Cuticle Formation in Parasitic Nematodes: 
Ultrastructure of Molting and the Effects of Actinomycin-D

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Received December 11, 1974, and in revised form January 29, 1975

The ultrastructure of cuticle formation and the effect of actinomycin-D on cuticle formation were examined in the second molt of Nippostrongylus brasiliensis. At the end of the first molt the rough endoplasmic reticulum of the hypodermis (epidermis) had narrow cisternae. As cuticle formation began the RER cisternae dilated and accumulated a fibrous material. These large fibrous material-containing vesicles then decreased and by the time cuticle deposition was completed they had disappeared. Actinomycin-D prevented the proliferation of RER and the formation of fibrous material-containing vesicles. Actinomycin-D induced several ultrastructural alterations in the hypodermis, the most prominent of which were large crystalline-like inclusions. Other alterations involved the configuration of RER and structure of nucleoli. These morphological aspects of cuticle formation were correlated to the molecular events that control molting.

The nematode body wall is composed of a limiting cuticle underlain by a syncytial or cellular hypodermis (epidermis). The hypodermis extends into the body cavity to form dorsal, ventral, and two lateral chords and these chords are connected by thin interchordal strands of cytoplasm. Hypodermal nuclei are confined to the chords and mitochondria, Golgi complexes and endoplasmic reticulum are largely concentrated in the chords (4, 5, 12, 16).

Recent ultrastructural and cytochemical studies have investigated the process of cuticle formation (5, 6, 13, 14, 17, 21). These studies indicate that the hypodermis is the site of cuticular protein biosynthesis. Prior to the initiation of cuticle formation, the hypodermis displayed comparatively little rough endoplasmic reticulum (RER), and few Golgi complexes, mitochondria, and vesicles. As cuticle formation began, the organelles increased in size, complexity, and number. These ultrastructural changes were similar to those seen in cells that synthesize and secrete collagen (20).

This reflects the fact that nematode cuticle contains large amounts of collagen (1). Nematodes form five cuticles during development from egg to adult. In some cases the cuticles are structurally different, as in the second, third, and fourth stages of N. brasiliensis (5, 6). These structural differences may reflect the presence of distinct proteins in each cuticle. Indeed, hemoglobin is present in the cuticle of adult N. brasiliensis (17), but it has not been detected in second and third-stage cuticle. Thus, it is likely that the biosynthesis of nematode cuticle is a result of differential gene expression and the presence of structurally different cuticles is a reflection of distinct gene expression.

Recent studies in our laboratory (3) have provided evidence to support the hypothesis that formation of nematode cuticle is under direct gene control. These investigations indicated that the RNA necessary for the synthesis of third-stage cuticle were transcribed approximately 90 hr after egg deposition. Actinomycin-D and α-amanitin inhibited the biosynthesis of those RNA and these transcription inhibitors prevented cuticle formation. The present
studies were designed to explore the ultrastructural manifestations of the genetic control of cuticle formation and to relate them to the molecular aspects of molting. These events were examined for the second molt of *N. brasiliensis*.

**MATERIALS AND METHODS**

A brief description of the life history of *N. brasiliensis* is appropriate to aid in following the events in cuticle formation. The adults of this trichostrongyle nematode parasitize the small intestines of rats. Eggs are passed in the feces and hatch as first-stage larvae. The first-stage larvae molt to the second stage and a second molt gives rise to the third stage. The third stage is the infective form and it penetrates the skin of the rat, migrates to its lungs, and there molts again. This fourth-stage larva then migrates to the small intestine, undergoes a fourth molt, and becomes the fifth-stage adult.

The isolation of eggs and cultivation of *N. brasiliensis* have been described elsewhere (2). Axenized eggs (10⁶/ml) were inoculated into 500-ml Erlenmeyer screw-cap flasks in 10-ml Krebs-Ringer solution buffered with 0.01 M Tris-HCl (KRT; pH 7.4). After 24 hr incubation at 25°C, eggs had hatched and first-stage larvae were transferred to *Esherichia coli* K-12 medium containing 100 μg/ml cholesterol, 100 units/ml penicillin, and 100 μg/ml streptomycin. *E. coli* K-12 medium was prepared by the methods of Bolla *et al.* (2), except that the concentration of *E. coli* was doubled. Cultivation was carried out in 50-ml stoppered microfermbach flasks containing 5 × 10⁴ organisms/2 ml medium. Previous studies (2) have indicated that 24 hr of incubation in KRT (a non-nutrient medium) established a relatively high level of developmental synchrony in first-stage larvae. Thus, when worms were transferred to *E. coli* K-12 medium subsequent development was rather uniform.

In control experiments, worms were removed from culture at various times from 60-150 hr; inoculation of eggs into KRT was designated time zero. This time interval was selected when preliminary experiments showed that the first molt was completed by 66-70 hr and the second molt was completed by 147 hr.

In other experiments, actinomycin-D (50 μg/ml) and α-amanitin (10 μg/ml) were added to cultures at 69 hr and worms were removed from the culture at 86-90 hr. All worms were placed in ice-cold 3.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) containing 0.25 M sucrose and 2.0 mM CaCl₂. After fixation for 2 hr, specimens were washed overnight in buffer, postfixed in 2.0% osmium tetroxide, washed in buffer, dehydrated in ethanol, and embedded in a low-viscosity medium (22). Thin sections were stained with uranyl acetate and lead citrate.

**RESULTS**

**Formation of third-stage cuticle.** Cultivation of *N. brasiliensis* from egg to third stage was accomplished in formalin-killed *Esherichia coli* medium. Ultrastructural examination of worms indicated that the first molt was usually completed by 66–70 hr after isolation of the eggs. The second molt, during which the third-stage cuticle was formed, was completed by 147 hr. Examination of worms during the second molt revealed minor variations in the time of development. For example, the most advanced worms at 96 hr had occasionally progressed further in the molting sequence than the least developed worms at 102 hr. This variation is not unusual and has even been observed during in vivo development of *N. brasiliensis* (6). These minor variations were seen in approximately 10–20% of the worms. To accurately standardize observations, the events observed in the greatest number of organisms at any designated time were regarded as representative of the entire population.

By 66 hr, the majority of larvae had a fully formed second-stage cuticle immediately subjacent to the uncast first-stage cuticle. The hypodermis was characterized by a well-developed rough endoplasmic reticulum (RER). The dilated cisternae of the RER contained a fibrous component. Golgi complexes were also prominent in the hypodermis.

By 68 hr, the overlying first-stage cuticle had been shed. The cisternae of the RER had decreased in size and no longer contained fibrous material (Fig. 1). Golgi complexes were less frequent and during the remainder of the molting period they were rarely seen. Hypodermal nuclei ranged in size, and the ribosomes and cisternae of RER are comparatively narrow. Note the second-stage cuticle (C2), × 34 000.

**Fig. 1.** Section through hypodermis of a young second-stage larva (75 hr). The hypodermis displays many free ribosomes and the cisternae of RER are comparatively narrow. Note the second-stage cuticle (C2), × 34 000.

**Fig. 2.** An electron micrograph of the hypodermis from a 90-hr control larva. In comparison to Fig. 1, the cisternae (*) of RER are considerably larger and contain fibrous material. These large fibrous material-containing vesicles of RER are considered to accumulate precursor cuticular protein. × 35 000.
appearance from spherical to irregular in outline and usually contained a single prominent nucleolus.

Between 75 and 96 hr, the hypodermis began to exhibit characteristic changes related to preparation for new cuticular protein biosynthesis. The most prominent changes involved the RER. There was a progressive increase in the number of RER profiles. The cisternae became dilated and accumulated fibrous material. These large, vesicular cisternae were generally distributed throughout the cytoplasm (Fig. 2).

The initial signs of cuticle deposition (110 hr) were marked by the appearance of a thin striated layer immediately subjacent to the second-stage cuticle (Fig. 3). As cuticle deposition continued (Fig. 4), the vesicular cisternae decreased in size and finally disappeared. In some instances, vesicles that contained fibrous material did not have ribosomes attached to the limiting membrane. Neither the vesicular cisternae or vesicles were observed to fuse with the hypodermis plasma membrane.

Effect of actinomycin-D on the hypodermis. When actinomycin-D was added to cultures at 69 hr and worms were examined at 75, 81, and 87 hr no obvious ultrastructural alterations were observed (Fig. 5). However, these worms, which were exposed to actinomycin-D for 18 hr or less, were not comparable to control worms of the same age. Worms that were treated with actinomycin-D for 18 hr displayed a hypodermal ultrastructure comparable to that seen in younger worms at 69–75 hr. Compare Fig. 5 from an actinomycin-D treated worm (87-hr larva) with a 75-hr control worm (Fig. 1). It can be seen that the hypodermis of both worms was similar and displayed a few RER profiles with narrow cisternae. When worms treated with actinomycin-D for 18 hr (87-hr larva, Fig. 5) were compared with control worms of the same age (90-hr larva, Fig. 2), it was observed that the hypodermis of treated worms did not display large vesicular RER cisternae.

By 115–120 hr, the vast majority of control larvae were depositing the third-stage cuticle. Conversely, the majority of larvae treated with actinomycin-D showed no signs of cuticle formation nor did these worms exhibit any of the ultrastructural manifestations of protein biosynthesis described for control larvae (Figs. 6 and 7). These worms, which were treated for 48 hr, displayed a hypodermal ultrastructure similar to that of younger control larvae.
(compare Figs. 6 and 1). In all cases, worms treated with actinomycin-D never progressed beyond that stage in development which was comparable to early second-stage control larvae at 69–75 hr, i.e., actinomycin-D inhibited those ultrastructural features associated with cuticle formation which were seen in control worms. Similar observations were made on worms treated with α-amanitin, although a detailed analysis was not performed.

Larvae exposed to actinomycin-D for longer than 18 hr exhibited several ultrastructural alterations. The most prominent and consistent change was the appearance of crystalline-like inclusions (Figs. 7–9). The inclusions were equally prominent in the nucleus and cytoplasm. The inclusions did not appear to result from condensation or other degenerative processes since the surrounding cytoplasm or nucleoplasm was unaltered. The majority of inclusions appeared as tightly packed aggregations of electron-dense subunits with a diameter of approximately 100 Å. Both tubular and rod-like subunits were observed. Occasionally the subunits were arranged in sectors (Fig. 9), each sector being oblique to others. This was particularly true in worms treated with α-amanitin (10 μg/ml). The initial appearance of inclusions was chronologically consistent in that 18 hr of exposure to actinomycin-D was necessary for their induction.
Fig. 7. This image shows a hypodermal nucleus from a worm exposed to actinomycin-D for 48 hr. Observe the electron dense inclusion in the nucleus and normal nucleolus. × 70 000.

Exposure to actinomycin-D for 18 hr induced other ultrastructural alterations. Commonly, the RER would change configuration from randomly dispersed channels to restricted concentric arrays of cisternae (Fig. 10). In other cases, the RER concentrated around nuclei with the cisternae oriented parallel to each other. Other occasional alterations included segregation and fragmentation of nucleoli.

DISCUSSION

The ultrastructure of the hypodermis seen in *N. brasiliensis* during the second molt was similar to that observed in other molts (5, 6, 17) and was similar to other collagen producing cell systems with respect to the development and form of the RER and vacuole systems. However, some notable differences were observed. First, in the fourth molt of *Nematospirodis dubuis*...
and the third and fourth molts of *Nippostrongylus brasiliensis* (5, 17) the hypodermis progressed from a stage containing very few ribosomes to a point where free ribosomes were abundant. These free ribosomes then attached to a proliferating ER. In the molt studied here, there did not appear to be a stage when ribosomes were sparse in the hypodermis. It may be that ribosomes were carried over from the first molt. Indeed, the time between the first and second molt is shorter than the intermolt period for the other molts. Second, the number and size of Golgi complexes was quite reduced in this second molt of *N. brasiliensis* as compared to the third molt (5). The significance of that observation is not known but may reflect the synthesis and secretion of different proteins.

Recent investigations (3) have demonstrated that the second molt of *N. brasiliensis* is dependent on new gene transcription. These studies indicated that cuticle formation was significantly inhibited when actinomycin-D or α-amanitin was added to cultures before 96 hr. Cuticle formation was not prevented when the inhibitors were added at 96 hr or later. Biochemical evidence indicated that RNA specific for cuticle formation was synthesized at 90 hr and actinomycin-D and α-amanitin inhibited the synthesis of essential RNA.

Biochemical observations on gene regulation of development are most meaningful when they can be correlated with specific morphogenetic events. The present results have related the above-mentioned molecular aspects of the genetic control of molting with the ultrastructural features of that morphogenetic event. Thus, the present studies indicated that prior to the initiation of cuticle formation the hypodermal RER displayed narrow cisternae and no fibrous material containing vesicles. As the molting process progressed, the hypodermis displayed many ribosome-limited vesicles containing fibrous material. It is suggested that the accumulation of fibrous
material, which may be protocollagen, within the dilated cisternae of RER represented an ultrastructural manifestation of the translation of molting-specific mRNA synthesized at or before 90 hr. Recent studies (7, 15, 18) have demonstrated that protocollagen is synthesized on polysomes, transported to the RER cisternae and selected proline residues are hydroxylated by prolylhydroxylase located in the cisternae. In previous studies on cuticle formation (4, 6, 17) evidence indicated that cuticular protein precursors were secreted from the hypodermis. In one study (5), worms were examined at 2-hr intervals. That the secretion of cuticular material was not observed in this study cannot be explained, but may be related to the fact that organisms were examined at 6-hr intervals.

The fact that actinomycin-D and α-amanitin inhibited molting and synthesis of mRNA specific for molting (3) was reflected in the ultrastructure of the hypodermis in worms treated with those drugs. Thus, in treated worms the development of RER and the subsequent accumulation of fibrous material in RER cisternae was inhibited. Further, the ultrastructure of the hypodermis of treated worms never progressed beyond a stage comparable to young second-stage larvae. Ultimately, worms treated with actinomycin-D or α-amanitin never formed a third-stage cuticle nor did they display hypodermal ultra-

**FIG. 10.** The concentric arrangement of RER cisternae can be appreciated in this micrograph from a worm treated with actinomycin-D for 48 hr. × 64 000.
structure that represented any stage of cuticular protein biosynthesis.

To our knowledge the crystalline-like inclusions seen in worms treated with both actinomycin-D and α-amanitin have not been reported in other studies employing these inhibitors. These inclusions were seen in well over 100 treated worms and were never seen in control worms. It would appear that the inclusions represented a morphological expression of some underlying biochemical action of the inhibitors. Whether this was a direct reflection of the inhibition of transcription or a more likely secondary effect is not known.

The following observation concerning crystalline-like inclusion is pertinent. During the course of other studies on the infection process, third-stage worms grown in tissue culture medium 199 plus 20% calf serum were examined by electron microscopy. These worms were never treated with actinomycin-D or α-amanitin nor were they ever exposed to glassware that contained those inhibitors. A few of these worms displayed inclusions that were indistinguishable from those seen in worms treated with drugs during the second molt. The possibility exists that the inclusions resulted from some unknown stress. In one case, the stress was from drug treatment. In the other, it may have originated from the tissue culture environment.

To determine whether a generalized stress would induce the appearance of inclusions, worms were cultured in *E. coli* K-12 medium as usual. At 45 hr, worms were removed from the *E. coli* medium and transferred to KRT. At 90 hr, worms were processed and examined by electron microscopy. In no case were inclusions seen in these starved worms. Foor (8) has described inclusions from *Trichosomoides crassicauda*, a nematode parasite of rat urinary bladder, that are somewhat similar to those seen in this study. It was suggested that the inclusions in *T. crassicauda* were virus-like particles. The nature of the inclusions observed here remains to be determined.

The reorganization of RER and segmentation and fragmentation of nuclei seen in *N. brasiliensis* have been reported in other cells treated with actinomycin-D (9, 10, 19).

REFERENCES