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Immunological Responses of Mice After Treatment with Ocimum Americanum Hexane and Bridelia Micrantha Water Plant Extracts

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Abstract :
Background*-The T helper 1 (TH1) and TH2 dichotomy was first shown in murine CD4+ lymphocytes clones and these cells could be differentiated in terms of the cytokines they secrete. The TH1 subsets produce interleukin 2 (IL-2,) interferon gamma (IFN-γ) and lymphotoxin, TH2 subsets produce IL-4, IL-5, IL-6 IL-10 and IL-13. An important function of the TH2 response during infection is to produce cytokines that can prevent or dampen the production or effector functions of potentially dangerous inflammatory mediators.* ***Results*** *The results obtained showed that Ocimum americanum hexane (OAH) and Bridelia micrantha (BMW) water extract had antischistosomal activity. This was indicated by low worm recovery, high worm reduction, and reduced gross pathology with histopathology showing no or few granulomas in the liver tissue, which was similar to Praziquantel (PZQ). The two extracts had both cellular and humoral responses as demonstrated by IFN-γ, IL-5 and IgG responses. OAH and BMW were significantly similar to PZQ; however BMW had higher IgG responses. BMW had higher IFN-γ responses for both spleen and lymph node cells.* ***Conclusion*** *this implied that treatment groups were able to produce the TH-1 response which is important for cell mediated immunity. Although both extracts induced production of IL-5 for both lymph node and spleen cells, OAH generated more IL-5.*

Keywords*: T helper cell, Cytokines, Interleukin, Interferon gamma, Humoral, Cell mediated*

I. INTRODUCTION

Schistosomiasis causes a range of morbidities, the development of which seems to be influenced to a large extent by the nature of the induced immune response and its effects on granuloma formation and associated pathologies in target organs. Granuloma formation occurs in response to chronic inflammatory stimuli. This pattern of inflammation is initiated and maintained by sensitized T helper 1 (TH1) cells **(1)**. Field studies in endemic areas, combined with animal experiments, have led to the view that host genetics, infection intensity, in utero sensitization to schistosome antigen and co infection status all influence the development of the immune response and, so, disease severity. Interestingly, the severe disease is not related to increased parasite burden, but, rather, seems to be linked to the immunological consequences of the absence of TH2 cytokines. Schistosoma infection stimulates production of antibodies; IgG1, IgG2, IgG4, IgA , IgE and IgM **(2,3)**.

II. MATERIALS AND METHODS

2.1 Plants materials extraction

Ocimum americanum (whole plant) and Bridelia micrantha (bark) were collected and placed in plastic bags. The plants were dried at room temperature for 2 months and crushed into tiny particles using Mekon Micromealer Single Phase and passed through a 0.5 mm mesh to standardize the particles. The ground plant materials were extracted using hexane then water. The plant extracts obtained were as follows; Ocimum americanum hexane extracts (OAH) and Bridelia micrantha water extract (BMW).

2.2 Parasite

Schistosoma mansoni isolate used in this study originated from infected baboons at the Institute of Primate Research (IPR), Karen, Nairobi and maintained in Biomphalaria pfeifferi snails collected from Mwea in Kirinyaga, Kenya. The snails were placed individually in each well of a 24-culture plate. Each snail was infected with 3-6 miracidia. The miracidia were left for 30 minutes to penetrate. The infected snails were placed in plastic tanks containing un-chlorinated water (snail water), sterilized sand and pebbles. Daphnea were included for aeration. The snails were fed on lettuce and maintained at the IPR Malacology laboratory. Four

weeks post infection the snails were covered with a dark cloth to prevent shedding of cercariae. Five weeks after infection, snails were placed under strong light to induce shedding of cercariae for mice infection.

2.3 Definitive host

The Swiss mice were obtained from the Kenya Medical Research Institute, Nairobi, Kenya and maintained in IPR at Rodent house at Animal Science Department and housed in cages, in groups of twenty per cage.

2.4. Infection of mice with Schistosoma mansoni

The mice to be infected with S. mansoni were anaesthetized using 0.02 ml Ketamine/Rompum mixture (ratio of 3:1) after been divided into five categories of 12 mice each. Each mouse received approximately 200 cercariae of S. mansoni through intact skin penetration by abdominal skin exposure using the ring method **(4)**. Treatment was done at week 4 post-infection with two doses two days apart. Each dose was 300 mg/kg body weight of the plant extracts. There were two groups of plant extracts; OAH and BMW. There were two control groups (PZQ, IC and naive) PZQ was treated with 900 mg/kg body weight of Praziquantel **(5)**, and the others were infected-untreated group.

2.5. Perfusion, Worm Recovery, Gross pathology

At week 6 the abdominal cavity of various mice were 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 **(9, 10)**. 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 phosphate buffered saline (PBS) and then counted. Any worm still left in the mesenteries/ liver were recovered by soaking the mouse in a Petri dish containing PBS for at least 1h, to allow the worms to crawl out. Percentage reduction for each group was calculated as shown in the formulae below.

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

2.6 Preparation of antigens

0-3 hour release protein was prepared by artificially transforming cercariae **(6)** and separating the heads from tails on a discontinuous Percoll gradient **(7)**. The heads were cultured in Complete medium [RPMI 1640 (Sigma), containing 10% foetal calf serum (Gibco BRL, Germany), Gentamycine 2mM (Sigma) and 1% β-mercaptoethanol] for 3 h. The schistosomules were pelleted, and the supernatant containing the proteins released during penetration obtained. Protein concentration was determined using Bradford **(8)** method. Schistosoma mansoni Soluble worm antigen (SWAP) was prepared from adult worms as described by **(9)**.

2.7 Preparation of serum

At weeks 2, 4 and 6 post-challenge, blood was obtained from anaesthetized mice (mixture of Rompun and Ketamine in ratio of 20:1) OAH and BMW, PZQ, 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).

2.8 Preparation of lymph nodes and spleen cells and cell culture

Spleen and mesenteric nodes, which drain the spleen and intestines, were obtained from the mice. 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, and USA) were used for culture and 6 x 10^5 cells were dispensed in each well. Duplicate wells were set for each regime. Negative control had only complete medium and cells. Positive control had 1µg/ml of Concanavalin A, while test wells had 10µg/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 37^oC, 5% CO_2 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.

2.9 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 by 1 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 for 1 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, Marxi Kinetic Microplate reader (Molecular Devices, Palo Alto, England).

2.10 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, Marxi Kinetic Microplate reader (Molecular Devices, Palo Alto, England).

III. RESULTS

3.1. Worm Reduction

There was no significant difference between Praziquantel (PZQ) (58.7) when compared with OAH (57.8%) and BMW respectively (P >0.05). OAH had closest worm reduction to PZQ. This was significantly different when IC was compared with PZQ, OAH and BMW (P< 0.05).

3.2. Gross pathology

For PZQ, OAH and BMW groups, showed no presence of/ or few granulomas while IC had moderate and severe granuloma. IC group had the most severe gross granuloma pathology, while PZQ had the least pathology.OAH was similar to PZQ with low pathology.

3.3 Histopathology

Granuloma sizes all the groups were subjected to T- test. PZQ compared with OAH and BMW groups showed no significant difference (P >0.05). When IC group was compared with plant extracts, OAH, BMW and PZQ there was significant difference (P < 0.001). The IC group had the highest number of granulomas.

3.4 Cytokines responses of mice after treatment with two plant extracts

a) IFN-γ responses to 0-3hr and SWAP antigen in Lymph nodes cells

When responses of PZQ was compared to OAH, BMW and IC with 0-3 antigen, showed no significant difference (P >0.05), however the response was higher for IC.When PZQ was compared to OAH, BMW and IC with SWAP antigen showed no significant difference (P >0.05), however the responses was higher for BMW. The response was high with CONA antigen therefore confirming the viability of the cells.

b) IFN-γ responses to 0-3hr and SWAP antigens in Spleen cells

There was no significant difference between PZQ and the two plants extract OAH and BMW (P >0.05) for 0-3 hr antigen. The same result was indicated between IC the two plants extracts. SWAP antigen responses showed no significant difference between PZQ and OAH, BMW (P >0.05). However IC and PZQ indicated significant difference (P < 0.05). The response was high with CONA antigen therefore confirming the viability of the cells.

c) IL-5 responses to 0-3hr and SWAP antigens in Lymph nodes cells

There was no significant difference between controls (PZQ and IC) and the two plant extracts (P >0.05) for 0-3 hr antigen, however the responses was low for BMW group. SWAP antigen responses showed there was significant difference between PZQ and IC (P < 0.05). On the other hand there was no significant difference between PZQ and the two plant extracts (P >0.05).High responses were recorded for CON A antigen.

d) IL-5 responses to 0-3hr and SWAP antigens in Spleen cells

IL-5 responses using 0-3hr antigen showed significant difference between PZQ and OAH, BMW and IC (P < 0.05). However there was no significant difference between IC and two plants extract OAH and BMW (P >0.05).SWAP antigen responses showed no significant difference between PZQ and the two plants extracts, OAH and BMW (P >0.05), On the other hand there was no significant difference between IC and BMW (P >0.05). High responses were recorded for CON A antigen.

IgG responses using 0-3 hr and SWAP antigens

For both 0-3hr and SWAP antigens, PZQ had the lowest IgG response which was significantly different to infected control ($P< 0.05$). However there was no significant difference between PZQ and the two plant extracts OAH and BMW ($P >0.05$). However BMW had a higher OD than the PZQ and OAH respectively.

IV. DISCUSSION

The two plant extracts (OAH and BMW) when compared with PZQ were significantly similar in reduction. This was an indication of animal protection. PZQ compared with IC showed few or no granulomas, while IC showed moderate and severe granulomas which were an indication of heavy worm burden due to the fact that the animals were unprotected from the infection. The plant extracts of OAH and BMW were similar to PZQ in terms of no or few granuloma in gross pathology. This meant that the two extract groups were very effective in reducing gross pathology at a similar level to PZQ. Granuloma sizes of OAH and BMW groups was significant similar to the PZQ, however significantly different to IC group. This means that the two plant extracts were effective in reducing granuloma sizes. BMW extract had high IFN responses for both spleen and lymph node cells. This implies that BMW was able to produce the TH-1 which is important profile for cell mediated immunity which would fight the infection. However IC had higher IFN responses for lymph node cells than the rest of the groups. This was probably due to high worm antigen concentration in circulation due to higher number of worms. Interferon-γ is used to treat chronic granulomatous disease **(10)**. PZQ and the treatment groups, BMW and OAH, were able to protect the mice from infection and produce cell mediated immunity. IC group produced high response due to high number of worm. All extracts induced production of IL-5. When PZQ was compared with BMW and OAH there was no significant difference but OAH was higher in response. From the observation the OAH was able to generate more IL-5 compared with the rest of the groups. IL-5 is able to the elimination antibody bound parasites through the release of cytotoxic granule proteins. Given that eosinophils are the primary IL-5-expressing cells this is an important fact in that eosinophils play an important role in regulating parasitic infections **(11)**. IL- 5 in IC could have occurred due to stimulation of high levels of Schistosome worm antigens. Serum antibody analysis indicated PZQ had lower IgG responses which were significantly different from IC. PZQ and the two plant extracts OAH and BMW were significantly similar; however BMW had higher IgG responses. This is an indication that BMW and OAH plant extract were able to fight infection using humoral immunity **(12)** in a similar manner to PZQ, with BMW being better. The high response in IC may be associated to high worm burden and resulting to more antigens being released inducing non-specific response, which was not protective.

V. CONCLUSION

Ocimum americanum hexane extracts and Bridelia micrantha water extract plant extracts were significantly similar to PZQ in terms of worm reduction and reduced pathology, an indication that the extracts were able to fight the parasitic infection. OAH and BMW plant extracts were able to generate IL-5, IFN- γ and IgG responses which were an indication that the extracts were able to fight infection using both humoral and cell mediated immunity.

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