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In vitro effects of *Trichinella spiralis* on muscle cells

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Abstract

Introduction of excretory/secretory (ES) products of both infective-stage and newborn larvae of *Trichinella spiralis* into cultures of primary rat myocytes elicited morphological and structural changes in the myotubes. They appeared more granular, thinner, and failed to form networks. The most prominent lesion was the formation of 'nodular' structures, each bearing an enlarged nucleus, along the myotubes. Each node contained numerous cavities enclosed by an intact sarcolemma. Co-culture of myocytes with newborn larvae also elicited nodular formation but each node contained a large central cavity encircled by smaller ones. An immunocytochemical study using IFAT and laser confocal microscopy showed the presence of parasitic epitopes inside the nodes. However, ES products from adult worms did not affect the myotubes.

Introduction

Trichinella spiralis can completely transform the striated muscles of mammals into a new type of syncytium known as the 'nurse cell complex' (Purkerson & Despommier, 1974; Lee & Shivers, 1987). After invasion by the newborn larvae, the muscles undergo major structural and biochemical changes. Myofilaments lose their cross-striations, become disorganized and eventually disappear. Extensive oedema occurs in surrounding tissues (Adams, 1975). A fully developed nurse cell consists of a cytoplasmic region with numerous hypertrophic myonuclei, endoplasmic reticulum, secretory vesicles, mitochondria and a cavity containing 1–4 worms. Cells and small nuclei of unknown origin are also present. The entire complex is circumscribed by collagenous fibres (Ko *et al.*, 1994). However, the mechanisms involved in the process of reorganization are unknown (Lee *et al.*, 1991).

Ko *et al.* (1994) reported that infective-stage larvae released from muscles could induce the formation of nurse cells after injection subcutaneously into rats. A parallel study also showed that intramuscular injections of excretory/secretory (ES) products from the infective-stage larvae into mice elicited extensive lesions including myotube formation, muscle bundles becoming rounded,

and an increase in endoplasmic reticulum. The authors suggested that the invasion of muscles by trichinellids elicits two independent events, i.e. a general degenerative/regenerative response of muscles and a specific change in the genomic expression of myonuclei. The two events are probably mediated by different effector molecules.

As an extension of the above *in vivo* experiments, the present study was undertaken to develop an *in vitro* model which can further document the morphological and structural changes of myotubes in the presence of various stages of worms and ES molecules.

Materials and methods

Production of ES materials

Infective-stage larvae of *T. spiralis* were recovered from muscles of experimentally infected ICR mice by the standard pepsin digestion method. The worms were maintained for 24 h in RPMI 1640 medium (Gibco-BRL), following the protocol of Gamble *et al.* (1983) and Ko & Yeung (1989). ES products in the culture medium were concentrated by ultrafiltration (Spectrum, 5 kDa molecular cut-off filter). Protein concentrations were determined by a protein assay kit (BioRad). Bovine serum albumin (BSA) was used as the standard. The concentrated samples were checked for the presence of specific antigens by SDS-PAGE

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in an automatic electrophoresis system (Phast System, Pharmacia). The gels were stained using a silver staining kit (Pharmacia).

Primary muscle culture

The method of muscle culture was modified from that of Tsim *et al.* (1992) for chick muscle cells. Skeletal muscles were obtained from 19-day embryos of Fischer rats which were killed by giving an overdose of ether. Only hind limbs of the animals were used because they contain the highest muscle to bone ratio. They were removed from the embryos under a stereomicroscope. After soaking the limbs in PBS for 5 min, the epidermis was removed prior to muscle extraction. Muscle blocks, which were separated from bones using a pair of sterile forceps, were cut into 1 mm³ pieces. They were then squashed using a cell mortar (Sigma) before being suspended in 50 ml of PBS solution containing 300 µl of 2.5% trypsin (Gibco-BRL). After incubation for 20 min at RT, the suspension was treated with 10 ml of DMEM medium (Gibco-BRL), supplemented with 10% heated inactivated horse serum (HHS, Gibco-BRL). It was centrifuged for 15 min at 1000 rpm to pellet the dissociated myocytes. The myocytes were resuspended in 10 ml of culture medium (10% HHS-DMEM). The mixture was filtered through a nylon mesh to remove undigested tissues and cell debris. It was preplated onto 100 mm culture dishes (Falcon) for 15 min at 37°C. Fibrocytes, which are more anchorage-dependent than myocytes, would then settle more readily onto the culture dishes. The medium containing mostly myocytes was aspirated and used for cell plating. The cell concentration was estimated using a cytometer (Sigma). Approximately 3 × 10⁵ cells were plated on each 35 mm culture dish (Falcon). The culture medium was changed every other day. The cultures were maintained for 12 days at 37°C and 5% CO₂ in an incubator (Nuair). They were used for experiments on day 3 or 5 after cell plating.

Effects of ES products on myotubes

Days 3 and 5 muscles were incubated for 7 days with either crude ES products (1 mg/ml), crude somatic extracts (1 mg/ml) or 45–53 kDa antigenic molecules (100 µg/ml) isolated from the ES products of the infective-stage larvae. The latter molecules were obtained by purifying the crude ES products using a Preparative Cell (Biorad) (Leung, 1995). The culture medium containing the protein samples was changed every second day. Morphological changes of the muscles were studied daily under an inverted phase-contrast microscope. A parallel study using crude ES products from the adult worms or newborn larvae was also undertaken.

Heat-denatured (95°C for 10 min) samples and culture medium containing only SDS (1 µg/ml) served as negative controls. The last control was necessary because the isolated 45–53 kDa molecules were originally coupled with SDS during purification.

Effects of living worms on muscle culture

Day 5 muscles were co-cultured for 7 days either with adult worms, infective-stage or newborn larvae. The culture medium was changed every third day. The

morphological changes in muscles and the activity of the parasites were studied daily under the microscope.

Three different methods had been tried to provide a closer contact between the worms and myotubes. The first attempt involved using a nylon netting, with 200 µm pore size, to confine the worms onto muscles which were attached to the bottom of the culture dish. Alternatively, the culture medium was reduced to only about 1 ml, i.e. just sufficient to cover the muscles. The cultures were inspected at a 4 h interval to ensure that no dehydration would occur. Thirdly, the parasites were sandwiched between two layers of muscle cells. Day 5 muscles were first collected using a cell scraper (Coaster). They were then gently pelleted by centrifugation for 5 min at 500 rpm before approximately 30 worms were added. This was followed by another layer of muscle cells.

In the control experiment, day 5 muscles were co-cultured with heat killed worms. As control for traumatic lesions, the muscles were mechanically damaged using the tip of a glass pipette.

Indirect fluorescent antibody test (IFAT)

Day 10 muscle samples from the co-culture and ES treatment experiments were fixed in Bouin's solution for 24 h before being embedded in paraffin and sectioned at 6 µm. The sections were dewaxed using xylene and then rehydrated through a series of 100%, 95% and 75% ethanol alcohol. After rehydration in distilled and deionized water, the sections were rinsed in 0.01M PBS (pH 7.4). They were incubated with 200 µl of normal mouse serum (1:30 in PBS) for 30 min before anti-*T. spiralis* serum (1:100 in PBS) was applied for 2 h at RT. Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (heavy and light chains specific, Cappel) served as the developing antibodies. The distribution of antigenic epitopes was studied by a laser confocal microscope (MRC 1000, BioRad). The optimal dilutions of serum and FITC conjugates were determined by chequer-board titrations, with reference to positive and negative sera.

Results

Conditions of primary muscle culture

Although it is extremely difficult to culture mammalian muscles, we succeeded in producing and maintaining viable myotubes for more than one week. Therefore, the conditions of the primary muscle culture based on rat myocytes is described briefly below.

On days 1 and 2, only individual round cells were seen. On day 3, cell aggregations appeared. Irregular cell colonies which were interconnected by cell processes were formed. At this stage, myocytes could not be clearly separated from fibrocytes. On day 4, a substantial increase in the number of fibrocytes occurred. They produced long and slender processes but cell fusion was not observed. In contrast, myocytes started forming myotubes, some of which were multicellular in thickness, with side branches and nuclei. Others were one cell thick, consisting of a row of myocytes aligned along the myotubes. During days 5–8, the myotubes grew substantially both in length and thickness. Extensive networks were formed and spontaneous contractions were

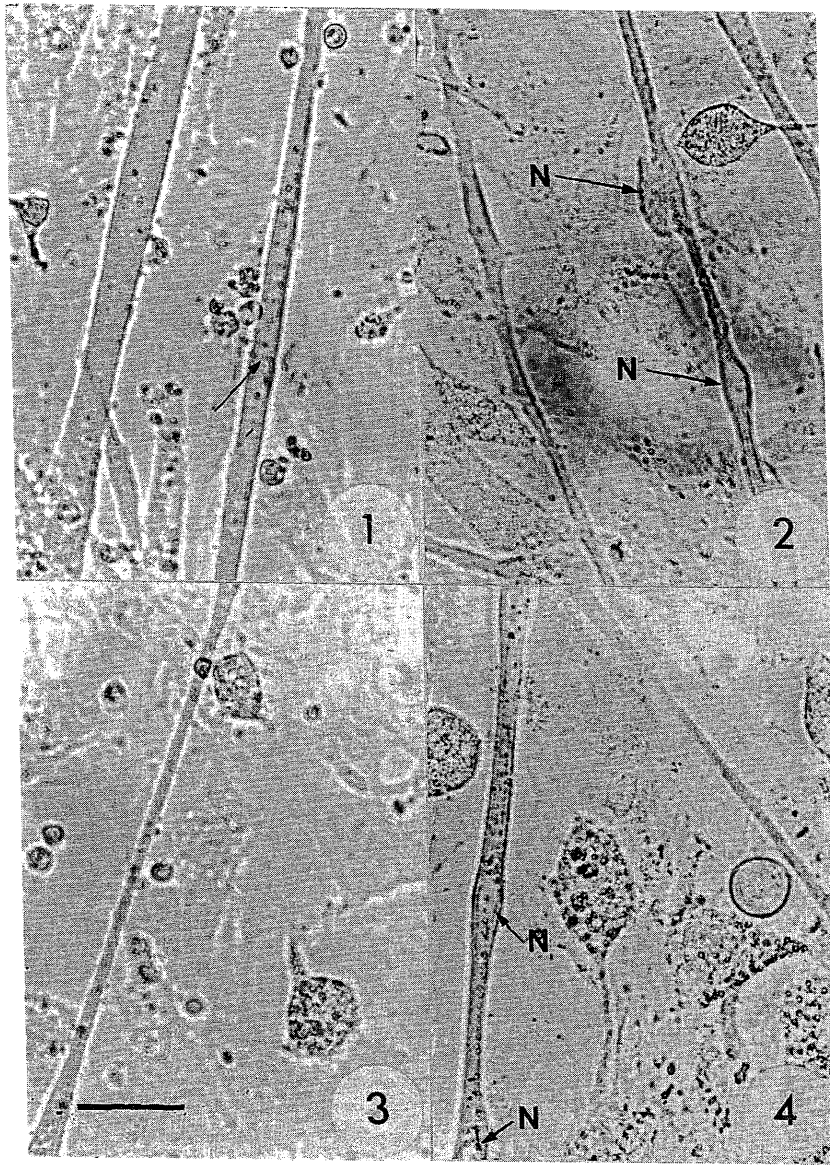


Fig. 1. Day 5 normal myotubes showing fusion of myocytes and occurrence of cross-striations (arrow). Fig. 2. Photograph showing the effect on myotubes after addition of ES products of infective-stage larvae into day 5 muscle culture. Note the presence of nodular structures (N) along the myotubes. A nucleus is visible in the lower node. Fig. 3. Photograph showing the effects of ES products from newborn larvae on day 3 prefused myocytes. Note the drastic reduction in diameter of the myotube. Fig. 4. Occurrence of nodular structures (N) on myotube after co-culture with newborn larvae. Note the granular appearance of the myotube. (Scale bar = 25 μ m for all figs.)

commonly observed. The myotubes assumed a smooth tubular appearance, with cross striations and distinct cell nuclei (fig. 1). From day 10 onwards, however, cell death was common. A significant degeneration of myotubes was noted on day 12.

Effects of ES products on muscle culture

The addition of ES products from the infective-stage larvae to day 5 culture induced drastic morphological and structural changes in the myotubes. Distinct nodular

structures were formed at intervals along the slender myotubes (fig. 2). Each node bore a prominent nucleus. Histological studies revealed that the node contained large cavities enclosed by an intact sarcolemma. Also as compared to the control, the myotubes, which became granular, seemed to have become reduced in diameter (fig. 3).

Similar lesions were also observed after ES products from the newborn larvae were added to the day 5 muscle culture. However, ES products from adult worms, heat denatured samples, crude somatic extracts, the 45–53 kDa

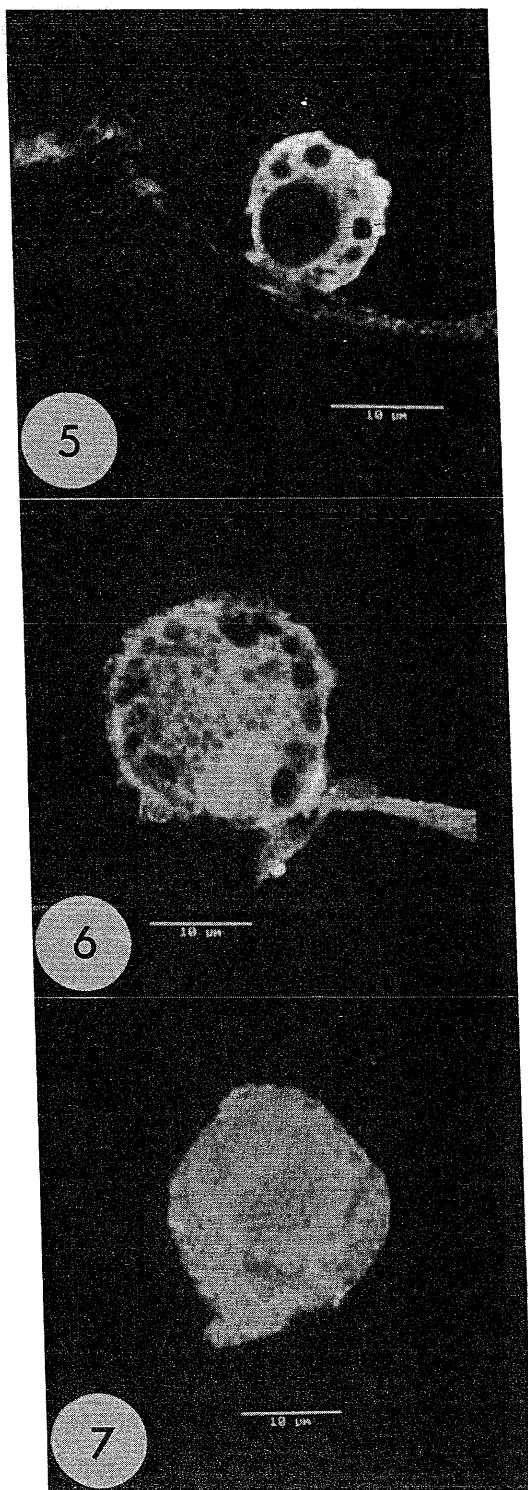


Fig. 5. FITC stained cross-section of the nodular structure of myotube formed after addition of newborn larvae into culture. Positive immunofluorescence occurred on the sarcolemma and sarcoplasm. Note the presence of a large central cavity circumscribed by smaller ones. Fig. 6. FITC stained cross-section of the nodular structure of a myotube formed after treatment with ES products of infective-stage larvae. Positive immunofluorescence occurred on the sarcolemma and sarcoplasm. Note the presence of numerous cavities around the peripheral region of myotube. Fig. 7. Cross-section of a myotube from the control experiment, showing a solid internal structure and smooth sarcolemma. (Scale bar = 10 µm for all figs.)

molecules (isolated from the ES products of the infective-stage larvae) and SDS failed to elicit any change.

ES products from the two larval stages also affected the development of myotubes after they were added into day 3 culture containing prefused myocytes. Only a small number of myotubes with indistinct cross striations were formed. They were mostly short and slender (fig. 4) and did not develop into networks.

Crude worm extracts and the 45–53 kDa molecules had no effect either on the fusion or elongation of myotubes. Negative results were also obtained using heat-denatured protein samples and SDS.

Effects of worms on muscle cells

Addition of newborn larvae into the cell culture seemed to prolong the lifespan of myotubes. The larvae remained highly active for seven days (i.e. day 12 of the myotube culture), although they did not penetrate into the myotubes. The myotubes survived until day 16 of the muscle culture. The myotubes developed similar nodular structures as in the ES products experiments.

However, adult worms or infective-stage larvae could survive for only three days after introduction into the muscle culture. The worms remained highly active for only 1–2 days and failed to enter the myotubes. No changes in the myotubes were observed during this period.

The three methods (i.e. nylon mesh, culturing with minimal medium, and worm packing) used to increase the contact between worms and cell cultures failed to induce the worms to penetrate into the myotubes. However, the nylon mesh method was most effective in limiting the mobility of the parasites in the liquid culture medium, while producing a better survival rate.

Myotubes which were punctured at different levels by a glass pipette did not develop nodular structures.

Histological and immunocytochemical studies

Histological sections of the nodular structures on the myotubes co-cultured with newborn larvae showed that each node contained a large central cavity surrounded by smaller ones dispersed in the sarcoplasm (fig. 5). The outer sarcolemma was rugged in appearance. The sarcoplasm and the sarcolemma both showed a strong fluorescence when tested by IFAT for the presence of *Trichinella* antigens.

Cavities were also found inside the myotubes which had been treated by ES products of either infective-stage (fig. 6) or newborn larvae. However, the cavities were more numerous than those initiated by newborn larvae and seemed to be confined to the peripheral region. There was no large central cavity. The sarcolemma and sarcoplasm also yielded positive IFAT results.

The control myotubes showed a smooth outer surface and without any internal cavity (fig. 7). They yielded a negative IFAT reaction.

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Discussion

Effects on growth and survival of myotubes

The present study has shown for the first time that it is possible to produce an *in vitro* model to elucidate the host cell-parasite interactions in trichinellosis. Our observations clearly indicate that effector molecules secreted by the first-stage *Trichinella* larva can affect the growth, development and internal structure of muscle cells.

Structural changes in myotubes

The presence of newborn first-stage larvae elicited prominent morphological changes in the myotubes which became more slender and with inconspicuous cross-striations. These may represent a form of degenerative lesion. However, the most distinctive lesion was the formation of nodular structures with internal cavities along the myotubes. Since similar transformations could also be induced by the crude ES products from the newborn or infective-stage larva (which is basically also a first-stage larva), the changes were most likely to be chemically mediated.

ES products of *Trichinella* are known to contain a complex mixture of molecules, e.g. proteases (Criado-Fornelio *et al.*, 1992; Lai & Ko, 1994; Todorova *et al.*, 1995), DNA-binding proteins (Leung, 1995), heat shock proteins (Ko & Fan, 1996), *n*-butylamine (Zenka *et al.*, 1980). Therefore, the presence of cavities inside the nodular structures was probably a result of enzymatic digestion, probably by a protease secreted by the nematode. The presence of parasitic epitopes inside the sarcoplasm of the nodes was confirmed by the immunocytochemical data. The most intriguing question is how the enzyme can selectively digest the sarcoplasm but not the sarcolemma of the myotube.

One speculation is that the protease recognizes a specific receptor on the surface of the membrane. After binding, receptor mediated endocytosis may occur. This phenomenon has been observed in *Leishmania mexicana* involving a surface metallo-protease, gp 63 (Chaudhuri & Chang, 1988). Another possibility is that the protease involved may be highly substrate specific towards the sarcoplasm of the myotubes. Such proteases have been reported in *Schistosoma mansoni* (Mckerrow *et al.*, 1985) and *Plasmodium falciparum* (Cooper & Bujard, 1992). The former parasite produces a specific collagenase which only cleaves collagens of the basal membrane but not those of the interstitial structures.

The failure of ES products from adult worms to elicit any lesion suggests that their chemical composition is probably different from that of the first-stage larvae. Similar failure of the purified 45–53 kD glycoprotein molecules may imply that either they are not directly involved in the process of muscle reorganization, or the concentration (i.e. 100 µg/ml) used in the experiment was below the threshold level of activity. Moreover, since these molecules were originally coupled with SDS during the fractionation procedure, their biological activities might have been inhibited. Therefore, further studies using native proteins are necessary to elucidate their roles.

Introduction of living infective-stage larvae into the cultures also failed to induce nodular formation in the myotubes. This may be due to the short survival time (about 3 days) of the worms under *in vitro* conditions.

ES products of newborn and infective-stage larvae appeared capable of affecting the development of prefused myocytes. Shorter and more slender myotubes were formed when ES products were added into day 3 muscle culture. Network formation was also inhibited.

A speculative explanation is that the ES products contain negative regulators on muscle development. Blau (1992) suggested that differentiation of myocytes into myotubes are modulated by positive and negative regulators. The former include MyoD1, myogenin, Myf-5 and Myf-6 which are DNA-binding proteins having a helix-loop-helix motif. (Davis *et al.*, 1987). They recognize consensus sequences (e.g. E-box) in the muscle genes and enhance the production of muscle specific proteins in myocytes. Some negative regulators (e.g. dystrophin) are also DNA-binding proteins. Others are serum proteins (e.g. fibrocyte growth factors, transforming growth factors) which have specific receptors on the membranes of myocytes (Kelvin *et al.*, 1989). They inhibit the fusion of myocytes and elongation of myotubes by altering the affinity of polymerases to genes that encode muscle specific components. Nevertheless, whether any of these regulators corresponds to the protein(s) in the ES products that depressed myocyte development requires further experimentation.

Another interesting phenomenon is that co-culturing with newborn larvae appeared to prolong the survival time (about 6 more days) of the myotubes in culture. One possible explanation is that in the presence of these larvae, the myocytes showed a slower growth rate. The reduction in metabolic demand may, in turn, prolong the survival of the cells under culture. Alternatively, living newborn larvae may provide some sustaining factors which help to extend the life span of myotubes. According to McLennan (1990), the histogenesis of myotubes in vertebrates are growth factor dependent. Some factors are required for maintenance and repair. In mice, they include insulin, catecholamines and prostaglandins (Platzer, 1978; Ontell *et al.*, 1988).

However, both of the above explanations are based on speculations. It is hoped that the present report may help to stimulate further research on the reorganization of host muscles by *Trichinella spiralis*.

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