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Ultrastructural localization of Ani s 1, a major allergen from the fish parasite *Anisakis simplex*

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Abstract No data about the nature or function of the first major *Anisakis simplex* allergen, named Ani s 1, is available. The aim of this study was to investigate the ultrastructural localization of this protein, to obtain further information about it. Ani s 1 was detected in secretory granules of the excretory gland and occasionally lining the main excretory canal. Our results suggest that Ani s 1 could be a secretory product and could have an enzymatic function related to mechanisms of infection.

Introduction

Anisakis simplex is a nematode parasite frequently found in a great diversity of fish and cephalopod species commonly consumed by man. In infected patients, *Anisakis* may produce a gastrointestinal disease or acute allergy symptoms, such as urticaria, angioedema and anaphylactic reactions (Ishikura et al. 1993).

In our previous work, a clinically relevant allergen, Ani s 1, was isolated from *A. simplex* larvae (Moneo et al. 2000). No data about the nature or function of this protein were obtained, because of the lack of similarity to other proteins when studying the amino acid sequence. An optical immunohistochemical localization of

Ani s 1 was performed with the serum of a monosensitized patient; and it was found only in the excretory gland of the parasite. The aim of the present study was the ultrastructural localization of this major allergen, using a specific polyclonal antibody in order to obtain further information about this interesting protein.

Materials and methods

Antiserum production

For the preparation of experimental anti-Ani s 1 immunoserum, immunization was performed in adult male white New Zealand rabbits. Subcutaneous injections of 30 µg of purified Ani s 1, as described by Moneo et al. (2000), with complete Freund's adjuvant were given fortnightly. After four injections, the animals were bled and the sera collected.

A *Pseudoterranova* sp. extract was prepared with larvae obtained from muscle tissue of Atlantic halibut (*Hippoglossus hippoglossus*). About 200 larvae were ground in a mortar with 10 ml of PBS. The mixture was centrifuged for 5 min at 1,000 g. The supernatant was mixed with anti-Ani s 1 antiserum at a 1:1 ratio and incubated for 1 h at room temperature before the immunohistochemical reaction, in order to enhance its specificity and avoid cross-reactivities. After absorption, this antiserum recognized only Ani s 1 in a total crude parasite extract, as shown by immunoblotting.

Electron microscopy

Anisakis simplex larvae, obtained from muscle tissue of *Micromesistius poutassou*, were chopped into 2-mm pieces and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 h at 4 °C. After fixation, the larvae were washed for 4 h in cold buffer and postfixed in 1% osmium tetroxide in cacodylate buffer for 2 h. After dehydration, the larvae were embedded in Epon 812 resin. Ultrathin sections were mounted in grids, stained with uranyl acetate and lead citrate and observed with a Hitachi 7000 electron microscope.

Electron microscopy immunohistochemistry

Fresh *A. simplex* larvae were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PBS (pH 7.2) for 24 h at 4 °C, dehydrated with an ethanol series and embedded in LR White resin

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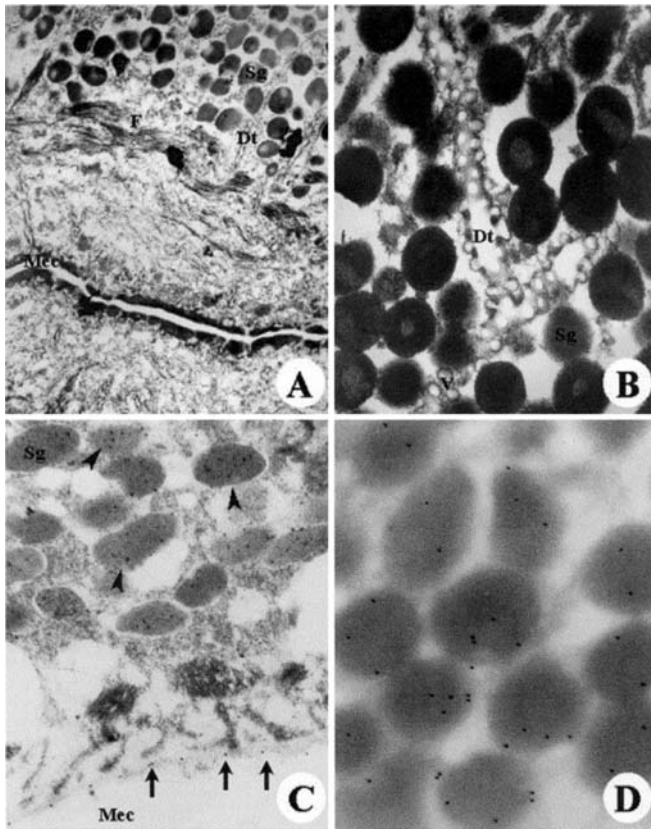


Fig. 1a–d Electron micrographs of excretory gland of *Anisakis simplex*. **a** Section through portion of cytoplasm showing secretory granules (Sg), the main excretory canal (Mec) and filaments (F). **b** High magnification of secretory granules (Sg), drainage tubules (Dt) and vesicles (V). **c** Immunogold staining of Ani s 1. Secretory granules are positive (arrowheads); and a weakly positive reaction is occasionally seen lining the lumen of the main excretory canal (arrows). **d** High magnification of secretory granules showing a positive reaction to Ani s 1

(London Resin Co., UK; Newman et al. 1983). Ultrathin sections, supported on copper grids, were incubated with anti-Ani s 1 polyclonal antibody diluted 1:100 in PBS containing 0.5% BSA and 0.05% Tween 20 for 1 h at 37 °C. After washing with PBS, the sections were incubated first with gold buffer (20 mM Tris, 225 mM sodium chloride, 0.1% Tween 20, 0.1% BSA, 20 mM sodium azide, pH 8.2) for 15 min at 37 °C and then with an anti-rabbit antibody conjugated with 15-nm gold particles (Biocell Research Laboratories, UK) diluted 1:50 with gold buffer for 1.5 h at 37 °C. Finally, the grids were washed with gold buffer and distilled water, stained with 1% uranyl acetate and lead citrate and were observed with a Hitachi 7000 electron microscope.

Simultaneously, negative controls were processed with non-immune rabbit serum instead of primary anti-Ani s 1 antibody.

Results and discussion

Ani s 1 recognised by polyclonal antiserum was present in secretory granules of the excretory gland (Fig. 1). The main excretory canal was usually negative and only occasionally was there a weakly positive lining of the lumen. Drainage tubules, vesicles, filaments and other structures of the excretory gland were not stained by the

immunogold. No positive reaction was observed in larval structures outside of the excretory gland. In the negative controls, no structure showed immunostaining for Ani s 1.

At present, the molecular and biochemical characterization of secretory/excretory proteins is a key point to understanding the pathology induced by *Anisakis simplex* (Kennedy 2000). In our previous work, we could demonstrate that allergen Ani s 1, a 24-kDa protein, had a high clinical relevance. Of 20 symptomatic subjects, 85% had detectable levels of specific IgE directed against this protein. Furthermore, around 5% of these individuals only recognized this protein in the crude parasite extract (Moneo et al. 2000). According to these data, this protein was considered as a major allergen, because an allergen is considered major when recognized by more than 50% of sensitized subjects (Liebers et al. 1996).

In spite of the clinical relevance of Ani s 1, no data about its biological function are available at the moment. The study of its N-terminal amino acid sequence and comparison with protein sequences in databases did not clarify its role (Moneo et al. 2000).

Ultrastructural studies of the excretory gland of *Anisakis* provide morphological evidence to suggest osmoregulatory and/or excretory functions (Lee 1973). Results obtained in our present work suggest that Ani s 1 could be a secretory product excreted by the parasite under certain stimuli. Taking into consideration that several hydrolases have been characterized in *Anisakis* (Morris and Sakanari 1994) and other nematodes with a similar excretory system (Romanowski et al. 1973), it can not be discarded that Ani s 1 could have an enzymatic function related with infection, migration, or survival mechanisms for the parasite.

References

- Ishikura H, Kikuchi K, Nagasawa K, Ooiwa T, Takamiya H, Sato N, et al (1993) Anisakidae and anisakidosis. *Prog Clin Parasitol* 3:43–102
- Kennedy MW (2000) Immune response to *Anisakis simplex* and other ascarid nematodes. *Allergy* 55 [Suppl 59]:7–13
- Lee H-F, Chen I-L, Lin R-P (1973) Ultrastructure of the excretory system of *Anisakis* larva (Nematoda: Anisakidae). *J Parasitol* 59:289–298
- Liebers V, Sander I, Van Kampen V, Raulf-Heimsoth M, Rozynek P, Baur X (1996) Overview on denominated allergens. *Clin Exp Allergy* 26:494–516
- Moneo I, Caballero ML, Gómez F, Ortega E, Alonso MJ (2000) Isolation and characterization of a major allergen from the fish parasite *Anisakis simplex*. *J Allergy Clin Immunol* 106:177–182
- Morris SR, Sakanari JA (1994) Characterization of the serine protease and serine protease inhibitor from the tissue penetrating nematode *Anisakis simplex*. *J Biol Chem* 269:27650–27656
- Newman GR, Jasani B, Williams ED (1983) A simple post-embedding system for the rapid demonstration of tissue antigens under the electron microscope. *Histochem J* 15:543–555
- Romanowski RD, Rhoads ML, Malakatis GM (1973) The secretory nature of the excretory gland cells of *Stephanurus dentatus*. II. Presence of hydrolytic enzymes. *Exp Parasitol* 34:40–43