



Research Journal of **Parasitology**

ISSN 1816-4943



Academic
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Scanning Electron Microscopic Observations of Adult *Fasciola gigantica* after Immunization with Glutathione S-Transferase in Goats

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Abstract: A complete understanding of the micromorphological features of the tegument is important in developing any vaccine that can damage the parasite's tegument. The present study had been carried out to assess tegumental changes in adult *Fasciola gigantica* recovered from goats after immunization with Glutathione S-Transferase (GST) antigen by means of Scanning Electron Microscopy (SEM). The GST in *F. gigantica* (FgGST) was isolated by affinity chromatography, by which highly purified enzyme was obtained and evaluated as a vaccine in combination with Freund's adjuvant. The statistically significant results were obtained when the goats were immunized with GST and challenged with 120 *F. gigantica* metacercariae; 2 weeks after last immunization, revealing 64.1% worm burden reduction over adjuvant control. Equally important, GST elicited highly significant ($p < 0.0001$) decrease in the size of the recovered flukes. SEM analysis of these flukes revealed evidence of severe swelling of the tegumental surface covering and between the spines in the apical cone and mid-body regions in the majority of the specimens examined. This tegumental swelling showed regional variations in its severity. In extreme cases, there was severe disruption of the apical cone region as well as the spines. These results might raise the possibility of being GST as a prospective vaccine candidate against fasciolosis in goats.

Key words: *Fasciola gigantica*, glutathione S-transferase, vaccine, goats, scanning electron microscopy

INTRODUCTION

Tropical fasciolosis caused by infection with *Fasciola gigantica* is regarded as the most important single helminth infection of ruminants (Meaney *et al.*, 2002) and still constitutes as one of the major economic and health problems. It is considered an important limiting factor for livestock productivity (Khan *et al.*, 2009). Parasitism by *F. gigantica* costs an estimated U.S. \$3.2 billion annually (Spithill *et al.*, 1999). In Egypt, the economical losses have been determined at 484.5 million LE per year, that value is likely to be much higher now. Humans are often infected in communities where there is close human-ruminant interaction, such as in some South American communities, Egypt and Iran (Mas-Coma, 2005). A value of 830,000 individuals in Egypt is the estimated number of fasciolosis cases

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(Haseeb *et al.*, 2002). It is worth mentioning that the number of reported clinical cases and of infected people identified during epidemiological surveys has been increasing since, 1980 (Dalton, 1999).

Fasciolosis could be partially controlled by periodic treatment of animals in endemic areas with drugs, among which triclabendazole was reported to be highly effective against both juvenile and adult flukes (Fairweather and Boray, 1999) even though resistance has been observed in sheep and cattle from different parts of the world (Alvarez-Sanchez *et al.*, 2006; Keiser *et al.*, 2005). In view of the cost and possible emergence of drug resistance, a better preventive measure would be the development of vaccines which could either completely prevent the infection or arrest the development of the parasites at certain stages of their life cycle. Recent vaccine trials have produced promising and interesting results. There are some groups of antigens that have demonstrated certain protective capacity (Spithill and Dalton, 1998). One of the most promising candidates has been Glutathione S-Transferase (GST) which is enzyme with a detoxifying activity (Sexton *et al.*, 1990; Morrison *et al.*, 1996). The GSTs from *Fasciola hepatica* had been found to be effective for vaccinating sheep and cattle against fasciolosis. This helminth contained at least seven GST isoforms, of which four had been cloned. The cloned isoforms (Fh51, Fh47, Fh7 and Fh1) all belonged to the mu class of GSTs (Rossjohn *et al.*, 1997).

In earlier study, the researchers had evaluated, with positive results, the protective capacity of *F. gigantica* GST against experimental infection in goats (Degheidy *et al.*, 2008). Immunization with native *F. gigantica* GST had very variable effects, ranging from no significant reduction in fluke burden in cattle (Estuningsih *et al.*, 1997) to lowering it by more than 60% in goats (Degheidy *et al.*, 2008). This difference in efficacy of GST formulations might be attributed to host species, whereby each host/parasite system was unique and could require its particular vaccination formulation and application protocol (Degheidy *et al.*, 2008).

Reasons for the variability and the mechanism by which immune responses to *F. gigantica* GST lower fluke burdens are unclear. On the other hand, the tegument of the parasite is one of the major targets for vaccines since it produces and releases a number of antigens that can stimulate the immune responses in hosts (Sobhon *et al.*, 1998). Furthermore, the tegument plays roles in maintaining the parasite's homeostasis, such as the absorption and exchange of nutritive and waste molecules and the regulation of ionic equilibrium between the interior of the parasite and the surrounding host fluid (Fairweather *et al.*, 1999). It often exhibits various immune evasion strategies such as antigenic variation and molecular mimicry (Crampton and Vanniasinkam, 2007). A complete understanding of the micromorphological features of the tegument is hence important in developing any vaccine that can damage the parasite's tegument. As a consequence of the previous results obtained using *F. gigantica* GST vaccine in goats, the present study had been carried out to assess, for the first time, tegumental changes in adult *F. gigantica* recovered from goats after immunization with GST antigen by means of Scanning Electron Microscopy (SEM).

MATERIALS AND METHODS

The study was undertaken during the period from March 2009 to November 2009, at National Research Center, Egypt.

Purification of *F. gigantica* GST Antigen (FgGST)

Adult *F. gigantica* worms were collected from infected buffaloes slaughtered at Cairo abattoirs and were used for GST preparation as previously described by Degheidy *et al.*

(2008). Briefly, adult worms were homogenized on ice in triton-EDTA buffer, pH 8. The suspension was centrifuged at 15000 xg for 30 min and the supernatant was used for the purification of GST by affinity chromatography on glutathione sepharose 4B (Pharmacia Biotech). The worm extract was filtered through the column and the bound FgGST was eluted with 5 mM glutathione in 150 mM tris buffer; pH 9.6. The FgGST was neutralized to pH 7.0 by adding drop wise 2 M tris, pH 6.0 (5 drops per 1.5 mL fraction) and dialyzed against PBS and protein concentration was determined (Lowry *et al.*, 1951).

Immunization of Goats

Eight goats, 5-6 months old, were used in this study. These animals were from a farm free of fasciolosis. Nonetheless, faecal samples were examined to confirm the absence of infection of *F. gigantica* as well as other helminthes. The goats were divided into two groups. The first group comprised four goats, considered as adjuvant control, while the second group comprised four goats for immunization with GST antigen. For the first and second immunization (4 weeks-apart), GST (triton-EDTA buffer for control goats) were emulsified in complete and incomplete Freund's adjuvant (Sigma Chemical Co., USA), respectively, according to Ramajo *et al.* (2001). Each goat of Group 2 received 100 µg GST/goat/immunization, intramuscularly, in two different sites.

Challenge

One hundred and twenty five *F. gigantica* metacercariae were inoculated by the oral route into each goat; two weeks post second immunization. The metacercariae were prepared in *Lymnaea cailliaudi*, intermediate host of *F. gigantica* previously infected with *F. gigantica* miracidiae at Parasitology and Animal Diseases Department, National Research Center Cairo, Egypt. All goats were humanely slaughtered for fluke burden determination, 15 weeks after parasite challenge, according to Nambi *et al.* (2005).

Measurements Technique

Measurements, including body length and width, of adult flukes recovered from both groups were made according to method proposed by Valero *et al.* (1996) using a computer image analysis system (ELICA QWin 500, Cambridge, England).

SEM Observations

The apical cone and mid-body region of adult flukes were fixed intact for 12 h in a 3:1 mixture of 4% (w/v) glutaraldehyde in 0.12 M-Millonig's buffer, pH 7.4 and 1% aqueous osmium tetroxide. The specimens were washed repeatedly in cold double-distilled water, dehydrated through a graded series of acetone, dried in a critical point drying machine using liquid carbon dioxide, mounted on aluminum stubs and coated with gold-palladium. The specimens were viewed in a Jeol scanning electron microscope (Jeol Corp., Mitaka, Japan) operated at 15 kV.

Statistical Analysis

Student's t-test was used to analyze the statistical significance of differences between mean values from experimental and control values.

RESULTS

Fluke Recoveries and Sizes

As shown in Table 1, the statistically significant results were obtained when the goats were immunized with GST and challenged 2 weeks after last immunization revealing 64.1%

Table 1: Protective value of GST against challenge *F. gigantica* infection in goats

Animal group	Mean±SD			
	Worm burden	Worm length (mm)	Worm width (mm)	Worm reduction (%)
Adjuvant control	25.75±3.31	43.33±1.35	10.0±1.0	-
GST immunized	9.25±1.26*	26.00±1.0*	4.5±0.5*	64.1

*Highly significant in comparison with adjuvant control group ($p < 0.0001$, t-test)

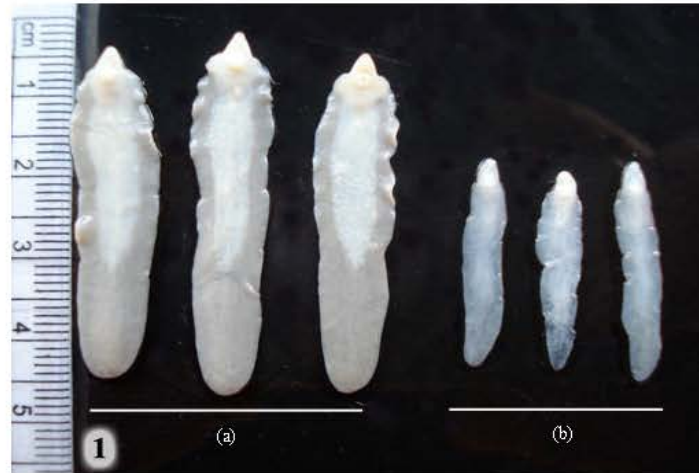


Fig. 1: Comparative size of adult flukes in GST immunized and adjuvant control goats (a) adjuvant control and (b) GST immunized

worm burden reduction over adjuvant control. Equally important, GST elicited highly significant ($p < 0.0001$) decrease in the size of the recovered flukes (Fig. 1a, b).

SEM of Adult Flukes

Control Specimens

All *F. gigantica* adult worms recovered from unvaccinated goats had a normal appearance and no alteration of the oral and ventral suckers or the tegument was visible (Fig. 2a-d). The surface morphology of the adult flukes was recently described in the earlier study (Shalaby *et al.*, 2009). However, a brief description of some of the important features was necessary to assess the changes resulting from immunization with GST. The SEM of the apical cone surface of the normal adult fluke revealed smooth oral and ventral suckers with thick rims covered with transverse folds (Fig. 2a, b). The entire surface of the tegument, with the exception of the rims of both suckers, was seen to be covered in spines. The spines from the anterior regions had finger-like protrusions at their tips (Fig. 2c,d).

Effect of GST Vaccine

The flukes recovered from the bile ducts of GST vaccinated goats were moving less than normal, appeared pale with no evidence of gut contents (Fig. 1). The SEM analysis of these flukes revealed evidence of severe swelling of the tegumental surface covering and between the spines in the apical cone region in the majority of the specimens examined. This tegumental swelling showed regional variations in its severity (Fig. 3a). Moderate swelling

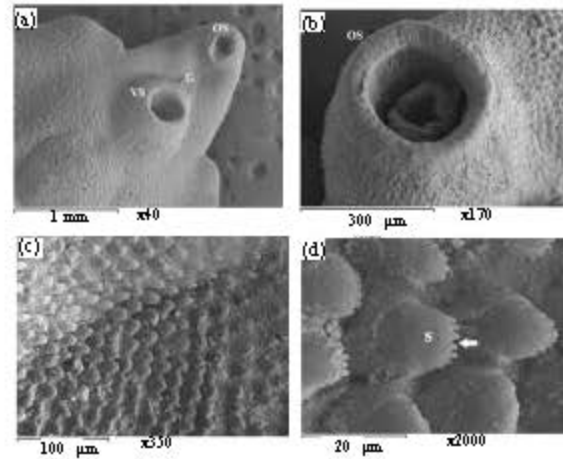


Fig. 2: Scanning electron micrographs (SEMs) of adult *F. gigantica* recovered from adjuvant control goat. (a) SEM of the apical cone and the anterior mid-body regions showing smooth oral and ventral suckers with thick rims. The surface of the fluke is covered with spines. OS, oral sucker; VS, ventral sucker; G, gonopore. (b) SEM of the oral sucker showing tegumental spines projecting beyond the surface. OS, oral sucker. (c) SEM of the tegument at the lateral margin of the oral cone showing broad and flat numerous spines. (d) SEM of the spines at higher magnification showing finger-like protrusions at their tips. S: spine

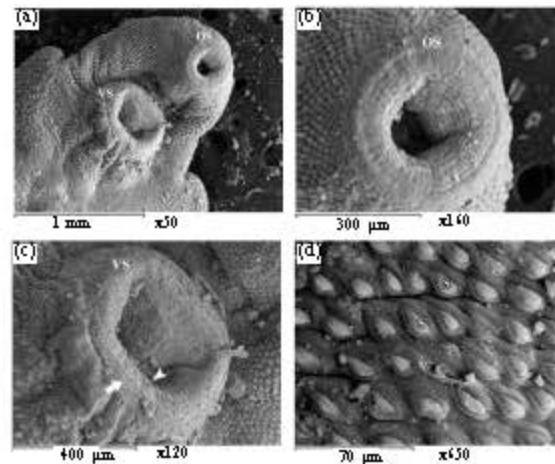


Fig. 3: SEMs of adult *F. gigantica* recovered from GST immunized goat. (a) SEM of the apical cone and the anterior mid-body regions revealing tegumental swelling with regional variations in its severity. OS, oral sucker; VS, ventral sucker. (b) SEM of the oral sucker. Note a moderate swelling of the tegument surrounding the sucker. OS, oral sucker. (c) SEM of the ventral sucker showing irregular swellings and a number of lesions (arrow) that was surrounded by an area of considerable blebbing (head arrow). VS, ventral sucker. (d) SEM of the central region of the oral cone. Note a severe tegumental swelling between the spines and also that covering the spines so that only the tips of the spines were visible. S: spine

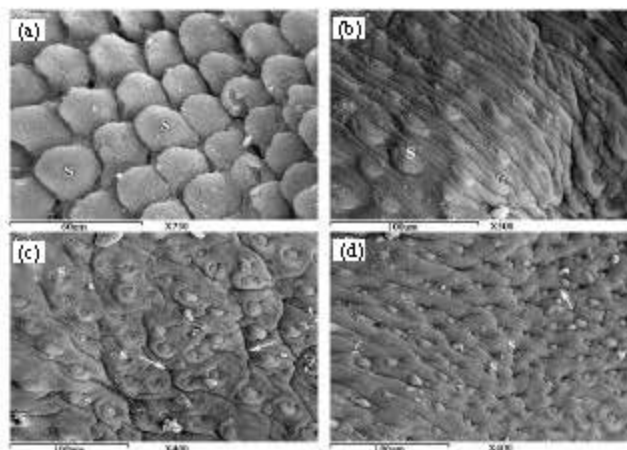


Fig. 4: SEMs of adult *F. gigantica* recovered from GST immunized goat (a) SEM of the lateral margin of the oral cone showing less severe swelling along the lateral margin and the spines themselves were barely visible. S, spine. (b) SEM of the anterior mid-body region, directly behind the ventral sucker. In this specimen, no spines were visible as they had become completely submerged by the extremely swollen tegument. S, spine. (c, d) SEM of the mid-body region and its lateral margin, respectively, revealing widespread swelling and extensive furrowing (arrows) of the tegument. The spines were almost submerged by the swollen tegument surrounding them. S: spine

of the tegument surrounding the oral sucker was observed (Fig. 3b). While, the most severe disruption was observed in the ventral sucker and took the form of irregular swellings and blebs scattered over the tegument surrounding the sucker (Fig. 3c). In addition, it might bear a number of lesions and was surrounded by an area of considerable blebbing (Fig. 3c). In the central region of the oral cone, the tegument between the spines and also that covering the spines was severely swollen so that only the tips of the spines were visible (Fig. 3d). The swelling was less severe along the lateral margins of the oral cone. At a time, the spines themselves were barely visible and some of them showed blebbing on their upper surfaces (Fig. 4a). In the anterior mid-body region, directly behind the ventral sucker, no spines were visible as they had become completely submerged by the extremely swollen tegument (Fig. 4b). The mid-body region (Fig. 4c) and lateral margins (Fig. 4d) of both surfaces displayed widespread swelling and extensive furrowing of the tegument. The spines were almost submerged by the swollen tegument surrounding them. On the dorsal surface, the disruption was of a similar severity to that seen on the ventral surface. In extreme cases, there was severe disruption of the apical cone region (Fig. 5a). The oral sucker showed extensive furrowing and broken and flaky tegument (Fig. 5b). Damage to gonopore was so extreme that little recognizable structure remained (Fig. 5c). The tegument surrounding the ventral sucker was so swollen that it was difficult to distinguish the spines as they had become completely submerged beneath the surrounding tegument (Fig. 5c). This gave the surface an extremely smooth appearance. On the other hand, the spines displayed extreme disruption; those in the lateral margins of the apical cone were ruptured (Fig. 5d), while spines in the anterior mid-body region appeared to be flaking off (Fig. 5e) and also showed severe blebbing over their upper surfaces with distortion of their tips (Fig. 5f).

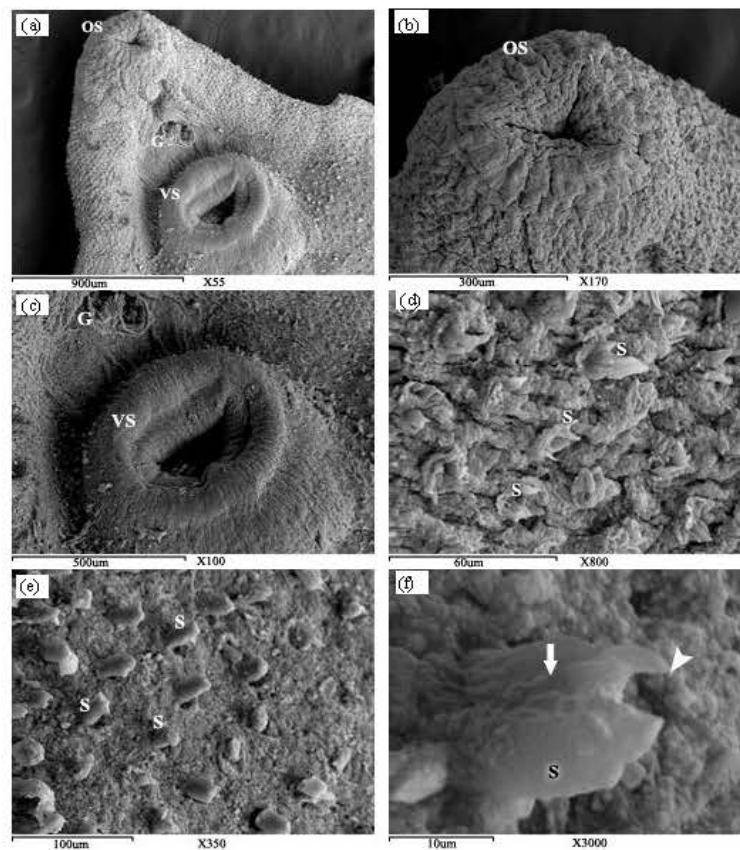


Fig. 5: SEMs of extreme cases of adult *F. gigantica* recovered from GST immunized goat (a) SEM of the apical cone region showing severe disruption. OS, oral sucker; VS, ventral sucker; G, gonopore. (b) SEM of the oral sucker showing extensive furrowing and broken and flaky tegument. OS, oral sucker. (c) SEM of the ventral sucker showing extremely smooth surface. Damage to gonopore was so extreme that little recognizable structure remained. VS, ventral sucker; G, gonopore. (d) SEM of the lateral margins of the apical cone showing ruptures of spines. S, spine. (e) SEM of the anterior mid-body region. In this specimen, the spines appeared to be flaking off and the tegumental syncytium appeared to be distorted. S, spine. (f) A higher power SEM of the spine showing severe blebbing over its upper surface (arrow) with distortion of its tip (head arrow). S: spine

DISCUSSION

Earlier studies showed that Glutathione S-Transferases (GSTs) were found in high levels in *Fasciola* spp. parasite and the level of this enzyme was approximately 4% of the total soluble protein (Brophy *et al.*, 1990; Howell *et al.*, 1988). Besides, immunolocalisation studies using antisera generated to native GST initially showed that GSTs were distributed in the parenchyma, tegument and gut tissues of adult fluke (Howell *et al.*, 1988; Wijffels *et al.*, 1992). But, was how a cytosolic protein that was not secreted by the fluke became exposed

to the host's immune system. Juvenile and adult flukes were thought to continually shed their teguments to evade the host immune system (Hanna, 1980). As GST was located at the surface or just below the surface of the fluke, it might be released into the circulation as the tegument was sloughed. Tegumental turnover at an elevated rate facilitated the release of GST. The ES proteome from dead cultured *F. hepatica* fluke confirmed that the release of GST *in vitro* was not a specific biological secretion but a physical degradation process (Morphew *et al.*, 2007). Such high levels of GSTs inferred an important role for these enzymes in helminth homeostasis and survival and that might be related to the naked tegument of helminth parasites and their potential exposure to a wide range of xenobiotics. Apart from reaction from their endogenous metabolism, GSTs of helminth parasite might protect against exogenous free radical damage or xenobiotics as a result of immune effector mechanisms from the host directed at the parasite (Brophy and Pritchard, 1994). The present study confirmed earlier results that *F. gigantica* GST antigen displayed significant reduction in both worm burden and development in vaccinated goats (Degheidy *et al.*, 2008). The reduction in the size of the worms in the vaccinated animals must be interpreted as a sign of protection because it implied a deficiency in their normal physical development (Ramajo *et al.*, 2001). These deficiencies, translated to a decrease of the mass and corporal dimensions of the worms were accompanied, with great frequency, by deficiencies in the biological development. This was reflected in the delay in maturation and in a decrease in the number, or absence, of eggs in the uterus and consequently a reduction in the number of eggs eliminated through the bile and faeces (Degheidy *et al.*, 2008).

In that sense, Preyavichyapugdee *et al.* (2008) used recombinant *F. gigantica* GST (rFgGST26) as a vaccine in combination with Freund's adjuvant to evaluate the induction of immune responses and protection against *F. gigantica* infection in mice. Mice were immunized via subcutaneous, intramuscular or intradermal routes. Strong protection (77-84%) against *F. gigantica* was observed in all routes. Passive intraperitoneal transfer of IgG1 predominating antisera from subcutaneous rFgGST26-immunized donors to naïve recipient mice resulted in 47% protection against *F. gigantica* infection. This suggested that the mechanism of resistance depended on the presence of specific antibody against rFgGST26. Piedrafita *et al.* (2007) demonstrated that resistance to *F. gigantica* infection by Indonesian thin-tail sheep was mediated by parasite specific antibody and effector cells such as monocytes/macrophages and eosinophils in the peritoneal cavity. Moreover, El Ridi *et al.* (2007) found that immunization of apparently naïve sheep with *F. gigantica*-derived Excretory-Secretory Products (ESP) induced antibodies bound to the surface of Newly Excysted Juvenile (NEJ) worms, supporting the contention that many ESP molecules were derived from the surface tegumental membrane and that ESP of juvenile and adult worms were antigenically cross-reactive. More importantly, ESP-specific antibodies were able to mediate attrition of NEJ worms via Antibody-Dependent Cell-mediated Cytotoxicity (ADCC). They added that the protective capacity of ESP could be related to its content of GST. Damage to NEJ worms by ADCC and cytokine-mediated inflammatory responses might provide an explanation for the GST-related highly significant ($p < 0.0001$) decrease in number and reduction in size of challenge *F. gigantica* worms.

Scanning electron microscopy of adult flukes recovered from the bile ducts of GST vaccinated goats revealed evidence of severe swelling of the tegumental surface covering and between the spines. This swelling was believed to be part of the general response of the fluke to a stress situation representing increased efforts on the part of the parasite to shed and replace damaged surface membrane (Stitt and Fairweather, 1993). In this case the stress had been applied by host's immune response following GST immunization. Indeed, such

swelling had been seen after attack by immunoglobulins (Bennett *et al.*, 1980) where, adult *F. hepatica* were placed intraperitoneally into rats which had been sensitized by an oral infection of 20 metacercariae given 3-5 weeks previously. Moreover, tegumental damage, in closely related trematode, *Schistosoma japonicum* adult worms, obtained from mice orally vaccinated with recombinant SjpGST26 was recorded by Yang *et al.* (1997). They noticed varying degrees of tegumental damage in all examined male and female worms obtained from the vaccinated mice, but they did not investigate whether the damage was widespread over the entire surface or localised to discrete regions of the tegument. Secretory granules in the tegument of fluke were thought to be associated with the formation and turnover of surface membrane and the glycocalyx (Hanna, 1980). The parasite apparently reacted to the immune situation by a rapid translocation of tegumental granules from the cells to just below plasma membrane. This might be the parasite's way of releasing damaged membrane by some form of capping mechanism (Bennett *et al.*, 1980). This obvious replacement of surface membrane by the tegument might, therefore, represent the prime mechanism whereby the parasite avoids the host's immune response. However, Fairweather *et al.* (1987) suggested that the area around the spines represented weak spots in the tegument. Where, it had been shown that tubulin, the main component of microtubules, was present in the tegumental syncytium, stretching vertically from the base to the apical surface and horizontally beneath the apex (Stitt *et al.*, 1991). The exception to that was around the spines, where microtubules were only present as a horizontal band beneath the apex. Since transport of tegumental secretory bodies was dependent on microtubules, this area might represent a weak point in the tegumental membrane and the spines tips would show the greatest disruption (Meaney *et al.*, 2002). This might be the cause of spine damage, as evident in this study. The integrity of the apical plasma membrane was essential for nutrient uptake, immunoprotection and osmoregulation (Fairweather *et al.*, 1987) so, any disruption would have serious consequences for the fluke. Once the tegument of the parasite was disrupted, the surfactant properties of bile would also have an effect on the parasite.

What was evident from this study and those cited above was that *F. gigantica* GST antigen had a protective value against fasciolosis in goats. The reduction in fluke burden/size and surface changes might raise the possibility of being GST as a prospective vaccine candidate against fasciolosis.

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