Research Note

Anisakis simplex (Nematoda: Ascaridoidea): Formation of Immunogenic Attachment Caps in Pigs

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ABSTRACT: Anisakis simplex larvae maintained in simple salt solutions were observed attached to the surface of a petri dish in vitro by a translucent cap. Previous reports attributed the formation of these caps to contributions by human serum. Dissolved caps reacted in Western blot with the sera of rabbits sensitized with A. simplex excretory–secretory products. Caps were demonstrated in vivo in the mucosae of experimentally infected miniature swine; they were partially composed of larval excretory–secretory products and remained embedded in stomach mucosae after removal of the invasive larvae.

KEY WORDS: Nematoda, Ascaridoidea, anisakiasis, Anisakis simplex, nematode larvae, attachment cap, calotte, excretory–secretory products, immunogenic, miniature swine, Sus scrofa, stomach mucosae.

Formation of a calotte or cap by approximately 16% of Anisakis simplex (=A. marina) larvae isolated from herring and incubated in tubes of human serum at 37°C was described by van Thiel (1967). On the basis of acrolein–Schiff staining, the clear cap was identified as consisting of albumin (P. van Duyn in van Theil, 1967). The cap was found with sera from persons unlikely to have experienced any exposure to anisakine nematodes and with the sera of anisakiasis patients. Noting that parasite excretory products were granular, van Thiel postulated that the clear cap was at least partly of host origin. We have frequently observed the formation of similar structures in vitro in simple salt solutions such as phosphate-buffered saline (PBS) or Ringer’s solution and in more complex media such as Medium 199 (with and without 10% fetal bovine serum). This observation suggested that structures similar to van Thiel’s “calotte” could be formed by parasite products alone and raised the question as to whether formation of these structures accompanied attachment to and invasion of stomach mucosae by A. simplex larvae.

In this report, culture conditions were the same as those described previously (Raybourne et al., 1986), in which PBS (pH 7.2) with 1% glucose was used. For experimental exposure, Hormel–Hanford miniature pigs (Sus scrofa) were maintained in masonry kennels with indoor–outdoor runs (indoor winter heat), fed USDA ration 1160 at 25 g/kg/day, and allowed water ad libitum. The pigs were treated with the anthelmintic Atgard (Fermenta Animal Health Products, St. Louis, Missouri) at least 2 wk before experimental exposure. Third-stage larvae of A. simplex were isolated from the viscerae of bocaccio (Sebastes paucispinis) by digestion with 2% pepsin-HCl (pH 2) at 35°C. Larvae were maintained at 2–6°C before pigs were experimentally infected. Food was withheld from the pigs for 24 hr before exposure. Each pig was then fed 100 larvae by stomach tube. Pigs were necropsied 24 hr after exposure; tissues were fixed in Bouin’s fluid, dehydrated, and sectioned by the method of Hinton et al. (1987).

Rabbit antiserum to larval excretory–secretory (ES) proteins was produced by subcutaneous injection of 60 μg ES protein in Freund’s complete adjuvant, followed in 2 wk by 60 μg ES protein in Freund’s incomplete adjuvant. Control serum was obtained from the rabbits before immunization. ES proteins used for immunization were collected as described previously (Raybourne et al., 1983).

Sections cut at 6–10 μm thickness were rehydrated in PBS and incubated on glass slides with a 1:40 dilution of rabbit anti-ES serum or control serum for 1 hr at room temperature. The sections were washed for 30 min in 3 changes of PBS and incubated with a 1:40 dilution of fluorescein-labeled goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Missouri) for 1 hr. The sections were rinsed in PBS, mounted in 90% glycerol–10% PBS, and examined with a Leitz fluorescence microscope. Photographs were taken with an automatic metering device designed to provide a uniform level of exposure (Wild, Heerbrugg, Switzerland).

Attachment caps adhering to the bottom of a petri dish were rinsed 3 times with PBS and made soluble by heating in PBS with 10% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. The samples were separated on a 12% SDS-PAGE

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Figures 1–4. Longitudinal sections through attachment caps of *Anisakis simplex* larvae in fundus of pig stomach. Scale bars = 0.1 mm. 1. Anterior end, 3rd-stage larva stained by modified Shorr’s stain after Vetterling and Thompson (1972). Note that cationic sites in attachment cap are deeply stained by Biebrich scarlet stain (bright orange area surrounding anterior end) and differentiated from the adjacent red blood cells (rust colored) stained by orange G. 2. Lesion showing attachment cap from which nematode was physically removed. Similar structures are encountered in mucosae of infected pigs. Azure A–Eosin B stain after Lillie (1965). 3. IFA staining of attachment site with rabbit antiseraum to *Anisakis simplex* and fluoresceinated goat antibody to rabbit serum. Exposure time 30 sec. 4. Same attachment site as in Figure 3 (5 intervening sections) treated with presensitization serum and goat fluoresceinated antibody to rabbit antibody. Exposure time 2 min 30 sec. Exposure demonstrates yellow autofluorescent material and background green fluorescence associated with IFA staining.
gel, electroblotted onto a nitrocellulose membrane, and reacted with a 1:50 dilution of anti-ES serum or control serum (Raybourne et al., 1986). Bands were developed with protein A peroxidase (Boehringer Mannheim, Indianapolis, Indiana) diluted 1:1,000. One lane of the gel/blot contained 10 µg ES material.

When crowded (> 100 larvae per 75-mm petri dish), a greater proportion of the worms appeared to form caps than when fewer organisms were present (≤ 50 larvae per petri dish). Grossly, all the larvae in a culture appeared to be attached to the petri dish by their anterior ends. Individual nematodes were extracted by pulling with forcepts, leaving the cap attached to the dish. Caps of nematodes that broke were not used. The caps, or groups of caps, were translucent and refractile, and could be scraped from the petri dish. They appeared to be similar to the structures described by van Thiel (1967) which formed in the presence of, and were thought to be partially composed of, normal human serum. The formation of caps by A. simplex larvae in media without serum or other protein additives suggests that caps may be formed entirely from material of parasite origin.

Similar structures apparently form in the stomach mucosa of experimentally infected pigs. In vivo, the cap surrounds the rudimentary lips and anterior end of the stage 3 larvae (Fig. 1). Caps were also observed around the lips and anterior ends of A. simplex stage 4 larvae in pigs. The flaring shape and knoblike handle at the apex suggested that the cap aids the parasite in maintaining attachment to the stomach mucosa. Some components of these structures remained at attachment sites that the worms vacated or from which they were removed mechanically (Fig. 2). Caps with nematodes are frequently observed in tissue sections of infected pigs. Caps were observed in more than 25 lesions with worms that were sectioned, stained, and examined in detail. At least some of the components of the cap were of parasite origin, as demonstrated by indirect fluorescent antibody (IFA) staining of the cap in vivo with anti-ES serum (Figs. 3, 4). Although the cap proteins could not be resolved on SDS gels as clearly as were the ES proteins, some of the components of the caps formed in vitro appeared to be equivalent to parasite ES proteins, based on their reactivity in Western blots (Fig. 5). These ES proteins, present in the supernatant fluid of larval maintenance cultures, are important immunogens in human anisakiasis (Raybourne et al., 1986). These caps may provide a residual of antigenic material that may prolong the inflammatory response observed after nematodes can no longer be detected in chronic human anisakiasis, or after surgical removal of the larva (Oshima, 1972).

The mechanism by which the caps are formed is not known. Proteolytic enzymes present in the anterior secretory structures and ES products of A. simplex larvae (Ruitenbergh and Londersloot, 1971; Mathews, 1982, 1984) may be involved. These proteases may contribute to the formation of attachment caps by a mechanism similar to that of the proteases which function to form a gel in the limulus amoebocyte lysate assay (Shikikura et al., 1983).

We thank Roger Mathews and the animal handlers at the Beltsville Research Facility of the Food and Drug Administration for their help in handling and caring for the pigs.

**Literature Cited**


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Research Note

Binding of Concanavalin A to Areas Compatible with the Locations of the Amphids and Phasmids of Larvae of Dirofilaria immitis (Nematoda: Filarioidea) and Toxocara canis (Nematoda: Ascaridoidea)

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ABSTRACT: Third-stage and 4th-stage larvae of Dirofilaria immitis and infective-stage larvae of Toxocara canis were fixed in 10% formalin and incubated with the fluorescein-labeled lectin, concanavalin A. The larvae were then washed, mounted on slides, and examined using an epifluorescent microscope. Upon examination, each larva was found to have 2 bright areas of fluorescence on both the anterior and posterior ends; these areas of fluorescence were not present on worms that had been incubated with fluorescein-labeled concanavalin A that had been preincubated with methyl α-D-mannopyranoside. The locations of the areas of fluorescence on these worms were found to be consistent with the described locations of the amphids and phasms of these larval stages.

KEY WORDS: concanavalin A, amphids, phasmsids, Dirofilaria immitis, Toxocara canis, Nematoda, Filarioidea, Ascaridoidea, lectins, chemoreception, cuticle, morphology.

As part of a study on various surface characteristics of larval nematodes, 3rd- and 4th-stage larvae of Dirofilaria immitis and infective larvae of Toxocara canis were incubated with the fluo-