

Penetration Sites and Migratory Routes of *Angiostrongylus costaricensis* in the Experimental Intermediate Host (*Sarasinula marginata*)

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The intermediate hosts of Angiostrongylus costaricensis are terrestrial molluscs, mostly of the family Veronicellidae. The present work aimed at clarifying more accurately the sites of penetration and the migratory routes of A. costaricensis in the tissue slugs and at verifying the pattern of the perilarval reaction at different times of infection. Slugs were individually infected with 5,000 L₁, and killed from 30 min to 30 days after infection. From 30 min up to 2 hr after infection, L₁ were found within the lumen of different segments of the digestive tube having their number diminished in more advanced times after exposition until complete disappearance. After 30 min of exposition, percutaneous infection occurred, simultaneously to oral infection. Perilarval reaction was observed from 2 hr of infection around larvae in fibromuscular layer, appearing later (after 6 hr) around larvae located in the viscera. A pre-granulomatous reaction was characterized by gradative concentration of amebocytes around larvae, evolving two well-organized granulomas. In this work we confirmed the simultaneous occurrence of oral and percutaneous infections. Perilarval reaction, when very well developed, defined typical granulomatous structure, including epithelioid cell transformation. The infection also caused a systemic mobilization of amebocytes and provoked amebocyte-endothelium interactions.

Key words: *Angiostrongylus costaricensis* - *Sarasinula marginata* - intermediate host - migratory routes - amebocytes - granuloma

The intermediate hosts of *Angiostrongylus costaricensis* Morera & Céspedes 1971, the aetiological agent of abdominal angiostrongyliasis (Morera & Céspedes 1971) are terrestrial molluscs, mostly of the family Veronicellidae: Costa Rica (Morera & Ash 1970), Ecuador (Morera et al. 1983), Honduras (Kaminsky et al. 1987, Morera et al. 1988), Nicaragua (Duarte et al. 1992) and Brazil (Graeff-Teixeira et al. 1989, Rambo et al. 1997). Molluscs from other groups, like *Limax maximus*, may play an important role as intermediate hosts for the parasite in some regions (Graeff-Teixeira et al. 1993). Morera (1973), describing for the first time the parasite's life cycle within the intermediate host, reported ingestion of L₁ as a way of infection (oral infection). Thiengo (1996) showed the passage of first stage larvae across the

tegument of *Sarasinula marginata* Semper, 1885 (tegumental or percutaneous infection).

Conejo and Morera (1988) studied the perilarval reaction in slugs of different ages, either in primary *A. costaricensis* infections or reinfections, showing for the first time in the literature an amebocytic reaction of the intermediate host to this parasite.

The present work aimed at clarifying more accurately the sites of penetration and the migratory routes of *A. costaricensis* in the slugs and at displaying the characteristic of the perilarval reaction at different times of infection, specifying its cellular composition.

MATERIALS AND METHODS

Twenty five slugs of *S. marginata* were individually infected with 5,000 larvae (L₁), killed at 30 min, 1, 2, 4, 6, 8 hr, and 1, 2, 4, 5, 6, 8, 10, 11, 12, 14, 15, 16, 20, 21, 22, 25, 26, 28 and 30 days after infection, and fixed in Carson's Formalin-Millonig (Carson et al. 1973). Serial cross-sections were stained with Hematoxylin-Eosin (HE), PAS-Alcian Blue pH 1.0 and 2.5, Phosphomolybdic-Picrosirius Red (PMA-PS) (Dolber & Spach 1993)

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and examined by brightfield and confocal laser microscopes (LSM-410, Zeiss) using transmission and reflected modes. For documentation, selected slides were analyzed in LSM-410 and in Photomicroscope III (Zeiss) and the images were transferred from the microscopes to Microsoft Imager™ and Corel Draw 6.0™ for final adjustments of contrast, brightness and gamma-correction, and then printed in a Codonics™ NP1600 printer.

RESULTS

Oral infection - From 30 min to 2 hr many larvae L_1 were found inside the lumen of different segments of the digestive tract (buccal bulb, esophagus, crop, stomach, intestine and digestive gland) (Fig. 1). The number of larvae diminished substantially at subsequent times and disappeared at these sites, two days after infection.

The penetration through the epithelium of the digestive apparatus occurred in three different steps: (1) adhesion of L_1 to epithelium cells, causing a depression of the epithelium and thinning the cuticular layer (Fig. 2); (2) trans-epithelial migration until reaching the basal membrane (Fig. 3); (3) transposition of basal membrane and invasion of the connective subepithelial layer (Figs 3, 4). Afterward, larvae could be detected within different organs like salivary, pedal, penial, and albume glands, penis, sheath of penis, ovariolestis, prostate, cerebral ganglia, copulation purse, heart and kidney. Sometimes, larvae were found in the pericardiac coeloma.

Cutaneous via - Percutaneous infection was found to occur simultaneously to the oral infection, from 30 min onward, after exposition. Large amount of larvae was found in the ventral surface and foot, and the preferential site of external penetration appeared to be the excretory ducts of the mucous glands (Figs 7, 8, 9). After penetration of the epidermis or the ductal epithelium, the larvae remained within the fibromuscular layer (Figs 9, 12), where they were gradually surrounded by amebocytic reaction (Figs 9, 12, 14, 15, 18, 19).

Perilarval reaction - It was observed 2 hr after infection, around larvae located in the fibromuscular layer of the body, starting lately (6 hr) around larvae trapped within the viscera (Fig. 13). The reaction consisted exclusively of amebocytes (hemocytes or celomocytes) which initially formed an involving cell monolayer around the larvae (Fig. 11) with formation of subsequent layers (Figs 10, 12, 14), characterizing pre-granulomatous reactions (Fig. 14). These lesions culminated on the 4th day of infection as organized granulomas, forming two well defined strata: (1) the inner stratum, composed of several concentric layers of amebocytes, with or without epithelioid transformation, and (2) the

outer stratum, formed by flattened or fibroblastic-like cells, which constituted a pseudocapsule (Fig. 18). According to the time of infection and the site of reaction, there were: (a) intense and scattered infiltrate around the larvae without organoid aspect (Fig. 13); (b) clusters of spherical amebocytes surrounding the larvae within vessels (pre-granulomatous reaction) (Fig. 16); (c) fibroblastic-like amebocytes (Fig. 17); and (d) vacuolization of amebocytes (Figs 19, 23). There was no evidence of collagen fibers permeating the amebocytic infiltrate even in the 30 days-granulomas (Figs 15, 22). After 30 days of infection the granulomas were seen mostly within vessel cavities (Fig. 20-22) producing emboli and causing vascular ectasia (Figs 20, 21). Larvae always appeared very well preserved, independently of the developmental stage: L_1 (Fig. 12), L_2 (Fig. 18) and L_3 (Figs 19, 23). Degenerating parasites were not found during all the time of experiment.

Systemic intravascular reaction - Concomitantly with the intensification of the perilarval reaction, occurred also a significant increase in the number of circulating amebocytes not directly related to the presence of intravascular larvae. These amebocytes showed adhesion between them and margination due to adherence to endothelium (Fig. 24). Sometimes, in small vessels, amebocytes were adhered to the endothelial region faced to contiguous perilarval reaction (Fig. 14).

DISCUSSION

In this work we confirmed the simultaneous occurrence of oral and tegumental or *percutaneous* infection by L_1 *A. costaricensis* in intermediate host, *S. marginata*. We displayed the mode and the sites of L_1 penetration, the involvement of several viscera in the routes of parasite migration, and characterized the patterns of perilarval (pre-granuloma and granuloma) and systemic (hemocytosis) amebocytic reactions.

The penetration of L_1 across the molluscan epithelium of the digestive tract reproduced the same events observed in the penetration of L_3 across the intestinal epithelium of murine (definitive) host (EM Mota & HL Lenzi manus. in prep.). In both hosts, the penetration process happened rapidly, without induction of any inflammatory reaction, and appeared to partially depend on unknown proteolytic mechanisms. The L_1 penetration occurred at different levels of the molluscan digestive tract, showing no preference to specific segments.

Our observations indicated that, in the tegumental infection, L_1 gained entry into the mollusc preferentially through the excretory ducts of the mucous glands, where the epithelium is thinner

than the epidermal cells. These ducts drain the secretion of several individual mucous cells rich in carboxylated and non-sulfated proteoglycans (hyaluronic acid?) and neutral glycoproteins. We do

not know yet if the mucous has some chemotactic effect or if the cuboid ductal epithelial cells present larger number of "counter or complementary receptors" to L_1 than the tegumental epidermal cells.

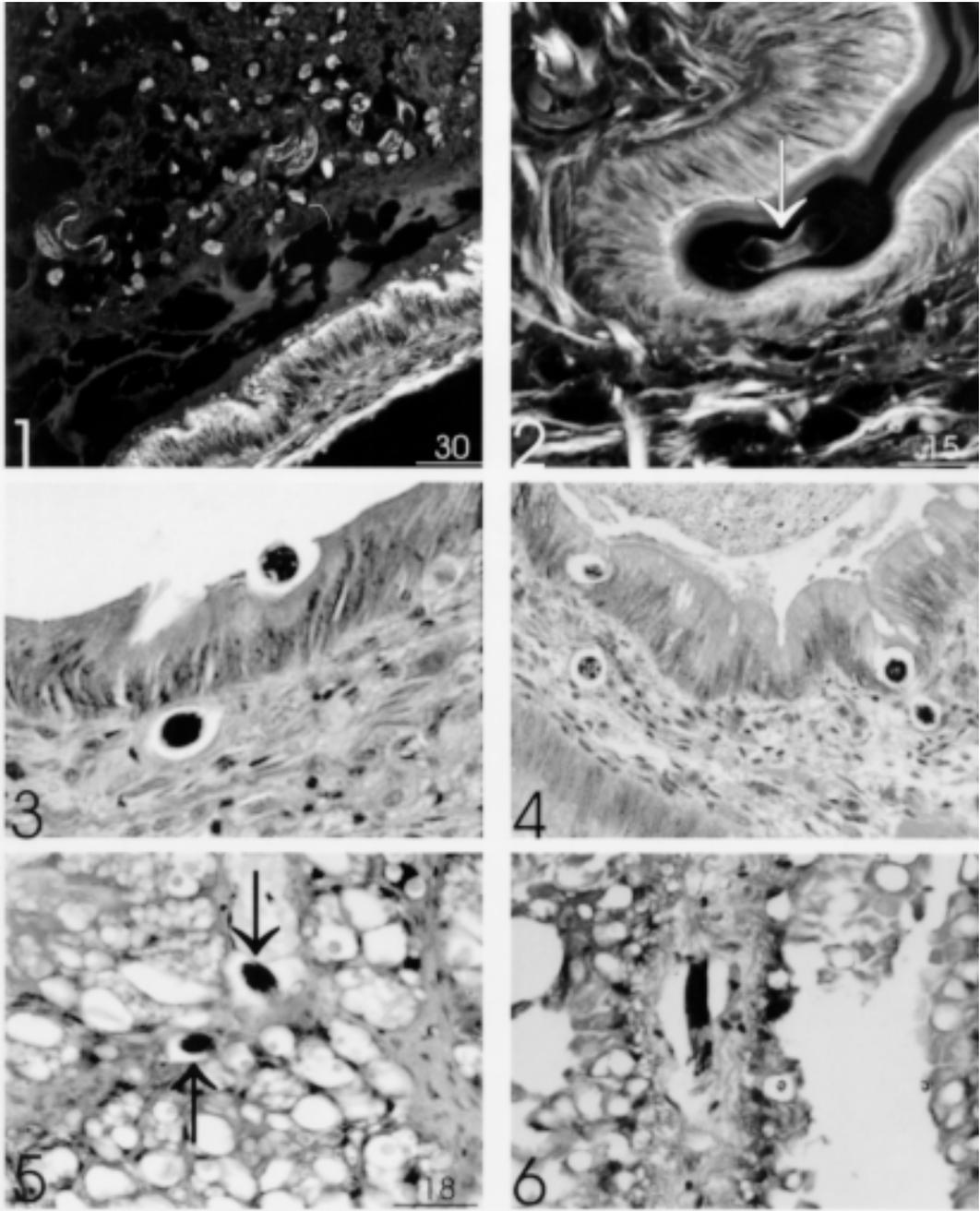
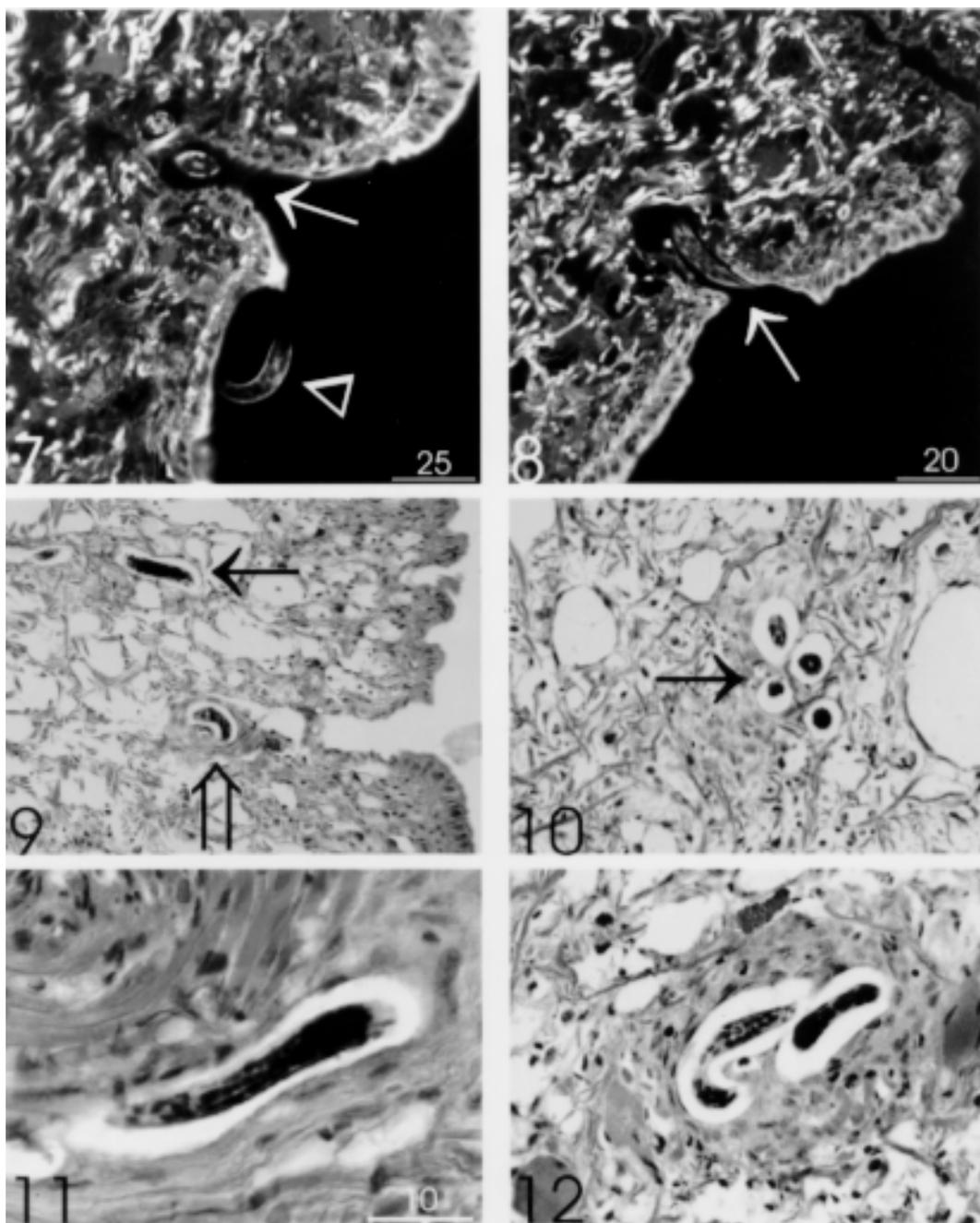


Fig. 1: several L_1 in the lumen of stomach (time of infection: 30 min) (HE - CLSM reflected mode. Bar = 30 μ m). Fig. 2: larva (L_1) (arrow) in contact with bulb buccal epithelium, producing depression in its surface and thinning of the cuticular layer (time of infection: 30 min) (HE - CLSM reflected mode. Bar = 15 μ m). Fig. 3: stomach showing one L_1 on the initial phase of penetration in the epithelium, and another one in the interstice, just below the basement membrane (time of infection: 2 hr) (HE, 640x). Fig. 4: intestine showing L_1 on final phase of intraepithelial penetration (supra-basement membrane location) and in the subepithelial connective tissue (time of infection: 2 hr) (HE, 400x). Fig. 5: larvae (L_1) in the digestive gland (arrows) (time of infection: 1 hr) (HE, CLSM. Bar = 18 μ m). Fig. 6: larvae (L_1) in the interstice of kidney with few contiguous amebocytes (time of infection: 8 hr) (HE-500x).

Independently of the penetration sites, L_1 showed a tendency to migrate to the fibromuscular layer where they moulted from $L_1 \rightarrow L_2 \rightarrow L_3$. The fibromuscular layer differentiates from the rest of the

organism due to considerable vascularity, vigorous fibromuscular tissue, intense innervation and abundant secretory glands. Probably the fibromuscular layer represents a complex microenviron-



Figs 7, 8: larvae (L_1) near the tegumental epithelium (head of arrow) and inside excretory ducts of mucous glands (arrow) (time of infection: 30 min) HE, CLSM. Bar = 25 μ m (Fig. 7), Bar = 20 μ m (Fig. 8). Fig. 9: larvae in the fibromuscular layer, one of them near an excretory duct presenting initial phase of perilarval reaction (double arrow) and another one free of reaction (arrow) (time of infection: 6 hr) (HE, 260X). Fig. 10: larvae in the fibromuscular layer (arrow), partially surrounded by amebocytes and next to hemolymph vessels (time of infection: 6 hr) (HE, 400x). Fig. 11: larva in the fibromuscular layer partially surrounded by a monolayer of amebocytes, some of them showing spindle nuclei similar to fibroblasts (fibroblast-like cells) (time of infection: 2 hr) HE, CSLM. Bar = 10 μ m. Fig. 12: two larvae in the fibromuscular layer, surrounded by several layers of amebocytes (pre-granulomatous reaction) near superficial vessels (time of infection: 6 hr) (HE, 500x).

ment, which is suitable to larval homing (ecotaxy) and moult. Indeed, the majority of the larvae was found at this level.

The migration of L_1 and L_3 is an active process involving larva movements, which appears to be associated with the participation of proteolytic enzymes. This phenomenon is apparently exem-

plified by the presence of an empty perilarval space, even around the immobile L_2 (Figs 3-6, 20).

The larva invasion of several viscera occurs by migration across organ to organ or through anatomophysiological ducts, coeloma or body cavity. The invaded organs operate as intermediate steps (or way of passage) to the larvae before reaching the final

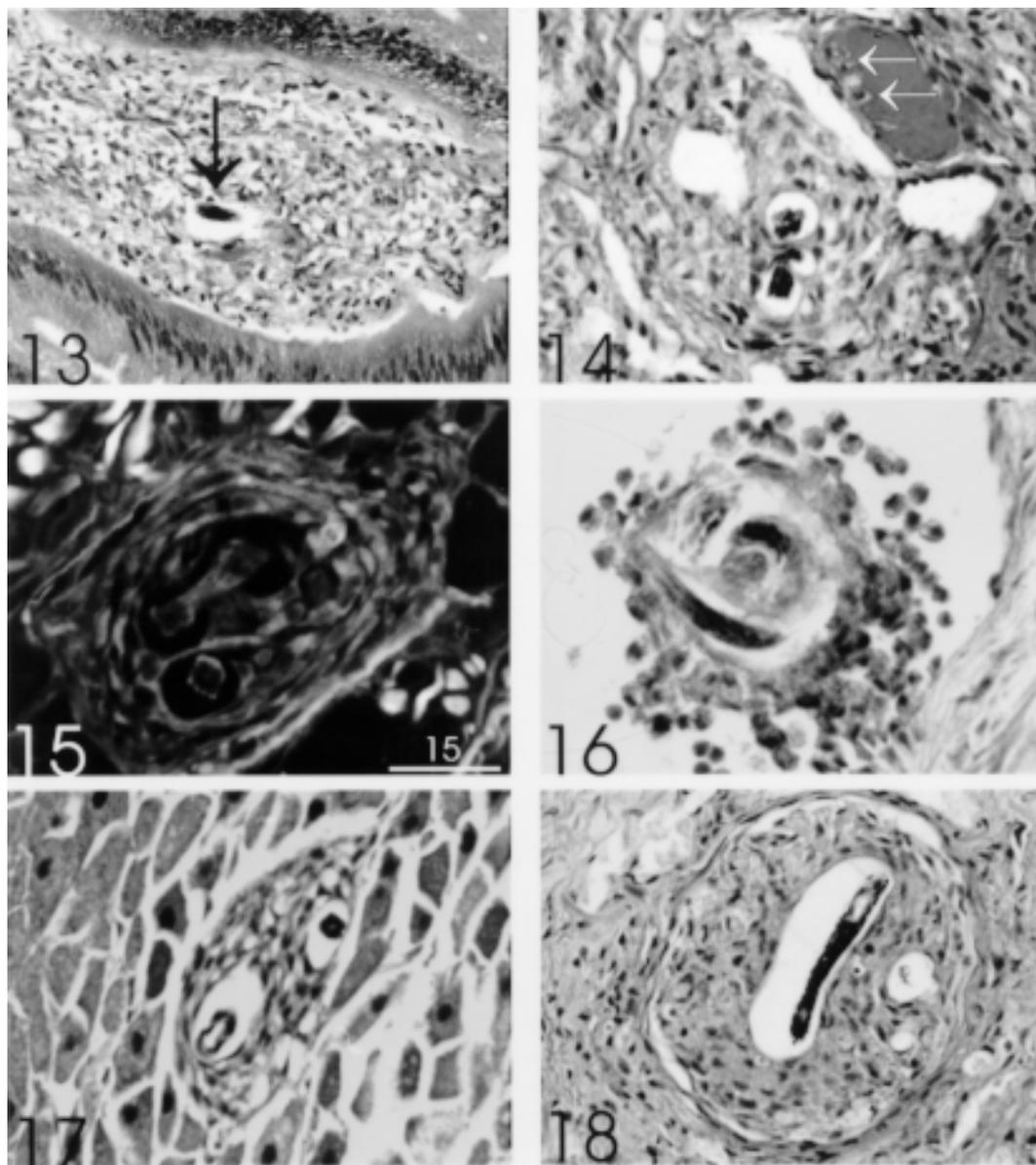


Fig. 13: non organoid scattered reaction of amebocytes around L_1 (arrow) in subepithelial connective tissue of copulation purse (time of infection: 8 hr) (HE, 310x). Fig. 14: pre-granulomatous reaction around L_1 in the periphery of the fibromuscular layer near to a superficial vessel, which presents amebocytes stuck to the endothelium (arrows) side that looks on to perilarval reaction (time of infection: 8 hr) (HE, 500x). Fig. 15: pre-granulomatous perilarval reaction in the fibromuscular layer without evidence of collagen fibers within it (time of infection: 24 hr) PMA-PS, CLSM. Bar = 15 μ m. Fig. 16: intravascular perilarval reaction showing centripetal gradient of cellular adhesion (time of infection: 24 hr) (HE, 500x). Fig. 17: pre-granulomatous perilarval reaction in the interior of pedial gland showing fibroblast-like amebocytes (time of infection: 2 days) (HE, 500x). Fig. 18: perilarval granuloma (L_2) presenting two well defined strata: the internum, bearing epithelioid amebocytes, and the externum thinner, which forms a pseudo-capsule constituted by fibroblast-like cells (time of infection: 5 days) (HE, 310x).

habitat, the fibromuscular layer. The kidney appears to provide the main passage way for orally delivered larvae to reach the fibromuscular layer. In fact, this organ was found to be parasited in all slugs in significant amounts when compared with other organs. Contingently, some larvae are arrested inside the viscera by the inflammatory reaction (Figs 13, 17). However, as in vertebrates, the inflammation predominates in the fibromuscular layer which is intensely vascularized. The amebocytes, within the

vessels, showed margination and increased adhesiveness and sticking to the endothelial cells, pointing out to the phylogenetic precocity of this capital event of the inflammatory process (Figs 14, 24). Amebocytes also stick to each other, revealing that the surface changes are not limited to the endothelium (Figs 16, 20, 24).

Larvae that have penetrated by tegumental or percutaneous infection got immediate access to the fibromuscular layer, where there is a network of

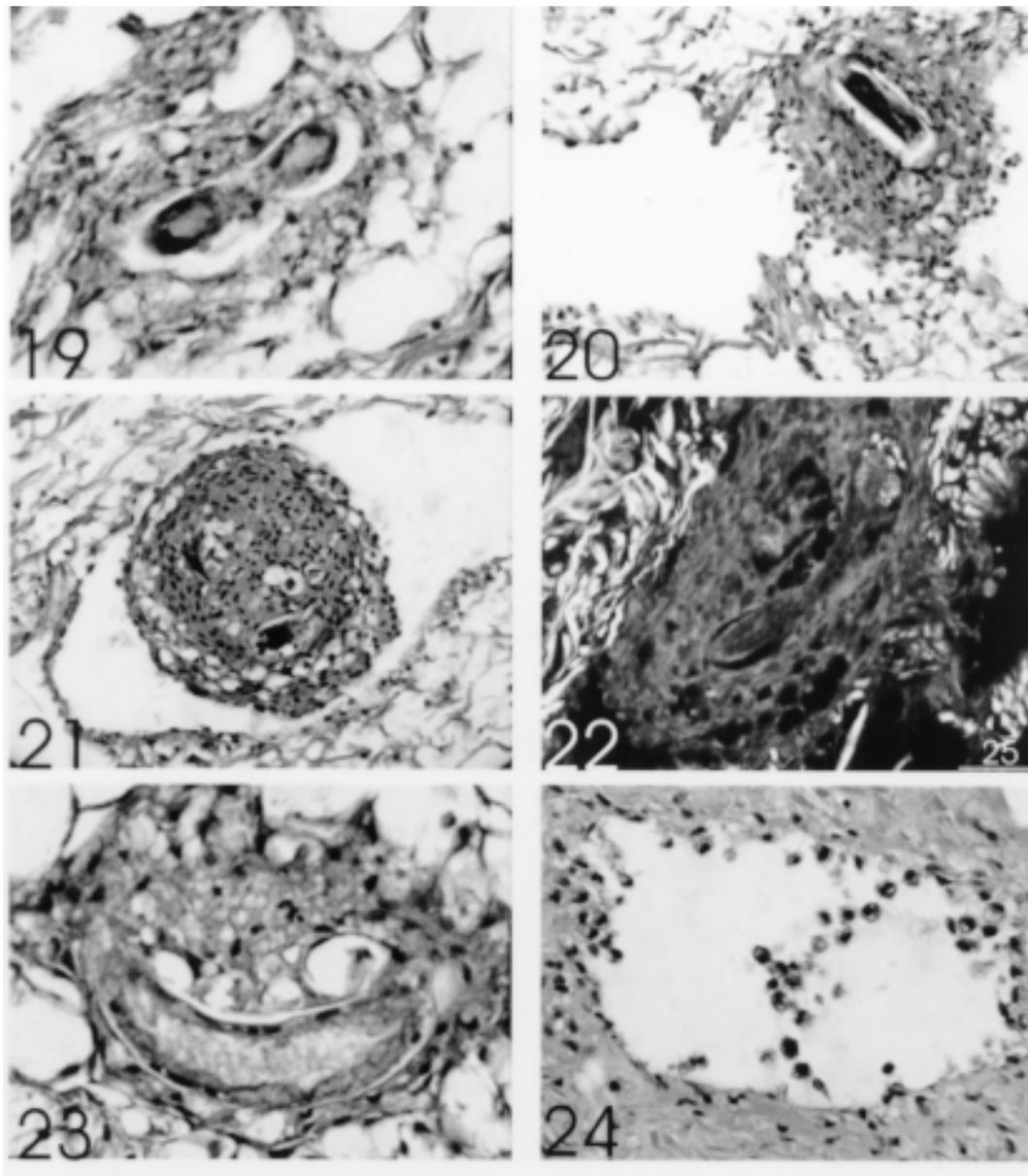


Fig. 19: vacuolate amebocytes surrounding transitional larvae (L_2 - L_3) (time of infection: 8 days) (HE, 500x). Figs 20, 21: intravascular granulomas surrounding L_3 in dilated hemolymph vessels (time of infection: 30 days) (HE, 260x). Fig. 22: absence of collagen in perilarval granuloma (L_3) (time of infection: 30 days) (PMA-PS, CLSM. Bar = 25 μ m). Fig. 23: larva (L_3) eccentrically surrounded by intensely vacuolate amebocytes (time of infection: 30 days) (HE, 500x). Fig. 24: hemolymph vessel presenting large number of amebocytes marginally stuck to the endothelium or forming clusters (time of infection: 5 days) (HE, 400x).

subepithelial vessels which facilitates the amebocyte migration, provoking rapid amebocytic encapsulation around larvae. This event made possible to discriminate the larvae originated from each route of infection. For instance, comparing the perilarval reaction in Fig. 6 (8 hr of oral infection), with Fig. 10 (6 hr of percutaneous infection) it is possible to observe almost absent or minimal intrarenal perilarval reaction in the former (oral infection), and large amount of amebocytes in the latter, surrounding intra-fibromuscular larvae (percutaneous infection).

The amebocyte reaction around invaded parasites has been considered in the literature as an encapsulating phenomenon (Pan 1965, Richards & Merritt 1967, Cheng & Rifkin 1970, Harris 1975, Harris & Cheng 1975, Lie & Heyneman 1975, Rachford 1976, Krupa et al. 1977), or as a collection of amebocytes (Conejo & Morera 1988), or as a granulomatous structure (Pan 1965, Souza et al. 1995, 1997). In this work we considered the very well developed perilarval reaction as typical granulomas, mainly when there was formation of epithelioid cells (Fig. 18). The granulomas were constituted by compact or organized collection of amebocytes showing, sometimes, the definition of two strata. The granulomatous stage was preceded by mobilization and aggregation of amebocytes around larvae (pre-granulomatous stage) (Fig. 16). Multinucleated or giant cells were never detected in the granulomas, confirming similar observation made by Pan (1965) in *Australorbis glabratus* infected with *Schistosoma mansoni*. After 30 days of infection, most of the larvae were located within vessels, evidencing, even in invertebrate host, the preference of Angiostrongylidae for intravascular habitat. During this period, and sometimes earlier, intravascular pre-granulomatous reaction or granulomas formed free (Fig. 16) or trapped (Figs 20, 21) emboli, causing vascular dilatation due to blockage of hemolymph circulation. Pan (1965), in *S. mansoni* infected *A. glabratus*, also observed, during the period of heavy emergence of cercariae, intravascular infiltration of hypertrophic amebocytes around them, forming "cercarial emboli".

We did not detect presence of interstitial collagen in the granulomas (Figs 15, 22), reinforcing the assumption that the fibroblastic-like cells are flattened amebocytes (Sminia et al. 1974, Harris 1975, Cheng & Garrabrant 1977, Locker 1979), instead of fibroblast or myofibroblast. Granulomas in *S. marginata*, at least during the period of this experiment, appears not to kill the larvae and may even contribute to the process of larval moulting. Harris and Cheng (1975) also observed that *A. cantonensis* larvae are not destroyed by *Biomphalaria glabrata* amebocytes, and, on the con-

trary, they successfully complete their metamorphosis despite rapid encapsulation which give a strong staining reaction for acid phosphatase, β -glucuronidase, and non-specific esterases. Such capsules persist for long periods and viable first-stage larvae have been recovered from *Biomphalaria* up to 12 months after infection (Richards & Merritt 1967).

According to Conejo and Morera (1988), a rupture produced by muscular contraction is required for L_3 evasion of the encapsulating reaction, allowing them to escape from the intermediate host. Then, the fibromuscular layer, besides being the preferred environment for the larval development, independently of L_1 portal of entry, it also favours the larvae extrusion due to muscular contraction. This is another example in the literature that shows exploitation of the host response by the parasite, aiming at continuing its life cycle (Damian 1987, Lenzi et al. 1997).

Histological observations, without quantitative analysis, suggest that there is an increased number of circulating amebocytes (hemocytosis) in the infected *S. marginata*. The mesothelial lining of the pericardial coeloma appears to be the main source of *S. marginata* amebocytes (coelomocytes), as it was suggested by the observations of serial sections (data not shown). Bilej et al. (1992) found that, in stimulated earthworms by infecting arsanilic acid coupled to human serum albumin in 3% agar gel (ARS-HSA), the cells of the lining of the coelomic cavity respond immediately to stimulation as measured by [3 H]TdR uptake, indicating mitotic activity of coelomocytes.

In conclusion, this paper shows that *A. costaricensis* infection in *S. marginata* provokes systemic amebocyte mobilization (hemocytosis) and perilarval granulomatous reaction, with participation of epithelioid cells. It emphasizes the concept that the granuloma is intrinsically a macrophage phenomenon, phylogenetic ancient, that prescinds from B and T lymphocytes. It operates as hybrid interface (composed by host and parasite components) between two different phylogenetic beings, which try to favour a symbiotic cohabitation between them.

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