J. Egypt. Soc. Parasitol. (JESP), 46(1), 2016: 217- 222

LYMPHO-PROLIFERATIVE RESPONSES TO VARIOUS *FASCIOLA HEPATICA* WORM'S ANTIGENS: AN *IN VITRO* STUDY

By

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Abstract

Fascioliasis is an important zoonotic disease with approximately 2-4 million people infected worldwide and a further 180 million at risk of infection. *F. hepatica* can survive within the bile ducts for many years through its ability to suppress the host immunity with *Fasciola* cathepsin L1 cysteine protease and Glutathione S transferase playing an important role. The aim of the present study is to investigate the *in vitro* lympho-proliferative responses of hepatic hilar lymphocytes (HLN) of infected sheep in response to different *F. hepatica* antigens. The suppressive effects of *Fasciola* excretory/secretory (ES) and tegument (TEG) and their fractions were also investigated. Our results showed that both ES and TEG had significant suppressive effects on lympho-proliferation, up to 74% and 92%, respectively. When these antigens were fractionated, fraction 3 (MW of >10000-30000) of both ES (64%) and TEG (59%) in addition to fraction 4 (MW of ≤ 10000) of TEG (38%) inherited the suppressive effects. Identification of the potential molecule(s) with such suppressive effects on lymphocytes in TEG fraction 4 could reveal vaccine candidates.

Keywords: *Fasciola hepatica*, tegument, excretory/secretory, lympho-proliferation.

Introduction

The parasitic trematode *Fasciola hepatica* (*F. hepatica*) infects a wide variety of mammals, including cattle, sheep, and humans, causing liver fluke disease, or fasciolosis. Infection is usually acquired by ingestion of water vegetation on which the infective metacercariae have encysted. The metacercariae excyst in the intestine, burrow through the gut wall of the mammalian host, and migrate across the body cavity to the liver, where the parasite causes extensive damage and finally settle down in the biliary tree (Joyce and Dalton, 1999). Fascioliasis is an important zoonotic disease with approximately 2-4 million people infected worldwide and a further 180 million at risk of infection (McManus and Dalton, 2006). *F. hepatica* can survive within the bile ducts for many years through its ability to suppress the host immunity. The infection promotes strong Th2/Treg T-cell-mediated immune responses and can suppress Th1 inflammatory responses (Moreau and Chauvin, 2010). These immunomodulatory abilities enable the worm to persist in the host and interact with inflammatory and immune mechanisms involved in other infections or in vaccination or in allergic and autoimmune diseases (Maizels *et al*, 2004).

The Denderitic cells (DCs) are potent antigen presenting cells that possess the ability to stimulate naive T cells. In response to infectious agents DCs undergo a process of maturation and migration to secondary lymphoid organs. In these lymphoid organs, DCs present captured antigens to naive T cells to trigger specific immunity (Steinman, 2012). *F. hepatica* secretes several molecules, excretory secretory products (ES) which are able to modulate the immune response. These ES can depress sheep and rat lymphocytes stimulation (Moreau *et al*., 2002). *Fasciola* Cathepsin L1 cysteine protease, one of the predominant secreted products, has been known to suppress the onset of protective Th1 immune responses to bacterial infections in mice and to prevent the development of a Th1 response to vaccination against bacteria (Collins *et al*, 2004).

Fasciola cathepsin cysteine proteases are vaccine candidates offering up to 83% protection against *F. hepatica* challenge infection in rats (Jayaraj *et al*, 2010). Glutathione S transferase (GST) is another major antigen secreted by *Fasciola* and could be involved in evasion of the parasite from immune response of its hosts (Cervi *et al*., 1999). *Fasciola* GST is another vaccine candidate that offered a 49-69% reduction in fluke number in cattle vaccinated with GST in different adjuvants (Morrison *et al*, 1996). On the other hand, *Fasciola* tegument (TEG) has immune-modulatory effects on the immune system of host. DCs cultured with *Fasciola* tegument was shown to maintain immature state, impairing their function and the subsequent development of adaptive immunity (Hamilton *et al*., 2009). In the present study, we investigated the lympho-proliferative responses of hepatic hilar lymphocytes (HLN) of infected sheep in response to different *F. hepatica* antigens. The suppressive effects of ES and TEG were also investigated in this study. Fractionations of ES and TEG were carried out according to the molecular weights. The suppressive responses of these fractions were also studied.

Materials and methods

The HLN were dissected and collected in sterile universal tubes containing RPMI-1640. After smashing and digestion of HLN, lymphocytes were collected using the Lymphoprep (1.077±.001g/ml) method (Nycomed Pharma), resuspended in the RPMI-1640. The cells were counted using an improved Neubauer haemocytometer and their concentration was adjusted at $1x10^7$cells/ml.

For preparation of soluble worm antigens (SWA), adult liver flukes, obtained from the infected livers of cattle from a local abattoir, were washed four times with phosphate-buffered saline (PBS), pH 7.0. Worms were homogenized in PBS on ice and then sonicated with 30 sec on and 30 sec off for 10 min. After centrifugation at 10,000rpm for 30 min, the supernatant was sterilized with filter (pore size = 0.22µm) and stored at -20°C. For preparation of *F. hepatica* tegument antigens (TEG), *F. hepatica* adult worms were washed in sterile PBS and incubated in 1% Triton X-100 (Sigma Aldrich) in PBS with gentle shaking for 1hour. The detergent was removed from supernatant using extracti-gel D detergent removing gel (Pierce). TEG was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was filtered with 0.22µm pore size filter and stored at –20°C. For preparation of *F. hepatica* excretory-secretory (ES), live *F. hepatica* adults were recovered from the livers of slaughtered sheep. These flukes were washed five times and incubated for 3 hours at room temperature in 0.9% NaCl (1 ml/fluke). The medium was then recovered and centrifuged at 10,000 rpm for 15 min. Finally, the supernatant was filter sterilised (pore size = 0.22 µm) and stored at –20 °C. Fractionation of ES and TEG was adopted using centricon centrifugation device of nominal molecular weight limit of 10000, 30000 and 50000 (Millipore, Ireland). Accordingly, each was fractionated into four fractions; fraction 1 of molecular weight (MW) of ≥ 50000, fraction 2 of MW of >30000-50000, fraction 3 of MW of >10000-30000 and fraction 4 of MW of ≤ 10000.

Lymphocytes from each group were cultured in 96-well tissue culture plates. Proliferation was assessed by lymphocytes incubation at different concentrations (1, 5 & 10µg/ml) of Concanavalin A (ConA) as a positive control, SWA, ES, TEG, SWA +ES & SWA+TEG. A 25µl of cell suspension was added to each type and well are completed to reach 200 µl complete RPMI-1640 media. Wells with 25µl of unstimulated cells+175µl of complete RPMI-1640 were used as control. Assays were run in triplicate. The plates were incubated at 37°C, 5% C02 and humidity. After one

day, 0.2µCi of the radioactive-thymidine (Amersharn Biosciences, UK) was added to each well and further incubated for 18 hours. At the end of incubations, the plates were harvested and isotype incorporation by the cells measured using a liquid scintillation counter (Wallac 1450 Microbetay Trilux). For antigen fractions experiments, the proliferative effects of SWA either alone or combined with different ES/TEG fractions were investigated at concentration of 10µg/ ml. All statistical tests were done by means using tanalysis of variance (ANOVA) test with P<0.05 as significance. When there was a significant difference it was followed by the student t-tests for pair wise comparisons between different groups. The SPSS software (version 16) was utilized for this purpose.

Results

Proliferation of HLN of non-infected sheep: Cells from non-infected sheep proliferated significantly in response to Con A only. Other antigens exhibited no significant proliferation. Proliferation of HLN of infected sheep was shown in (Figure 1). The highest proliferation was reported with SWA antigen, followed by Con A, ES and TEG at all concentrations used. When ES antigen was added together with SWA, a significant reduction ($P < 0.05$) in the proliferation was shown. This reduction was most evident with concentration of 10µg/ml (74% reduction). When TEG antigen was added in parallel with SWA, a significant reduction ($P < 0.05$) in the proliferation response was observed. Uercentage of reduction was 87%, 92% with 5µg/ml & 10µg/ml, respectively.

When HLN was incubated with different fractions of ES with SWA (figure 2), fraction 3 (MW of >10000-30000) showed the greatest reduction of the proliferative effect of SWA (64% reduction) When HLN was incubated with different fractions of TEG with SWA, fraction 3 (MW of >10000-30000) showed the greatest reduction of the proliferative effect of SWA (59% reduction), followed by fraction 4 (MW of ≤ 10000) with 38% reduction.

Discussion

In the current study, the lympho-proliferative responses of HLN of *Fasciola*-infected sheep in response to different worm antigens were investigated. The highest proliferation was reported in response to SWA, followed by Con A antigens. When HLN were incubated with SWA and ES together, ES caused a significant reduction in lympho-proliferative responses than when incubated with SWA alone. In accordance with this, *F. hepatica* ES caused significant levels of suppression of human and sheep lymphocyte proliferation responses, compared to cells treated with Con A alone, consistent with previous report (Jefferies *et al*, 1996). *F. hepatica* ES inhibited the ConA-induced stimulation of sheep peripheral blood mononuclear cells and T lymphocyte subsets (Moreau *et al*, 2002). Furthermore, ES of *F. hepatica* modulated the maturation and function of DC as part of a generalized immunosuppressive mechanism that involves a bias towards a Th2/ Treg response, in agreement with Falcon *et al*., 2010). However, at high doses, ES inhibited proliferation of lymphocytes of all the animal species tested while at low doses these antigens inhibited the proliferation of sheep lymphocytes and increased the proliferation of buffalo and mouse lymphocytes (Zhang *et al.*, 2005).

In the present study, when different ES fractions were incubated with SWA, fraction 3 (MW of >10000-30000) inherited the suppressive effects of ES. These suppressive effects could be attributed to cathepsin L cysteine protease or GST, both of which lie in the molecular weight range of ES fraction 3. GST is housekeeping enzyme found in nearly all eukaryotics including a number of parasites. The dimeric form of GST present in the *F. hepatica* ES exerted a significant inhibition of rat T cell proliferation *in vitro* and causes a down-

regulation of nitric oxide (NO) production by normal peritoneal macrophages, suggesting a role for GST in the host immune response evasion exhibited by *Fasciola* (Cervi *et al*, 1999). On the other hand, Prowse *et al.* (2002) investigated the effect of ES fractions, separated by gel filtration, on the proliferation of sheep T cells *in vitro*. A major proportion of the suppressive activity in ES was attributed to the cathepsin L proteases which reduced surface CD4 expression on both human and ovine T cells. Moreover, comparison of cattle HLN response to a variety of *F. hepatica* antigens revealed that proliferation was less after culture with cathepsin L, than with a high MW fraction of whole fluke antigens or ES antigen (Hoyle and Taylor, 2003).

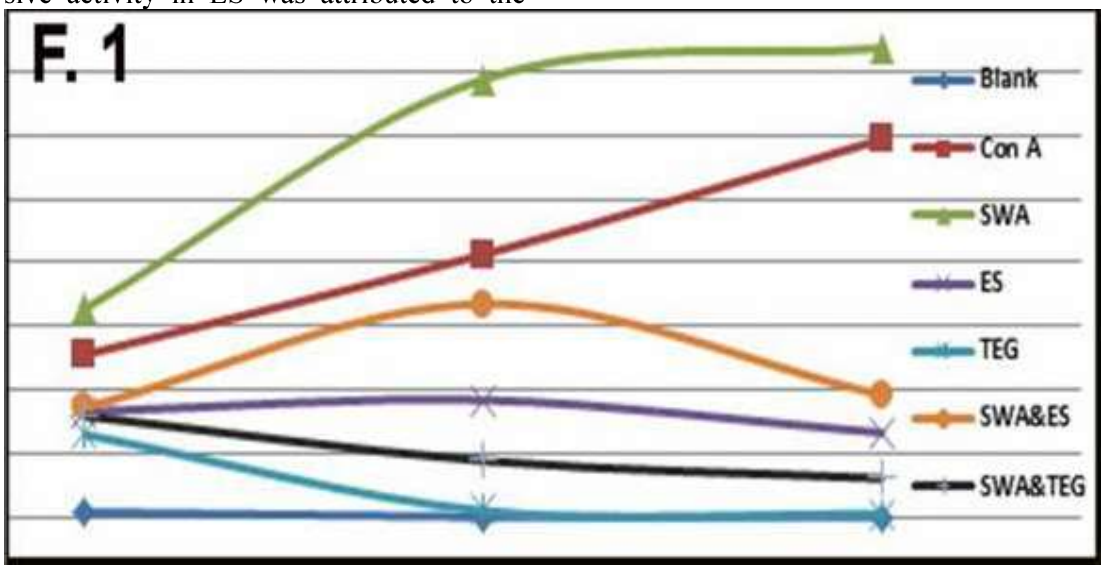

Fig. 1: Proliferative effects of different antigens, at different doses, on HLN of infected sheep

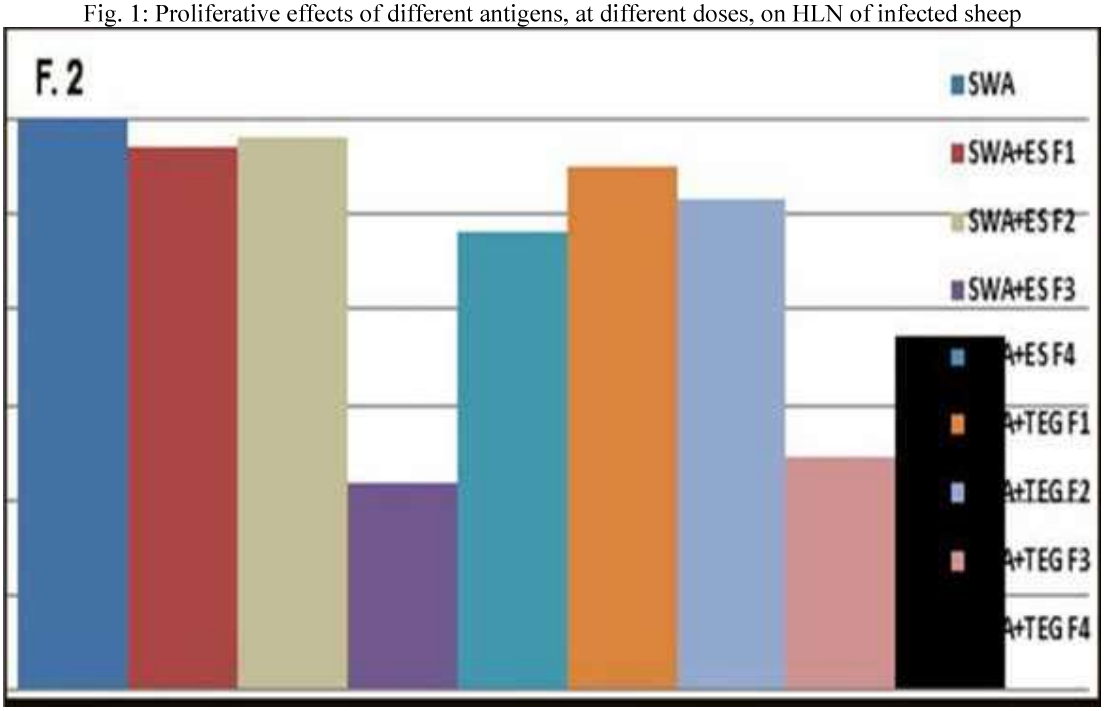

Fig. 2: Effects of different ES and TEG antigen fractions when combined with SWA on proliferative effect of SWA on HLN of infected sheep.

*F*asciola *hepatica* TEG is a metabolically active layer that is in intimate contact with the host tissues and body fluids. Studies have shown that by shedding its tegument, the fluke can also shed immune complexes that have formed on the surface, thus evading the damaging effect(s) of the immune response (Mulcahy *et al*, 1999). In the present study, *Fasciola* TEG caused non-significant proliferation of HLN of infected sheep. When TEG was incubated with SWA it caused significant reduction of the proliferative effects of SWA and this suppressive effect is a concentration dependent. In accordance with this, the isolated TEG antigen of *F. hepatica* suppressed DC maturation and function by inhibiting interleukin 6 (IL-6), tumour necrosis factor (TNF)-a, IL-10 and IL-12 production and CD80, CD86 and CD40 cell surface marker expression in Toll like receptors (TLR) stimulated dendritic cells. *Fasciola hepatica* tegumental coat antigen also impaired dendritic cell function by inhibiting its phagocytic capacity and its ability to prime T cells (Hamilton *et al*, 2009). Similarly, isolated *F. hepatica* TEG antigen demonstrated a suppressive effect *in vivo* by directly targeting mast cells, impairing their ability to drive Th1 responses (Vukman *et al*, 2013).

Moreover, the present study demonstrated that when different TEG fractions were incubated with SWA, both fractions 3 and 4 suppressed the proliferative response to SWA significantly. The suppressive effects of fraction 3 could be attributed to GST or other compounds. A proteomic approach identified enolase, aldolase, GST, and fatty acid binding protein as the major immunoreactive components of the TEG (Morales and Espino, 2012).

However, the suppressive effect of TEG fraction 4 was less profound than that of fraction 3. The responsible compound could be a Kunitz type molecule as well as other compounds. In relation to the present results, Falcon and colleagues demonstrated that after fractionation of *F. hepatica* total extract (TE), a fraction lower than 10 kDa was able to maintain the TE properties to modulate the DC pro- and anti-inflammatory cytokine production induced by lipopolysccharides (LPS). Furthermore, these investigators have showed that a Kunitz type molecule, present in this fraction, was responsible for suppressing pro-inflammatory cytokine production in LPS-activated DC, by causing tolerogenic features on DC that impaired their ability to induce inflammatory responses (Falcon *et al*., 2014). *F. hepatica* Kunitz type molecule was isolated from the worm as a single polypeptide of 58 amino acids and a *Mr* of 6751. Indirect immunofluorescence and immunogold labeling revealed that Fh-KTM is an abundant molecule localized to the gut, the parenchymal tissue and the adult *F. hepatica* TEG (Bozas *et al*, 1995). However, owing to our limited financial resources, we could not carry out further investigation with other approaches to identify the responsible compound(s) in TEG fraction 4 for the suppressive effect on lymphocytes. Identification of such compounds could lead to vaccine candidates.

Conclusion

Generally spraking, zoonotic fasciliassis is a worldwide health problem particularly in the sheep rearing countries.

The *F. hepatica* has an immunomodulatory effects by mean of their ES and their TEG. This immunomodulation could be attributed to well-known molecules such as GST and Cathepsin L proteases or could be attributed to other unknown molecules. Identification of such molecules could lead to the discovery of the vaccine candidates.

Further study is ongoing and will be publish in due time elsewhere.

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