Cellular Immune Responses in Sheep against Glutathione S-transferase of *Fasciola gigantica*

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Summary

Glutathione S-transferase (GST) is an important candidate for vaccination against temperate fasciolosis. For assessment of cellular immunity against GST of *Fasciola gigantica* (FgGST), sheep was immunized by FgGST combined with saponin and $Al(OH)_3$, as adjuvants. Cellular immune response, after three times immunization, was evaluated by flowcytometry of peripheral blood, lymphocyte transformation test (LTT) and delayed hypersesitivity skin test. Flowcytometry of peripheral blood showed no significant differentiation between treatment and control groups in the percent of $CD8^+$ lymphocyte, MHC class I^+ and MHC class II^+ leukocyte subsets. Proliferation of T cells against FgGST was detected in vaccinated groups; but stimulation index was lower than 2 in these groups. Erythema and induration of skin against FgGST in immunized groups were significantly greater than control. In conclusion FgGST appears to be only weakly antigenic for sheep T cells and it is therefore an unpromising candidate for inducing resistance to *F.gigantica*.

Key words: *Fasciola gigantica*, glutathione S-transferase, sheep, cellular immunity, vaccine

Introduction

The digenetic trematodes, *Fasciola hepatica* and *F.gigantica,* are important pathogens of sheep and cattle and cause economic loss estimated at US $2000 M per annum worldwide. Fasciolosis is increasingly recognized as causing significant human disease, with 2.4 million people infected. Because of recent estimates of more

than 10,000 cases and about 6 million at risk (Dalton 1999) human Fasciolosis is a problem in Iran. An alternative means of control may lie with the development of an effective vaccine against *Fasciola*. More recently, the search for a novel vaccine for trematodes has focused on essential enzymes and receptors. One of the most promising candidates has been the GST (Morrison *et al* 1996). GSTs (EC 2.5.1.18) are a family of enzymes that are involved in the cellular detoxification process. They primarily function by catalyzing the conjugation of the glutathione to a wide variety of electrophilic toxic substrates (Rossjohn *et al* 1997). GSTs of helminths role as immune defense proteins and have significant activity with lipid peroxidation-derived carbonyls and also have the potential to neutralize exogenously derived toxins such as anthelmintics (Brophy & Pritchard 1994). Based on substrate specificity and primary structure studies, cytosolic GSTs have been grouped into six classes: alpha, mμ, pi, theta, sigma and kappa (Pemble *et al* 1996). GSTs have been highly conserved throughout evolution and are particularly abundant in parasitic helminths (Brophy & Pritchard 1994). These molecules first achieved prominence as likely vaccine candidates following work performed using GSTs isolated from *Schistosoma* spp (Mitchell 1989). The homologous GST fraction, purified from F. hepatica, proved ineffective in a vaccination study in rats (Howell *et al* 1989). A trial in sheep indicated that a mean 57% reduction in worm burdens was possible (Sexton *et al* 1990). Significant reduction in fluke burdens (49-69%) were observed in cattle vaccinated with GST in Quil A/Squalene Montanide (Morrison *et al* 1996). A recent report of vaccination of cattle against *F.gigantica* with GST did not induce significant reduction in worm burdens (Estuningsih *et al* 1997). Vaccination of sheep against *F.gigantica* with GST did not induce significant reduction in worm burdens and egg production (Paykari 2000). Although, anti-FgGST Ab titer was elevated in vaccinated animals, but cellular immune responses were not assessed. Since cellular immune responses are important in protection against fasciolosis, we evaluated cellular immune response of sheep against FgGST.

Materials and Methods

Purification of FgGST. FgGST was purified by affinity chromatography on GSH-agarose beads (Sigma) according Sexton (1990).

Enzyme activity assay. Activity of GST enzyme in crude antigen and purified GST of *F.gigantica* was estimated by the spectrophotometric conjugation of glutathione and 1-chloro-2,4-dinitrobenzene according to the method of Habig (1981). The absorbance was read at 340nm at 10 S intervals.

Immunization protocol. Fifthy Male Balouchi cross sheep were obtained from Razi Research Institute and screened for the absence of worm infection. The animals were divided in 6 groups: Group 1 (n=10) received only 200µg FgGST. Group 2 (n=10) received 200µg FgGST with 3mg $Al(OH)_3$. Group 3 (n=8) received only PBS. Group 4 and 5 (n=12) received only 3mg $Al(OH)_3$ or 1 mg saponin, respectively. Group 6 (n=10) received 200µg FgGST with 1mg saponin. Each animal received subcutaneously 3 doses at 4 weeks intervals in volume of 1ml. Animals were killed 24-26 weeks after challenge.

Flowcytometry. One week after 3rd immunization, 100µl of whole blood of animals were mixed with 20µl FITC conjugated monoclonal antibodies (Serotec) to sheep cell surface antigens (CD8, MHC class I and MHC classII), separately. As negative control, 100µl of whole blood from each sample was used without any Abs. Samples were incubated for 15min at room temperature. 2ml FACS lysing solution were added and incubated for 10min at room temperature. Tubes were centrifuged at 300*g* for 5min and aspirated the supernatant. 2ml PBS was added and vortexed, then centrifuged at 200*g* for 5min. After aspirating the supernatant, 1ml isotonic solution was added to each tube and vortexed. Each sample with related control was analyzed by Becton Dickinson Immunocytometry System.

Lymphocyte transformation test (LTT). After immunization, 10ml heparinized whole blood of animals was mixed with 10ml RPMI-1640 medium. Lymphocytes were isolated from whole blood by density gradient centrifugation on Ficoll-Hypaque. After washing, the viability of lymphocytes was detected with 0.2% tripan blue. 2×10^5 viable lymphocytes in RPMI-1640 containing 15% FCS, were added to each well of round bottom 96-well plates. Assays were carried out in triplicate for each sample. 5µg FgGST per ml was used as antigen. Phytohemagglutinin (PHA, Sigma) was added in positive control wells. After 72h incubation in 37°C with 5% CO_2, 2.5

μCi [^{3}H] thymidine were added to each well and plates were incubated in the same condition for 16h. The cells were harvested and radioactivity was counted on a beta plate reader (Wallac). Results are presented as mean counts per minute (CPM) and standard deviation (SD). Stimulation index (SI) were obtained according the following formula:

SI=mean of CPM in immunized group / mean of CPM in control group

Skin test. After immunization, 4 doses of FgGST (5, 10, 20 and 30μg) were separately injected in 12 immunized and 12 non-immunized animals (3 sheep for each group). To assess delayed hypersnsitivity reaction, after 8, 24 and 48h, erythema and induration were measured with a ruler. Optimal dose of FgGST (20μg) was selected and injected in all groups and the reaction was measured with the same method.

Results

Antigen preparation. GST activity was detected in crude antigens and purified GST of *F.gigantica*. Specific activity in crude antigens and FgGST was 2 and 25.7 μmol. min^{-1}. mg protein^{-1} , respectively. SDS-PAGE of FgGST showed three bands with a Mr 24.5 - 26.5kD (Figure 1).

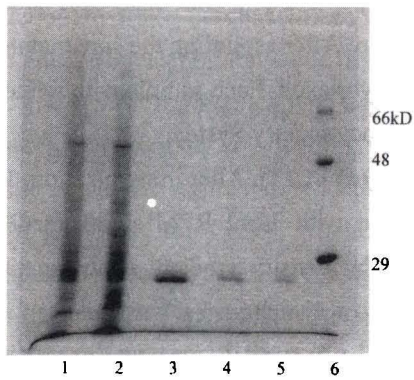

Figure 1. *SDS-PAGE of crude antigen (1, 2), GST of F.gigantica (3-5) and standard marker (6) with 66, 48 and 29 kD MW*

Flowcytometry. Results showed the number of $CD8^+$ lymphocytes in vaccinated groups were lower than control, but this alteration was not significant (Table 1).

Table 1. *Mean and SD of the percentage of $CD8^+$ lymphocyte, MHC class II^+ lymphocyte and MHC class II^+ monocyte in immunized (groups 1, 2 and 6) and control sheep (groups 3, 4 and 5)*

Group*	No.	% CD+ lymphocyte		% MHC class II^+ Lymphocyte		% MHC class II^+ monocyte	
		Mean	SD	Mean	SD	Mean	SD
1.GST	10	27.5	10.0	39.4	10.3	17.5	12.1
2.GST+Al $(OH)_3$	10	23.2	6.6	40.6	8.0	17.8	13.6
3.PBS	8	27.2	5.4	42.7	16.5	18.0	12.0
4.Al$(OH)_3$	6	29.6	6.4	40.9	12.1	9.0	5.6
5.Saponin	6	25.1	4.0	42.3	10.1	18.1	8.1
6.GST+saponin	10	22.9	12.3	40.7	6.5	13.6	9.0

* Groups 1, 2 and 6 were immunized three times with 200 µg FgGST with or without adjuvant.

Alterations of the number of MHC class II^+ lymphocytes, MHC class II^+ monocytes, MHC class I^+ monocytes, MHC class I^+ lymphocytes and MHC class I^+ granulocytes were not significant in all groups (Tables 1 and 2).

Table 2. *Mean and SD of the percentage of MHC class I^+ lymphocyt, MHC class I^+ monocyte and MHC class I^+ granulocyte in immunized (groups 1, 2 and 6) and control sheep (groups 2 and 6)*

Group*	No.	% MHC class I lymphocyte		% MHC class I monocyte		% MHC class I granulocyte	
		Mean	SD	Mean	SD	Mean	SD
1.GST	10	99.2	0.8	79.0	9.2	89.1	7.2
2.GST+Al $(OH)_3$	10	99.1	0.9	84.4	13.9	86.1	11.9
3.PBS	8	99.1	0.8	69.5	22.3	72.1	21.9
4.Al$(OH)_3$	6	99.5	0.5	74.2	20.6	72.7	22.4
5.Saponin	6	99.3	0.8	83.3	8.5	86.0	7.3
6.GST+saponin	10	98.8	1.1	87.8	19.6	89.8	17.0

* Groups 1, 2 and 6 were immunized three times with 200 µg FgGST with or without adjuvant.

Lymphocyte transformation test. LTT of peripheral blood indicated CPM in vaccinated groups (groups 2 and 6) was higher than control groups (groups 4 and 5) and this alteration was significant but SI, in all groups, was lower than 2 (Table 3).

Skin test. Erythema and induration of skin, caused by injection of FgGST, were significantly increased in vaccinated groups (groups 2 and 6). The results are indicated in table 4.

Table 3. *Mean and SD of counts per minute (CPM) and stimulation index (SI) in immunized (groups 1, 2 and 6) and control sheep (groups 3, 4 and 5)*

Group*	No.	CPM/min		SI	
		Mean	SD	Mean	SD
1.GST	4	659.7	298.4	1.37	0.62
2.GST+Al $(OH)_3$	7	$685.5^{(1)}$	223.8	1.42	0.47
3.PBS	7	$481.4^{(1)}$	99.8	1.00	0.21
4.Al$(OH)_3$	6	530.2	240.0	1.10	0.50
5.Saponin	5	$567.4^{(2)}$	212.2	1.18	0.44
6.GST+saponin	9	$800.6^{(1)(2)}$	66.7	1.66	0.35
Cell + PHA		1804.0	489.0		
Cell		444.0	121.9		
RPMI		200.0	53.9		

(1)(2) $P < 0.05$

* Groups 1, 2 and 6 were immunized three times with 200 µg FgGST with or without adjuvant.

Table 4. *Mean and SD of erythema and induration of skin at 3 stages after skin test in immunized (groups1, 2 and 6) and control sheep (groups 3, 4 and 5)*

Group*	No.	After 6h (mn)		After 24h (mn)		After 48h (mn)	
		Mean	SD	Mean	SD	Mean	SD
1.GST	10	1.50	1.2	8.00	6.3	6.00	5.6
2.GST+Al $(OH)_3$	10	2.00	1.6	10.50	6.9	$10.00^{(3)}$	7.1
3.PBS	8	1.25	1.2	6.25	5.9	$1.25^{(1)(3)}$	1.2
4.Al$(OH)_3$	6	1.67	1.6	5.80	4.9	$3.33^{(3)}$	2.6
5.Saponin	6	3.33	2.6	6.67	5.2	$2.50^{(2)}$	1.7
6.GST + saponin	10	2.00	1.6	11.50	7.1	$11.00^{(1)(2)}$	7.7

(1) $P < 0.005$

(2) (3) $P < 0.05$

* Groups 1, 2 and 6 were immunized three times with or without adjuvant.

Discussion

FgGST, as a vaccine in sheep, to be able to stimulate humoral immune response of sheep (Paykari 2000). Our results indicate FgGST stimulates cellular immunity

weakly. This failure may be responsible to nonprotection of FgGST vaccine in cattle and sheep (Estuningsih *et al* 1997, Paykari 2000).

GST from trematodes has been shown previously to induce significant reduction in parasite infection and or fecundity in vaccinated ruminants. Vaccination of cattle with native GST of *Schistosoma bovis* has been shown to induce anti fecundity effects against *S.bovis* (Bushara *et al* 1993). In contrast, in goats, vaccination with the recombinant 28 KD GST of *S.bovis* induced significant reduction in worm burdens, but not in egg production (Boulanger *et al* 1994). In sheep, vaccination with native GST of *S.japonicum* induced anti-fecundity effects against *S.japonicum* and also reduced worm burdens (Xu *et al* 1995). With native FhGST, vaccination of sheep reduced *F.hepatica* worm burdens but did not effect egg production (Sexton *et al* 1990). It is clear from data of Morrison *et al* (1996) that native FhGST is capable of producibly inducing high levels of protection against infection with *F.hepatica* in Bas taurus cattle, using Quil A/SM as adjuvant. Protection was shown to persist for at least 6 months post-challenge.

To date, there are two reports of the use of defined antigens as vaccine against *F.gigantica* (Estuningsih *et al* 1997, Paykari 2000). When the efficacy of GST from *F.gigantica* was assessed in Brahman-cross cattle, no significant reduction in worn burdens or faecal egg counts was observed, despite the use of the same adjuvants previously shown to induce protection against *F.hepatica* with GST (Estuningsih *et al* 1997). These results highlight the variability in the effect of vaccination with trematode GST in various ruminants and parasite species.

There are several possible explanations as to why FgGST did not induce the same high levels of protection in cattle and sheep seen previously with FhGST. One possibility is that *F.gigantica* may differ from *F.hepatica* in being resistant to bovine and sheep immune responses induced by vaccination with GST. Since *F.hepatica* is known to posses enzyme systems, which protect against inflammatory mediators, it is possible that *F.gigantica* has further developed enzymes or other protection systems, which enable it to escape the putative vaccine-induced immune effector response. However, it is recently founded that larvae of *F.gigantica* are susceptible to killing *in vitro* mediated by activated macrophages from rats and sheep. In addition, *in vivo*

challenge experiments have shown that *F.gigantica* is less effective than *F.hepatica* at establishing infection in rats and Indonesian thin tail or merino sheep. These observations argue against the notion that *F.gigantica* is a "supper parasite" which is more resistant to immune effector mechanisms. Another possibility is that the immune effector response induced in B.taurus cattle by vaccination with *F.hepatica* GST is missing or less effective in Brahman cross cattle vaccinated with FgGST. Although GST of these 2 parasites are similar in activity, NH_2-terminal sequence and antigenicity, it is possible there are epitopes on FhGST that have diverged in sequence during the evolution of *F.gigantica* resulting in the loss of protective epitopes in GST of this species. Variations in amino acid sequences in T and B cell epitopes have been described in GST of *Schistosoma* species. A third possibility is variation in immune responsiveness between host breeds, for example, Brahman cross breed does not efficiently present FgGST epitopes to the immune system to induce a protective immune response (Estuningsih *et al* 1997).

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References

Boulanger, D., Trottein, F., Mauny, F., Bremond, P., Couret, D., Pierce, R. J., Kadri, S., Godin, C., Sellin, E., Lecocq, J.P. and Capron, A. (1994). Vaccination of goats against the trematode *Schistosoma bovis* with a recombinant homologous schistosome-derived glutathione S-transferase. *Parasite Immunology* 16:399-406.

Brophy, P. M. Crowley, P. and Barrett, J. (1990). Relative distribution of glutathione transferase, glyoxalase I and glyoxalase P in helminths. *International Journal for Parasitology* 20:259-261.

Brophy, P.M., Pritchard, D.L. (1994). Parasitic helminth glutathione S-transferase: an update on their potential as targets for immuno- and chemotherapy. *Experimental Parasitology* 79:89-96.

Bushara, H.O., Bashir, M.E., Malik, K.H., Mukatr, M.M., Trottein, F., Capron, A. and Taylor, M.G. (1993). Suppression of *Schistosoma bovis* egg production in cattle by vaccination with either glutathione S-transferase or keyhole limpet haemocyanin. *Parasite Immunology* 15:383-390.

Dalton, J.P. (1999). *Fasciolosis*. CABI publishing, Walingford, UK.

Estuningsih, S.E., Smooker, P.M., Wiedosari, E., Widjajanti, S., Vaiano, S., Partoutomo, S. and Spithill, T.W. (1997). Evaluation of antigens of *Fasciola gigantica* as vaccines against tropical fasciolosis in cattle. *International Journal for Parasitology* 27(11):1419-1428.

Habig, W.H., Jakoby, W.B. (1981). Assays for differentiation of glutathione S-transferase. *Methods of Enzymology* 77:398.

Hillyer, G.V., Galanes, M.S. and Battisti, G. (1992). *Fasciola hepatica*: host responders and nonresponders to parasite glutathione S-transferase. *Experimental Parasitology* 75:176-186.

Howell, M.J., Board, P.G. and Boray, J.C. (1988). Glutathione S-transferase in *Fasciola hepatica. Journal of Parasitology* 74:715-718.

Miller, C.M.D., Howell, M.J. and Boray, J.C. (1994). Glutathione S-transferase as markers of salicylanilide resistance in isolates of *Fasciola hepatica. International Journal for Parasitology* 24(4):533-542.

Mitchell, G.F. (1989). Glutathione S-transferase: potential component of anti-schistosomal vaccines. *Parasitology Today* 5:34-57.

Morrison, C.A., Colin, T., Sexton ,J.L., Bowen, F., Wicker, J., Friedel, T. and Spithill, T.W. (1996). Protection of cattle against *Fasciola hepatica* infection by vaccination with glutathione S-transferase. *Vaccine* 14(17/18):1603-1612.

Paykari, H. (2000). Study on immune response of sheep against purified GST of *Fasciola gigantica*, Ph.D. Thesis, Tarbiat Modarres University, Tehran (In Persian).

Pemble, S.E., Wardle, A.F. and Taylor, J.B. (1996). Glutathione S-transferase class kappa: characterization by the cloning of the rat mitochondrial GST and identification of a human homologue. *Biochemical Journal* 319:749-754.

Rossjohn, J., Feil, S.C., Wilee, M.C.L., Sexton, J.L., Spithill, T.W. and Parker, M.W. (1997). Crystallization, structural determination and analysis of a novel

parasite vaccine candidate *F.hepatica* GST. *Journal of Molecular Biology* 273(4):857-72.

Sexton, J.L., Milner, A.R., Panaccio, M., Waddington, J., Wijfels, G., Chandler, D., Thompson, C., Wilson, L., Spithill, T.W., Mitchell, G.F. and Campbell, N.J. (1990). Glutathione S-transferase, novel vaccine against *Fasciola hepatica* infection in sheep. *The Jounal of Immunology* 145:3905-3910.

Sexton, J.L., Wilce, M.C.J., Colin, T., Wijffels, G.L., Salvatore, L., Feil, S., Parker, M.W., Spithill, T.W. and Morrison C.A. (1994). Vaccination of sheep against *Fasciola hepatica* with glutathione S-transferase, identification and mapping of antibody epitopes on a three-dimensional model of the antigen. *Journal of Immunology* 152:1861-1872.

Xu, S.T., Shi, F.H., Shen, W., Lin, J.J., Wang, Y.H., Ye, P., Tian, E., Qian, C.G., Lin, B.F., Shi, Y.J. and Zhang, Z.D. (1995). Vaccination of sheep against *Schistosoma japonicum* with either glutathione S-transferase, Keyhole limpet haemocyanin or the freeze/thaw schistosomula/BCG vaccine. *Veterinary Parasitolgy* 58:301-312.