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Gnathostoma binucleatum: Excretion–secretion antigen analysis obtained from advanced third-stage larvae in in vitro culture

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Abstract

The paper describes an introductory characterization of antigenic stimulation of excretion–secretion products (ESP) of *Gnathostoma binucleatum* advanced third-stage larvae cultured in vitro and proteinases present in this products. Excretory and secretory proteins were obtained after 10 larvae were maintained in 5% CO_2 RPMI medium. The supernatant was collected each week for two months. The proteins were dialyzed, concentrated, and separated in 10% SDS–PAGE gels under reducing conditions and transferred to nitrocellulose paper for immunoblot analyses. *G. binucleatum* immunized mice serum was used to determine protein antigenicity. Four proteins of 40, 80, 120, and 208 kDa persisted for two months and three proteins, 80, 120, and 208 kDa were recognized for antibodies of mice. In SDS–PAGE gelatin substrate gels ESP resolved as two proteins with molecular weight of 80 and 208 kDa that were sensitive to a metalloproteinase inhibitor, and thus it may be inferred that they might be used for diagnosis of gnathostomiasis.

Index Descriptors and Abbreviations: *Gnathostoma binucleatum*; Nematode; $AdvL_3$, advanced third-stage larvae; Protease; Cysteine protease; Serine protease; Metalloprotease; ESP, excretory–secretory products; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IgG, immunoglobulin G; 1-*trans*-epoxy succinyl-leucylamide-4-guanidinol-butane (E64); Benzamidina; EDTA, ethylenediamine tetraacetic acid

1. Introduction

Gnathostomiasis is a systemic parasitic disease transmitted by raw or uncooked meat of intermediate hosts such as crustaceans and freshwater fish, icthyophagous birds, amphibians, and reptiles that are infected with the advanced third-stage larva ($AdvL_3$) of the genus *Gnathostoma* which potentially may infect humans as an accidental intermediate host. We know that the disease in humans is a combination of signs and symptoms caused by the parasite, such as mechanical damage due to the migration of the larvae, release of toxic substances, and the inflammatory reaction of the host (Miyazaki, 1960) the larvae can migrate to any part of the body and symptoms will depend on the affected site. This process can cause the "*larva migrans*" syndrome (Beaver, 1969) and symptoms can be cutaneous, ocular, neurologic or a combination of all. It has been reported that the cutaneous symptoms are the most prevalent in Mexico (Díaz-Camacho et al., 1998, 2003; Martínez-Cruz et al., 1989; Ogata et al., 1998; Pérez Polito et al., 1995; Rojas-Molina et al., 1999).

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The clinical diagnosis of gnathostomiasis is made with a positive biopsy for the parasite, however, it is difficult to find the larva because of its migratory condition.

The host-parasite interaction in human gnathostomiasis, especially concerning the immune response, is not completely understood. Serology has some limitations that prevent standardization, since antibodies for immunological diagnosis come from whole extracts of the parasite (Akao et al., 1989; Parkhouse and Harrison, 1989; Tapchaisri et al., 1991).

Improvements in immunological diagnosis of parasitic infections have focused on the study of excretion–secretion products (ESP). The specific functions attributed to the ESP of nematodes are invasion and migration through host tissue, facilitation of feeding and evasion of host immune responses (Tort et al., 1999). Nevertheless, ESP have been most valuable for diagnosis because they detect specific antibodies avoiding cross-reactions with other parasites. They have also helped to have a better understanding of the way parasites escape detection by the immune system. The identification of products secreted by nematodes suggests that they usually are a mix of single antigens compared to multiple antigens present in the raw extracts (Lightowlers and Rickard, 1988). But the biochemical mechanisms of tissue invasion by the infective larval stages of the genus *Gnathostoma*, as well as other *larva migrans*, are not well understood, although they appear to depend on parasite-derived proteases. As helminth larvae migrate through connective tissue, they release proteases that degrade extracellular matrix macromolecules in vitro and presumably function in histolysis (Hotez et al., 1990; McKerrow, 1989).

In this study, we investigate the excretion–secretion products of *Gnathostoma binucleatum* $AdvL_3$ and larval protease secretion using an in vitro model system towards their possible use in immune diagnosis.

2. Materials and methods

2.1. Parasite

The advanced third-stage larvae were collected from livers of experimentally infected mice at 18 post-infection day. The mice were previously infected orally with the early third-stage larvae (EaL_3) of *G. binucleatum* (Almeyda-Artigas, 1991) which developed in cyclops, as mentioned by Almeyda-Artigas et al. (1995). Briefly, the cyclops were infected experimentally with the second-stage larvae of the parasite obtained from eggs expelled from the uteri of a mature female dissected from a stomach tumour of a naturally infected dog from Tlacotalpan, Veracruz, Mexico (Colección Helmintológica del Laboratorio de Sanidad Acuícola y Parasitología Molecular, UAM-Xochimilco catalog no. G638, 07/19/03).

2.2. Preparation of the excretion–secretion products

Gnathostoma binucleatum $AdvL_3$ were cultured in RPMI 1640 medium without supplement (Sigma, R0883) and prepared according to the manufacturer, containing 100 μg/ml penicillin (Sigma P7539) and 100 μg/ml streptomycin (Sigma P77539). The parasites were incubated for 24 h at 37 °C under 5% CO_2. They were maintained in the culture media for a week. Fifty milliliter conical tubes were used for collection changing the RPMI media every week. This procedure was performed throughout a month. Finally, the larvae were separated by centrifugation and the supernatant containing the excretion–secretion protein was dialyzed with distilled water overnight at 4 °C and concentrated to dryness by lyophilization and stored at −20 °C until needed. The lyophilized supernatants were resuspended in distilled water and used immediately or stored at −70 °C.

For substrate gel electrophoresis, lyophilized supernatants containing larval excretion–secretion were resuspended in 2 ml of Tris–HCl buffer 10 mM, pH 7.4, and AMICON UM-2 membrane filters were used for filtration and the content was used immediately. The total protein concentration was estimated by the Bradford Method (Bradford, 1976).

2.3. Immunized mice serum

Four 25 g Balb/c mice were injected in the peritoneum with 5 μg/mL of *G. binucleatum* larvae raw extract plus complete Freudás adjuvant v/v (100 μl) (F-5581). Eight days later the same amount of antigen plus incomplete Freundás adjuvant v/v (F-5560) were injected intradermically and repeated three more times, eight days apart, under the same conditions. Thus, the last immunization was given 30 days after the first one.

The mice were anesthetized and blood was drawn from the heart. Later they were killed. The serum was separated and kept at −20 °C until used.

2.4. Serum samples

Present investigation was carried out with 10 human normal sera controls and 10 human sera from patients with clinical manifestations suspected of having gnathostomiasis since they were found to have creeping eruption, migratory swellings, and history of eating raw meat of fishes. Diagnosis of one of the patients was made by a biopsy in which the larval gnathostomiasis was identified. Serum samples of 10 mice infected with *G. binucleatum* larvae were used. The mice were experimental infected with larvae ($AdvL_3$) of the parasite and sacrificed 10 day post-infection. The liver of every mouse was analyzed and the parasites was identified for the confirmation of the infection.