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Trichinella spiralis: Synthesis of Type IV and Type VI Collagen during Nurse Cell Formation

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POLVERE, R. I., KABBASH, C. A., CAPÓ, V. A., KADAN, I., AND DESPOMMIER, D. D. 1997. *Trichinella spiralis:* Synthesis of type IV and type VI collagen during Nurse cell formation. *Experimental Parasitology* **86,** 191–199. The portion of skeletal muscle fiber (Nurse cell) harboring *Trichinella spiralis* is surrounded by an acellular capsule susceptible to digestion with collagenase. Antibodies recognized type IV and type VI collagen in the capsule, while the periodic acid Schiff reagent stained the capsule differentially, revealing at least two distinct layers. RNA analysis showed that mRNA specific for type IV and type VI collagen was present in muscle tissue on Days 9 and 15, but not on Day 3, following intracellular infection. *In situ* hybridization showed that most of the mRNA for both types was within the Nurse cell, and all enlarged Nurse cell nuclei were transcriptionally active for those messages. Synthesis of type IV collagen mRNA was absent by Day 24. In contrast, type VI collagen mRNA was still present at 24 days and 8 months. These results support the hypothesis that *T. spiralis,* either directly or indirectly, influences the synthesis of these two collagen types throughout its own developmental cycle in the Nurse cell.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Trichinella spiralis;* collagen; *in situ* hybridization; host–parasite relationship; Northern; Nurse cell; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DAB, diaminobenzidine; DIG, digoxigenin; EtOH, ethanol; ISH, *in situ* hybridization; NBT, nitrobluetetrazolium; PAS, periodic acid Schiff; PBS, phosphate-buffered saline; UTP, uridine triphosphate.

INTRODUCTION

Trichinella spiralis is an intracellular parasite that lives within an altered portion of skeletal muscle fiber (Beckett and Boothroyd 1961; Despommier 1975), completing its developmental cycle in that niche within 20 days (Despommier *et al.* 1975). In the process it modulates a portion of host cell architecture (Despommier 1975; Jasmer 1990; Jasmer *et al.* 1991), disrupts the cell cycle (Jasmer 1993), and downregulates the myogenic program (Jasmer 1993) and transforms it into a new cell type whose function is to ensure the growth and development of the parasite (Despommier 1993); hence, its name, Nurse cell (Purkerson and Despommier 1974). It is surrounded by a collagen capsule (Ritterson 1965) of which type IV collagen is a component (Gabryel *et al.* 1993), but the full complement of collagen types in it have not been described, nor have their origin(s) of synthesis been determined. A circulatory rete (Humes and Akers 1952; Baruch and Despommier 1991) surrounds the capsule except at the poles, which remain attached to the unaltered portions of muscle cell. An intense inflammatory response consisting of mixed cellular types (macrophages, lymphocytes, neutrophils, and eosinophils) accompanies Nurse cell formation (Weatherly 1983) and is particularly intense at the poles. It is probable that the capsule is not derived from cells in the immediate vicinity of the Nurse cell because its formation can occur in the absence of inflammation, as observed in animals given intensive immunosuppressive therapy during infection (Walls *et al.* 1973). The parasite is surrounded by a parasitophorous vacuole within the cytoplasmic matrix. The vacuole consists of a double layer with a complex structure (Despommier and Kajima 1967; Phillip *et al.* 1980; Lee and Wright 1984). The cytoplasmic matrix itself consists of whorls of

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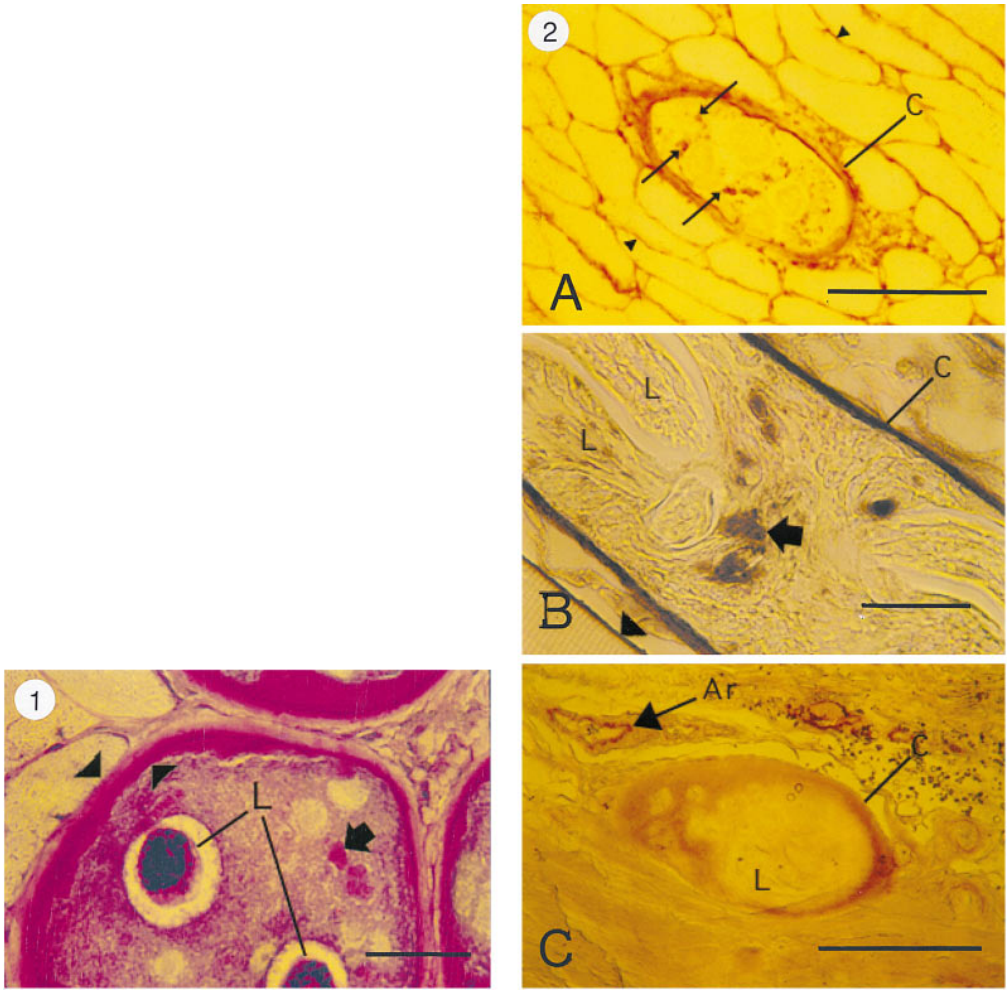

FIG. 1. Histochemistry. The PAS reagent stained the capsule differentially, with two main layers in evidence (arrowheads). Inclusions inside the Nurse cells also stained positive with PAS (arrow). L, larva. Bar, 50 μm.

FIG. 2. Immunohistochemistry. (A) Anti-type IV collagen antibody reacted with the capsule (c), some regions of Nurse cell cytoplasm (arrows), and the glycocalyx of normal muscle cells (arrowheads). Bar, 450 μm. (B) Anti-type VI collagen antibodies reacted with the capsule (c), some regions of Nurse cell cytoplasm (arrow), and the glycocalyx of normal muscle cells (arrowhead). L, larva. Bar, 50 μm. (C) Anti-type VI collagen antibodies also reacted with the outer capsule (c) of Nurse cells in human muscle tissue. Arrow, artery. Bar, 450 μm.

smooth membranes in which aggregates of dysfunctional mitochondria (Bozcon *et al.* 1989) and numerous enlarged nuclei are embedded (Despommier 1975; Despommier *et al.* 1991). Both Nurse cell and parasite are most likely anaerobic (Stewart 1983). Nurse cell nuclei may be transcriptionally active (Gustowska *et al.* 1989), although no direct evidence for this has been reported.

We sought to determine the composition of the capsule relative to its collagen content and to identify the cellular origin(s) of capsule collagen synthesis. Antibodies against various collagens were used to determine the type(s) of collagen present and the periodic acid Schiff reagent (PAS) served to indicate the relative degree to which each is glycosylated. *In situ* hybridization (ISH) with cRNA probes was used to localize the site of synthesis of collagen types identified by immunohistochemistry.

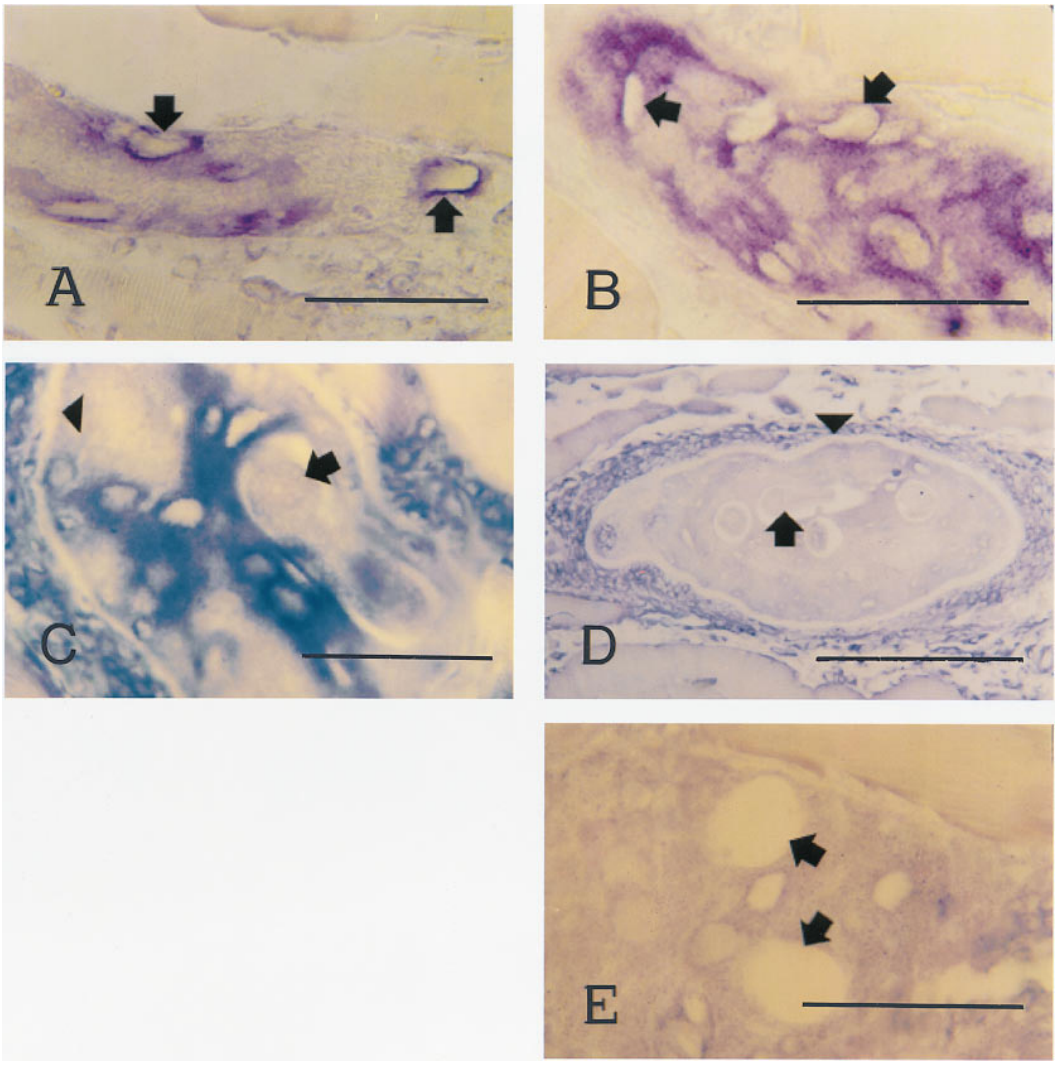

FIG. 4. *In situ* hybridization. (A) Hybridization signal of anti-sense cRNA specific for type IV collagen was positive and was first detected on Day 7 postinfection. Most of the signal localized around the enlarged nuclei (clear zones, arrows). Bar, 80 μm. (B) Signal was stronger on Day 9 of infection and also occurred adjacent to all enlarged nuclei (arrows). Anti-sense probe also localized in the periphery of Nurse cell cytoplasm, but to a lesser extent. Bar, 80 μm. (C) A pattern of hybridization similar to that observed on Day 9 was seen on Day 15 of infection. All enlarged nuclei observed were expressing message for type IV collagen. No hybridization occurred in the worm (arrow), capsule (arrowhead), invading cells, or normal muscle cells. Bar, 100 μm. (D) No hybridization occurred on the worm (arrow) or the cytoplasm on Day 24. Note the Nurse cell capsule (arrowhead). (E) Sense cRNAs did not hybridize with any of the above tissues. Arrows, parasite. Bar, 10 μm.

MATERIALS AND METHODS

Preparation of tissues. Synchronous infections (Despommier *et al.* 1975) were used throughout. Tissues used in histochemical and immunohistochemical reactions were fixed in 10% buffered formalin (pH 7.0) and routinely processed for paraffin sections (6 μm thick) (Vacca 1985). For the ISH the tissues were placed in 30% sucrose and 4% paraformaldehyde for 24 hr each, snap frozen in liquid nitrogen and kept at −70°C (Shaeren-Wiemers and Gerfin-Moser 1993).

Histochemistry. The PAS reaction was carried out as described elsewhere (Vacca 1985). Briefly, the deparaffinized sections were rehydrated in a series of degraded alcohols to

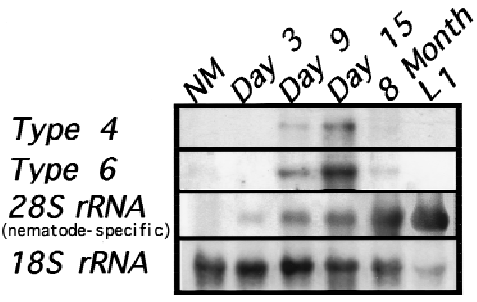

FIG. 3. Northern analysis. A cDNA probe specific for type IV a1 collagen gave no signal with normal muscle (NM), isolated larvae (L1), or at Day 3 after infection. Messenger RNA was detected at Days 9 and 15, and both gave strong signals. A small background level of mRNA was noted at 8 months after infection. Similar results were obtained when a cDNA probe specific for type VI a1 collagen was used. A cDNA probe specific for mouse 18S rRNA was weakly cross-reactive with parasite rRNA and demonstrated the presence of equivalent amounts of RNA in each lane. The nematode-specific 28S rRNA probe showed bands for each day of the infection. A shorter exposure time was used for lane 6 (L1 larva) because of the large amount of rRNA present in the purified *Trichinella* RNA, compared to the synchronously infected samples.

distilled water, rinsed in 70% EtOH, placed in aldehyde fuchsin for 15 min, rinsed in alcoholic bisulfite solution, counterstained, and washed in 70% EtOH, dehydrated, cleared, and permanently mounted.

Immunohistochemistry. Rabbit antisera were obtained from the following sources: Accurate Chemical and Scientific Corporation (Westbury, NY; type III and V) and Chemicon (Temecula, CA; types I and IV). Type VI was kindly given by Dr. Eva Engvall. Antibodies were applied to deparaffinized sections of infected mouse muscle tissue. The Pathology Department, Columbia University College of Physicians and Surgeons, generously provided the infected human tissue. The tissue was obtained from a patient with laryngeal cancer, and thus the age of the infection cannot be determined. *T. spiralis* infection was incidental to the patient's disease diagnosis. The tissue sections were deparaffinized and rehydrated in the same way as that described above. The specificity and optimal dilution of each of the antibodies was determined using type I, III, IV, V, and VI collagen standards (Sigma Chemical Company, St. Louis, MO) in dot blot configuration. Different dilutions of the antibodies were titered against all of the collagen standards. At low dilutions, some degree of cross-reactivity was demonstrated (whole serum and 1:50 dilution). In contrast, at 1:500, the dilution used on all tissue sections, all antibodies only reacted with the collagen species against which they were generated. All primary antisera were diluted in PBS and incubated at 4°C overnight. A conjugate of horseradish peroxidase (Sigma Chemical Co.) was used at 1:500 concentration at 20°C for 45 min. The reaction was developed using DAB reagent (Sigma Chemical Co.) (Vacca 1985). The sensitivity of the reaction was increased by adding 1% nickel chloride to the DAB developing solution (ibid).

Synthesis of RNA probes. All four cRNA probes were transcribed *in vitro,* from plasmid DNA, in the presence of DIG-labeled UTP as described by the manufacturer (Boehringer-Mannheim Biochemical, Indianapolis, IN; ''Genius System User's Guide for Filter Hybridization, Version 2.0,'' pp. 30–32). The mouse type IV a1 collagen antisense strand was synthesized using T3 RNA polymerase (Boehringer-Mannheim Biochemical), while the sense strand was synthesized using T7 RNA polymerase (Boehringer-Mannheim Biochemical). The antisense and sense strands of mouse type VI a1 collagen were generated using T7 and T3 RNA polymerases, respectively.

In situ hybridization. Frozen sections of synchronously infected mouse skeletal muscle were hybridized with sense and anti-sense cRNAs with specificities for type IV and type VI collagen mRNA using the protocol previously described (Shaeren-Wiemers and Gerfin-Moser 1993). Briefly, the sections were thawed, fixed again in buffered 4% paraformaldehyde, washed in PBS, acetylated with a mixture of triethanolamine, NaOH, and acetic anhydride for 10 min, washed in PBS, then blocked with hybridization buffer composed of 50% formamide (Fisher Scientific, Springfield, NJ), 5× SSC, 2% blocking solution (Boehringer-Mannheim Biochemical), 250 μg/ml torula yeast RNA (5 Prime to 3 Prime Inc., Boulder, CO), 500 μg/ml herring sperm DNA (5 Prime to 3 Prime). Slides were incubated overnight at room temperature. Hybridization was carried out at 62°C in the presence of 200–400 ng/ml of digoxigenin-labeled probe which was subsequently detected with an antibody anti-DIG marked with alkaline phosphatase enzyme (Boehringer-Mannheim Biochemical) used as indicated by the manufacturer and developed with the NBT/BCIP color substrate detection procedure (Boehringer-Mannheim). The endogenous alkaline phosphatase activity was blocked with 0.24 mg/ml levamisol. No counterstaining was used. The slides were mounted with Kaiser's glycerol gelatin (Vacca 1985). A few sections were reacted with sense probes specific for each collagen and served as negative controls for the anti-sense probes.

Northern analysis. A Northern blot containing total RNA was prepared from normal mouse muscle tissue, infected mouse tissue on Days 3, 9, and 15 after infection, an 8-month-old infection, and isolated *T. spiralis* larvae (L1). The filter was probed with ^{32}P-labeled cDNAs. Total RNA (10 μg) was isolated (Chomczynski 1993), blotted (Maniatis *et al.* 1989), and probed with ^{32}P-labeled cDNAs (Feinberg and Vogelstein 1983). The hybridization and washing of the blots was carried out at a temperature of 65°C, and the final wash was in 0.1 × SSC, 0.1% SDS. The molecular weights of the hybridizing bands were determined using RNA molecular weight size standards (Promega, Madison, WI). Ethidium bromide was added to all samples prior to electrophoresis to ensure equal loading, transfer, and visualization of the samples as well as the markers (Rosen and

Villa-Komaroff 1990). The molecular weights of the hybridizing bands were in agreement with published results (Kurkinen *et al.* 1983; Zhang *et al.* 1993).

Southern analysis. Southern blots (data not shown) containing digested genomic DNA from *Mus musculus* and *T. spiralis* were probed with ^{32}P-labeled cDNA clones encoding portions of the type IV and type VI collagen genes. Each probe gave the expected pattern on mouse DNA digests (Zhang *et al.* 1983; Solomon *et al.* 1987; McDonald *et al.* 1991) under conditions of high stringency (0.1 × SSC, 0.1% SDS, 65°C). Neither collagen probe hybridized with *T. spiralis* DNA digests, even under low-stringency (6× SSC, 0.1% SDS, 50°C) conditions. Additionally, each of the collagen clones was partially sequenced and verified the identity of those probes.

RESULTS

The capsule stained PAS positive, and the inner layer usually stained more intensely than the outer one (Fig. 1). In a few Nurse cells, the opposite pattern was noted. The cuticle of the parasite reacted weakly with antibodies against type I collagen, but not with antibodies against other types of mammalian collagen (anti-type III, IV, V, and VI) or normal serum (not shown). In contrast, anti-type IV and anti-type VI sera reacted positively with components of normal and infected host cells. Antisera against mouse type IV collagen reacted with the Nurse cell capsule (Fig. 2A) and the glycocalyx of noninfected muscle cells. An anti-human type VI collagen antibody also reacted with Nurse cell capsule (Fig. 2B) and glycocalyx of noninfected mouse muscle fibers (Hessle and Engvall 1984, Engvall *et al.* 1986). To verify antibody specificity, a section of infected human muscle was incubated with it and the capsule reacted positively (Fig. 2C). Type IV collagen is heavily glycosylated (10% by mass) (Burgeson and Nimni 1992); thus, immunohistochemical data, together with PAS staining patterns, show that the capsule is composed of at least types IV and VI collagen. Other components normally found on the surface of muscle cells (e.g., proteoglycans) were not tested for in this study. Deposits of apparently unprocessed types IV and VI collagen were detected in the cytoplasm of Nurse cells (Figs. 2A and 2B).

Northern analysis (Fig. 3) of total RNA from infected muscle on Day 3 (prior to maximum Nurse cell formation), Days 9 and 15 (during formation), and 8 months (after formation) gave the following results. Abundant RNA for both collagen type IV and VI was observed beginning on Day 9. A stronger signal was obtained on Day 15. Message for both types of collagen was greatly reduced in infected muscle at 8 months. No signal was obtained for normal muscle or in the larva (L1) with either collagen probe. In contrast, nematode-specific 28S rRNA was present in extracts of tissue throughout the infection period in increasing amounts and correlated with the rate of growth of the parasite (Fig. 3). The 18S rRNA component (probe generated from *Mus musculus* RNA) was detected in all tissue extracts, including the L1 larva of *T. spiralis*, but in the latter case to a much lesser extent, due probably to its low degree of cross reactivity.

Sense and anti-sense DIG-labeled cRNA probes for ISH were made from each cDNA clone (Shaeren-Wiemers and Gerfin-Moser 1993). Anti-DIG antibody labeled with alkaline phosphatase was used to reveal the anti-sense cRNA probes in tissue (ibid). Hybridization was performed on formalin fixed frozen muscle tissue at 7, 9, 15, and 24 days after infection (Figs. 4A–4D, 5A–5D). Sense cRNA probes were used in a similar fashion (Figs. 4E and 5E). Each anti-sense probe hybridized with areas of infected muscle cells beginning on Day 7 (Figs. 4A and 5A) and the signal increased in intensity on day 9 (Figs. 4B and 5B). Cytoplasmic regions immediately adjacent to all enlarged nuclei in all Nurse cells gave the most intense signal. On Day 15 the signal was still strong (Figs. 4C and 5C), while on Day 24 it was nearly absent for the probe specific for collagen type IV (Fig. 4D). In contrast, signal was still detectable for type VI collagen (Fig. 5D). Some signal for both RNA probes was also noted in the interstitial space immediately outside the Nurse cell at Days 15 and 24 and appeared to be associated with inflammatory cells. Anti-sense probes did not react with any portion of normal muscle cells. No mRNA was detected with sense cRNA probes for either collagen type (Figs. 4E and 5E).

DISCUSSION

T. spiralis takes 20 days to achieve its maxi-

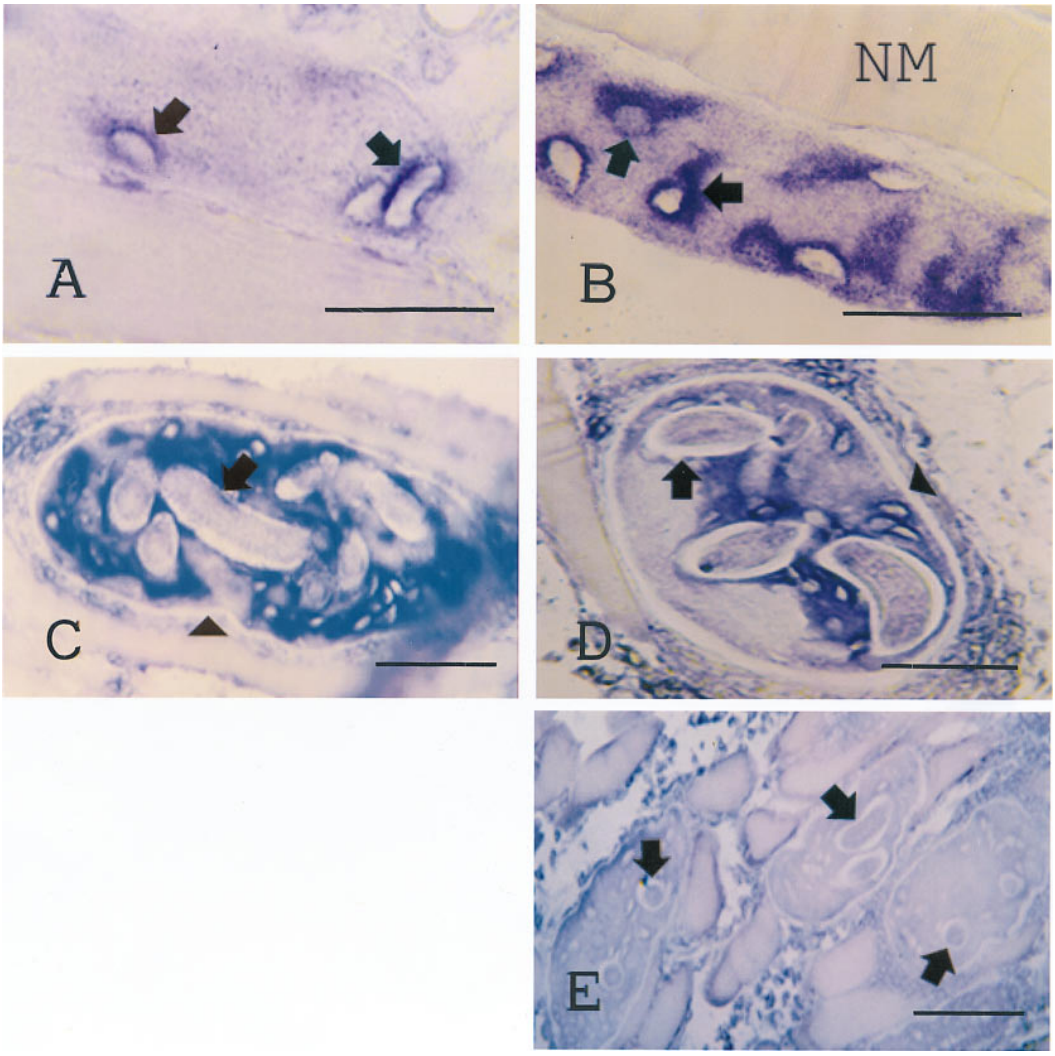

FIG. 5. *In situ* hybridization. Anti-sense cRNA probe specific for type VI collagen mRNA gave a pattern similar to that seen in Fig. 4. (A) The hybridization signal on Day 7 was observed around all enlarged Nurse cell nuclei (arrows). Bar, 70 μm. (B) A stronger signal was noted immediately adjacent to enlarged nuclei (clear zones, arrows) on Day 9. Normal muscle (NM) did not react with the probe. Bar, 70 μm. (C) On Day 15, all observed enlarged nuclei expressed message for type VI collagen. The signal was strong at this time and was never present over sections of worm (arrow). Note the Nurse cell capsule (arrowhead). Bar, 100 μm. (D) Some signal was still present around the Nurse cell nuclei at Day 24. Neither the worm (arrow) nor the capsule (arrowhead) reacted with the probe. The probe hybridized with some cells in the adjacent interstitial spaces around the developing Nurse cell. Bar, 100 μm. (E) Sense cRNA gave no signal for any tissue for any time point. Arrows, parasite. Bar, 200 μm.

mum size within the Nurse cell (Despommier *et al.* 1975), while formation of the host cell occurs mainly from Days 4 through 15. Collagen can first be detected by immunohistochemistry on the outside of the developing Nurse cell beginning on Day 10 following infection (Despommier 1975). *In situ* hybridization indicates that the genes for type IV and VI collagen

are expressed for different amounts of time throughout the infection, but both are first detected on Day 7. Most of the mRNA for both types of collagen was within the developing Nurse cell, as judged by the intensity of the reaction for each species of RNA probe. Although quantitative determination of mRNA could not be performed *in situ*, care was taken to insure that each tissue section was cut to the same thickness and that the same amount of probe was applied to the surface of each slide. Thus, differences in staining patterns should generally reflect the amount of specific mRNA present, allowing for comparison of relative amounts of message in each tissue section throughout the infection period.

Some mRNA specific for both types of collagen was associated with the adjacent inflammatory response near each Nurse cell. Earlier studies on immunosuppressed mice (Walls *et al.* 1973) have shown that capsule formation can proceed in the complete absence of inflammation. Furthermore, Nurse cells in nude mice also develop a thick collagen capsule (Gustowska *et al.* 1981). However, nude mice mount an inflammatory response, albeit reduced compared to heterozygous thymic litter mates, that consists almost entirely of eosinophils. This cell type has no known association with collagen deposition, either as an inducer of collagen synthesis or as an inhibitor of it. Fibroblast collagens (types I and III) have been observed near the capsule but are not components of it, as judged by electron microscopy (Despommier 1975). In the present study, anti-collagen type I or type III antibodies did not detect significant amounts of either of these molecules in any region of muscle tissue. Taken together, the above data argue in favor of the Nurse cell, alone as the single source of the collagen capsule.

Demonstrating type VI collagen in the capsule of the Nurse cell is a novel finding. Data from Northern analysis showed that this collagen is always being produced, up to 8 months after infection. This suggests that Nurse cell-associated collagen type VI turns over at a more rapid rate than most other host collagens, since the thickness of the capsule does not increase beyond Day 30 after infection. In contrast, synthesis of both collagen types was at an extremely low level in noninfected skeletal muscle as no mRNA was detected in any unaffected cells.

The present study also showed that all Nurse cell nuclei were transcriptionally active. There are some 30–60 nuclei in the majority of Nurse cells (Despommier *et al.* 1991), and each nucleus has 4 *N* DNA (Jasmer *et al.* 1993). Parasite amplification of the genome of the Nurse cell most likely reflects its need to develop rapidly. As the worm enters its log phase of volumetric growth (Days 4–19 after infection), demands for nutrients and elimination of wastes increase and most likely depend heavily on the construction of the circulatory rete. A solid substrate, the collagen capsule, provides the necessary scaffolding for it to grow around. Induction of rete growth occurs on Day 12 (Humes and Akers 1952), while collagen is deposited in the capsule commencing on Day 10.

The origin and nature of the parasite signal(s) that may regulate the synthesis of collagens and other Nurse cell functions is central to unraveling the way in which the worm influences its hypertrophic niche (Despommier 1993). In this regard, antibodies produced against the secreted proteins of the parasite identify a family of uniquely glycosylated peptides (Wassom *et al.* 1991) that are secreted by the L1 larva, only, during its developmental cycle in the Nurse cell. The majority of these antigens share a common epitope consisting of tyvelose (3,6-dideoxy-*D*-arabinohexose) (Wisnewsky *et al.* 1993), and it serves as the marker for stichocyte-secreted proteins, as that rare variety of sugar is unique to that cell type. Some tyvelosylated peptides localize to host cytoplasm, while others localize to enlarged nuclei (Despommier *et al.* 1990), and can be found there as early as Day 8 after infection. They are present in those locations thereafter. Attempts to identify specific tyvelosylated peptides in tissues have been largely unsuccessful. The use of monoclonal antibodies against the peptide backbone may help resolve this problem.

Since virtually all tyvelosylated proteins are

synthesized by the stichocyte cells, we hypothesize that stichosome-secreted L1 larval proteins could function to regulate the expression of host genes encoding these two collagens, since the presence of tyvelosylated secreted antigen in the cytoplasm and nuclei of the developing Nurse cell is temporally coincident with the synthesis of both collagens. Furthermore, we believe that the formation and maintenance of the Nurse cell is under the direct control of *T. spiralis* and involves mechanisms similarly dependent on stichocyte secretions.

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