

## Invasion of Intestinal Epithelia In Vitro by the Parasitic Nematode *Trichinella spiralis*

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**Studies of nematode establishment in intestinal niches has been hindered by the lack of a readily manipulated in vitro assay. In this report, experiments are described wherein the larval stage of the parasitic nematode *Trichinella spiralis* was shown to invade epithelial cell monolayers in vitro. Larvae penetrated cells and migrated through them, leaving trails of dead cells in their wake. Cells derived from five different species were susceptible to invasion, reflecting the broad host range of *T. spiralis* in vivo. Epithelial cells derived from large and small intestines and kidneys were susceptible. Fibroblast and muscle cells were resistant. Larvae deposited glycoprotein antigens in the cells they invaded. Although the function of these antigens is unknown, they are targeted by rat antibodies that cause *T. spiralis* to be expelled from the intestine. The model system described provides the means to further investigate this process as well as the mechanisms by which this parasitic nematode establishes its intestinal niche.**

*Trichinella spiralis* is a parasitic nematode that initiates infection in the small intestine of its vertebrate host. The larval and adult stages of *T. spiralis* are found in the intestinal epithelium, often at the crypt-villus junction, and do not appear to cross the basement membrane. Despite its relatively large size, the worm establishes an intracellular niche and appears to occupy several cells simultaneously (10, 23). The nematode is not sessile in this niche but instead migrates in a sinusoidal pattern, leaving trails of dead cells behind (22). The mechanisms by which infective larvae of *T. spiralis* recognize, invade, and migrate within the intestinal epithelium are unknown.

As much as 99% of an oral dose of infective *T. spiralis* larvae is expelled from the intestinal epithelium in appropriately immunized adult rats and in neonatal rats whose dams are immune (2, 5, 7, 13, 14, 21). This dramatic immune defense, called rapid expulsion, is mediated by antibodies (1). Monoclonal antibodies developed against *T. spiralis* excretory/secretory antigens effect rapid expulsion in passively immunized neonatal rats (3). Protective antibodies are specific for a carbohydrate epitope which is present on glycoproteins displayed on the surface of the parasite as well as in excretory/secretory antigens (collectively called TSL-1 antigens) (11). We have reported evidence that glycan-specific antibodies mediate expulsion by interfering directly with the parasite in the intestinal epithelium (17). This suggests that the target glycoproteins may mediate invasion or facilitate migration of the parasite in the epithelium.

Studies of nematode establishment in intestinal niches has been hindered by the lack of a readily manipulated in vitro assay. To investigate the mechanisms by which *T. spiralis* invades and migrates in its niche, we developed an in vitro assay with epithelial cell lines. This culture system allows the observation of the parasite's behavior during invasion, and our data

indicate that it accurately reproduces several parameters of parasite establishment in vivo.

### MATERIALS AND METHODS

**Abbreviations used in this paper.** TSL-1, *T. spiralis* larva type 1; FBS, fetal bovine serum; MEM, minimal essential medium; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; SD, standard deviation.

**Rats.** The life cycle of *T. spiralis* was maintained in adult AO, PVG, and DA strain rats. The rats were produced and maintained in the James A. Baker Institute vivarium in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care.

**Cells.** The following cell lines were obtained from the American Type Culture Collection: Caco-2, T84, HT-29, Henle-407, WI-38, and C2C12. The AA7 and BG12 clones of the MDCK cell line were a gift from William Young (University of Kentucky) (16). The cell type, tissue, and species of origin of each cell line are listed in Table 1.

Cells were cultured in MEM (Earle's salts) with L-glutamine, nonessential amino acids, and 10% FBS. The cells were passaged no more than 15 times before use in the experiments. Monolayers were dispersed by trypsinization (0.05% trypsin, 0.65 mM EDTA).

**Parasite.** *T. spiralis* (pig strain) infectious larvae were recovered from muscles of irradiated rats by digestion with 1% pepsin in acidified water (8). Donor rats had been infected at least 28 days prior to collection. For most experiments, these larvae were inoculated into the rats (from which feed had been withheld for 8 h) and recovered from the intestine 2 to 3 h later. Such larvae are subsequently referred to as intestine-recovered larvae. Specifically, the rats were lightly sedated with ether and then inoculated by gavage with 5,000 to 6,000 larvae in 0.3 to 1.0 ml 0.85% saline. To recover the intestinal larvae, the rats were killed by CO<sub>2</sub> inhalation and the intestines were removed immediately, rinsed with saline, opened, and incubated for 60 to 90 min in saline containing antibiotics (200 IU of penicillin per ml, 200 µg of streptomycin per ml, and 50 µg gentamicin per ml). The larvae were recovered on a 200-mesh sieve and then washed with saline containing antibiotics before being inoculated onto monolayers (see below).

**Incubation of larvae in bile or gut contents.** In some experiments, pepsin-digested larvae were treated with intestinal contents or bile rather than being inoculated into rats. Intestinal contents were prepared by rinsing the intestine of an adult rat (from which feed had been withheld for 8 h) with 5 ml of saline containing antibiotics. Debris was removed from the recovered fluid by centrifugation at 1,200 × g for 10 min. The supernatant fluid was diluted 1:2 with saline and frozen at -20°C. These intestinal contents were diluted 1:10 in saline before being used in experiments. Bile from sheep (obtained postmortem) or rats (provided by R. G. Bell, Cornell University) was diluted 1:20. Larvae were incubated with saline, diluted bile, or intestinal contents at 37°C for 2 to 3 h. This incubation time matched the time during which the larvae occupied rat intestines in other experiments. Following treatment, the larvae were rinsed three times with 15 ml of saline, incubated for 1 h in saline plus antibiotics (to mimic the gut

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TABLE 1. Semisolid medium is required for *T. spiralis* larvae to invade epithelial monolayers

Cell line	Damage to monolayer ( $10^3 \text{ mm}^2$ ) <sup>a</sup> in:				P
	Liquid medium		Semisolid medium		
	Medium control	Larvae	Medium control	Larvae	
MDCK-AA7	8 ± 1	7 ± 1	8 ± 3	31 ± 3 <sup>b</sup>	<0.0001
T84	28 ± 1	29 ± 2	24 ± 2	75 ± 11 <sup>b</sup>	<0.0001

<sup>a</sup> Damage to the monolayer is expressed as the mean ± 1 SD for three or four monolayers.

<sup>b</sup> The mean was significantly different from the medium control.

recovery procedure), and washed on a sieve before being inoculated onto monolayers (see below).

**Invasion assay.** Cells were grown to confluence in eight-well glass chamber slides (Nunc, Naperville, Ill.). Larvae were prepared as described above and suspended in 0.2 ml of medium without serum but including 15 mM HEPES and 1.75% agarose. Larval suspensions and media were held at 38 to 40°C, and the mixture was overlaid on the monolayer in one chamber. Cultures were incubated at 37°C in 5% CO<sub>2</sub> for 2 h. During the final 30 min of the incubation period, the number of larvae in contact with the monolayers was counted by using a phase-contrast inverted microscope and a 4× objective. This confirmed that monolayers in each treatment group had equivalent numbers of larvae. Inocula were prepared so that approximately 25 larvae would be delivered to the surface of the monolayer. Cultures that had high or low counts (significantly different by Scheffé's test) were discarded.

After the incubation period, the monolayers were stained for 2 min in 0.4% trypan blue (Sigma, St. Louis, Mo.), rinsed twice in Dulbecco's PBS plus MgCl<sub>2</sub> and CaCl<sub>2</sub>, and fixed for 20 min in 10% buffered formalin. After the slides were rinsed twice in distilled water, coverslips were applied with glycerol (Dako Corp., Carpinteria, Calif.). The area of stained (dead or damaged) cells was quantified by image capture methods. A total of 25 to 40 microscope fields from each monolayer were captured by using a 4× objective on a bright-field microscope (Labophot; Nikon) fitted with a black-and-white video camera (Cohu, Inc., San Diego, Calif.). A frame grabber captured the image, and the area of dead or damaged cells was determined with NIH Image 1.58 software.

**Statistical analysis.** The mean area of damage (trypan blue stain) per microscope field was estimated for each monolayer. Three to five monolayers were evaluated per treatment group. The mean results for the treatment groups were compared by analysis of variance, and significant differences between groups were identified by Scheffé's test. The same method was used to document that the numbers of larvae on monolayers in different treatment groups were equivalent.

**Electron microscopy.** Caco-2 cells ( $4 \times 10^5$  cells) were plated on 0.9-cm<sup>2</sup> cell culture filter inserts (pore size, 0.45 μm) that were pretreated with rat tail collagen type 1 ("Biocoat" Collaborative Biomedical Products, Becton Dickinson). The electrical resistance was monitored (Millicell-ERS; Millipore Corp., Bedford, Mass.), and the monolayers were inoculated with larvae 1 day following an increase in resistance that indicated the formation of tight junctions. At 30 to 60 min after inoculation, the monolayers were fixed for 2 h in 3% glutaraldehyde–2% formaldehyde in cacodylate buffer and postfixed in 2% osmium tetroxide. The filters were removed from their supporting structure after fixation and washed in cacodylate-sucrose buffer. After dehydration through an alcohol gradient, the monolayers were infiltrated with epoxy resin (Polybed; Polysciences, Warrington, Pa.). Sections (60 nm thick) were made, stained with uranyl acetate and Reynold's lead citrate, and examined with a Zeiss EM109 electron microscope at 80 kV.

**Fluorescence microscopy.** The cells were grown to confluence on glass coverslips. Monolayers were inoculated with larvae and incubated 1 to 2 h. The agarose was removed, and the coverslips were washed twice with MEM–10% FBS. The monolayers were covered with 0.03 mg of propidium iodide per ml diluted in MEM–50% FBS and incubated at 37°C for 2 min and then at 4°C for 30 min. After two washes with MEM–10% FBS, the cells were fixed in 2% paraformaldehyde in PBS (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 80 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl [pH 7.2]) for 30 min, washed in PBS, and then permeabilized for 15 min with 0.075% saponin in PBS. The monolayers were incubated with monoclonal antibody 18H (10 μg/ml), which is specific for the tyvelose and binds TSL-1 antigens (3) or with serum immunoglobulin obtained from normal rats and diluted in PBS. Anti-rat immunoglobulin G conjugated to FITC (Organon Teknika Corp., Durham, N.C.) was diluted in PBS containing 0.075% saponin, 10% normal goat serum, and 0.2% gelatin. (Saponin was included in the procedure because in some experiments the cells were stained for actin with fluorescently labeled phalloidin [data not shown].) Saponin treatment did not affect antibody staining of fixed monolayers [data not shown].) Antibody incubations were carried out for 45 min, and following each treatment the coverslips were washed three times with PBS. Upon completion of the staining procedure, the coverslips were

TABLE 2. Invasion by *T. spiralis* larvae is enhanced following passage of larvae in rat intestine or incubation in intestinal contents

Cell line	Damage to monolayer ( $10^3 \text{ mm}^2$ ) <sup>a</sup> following treatment of larvae with:			
	Medium control	Saline	Intestinal passage	Intestinal contents
Henle-407	29 ± 4	32 ± 3	60 ± 8 <sup>b,c</sup>	ND <sup>d</sup>
MDCK-AA7	5 ± 1	11 ± 3 <sup>b</sup>	20 ± 1 <sup>b,c</sup>	19 ± 2 <sup>b,c</sup>
Caco-2	51 ± 12	67 ± 12	100 ± 21 <sup>b,c</sup>	85 ± 6 <sup>b,c</sup>
T84	23 ± 3	56 ± 9 <sup>b</sup>	83 ± 13 <sup>b,c</sup>	83 ± 13 <sup>b,c</sup>

<sup>a</sup> Damage to the monolayer is expressed as the mean ± 1 SD for three or four monolayers.

<sup>b</sup> Mean significantly different from medium control ( $P \leq 0.01$ ).

<sup>c</sup> Mean significantly different from saline-treated larvae ( $P < 0.05$ ).

<sup>d</sup> ND, not done.

mounted (Vectashield; Vector Laboratories, Inc., Burlingame, Calif.) and examined on a Nikon Diaphot inverted microscope equipped with epifluorescence (Opti-quip, Highland Mills, N.Y.).

## RESULTS

**Parasite invasion in vitro requires semisolid medium.** Larvae recovered from the intestines of rats and suspended in semisolid medium caused significant cell death, while larvae suspended in liquid medium caused no significant damage to monolayers. Table 1 shows results obtained with two epithelial cell lines. Larvae in either medium browsed the surfaces of epithelial cells as they moved over the monolayer, but only larvae in agarose invaded the cells.

**Larvae exposed to the intestinal milieu are invasive.** Four cell lines were tested in 10 experiments to compare the invasiveness of larvae recovered from intestines with that of larvae treated only with saline following pepsin digestion (Table 2). In all the experiments, cell death in monolayers treated with intestine-recovered larvae was significantly greater than in control monolayers receiving only semisolid medium. In addition, intestine-recovered larvae caused significantly greater cell death than did saline-treated larvae. In some experiments, saline-treated larvae caused a significant amount of cell death; however, in other experiments, there was no significant difference compared with uninoculated monolayers. Due to the variability in the invasive properties of saline-treated larvae, intestine-recovered larvae were used in subsequent experiments.

Larvae incubated in intestinal contents for 2 to 3 h were as invasive as intestine-recovered larvae (Table 2). Furthermore, bile from sheep or rats could be substituted for intestinal contents. Larvae incubated in bile and then inoculated onto MDCK-AA7 cells also invaded monolayers aggressively [ $26 \pm 2 \times 10^3 \text{ mm}^2$  for larvae treated with sheep bile versus  $13 \pm 3 \times 10^3 \text{ mm}^2$  for the medium control [ $P = 0.0136$ ];  $33 \pm 5 \times 10^3 \text{ mm}^2$  for larvae treated with rat bile versus  $10 \pm 2 \times 10^3 \text{ mm}^2$  for the medium control [ $P = 0.0004$ ].

**Host range and cell specificity of *T. spiralis* in vitro.** Ten epithelial cell lines, of renal or intestinal origin and derived from five different species, were susceptible to invasion by *T. spiralis* larvae (Table 3). Fibroblast and myoblast lines were resistant.

**Parasite glycoprotein antigens are present in cells invaded by larvae.** Trails of dead cells were left by parasites that migrated through monolayers. This was documented by nuclear staining with propidium iodide (Fig. 1A) and also with trypan blue. In addition, the remnants of dead cells were heavily

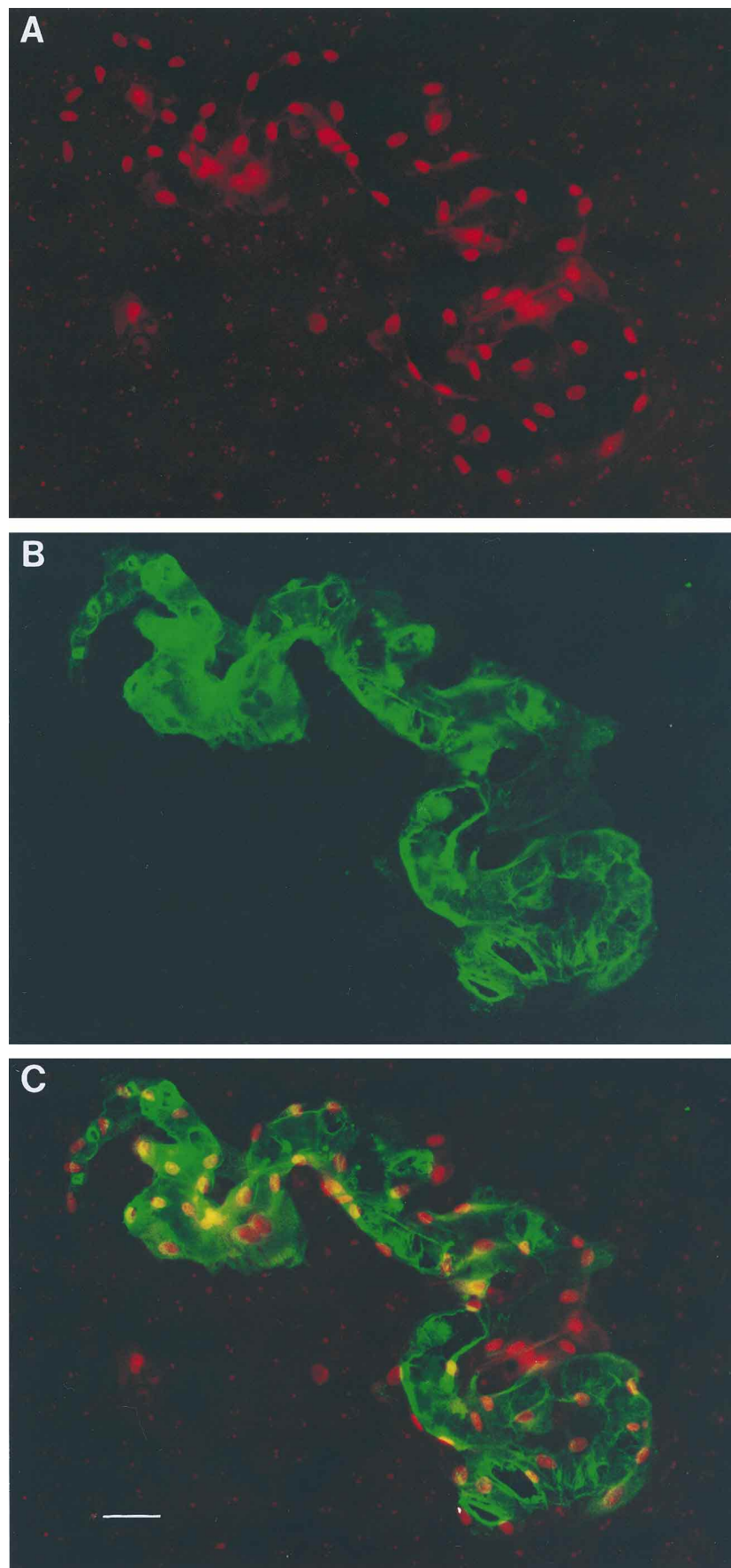




TABLE 3. *T. spiralis* larvae demonstrate broad host range and narrow cell specificity

Cell line	Cell type	Tissue of origin	Species	Damage to monolayer (10 <sup>3</sup> mm <sup>2</sup> ) <sup>a</sup> by:		P value	No. of expts
				Medium control <sup>b</sup>	Intestine-recovered larvae		
Henle-407	Epithelial	Small intestine	Human	29 ± 4	60 ± 8	0.0001	4
SLC44 <sup>c</sup>	Epithelial	Small intestine	Rat	3 ± 2	35 ± 11 <sup>d</sup>	0.0044	2
Caco-2	Epithelial	Colon	Human	29 ± 4	45 ± 4	0.0003	3
T84	Epithelial	Colon	Human	24 ± 2	75 ± 11	<0.0001	4
HT-29	Epithelial	Colon	Human	8 ± 3	22 ± 4	0.0005	2
MDCK-AA7	Epithelial	Kidney	Dog	8 ± 3	31 ± 3	<0.0001	8
MDCK-BG12	Epithelial	Kidney	Dog	33 ± 3	52 ± 8	0.0022	2
Vero	Epithelial	Kidney	Monkey	5 ± 1	11 ± 3	0.0157	3
PK-15 <sup>c</sup>	Epithelial	Kidney	Pig	7 ± 2	22 ± 5 <sup>d</sup>	0.0078	2
WI-38	Fibroblast	Lung	Human	16 ± 4	18 ± 2	0.2656	2
C2C12 <sup>c</sup>	Myoblast	Striated muscle	Mouse	16 ± 3	16 ± 6	0.9538	2

<sup>a</sup> Damage to the monolayer is expressed as the mean ± 1 SD for three or four monolayers. Values are presented for representative experiments.

<sup>b</sup> Monolayer incubated with semisolid medium without larvae.

<sup>c</sup> Experiments were conducted in 24-well plastic plates (Corning, Corning, N.Y.).

<sup>d</sup> Larvae were activated by treatment with intestinal contents (see Materials and Methods).

loaded with TSL-1 antigens recognized by monoclonal antibody 18H (Fig. 1B and C).

**Larvae travel through epithelial cells.** Although it was clear from light microscopic evaluation that larvae penetrated epithelial monolayers, the precise location of the parasite in the monolayer could not be determined. Specifically, we wanted to know whether the larva traveled beneath, between, or through cells. Caco-2 cells grown on filter membranes were well differentiated, with tight junctions and well-developed apical microvilli (Fig. 2). Examination of larvae embedded in Caco-2 cells revealed that they were surrounded by cellular cytoplasm (Fig. 2), and tangential sections showed that one larva occupied several cells simultaneously (Fig. 2A). Similar results were obtained with MDCK and T84 cells (data not shown). The space between the larval cuticle and host cell cytoplasm seen in Fig. 2 also was observed by Dunn and Wright (10) in thin sections of intracellular larvae in intestinal tissue and was considered by them to be an artifact of processing.

## DISCUSSION

Although some parameters of invasion and behavior of *T. spiralis* in vivo have been established, a detailed investigation of these processes has been hindered by difficulties in accessing and manipulating the intestinal niche of the parasite. Our results document the first successful in vitro method for studying the interaction of the worm with the host cell. We identified three pivotal requirements for invasion. First, larvae require a semisolid environment above the epithelial cell surface. We speculate that agarose may provide physical support for the worm so that it can propel itself into the cell layer. Alternatively, agarose may alter the microenvironment above the cell layer so that the parasite receives the sensory input necessary to trigger invasive behavior. These parameters are readily manipulated in our model system, and so these hypotheses can be tested experimentally.

The second requirement for invasion is the activation of the

larvae by exposure to the intestinal milieu, either in vitro or in vivo. This exposure has been described by Stewart et al. (18) to alter the behavior of the larva, changing the type of movement exhibited from coiling to serpentine. Serpentine movement appears to be advantageous for the worm to gain entry into the cell layer, and this is the type of movement displayed by the larva once it is in the epithelium (22; see above). We found that treatment of larvae with intestinal contents in vitro could substitute for inoculation into the intestine. Larvae enter the epithelium within minutes of inoculation in vivo (2), suggesting that exposure to the intestinal lumen for as little as 5 min is sufficient to promote invasive behavior. In contrast, exposure of larvae to intestinal contents for less than 1 h in vitro did not activate them reproducibly (data not shown). The basis for this difference is not clear.

The third requirement is the epithelial cell itself. Although larvae invaded a number of epithelial cell lines, they did not invade nonepithelial cells. The broad host range demonstrated by *T. spiralis* in vivo (reviewed by Campbell [6]) and in vitro (Table 3) suggests that the characteristics of the epithelial cell that are required for parasite invasion are conserved across species. The distinctive surface morphology created by apical microvilli may play a role. Alternatively, the parasite may require some epithelial cell-specific chemical signal to initiate invasion. The larvae used their heads to probe and poke at the surfaces of cells as they moved over the monolayer. Although this behavior preceded the invasion of susceptible cells, the larvae behaved this way when cultured on resistant cells and when cultured in liquid medium. This suggests that larvae recognize surfaces or receive feedback from the cells they probe. The worm has mechanical and chemical sensory receptors encircling the mouth that are positioned to respond to such signals (15); however, little is known of the stimuli to which they respond.

The L1 stage of *T. spiralis* has no oral appendages and does not possess a stylet. Without these rather obvious tools of invasion, *T. spiralis* appears to use more subtle devices. Fluo-

FIG. 1. MDCK-AA7 cells inoculated with *T. spiralis* larvae, labeled with propidium iodide, fixed, and stained with rat anti-tyvelose monoclonal antibody and goat anti-rat FITC as described in Materials and Methods. (A) Photomicrograph taken with 546-nm excitation and 580-nm barrier filters for imaging propidium iodide. Nuclei of the dead cells stain intensely and uniformly red. Nucleoli of the live cells in the surrounding monolayer are very lightly fluorescent. (B) Same field with 450- to 490-nm excitation and 520- to 560-nm barrier filters for imaging FITC. Tyvelose-bearing TSL-1 antigens stain green and are limited to the serpentine path traveled by a larva. (C) Same field with double exposure. Nuclei are pressed to the margins of the antigen-laden path. Bar, 50 µm.

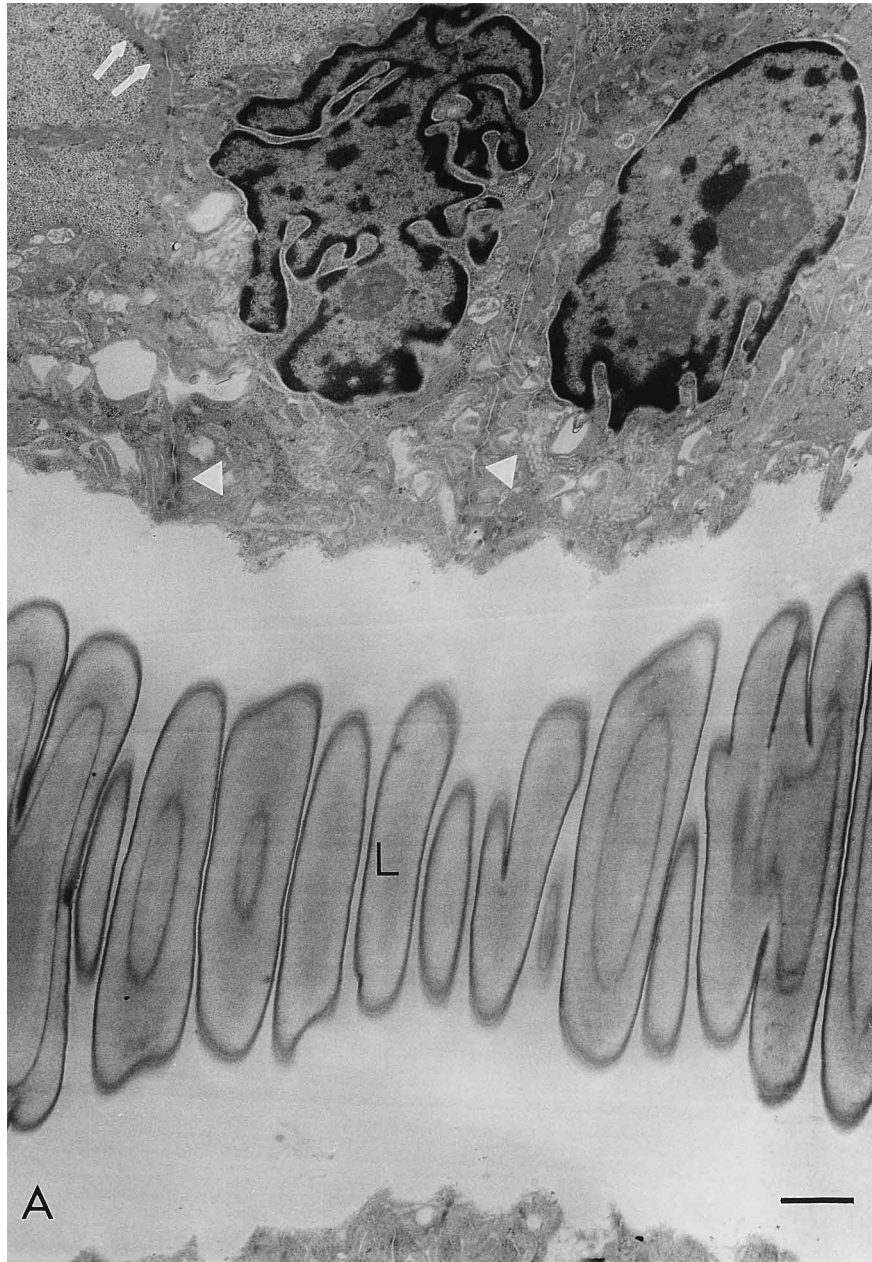


FIG. 2. Electron micrographs revealing the location of larvae in polarized Caco-2 monolayers grown on filter inserts. (A) Apical microvilli and tight junctions (arrows) provide evidence of epithelial cell polarization. A tangential section through the cuticle of one larva (L) shows that it occupies three or more cells. The cell margins are highlighted by arrowheads. Cytoplasm of the enterocytes is evident above and below the larva, compatible with a multi-intracellular location. Magnification,  $\times 10,298$ . Bar, 952 nm. (B) The basal aspects of cells are adjoined to the filter (F) (arrowheads). The cellular cytoplasm and nucleus separate the larva (L) from the filter. Magnification,  $\times 15,826$ . Bar, 625 nm.

rescent-antibody staining of infected monolayers showed intense TSL-1 glycan deposition in the paths of occupied cells. These glycans could be derived from the surface, the oral secretions, and the anal excretions of the parasite; all are sources of TSL-1 glycoproteins. Both the surface glycoproteins and the oral secretions would be positioned to facilitate entry into and transit through the epithelium; however, our results do not implicate one or the other. Functional activities have not yet been ascribed to the TSL-1 glycan. Although a few genes encoding excretory/secretory polypeptides have been cloned (4, 19, 20, 24), none of the deduced amino acid se-

quences show significant homology to any gene of known function. One protein has been reported to incorporate a DNA binding motif (20); however, a DNA binding function for the cloned gene product has not been described. A gene cloned from the parasitic nematode *Trichuris muris* has been reported to encode a secreted protein with fusogenic activity (9). The niche of *Trichuris* is similar to that of *T. spiralis* (12), and a fusogen may facilitate entry and transit of the worm through the epithelium. The existence of a fusogen in *T. spiralis* seems probable but has not been reported.

In summary, we have developed a simple method for study-



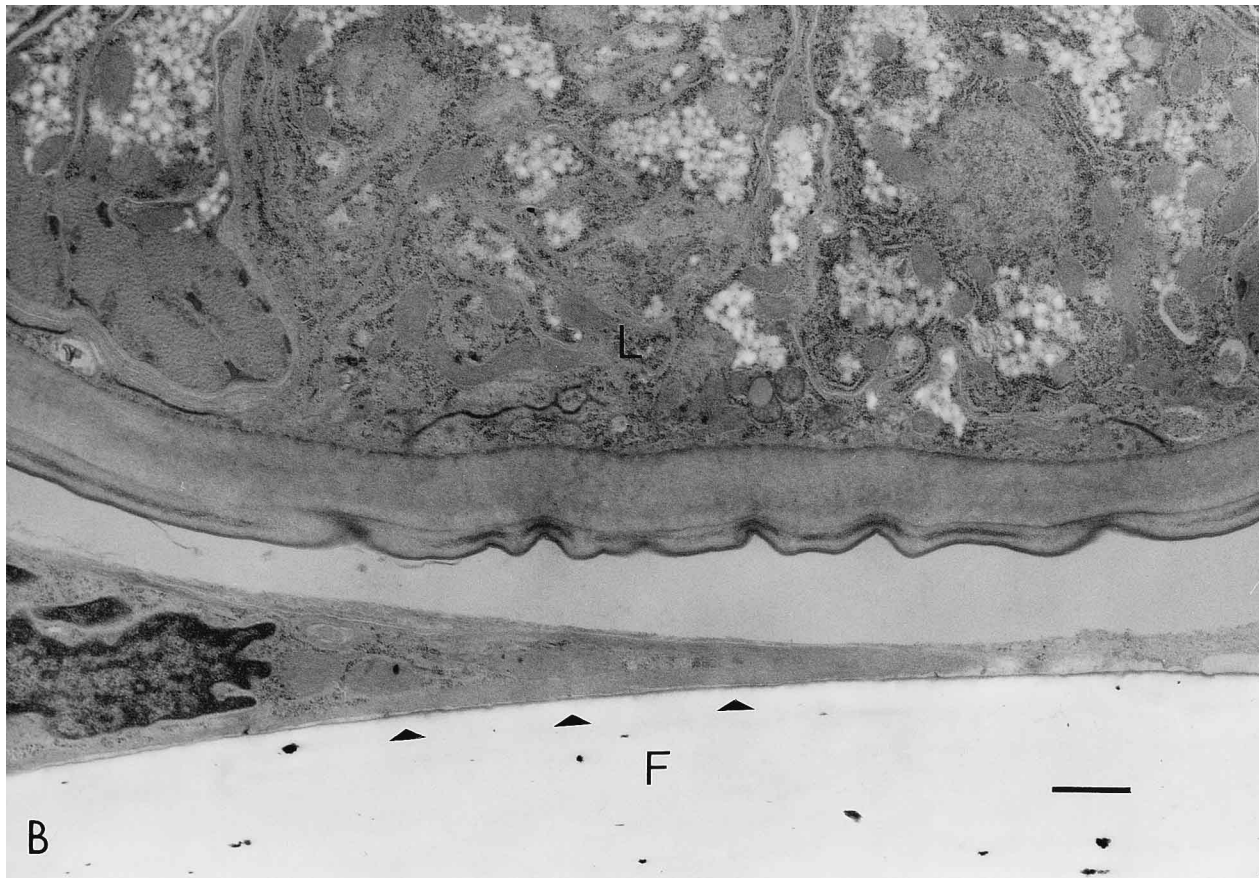


FIG. 2.—Continued.

ing the invasion of epithelial cells by the parasitic nematode *T. spiralis*. The assay reproduced the broad host range and restricted cell specificity of the parasite. Although worm occupation dramatically distended the epithelial cells, larvae in the monolayer were seen to be within cells, not beneath or between them. Thus, the assay confirmed the intracellular habitat of *T. spiralis* (23). Tyvelose-bearing glycans were shed or secreted by the larvae as they traveled through the cells, suggesting a role for these molecules or glycoproteins in invasion of and transit through the epithelium. The method holds considerable promise for further investigation of the niche of *T. spiralis*, as well as the mechanisms of immune system-mediated disruption of that niche.

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