ surface) ELISA plates were coated with 50 µl of 5 µg/ml solution of monoclonal anti-IFN/anti-IL-5 antibody and incubated overnight at 4°C. They were blocked by1 h incubation with 100 µl/well of 0.1% Bovine Serum Albumin (BSA). In specified wells, 50 µl/well of samples and mouse IFN/IL-5 standards were added in duplicate and plates incubated for 2 h. In each well, 50 µl of 3 µg/ml of rabbit anti-mouse IFN [Detection mAb (R4-6A2-Biotin)] or IL-5 [(Detection mAb (TRFK4-Biotin)] was added and incubated for1 h. Binding was then detected by adding 50 µl/well of Streptavidin-Horseradish peroxidase and incubating for 1 h. The incubations were at 37°C, and there were appropriate washes in between the steps. After the final wash, 50 µg/well of the substrate (Sure Blue ™ TMB) was added. The plates were incubated in the dark, at 37°C, for 30 minutes. Optical density was read at 630 nm in an ELISA microplate reader (Dynatech MRX).

SCHISTOSOME-SPECIFIC IGG ELISA

Nunc-Immuno™ plates (MaxiSorp ™ Surface) ELISA plates were coated overnight at 4°C with 50 µl of 10 µg/ml SWAP/ 0-3 hr release antigens. Non-specific binding sites were blocked by 1 h incubation with 100 µl 3% BSA. Diluted (1:200) 50 µl serum samples were dispensed into specified wells in duplicates and incubated for 1 h. IgG binding was detected by incubating for 1 h with 50 µl of 1:2000 peroxidase conjugated goat anti-mouse IgG (SIGMA Goat anti-mouse IgG peroxidase). The incubations were at 37°C, and there were appropriate washes in between the steps. In each well, 50 µl of substrate (Sure Blue ™ TMB) was added. The plates were incubated at 37°C in the dark for 30 minutes. Optical density was read at 630 nm in an ELISA microplate reader (Dynatech MRX).

PERFUSION, WORM RECOVERY, GROSS PATHOLOGY

At week 6 the abdominal cavity of DG, RT and IC was opened. The gross pathology of the liver was observed in terms of: inflammation, adhesions and presence of granulomas. Granulomas appear as raised pinheads sized foci distributed over the surface of the liver lobes. Severity of the granulomas was categorized as follows: 1-3 granulomas per lobe were considered few, 4-10, moderate and ≥ 10 severe.

The mice were perfused using the modified method of Smithers and Terry, 1965 (Yole et al. 2006). The perfusate was collected in a 20 cm glass Petri-dish. The perfusate containing the recovered worms were transferred into urine jar and topped with phosphate buffered saline (PBS). After the worms had settled, the supernatant was sucked out, and the settling procedure repeated three times. When the supernatant was clear, the worms were then placed on a Petri dish containing PBS and then counted. Any worms still left in the mesenteries/ liver were recovered by soaking the mouse in a Petri dish containing PBS for at least 30 minutes, to allow the worms to crawl out. Percentage worm recovery and reduction for each group was calculated as shown in the formulae below.

Figure 1

$$\text{Percentage worm recovery} = \frac{\text{Mean of worms from the experimental group}}{\text{Mean of worms from the IC}} \times 100$$

Figure 2

$$\text{Percentage worm reduction} = \frac{\text{Mean of worms from the IC - the mean of worms from the experimental group}}{\text{The mean of worms from the IC}} \times 100$$

RESULTS & DISCUSSION

WORM RECOVERY

The percentage worm recovery for RT group was 39.5% while that for DG group was 56.7% when perfused 6 weeks post infection. Both RT and DG had significantly lower worm burden than IC (t-test; $p < 0.05$). The difference between RT and DG was also significant ($p<0.05$) with the worm burden being lower in RT mice than DG. Worm reduction in RT was 60.5% while that of DG was 43.3%. This exceeded the World Health Organization goal of 40% protection for both proteins (WHO/TDR, 1996).

CELLULAR RESPONSES

Inteferon gamma is a THI cytokine involved in development of inflammatory foci, while Interleukin-5, a TH2 cytokine, is one of the cytokine which stimulate B cells to produce

antibodies.

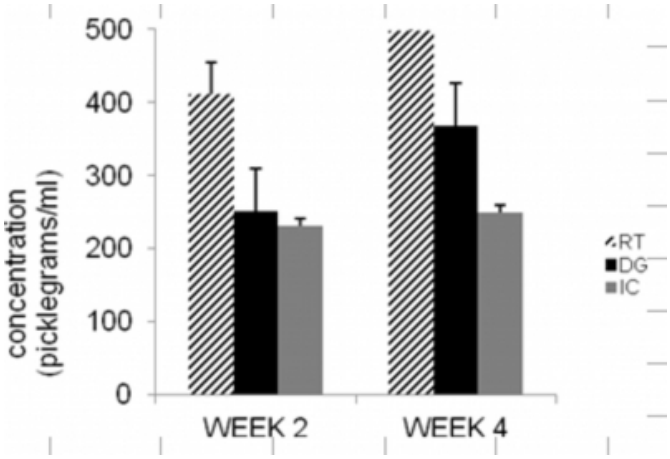
INTEFERON GAMMA RESPONSES

RT group of mice had significantly higher ($p < 0.05$) IFN gamma responses than both DG and IC at all time points for both lymph node and spleen cells and also for both antigens, SWAP and 0-3 hr release protein (Fig. 1). In most of the times, DG had higher responses than IC, for both cells and both antigens, but the difference was not always significant. This shows that RT vaccination had the stronger interferon gamma production, implying better Th1 protection against the parasite, produced by RT, compared to DG (Pearce & Sher., 1991).

Lymph node cells had a peak production of IFN gamma at week two post-challenge, which is in agreement with Pemberton et al., (1991). Spleen cells had peak responses at week four. This is expected since antigens take longer time to reach the spleen compared with the draining lymph nodes, which encounter parasite antigens presented by dendritic cells soon after the infection. The 0-3 hr release protein gave better responses than SWAP antigens. This is expected since sampling was done at early part of migration of parasites, before most worms had matured to adult worms, implying more shared antigens between 0-3 hr release protein and early stages of development of S. mansoni before maturation.

Figure 3

Fig. 1: Inteferon gamma responses in spleen cells stimulated 0-3 hr release protein



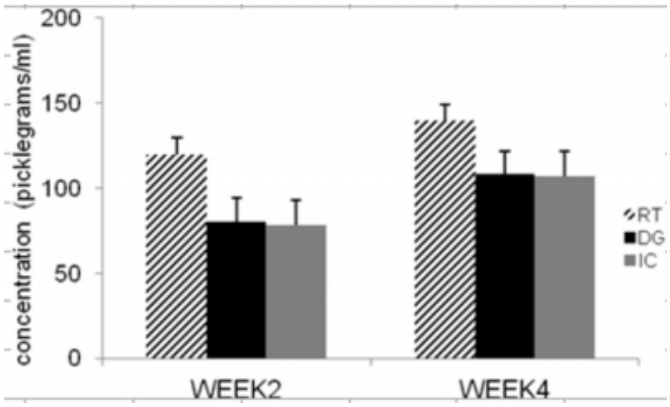
Key: RT - Mice immunized with soluble proteins derived from the rest of the body tissues of the intermediate host and then challenged; DG - Mice immunized with soluble proteins derived from the digestive gland of the Intermediate host then challenged; IC - Infected control

INTERLEUKIN-5 RESPONSES

RT mice had a significantly higher IL-5 ($p<0.05$) responses compared to DG and IC (Fig. 2) except for just one time point were the difference was not significant (LN cells, SWAP week 4). Except for responses to SWAP for the spleen cells, all other responses for RT were higher at week 2 when compared to week 4. DG and IC had similar IL-5 responses throughout the sampling period, for both organs and antigens. These results show that, in addition to IFN gamma, RT was able to stimulate production of IL-5, and hence invoke Th2 protection in Swiss mice. This phenomenon is reported in man, primates and rats (Pearce & Sher., 1991). Again, like in the case of IFN gamma, cells stimulated with 0-3 hr had higher responses than those stimulated with SWAP.

Figure 4

Fig 2: Interleukin-5 responses in mice spleen cells stimulated with 0-3 hr release protein



Key: RT - Mice immunized with soluble proteins derived from the rest of the body tissues of the intermediate host and then challenged; DG - Mice immunized with soluble proteins derived from the digestive gland of the Intermediate host then challenged; IC - Infected control

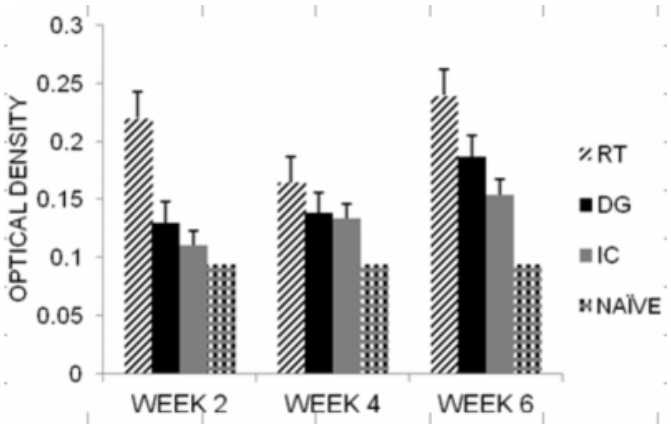
HUMORAL RESPONSES

RT had very strong IgG responses at week 2, which declined slightly at week 4, before rising again at week 6 (Fig. 3). The strong response at week 2 correlates with higher IL-5 responses at the same time point for this group. IL-5 is involved in the Th2 responses, which stimulates B cells to produce antibodies. RT had significantly higher IgG responses than both DG and IC at all sampling points ($p<0.05$). IgG responses for DG and IC showed an

increasing trend from week 2 to 6. There was no significant difference for IgG responses between DG and IC although DG responses for 0-3hr release protein were consistently slightly higher than those of IC. RT, DG and IC had significantly higher IgG responses than naïve control, which is expected. This shows that all the groups produced IgG which is involved in antibody dependent cell–mediated cytotoxicity (ADCC; Hagan et al, 1991). However, the strongest response was in RT, and this correlates very well with the greatest worm reduction recorded in this group.

Figure 5

Fig 3: 0-3 hr Schistosome specific IgG responses



Key: RT - Mice immunized with soluble proteins derived from the rest of the body tissues of the intermediate host and then challenged; DG - Mice immunized with soluble proteins derived from the digestive gland of the Intermediate host then challenged; IC - Infected control

NAIVE: Not infected

GROSS PATHOLOGY

Gross pathology was done by physical observation of the liver surface to detect inflammation; adhesions and presence of granulomas. None of the mice had granuloma. This could be attributed to a slower development of the parasite in the Swiss mice model compared to, for example, BALB/c mouse where granulomas were present at week 6 (Kanyugo et al., 2009).

Inflammation in the liver was categorized as slightly inflamed or inflamed. All the mice in RT group had slightly inflamed liver tissues. DG had 5 mice with slightly inflamed liver tissues and one mouse with inflamed liver. All the mice in IC had inflammed liver tissues. This shows that RT had the least pathology, while IC had the worst, with DG lying in between.

CONCLUSION

The results from this study showed that mice immunized with soluble proteins from the snail intermediate host, Biomphalaria pfeifferi, where protected against Schistosoma mansoni. Both cellular and humoral responses were involved in the protection, as shown by high production of Interferon gamma, Interleukin 5 and IgG. The soluble protein prepared from the rest of the snail body, RT, offered better protection than the one prepared from the digestive gland, DG. Worm reduction in RT was 60.5% while that of DG was 43.3%. RT stimulated significantly higher production of interferon gamma as compared to DG at all time points, implying that RT induced better protection related to Th1. RT IL-5 responses were significantly higher than the DG, meaning that RT had induced better Th2 protective responses. RT produced higher IgG responses than DG, implying it killed more worms using antibody-dependant cell-mediated cytotoxicity. This study shows that both cellular and humoral responses are involved in protection induced by RT in Swiss mice. It is supported by reduced worm counts and reduced gross pathology in this group. In conclusion therefore, although both soluble proteins were protective, RT was a better in terms of higher worm reduction, higher cellular and humoral protective responses and reduced pathology.

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