Identification of excretory-secretory products from larval stages of *Ostertagia circumcincta* cultured in vitro by SDS-PAGE and immunoblotting

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Summary

Exsheathing fluid (EF) and excretory-secretory products (ES) of infective third-stage cultured larvae of *Ostertagia circumcincta* were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Five and seven predominant proteins were found in the EF and ES products, respectively. Immunoblotting by sheep pre-infection serum did not react with any of the EF and ES proteins, but the post-infection serum recognized four proteins of 44.5, 41.5, 38 and 24 kDa of the ES products. None of the EF products was recognized by the post-infection serum. Protectivity of the four proteins remains to be determined.

Key words: *Ostertagia circumcincta*, ES products, Sheep, SDS-PAGE, Immunoblotting

Introduction

A major goal of the current immuno-parasitological research on gastrointestinal nematode parasites of sheep is to develop effective vaccines. *Ostertagia circumcincta* is the most frequent species affecting small ruminants in Iran (Eslami and Nabavi, 1976). These nematodes cause serious losses world-wide by impairing weight gain and wool production (Kaufmann, 1996).

The infective stages of *Trichostrongyle* parasites of ruminants are free-living third-stage larvae retaining the second molt cuticle. Upon ingestion by a suitable host, subsequent parasite development is initiated, through casting of the second molt cuticle (Gamble *et al*., 1990) and development (in 48-72 hrs) to the fourth-stage (L4) larvae. During this developmental period, which can be duplicated in vitro, larvae do not actively ingest nutrients (Sommerville, 1966) and do not grow appreciably in size. Following molting to L4, larvae begin feeding, grow rapidly in size, become metabolically active and can no longer be maintained on simple media. Nematode excretory-secretory (ES) products have been shown to have important biological effects (Hotez *et al*., 1990; McKerrow *et al*., 1990; Richer *et al*., 1992). Furthermore, the parasite ES products have been shown to be likely targets for immune (Miller, 1988) and biochemical (Ring *et al*., 1993) control of infections. The objective of the present study was to analyse the exsheathing fluid (EF) and ES products of *O. circumcincta* larvae by SDS-PAGE and to evaluate the immune response of sheep to these products by immunoblotting, following infection.

Materials and Methods

Infected (donor) animals and serum collection

Production of an *O. circumcincta* donor was carried out by implantation of adult parasites into the sheep abomasum through a surgically established cannula (Scott and Mckellar, 1988). Thereafter, 10,000 third-stage larvae (L3) collected from the sheep faecal cultures, were given to a 6-month-old

lamb (Arabian breed) by stomach tube. Pre- and post-infection (seven days after a second infection) sera of the lamb were collected and stored at -20°C.

Faecal culture

To collect L3 (2M), faeces were cultured at 25°C for 10 days. Then, L3 (2M) were recovered and washed by repeated bernannization through 63 µm mesh screens. Larvae were stored in 0.85% saline at 4°C up to 4 weeks (Gamble And Mansfield, 1996).

Preparation of EF and ES products

Larvae were washed in sterile 0.85% saline and Earles' balanced salt solution, supplemented with penicillin G (250 U/ml), streptomycin sulfate (250 µg/ml), chloramphenicol (350 µg/ml) and amphotericin B (0.2 µg/ml) (EBSS-antibiotic) as follows: by centrifugation at 700 × g, the larvae were washed in five changes of 0.85% saline and five changes of EBSS-antibiotic (Douvres and Malakatis, 1977). Larvae were exsheathed by a modification of the process described by Gamble and Mansfield (1996). They were gassed for 20 min with 100% CO_2, shaken at 37°C for 2 hrs and then incubated at 37°C for 18 hrs in the presence of 5% CO_2. This procedure yielded up to 99% exsheathment of the larvae. Exsheathed larvae were separated of cast sheaths and debris by active migration through 38-µm mesh screens and EF preparation was collected. Third-stage larvae, obtained in this way, were axenized again by four 1 hr washing in EBSS-antibiotic (as above) and shaking at 37°C. Axenized larvae were suspended in EBSS-antibiotics (as culture media), placed in a tissue culture flask (T-75) at an approximate concentration of 10,000 per ml and incubated at 37°C in 5% CO_2 for up to 12 days. At 24-hr intervals, culture media was recovered, larvae were washed for 1 hr in EBSS-antibiotic and placed back into the flask with fresh media. The EF and ES products were concentrated 300× and 200×, respectively first on a YM-3 filter (Amicon) by centrifugation at 3000 × g, and then under N_2 flow. Protein content of concentrated EF and ES products were estimated by dye (Coomassi blue G-250) binding protocol (Burtis and Ashwood, 2001). The EF and ES products were stored at -70°C.

Electrophoretic analysis

Samples of EF (300×) and ES (200×) products containing 100 and 90 µg protein/ml, respectively, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970) on a 7.5% separating gel. Prior to electrophoresis, the samples were mixed with an equal volume of a non-reducing sample buffer and boiled for 5 min. For electrophoresis a constant 100 V current was used for 5 hrs. SDS-polyacrylamide gel was stained for protein visualization with a silver staining method.

Immunoblotting

For immunoblotting, proteins of EF and ES products were first electrophoresed on 7.5% SDS-polyacrylamide gel. Western blot was carried out following established protocols (Towbin *et al*., 1979). Proteins were transferred onto a nitrocellulose membrane using tris-glycine-methanol buffer for 3 hrs at 60 V. The membrane was blocked in 1% albumin in PBS for 1 hr at room temperature. After rinsing three times in the washing buffer (PBS-T: PBS, 0.05% Tween20) and once in PBS, the membrane was cut in strips and incubated with pre- and post-infection sera of the donor, diluted 50 times in diluting buffer (PBS-T, 1% albumin) for 1 hr at room temperature and then washed as above. The strips were then incubated with peroxidase-conjugated anti-sheep IgG (Sigma) for 1 hr at room temperature and developed in a mixture of H_2O_2-diaminobenzidine.

Results

Protein profiles of EF and ES products

EF and ES products of cultured L3 of *O. circumcincta* were analysed by SDS-PAGE. The gel was stained with a silver impregnation method. As shown in Fig. 1, silver staining revealed the presence of several protein bands ranging in molecular weight from 200 to 18.4 kDa. However, the

predominant EF protein bands were at 154, 82, 57, 35.5, 31 and those of ES were at 154, 82, 57, 44.5, 41.5, 35.5 and 31 kDa.

Immunoblotting

The pre-infection serum did not react with any of the EF and ES protein bands, but as indicated in lane 4, the post-infection serum recognized four proteins of 44.5, 41.5, 38 and 24 kDa of the ES products. None of the EF products was recognized by the post-infection serum (Fig. 2).

Fig. 1: Protein profile of exsheathing fluid (EF) of the third-stage larvae and excretory-secretory products (ES) of cultured larvae *O. circumcincta.* ST represents the standard molecular weight proteins

Fig. 2: Immunoblot of exsheathing fluid (EF) and excretory-secretory (ES) products. Lanes 1 and 2: EF reacted with pre- and post-infection sera, respectively. Lanes 3 and 4: ES reacted with pre- and post-infection sera, respectively

Discussion

An important aspect of research on immunity to *O. circumcincta* is to identify which parasite surface/ES antigens influence the outcome of infection and to determine how the immunological effects of these antigens are medicated. McGillivery *et al*., (1989) identified and extracted a 31 kDa glycoprotein (Gp31) antigen of *O. circumcincta* by the serum of resistant sheep. Characterization of this antigen revealed that it is stage-specific, located internally in secretory organelles and appeared to be released by larvae during growth in vitro (McGillivery *et al*., 1990). Subsequent study showed that the Gp31 was immunogenic, but it conferred only up to 50% protection (McGillivery *et al*., 1992). Wedrychowicz *et al*., (1992) found that surface antigen of the third-stage larvae of this nematode conferred over 70% protection when administered with beryllium hydroxide as an adjuvant. To understand the immunogenic properties of surface proteins of L4 and adult parasites, Wedrychowicz *et al*., (1994) further studied these antigens. It revealed that surface antigens of both adult and L4 of the nematode induced high levels of serum antibodies. Relating on the number of infections, up to 12 proteins in adults ES products and one protein of 64 kDa in L4 ES product were recognized by infected sheep serum. In the present work, we studied the immunogenicity of EF and ES products of cultured L3 larvae. The results of immuno-blotting showed that at least four proteins of 44.5, 41.5, 38 and 24 kDa in ES products were recognized by infected sheep serum. Reaction of infected sheep serum with some of the ES products indicated that these proteins had been shed from the larvae in vivo. The active shedding of surface/ES proteins may have profound implication for the development of host protective immune response to helminth parasites. Therefore, the immunogenic ES products identified in the present study may be the potential candidates for molecular vaccine development. Further studies are necessary to assess the relevance of these ES antigens in protection.

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