Changes in host muscles induced by excretory/secretory products of larval *Trichinella spiralis* and *Trichinella pseudospiralis*

R. C. KO[1], L. FAN[1], D. L. LEE[2] *and* H. COMPTON[2]

[1] *Department of Zoology, University of Hong Kong, Hong Kong*
[2] *Department of Pure and Applied Biology, University of Leeds, Leeds LS2 9JT, UK*

SUMMARY

Excretory/secretory (ES) products obtained by *in vitro* culture of infective-stage larvae of *Trichinella spiralis* and *T. pseudospiralis* were injected intramuscularly at various intervals into mice. Mini-osmotic pumps containing *T. spiralis* ES products were also implanted subcutaneously and intraperitoneally into rats. The introduction of ES materials into muscles elicited extensive lesions which included dissolution of myofibres, mobilization of mononuclear and polymorphonuclear leucocytes, angiogenesis, hypertrophy of myonuclei, myotube formation, mitosis, muscle bundles becoming rounded and separated from each other, disappearance of Z, I and A bands of sarcomeres, increase in endoplasmic reticulum and Golgi complexes, decrease in glycogen and relocation of mitochondria. These are considered as degenerative/regenerative changes of muscles to injury. Immunodominant epitopes of specific 45–53 kDa glycoproteins in ES antigens of *T. spiralis* could not be detected in hypertrophic nuclei of injected muscles by using polyclonal and monoclonal antibodies and immunocytochemical methods. ES products of *T. spiralis* failed to stimulate unsensitized lymphocytes in the lymphocyte transformation test. Infective-stage larvae of *T. spiralis* released from muscles were found capable of forming nurse cells after injection subcutaneously into rats. It is postulated 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 genomic expression of myonuclei. The two events are probably mediated by different effector molecules.

Key words: epitopes, excretory/secretory products, muscle reorganization, nurse cells, *Trichinella*.

INTRODUCTION

Among the parasitic helminths, the trichinelloid nematodes exhibit some unique developmental adaptations. A well-known trichinellid, *Trichinella spiralis*, can form nurse cell complexes in striated muscles of mammals by completely transforming myofibres into a new syncytium after the invasion of newborn larvae (Despommier, 1983; Hulinska, Grim & Mackinnon, 1985; Lee & Shivers, 1987). A fully developed nurse cell complex consists of a cytoplasmic region with numerous hypertrophic and small nuclei, endoplasmic reticulum, secretory vesicles, mitochondria and a cavity containing 1–4 worms. Cells of unknown origin are also present. The entire complex is encapsulated by collagenous fibres. However, the inductive and regulatory mechanisms involved in the reorganization of host cells are poorly understood.

In a freeze-fracture study, Lee & Shivers (1987) suggested that the reorganization of myofibres is probably triggered by secretions from *T. spiralis*. The hypothesis appears to be supported by the work of Despommier (1990), Despommier *et al.* (1990) and Lee *et al.* (1991). Using immunocytochemical techniques, they showed that hypertrophic nuclei in the nurse cells were positively stained when either polyclonal or monoclonal antibodies against ES antigens were used as developing antibodies. The findings indicate that antigens secreted by the parasite may directly interact with host cells at the nuclear level.

An attempt has been undertaken to elucidate the mechanisms involved in host cell reorganization. The initial phase of the study was aimed at documenting the nuclear and cellular changes which could be induced by ES products of *T. spiralis* and related species.

In a recent short communication, we reported that intramuscular injections of EC products from infective-stage larvae of *T. spiralis* into a mouse could induce the formation of hypertrophic nuclei (Ko, Fan & Lee, 1992). Mitotic figures in some nuclei were also observed. However, more extensive experiments have since been undertaken and the results are presented in this paper. The following aspects were studied: effect of ES products on the structures of the muscle fibres, mitogenicity of ES, and ability of fully developed infective-stage larvae to induce nurse cell formation.

A parallel study using ES products from another trichinellid, *Trichinella pseudospiralis*, was also undertaken. Unlike *T. spiralis*, the nurse cell complexes formed by this species are not encapsulated by

collagenous fibres and the parasite can develop in both avian and mammalian hosts. Their ES products may therefore contain different effector molecules.

MATERIALS AND METHODS

Production of ES materials

Infective-stage larvae of *T. spiralis* and *T. pseudospiralis* 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), following the protocol of Gamble *et al.* (1983) and Ko & Yeung (1989). ES products 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 in an automatic electrophoresis system (Phast System, Pharmacia). The gels were stained using a silver staining kit (Pharmacia).

T. spiralis *experiments*

Injection of ES products. ES products and control materials were injected intramuscularly into 8-week-old ICR mice using a tuberculin syringe with a 27-gauge needle. The injection was made on the medial region of the thigh muscle. The animals were killed by an overdose of ether.

Seven experimental groups (2 animals/group) of mice were used. Animals in Groups I, II and III were each given a daily intramuscular injection of 0·1 ml of ES products (5 mg/ml) for 2, 5 and 8 days respectively. They were killed 24 h after the last injection. Animals in Groups IV, V, VI and VII were injected every other day and the last injection was given on day 14. The animals were killed on day 15, 17, 20 and 40 respectively.

Three different treatments were used as controls. Animals in control group A were each injected with 0·1 ml of concentrated RPMI medium whereas those in control group B were each injected with 0·1 ml of BSA (5 mg/ml) (Sigma, no. A-7030, 98–99 % albumin) in RPMI. The sample size of animals and the schedules of injections in control A and B groups were the same as those of corresponding experimental groups. In control group C, four mice were injected daily for 8 days, each with 0·1 ml containing 0·6, 1·2, 2·5 or 5 mg/ml of egg albumin in RPMI.

Implantation of mini-osmotic pump. Two 8-week-old Wistar rats were each implanted with a mini-osmotic pump (Alzet, model no. 2002, pump rate 0·6 μl/h) containing 240 μl of ES products from *T. spiralis* which were sterilized by passage through a bacterial filter (Millex-GV4, Millipore). The animals were anaesthetized with ketamine hydrochloride (Ketalar,

Parke Davis). A pump connected to a silicone cannula was implanted subcutaneously into one rat. The opening of the cannula was attached to the surface of the latissimus dorsi muscle by surgical threads. Another pump was implanted intraperitoneally into the second rat. The incisions were closed by surgical threads and Michel clips. The animals were killed 14 days after implantation.

Injection of newborn larvae. To compare the tissue changes elicited by worm development and by direct injection of ES, two additional groups (6 animals/group) of mice were each injected, either subcutaneously or intramuscularly (at the thigh region), with about 20000 newborn larvae. They were harvested from RPMI 1640 medium 24 h after culture of gravid female worms. Two mice from each group were killed on day 5, 14 or 20 after injection.

Injection of infective larvae. To determine whether fully developed infective-stage larvae can also form nurse cells, worms were recovered 50 days post-infection from experimentally infected mice by pepsin digestion. They were washed 5 times in sterile 0·8 % saline containing penicillin and streptomycin before being injected either intramuscularly or subcutaneously into the thigh region of 8 mice. Each mouse was injected with about 1500 worms and the animals were killed on day 5, 14 or 30 after injection.

T. pseudospiralis *experiments*

Five random bred, 10-week-old mice were each injected daily with 0·05 ml of ES (1 mg/ml) from *T. pseudospiralis* for 4–5 days and were killed on day 5 or 6. Two mice were also each injected with 0·05 ml of ES on alternate days and were killed on day 6. Four mice were each injected with 0·05 ml of ES on alternate days for 14 days and were killed on day 15. All animals were killed by cervical dislocation.

The control mice were injected either with 0·05 ml of 0·85 % NaCl solution or BSA (1 mg/ml) or RPMI 1640.

Fixation and embedding of tissues

At necropsy of animals given injections or implanted subcutaneously with a mini-osmotic pump, muscle samples were removed from sites of injection or around the tips of the cannula. However, the entire diaphragm and abdominal muscles were removed from the mouse implanted intraperitoneally with the osmotic pump.

For light microscopic study, the tissues were fixed in Bouin's solution, embedded in paraffin and sections cut at 6–8 μm. The sections were stained with haematoxylin and eosin. Some sections were stained with Periodic acid/Schiff reagent (PAS).
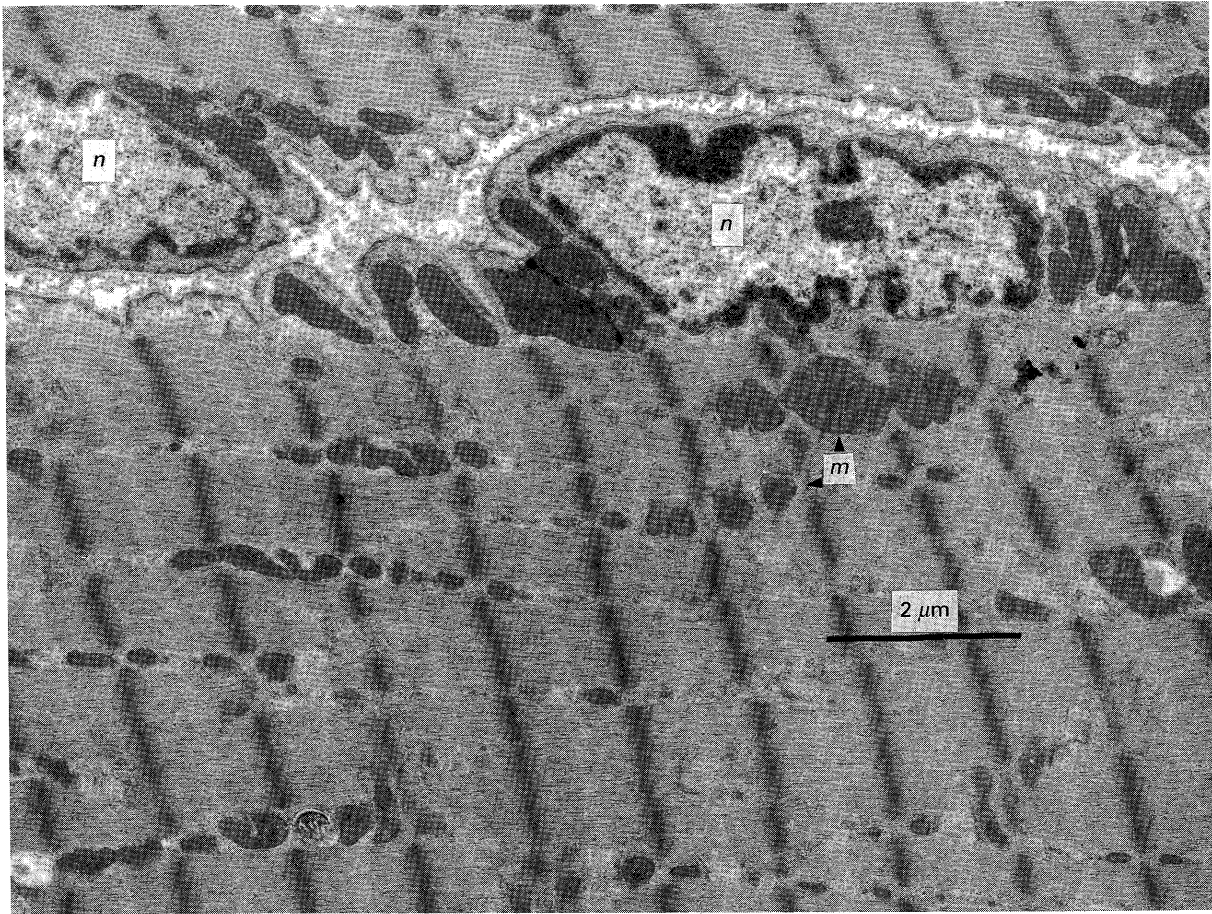
Fig. 1. Electron micrograph of a longitudinal section through thigh muscle of a control mouse injected for 14 days with RPMI 1640 medium without ES to show the normal appearance of two muscle fibres. Note the characteristic appearance and peripheral location of two myonuclei (*n*), the striated appearance of the closely packed myofibrils, the distribution of the mitochondria (*m*) and the small amount of sarcoplasm.

For electron microscopic study, small pieces of muscles were fixed in cold 2·5 % glutaraldehyde in 0·1 M cacodylate buffer (pH 7·2) for 24 h at 4 °C. The tissues were then transferred to 0·1 M cacodylate buffer at 4 °C for at least 24 h before being post-fixed for 1 h in 1 % osmium tetroxide in 0·1 M phosphate buffer (pH 7·2) (also at 4 °C). They were then rinsed in buffer, dehydrated through a graded series of ethanol, transferred to propylene oxide and embedded in EPON 812 or E-Mix. Tissues from *T. pseudospiralis* experiments were embedded in Araldite CY 212. Sections of 1 μm thickness were stained with toluidine blue in 1 % borax. Ultrathin sections, which were stained with uranyl acetate and lead citrate, were examined by a JEOL JEM 11SX electron microscope.

Immunocytochemical studies

The distribution of ES antigens in muscles from *T. spiralis* experiments was studied by immunoperoxidase labelling and the indirect fluorescent antibody test (IFAT), following the methods of Lee *et al.* (1991). An IgM monoclonal antibody $7C_2C_5$ (against specific epitopes of ES antigens) and rabbit anti-ES

polyclonal antibodies were used as developing antibodies. The monoclonal antibody was originally produced by Gamble & Graham (1984) and the hybridoma was purchased from the American Type Cell Collection. The polyclonal antibodies were produced in rabbits according to Ko & Yeung (1989). For the peroxidase method, diaminobenzidine tetrahydrochloride (Sigma) was used as substrate.

Lymphocyte transformation test

The mitogenicity of ES products from *T. spiralis* was studied by the lymphocyte transformation test. Human lymphocytes, which were separated from defibrinated blood by Ficoll, were cultured for 72 h in RPMI 1640 supplemented with sodium bicarbonate, HEPES, glutamine, penicillin and streptomycin. The final concentration of lymphocytes was 1×10^6 cells/ml. The concentrations of phytohaemagglutinin (PHA) and ES products varied from 3·125 to 12·5 μg/ml and 1 mg/ml–12·5 μg/ml respectively. PHA-stimulated cultures contained 20 % normal human AB serum whereas the serum concentration for ES cultures was 5 %. [³H]thymidine
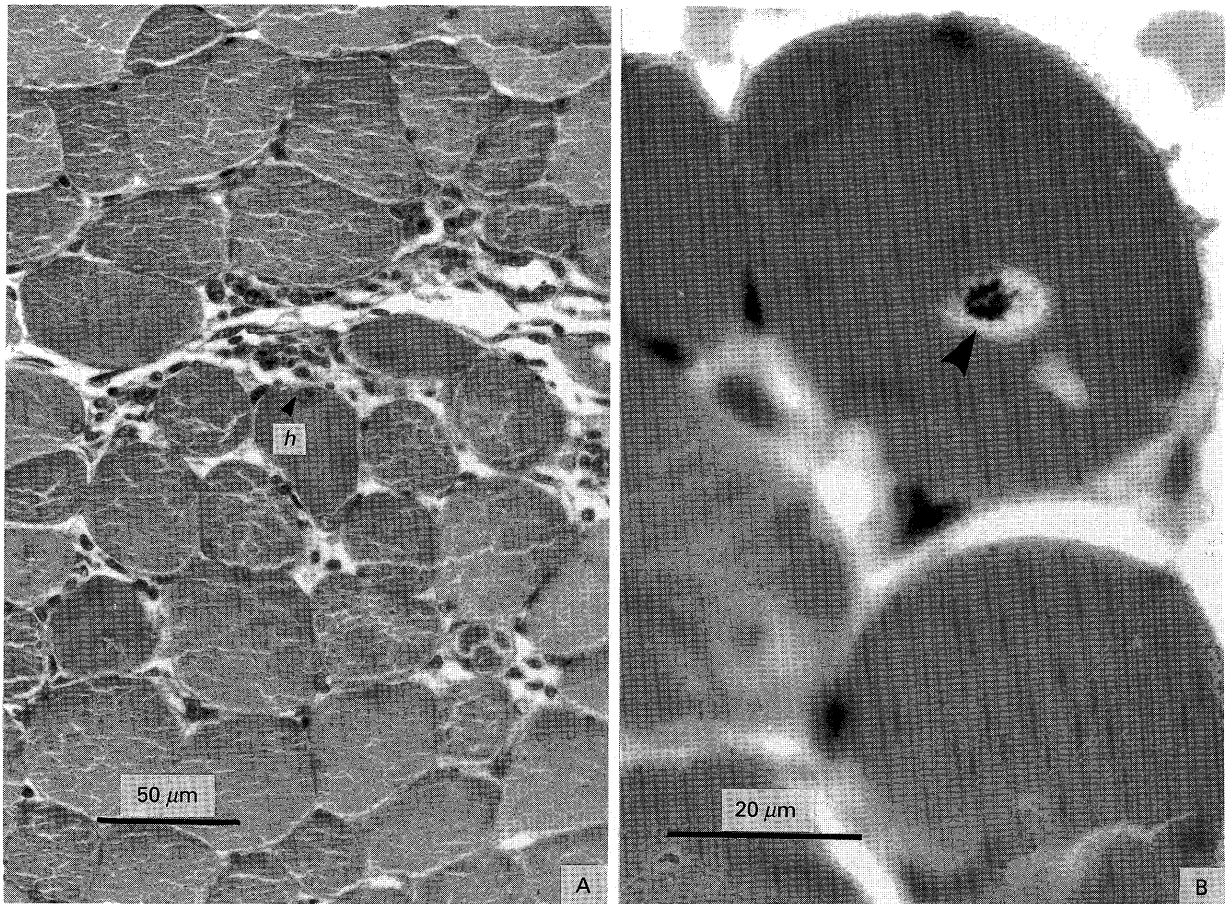
Fig. 2. Photographs of cross-sections of thigh muscle of mice injected with BSA or ES products of *Trichinella spiralis*. (A) Effects of injecting BSA for 14 days. Note the presence of hypertrophic nuclei (*h*) in only three of the muscle bundles. Only a mild cellular infiltration occurs in the interfibre region. (B) Unknown nucleus undergoing mitosis (arrow) inside a muscle bundle. The mouse was injected daily with ES products for 5 days.

at a concentration of $1 \mu Ci/ml$ was used for radio-isotope labelling, incorporation being counted by a liquid scintillation counter.

RESULTS

T. spiralis *experiments*

Changes induced by injecting ES. Muscle samples from control animals injected with RPMI generally showed a slight infiltration of polymorphonuclear leucocytes, pyknosis, hyaline and other mild degenerative changes (indicative of limited traumatic lesions) which were localized near the site of injection. Nuclei of muscle fibres were normal in number, size and distribution. The myofibrils, sarcoplasmic reticulum, sarcoplasm, mitochondria and other components of the muscle fibres retained their normal appearance and position (Fig. 1).

In muscles from animals injected with BSA or egg albumin, the degree of cellular infiltration was similar to those injected with RPMI. However, the nuclei of a few muscle bundles appeared to be hypertrophic and were centrally located inside the bundles (Fig. 2A). Occasionally, 3–4 nuclei occurred in a chain form. The mean size of the hypertrophic

nuclei was $9.6 \times 7.4 \mu m$ ($n = 30$). Mitosis was rarely seen but it was noted in a few granulated or non-granulated cells of unknown origin in the interfibre region. The average size of muscle nuclei from uninjected tissues was $10.6 \times 4.7 \mu m$ ($n = 30$). A more detailed comparison of lesions in control and experimental animals will be given later in the Discussion section.

A drastic reorganization of muscle fibres and extensive tissue reactions was observed in animals given intramuscular injections of ES products. The lesions were generally localized near the sites where ES products were introduced. Our observations are summarized below.

In the 2 day treatment an extremely heavy cellular infiltration dominated by polymorphonuclear leucocytes occurred in the interfibre regions near the site of injection. Myofibres of numerous muscle bundles had disappeared and were replaced by leucocytes. A short distance from this area, muscle bundles each bearing 2–4 hypertrophic nuclei were observed. A few cells of unknown origin were undergoing mitosis inside muscle bundles.

In the 5-day treatment the lesions were similar to those seen on day 2. A few muscle nuclei had

Fig. 3. Electron micrograph of a transverse section through thigh muscle of a mouse injected daily with ES products of *Trichinella spiralis* to show a small cell partly embedded in an altered muscle fibre. Note the small amount of cytoplasm surrounding the nucleus (*n*) of the small cell, the slightly dispersed myofibrils and Golgi complex (*G*) of the altered muscle fibre and the normal appearance of the adjacent muscle fibre.

migrated from the periphery towards the inner region of muscle bundles which had become rounded and were separated from each other. In cross-sections, 1–2 nuclei could usually be observed in the central region of the muscles. Mitotic figures were observed in some muscle bundles (Fig. 2B). In the interfibre regions, there were extensive infiltrations of polymorphonuclear leucocytes. Some areas were also oedematous, hyperaemic and necrotic. Fibroplasia was also present.

Ultrastructural studies showed that in the altered fibres, the bundles of myofibrils had become loosely packed and less well defined. In some fibres, it was difficult to recognize the sarcomeres. There was
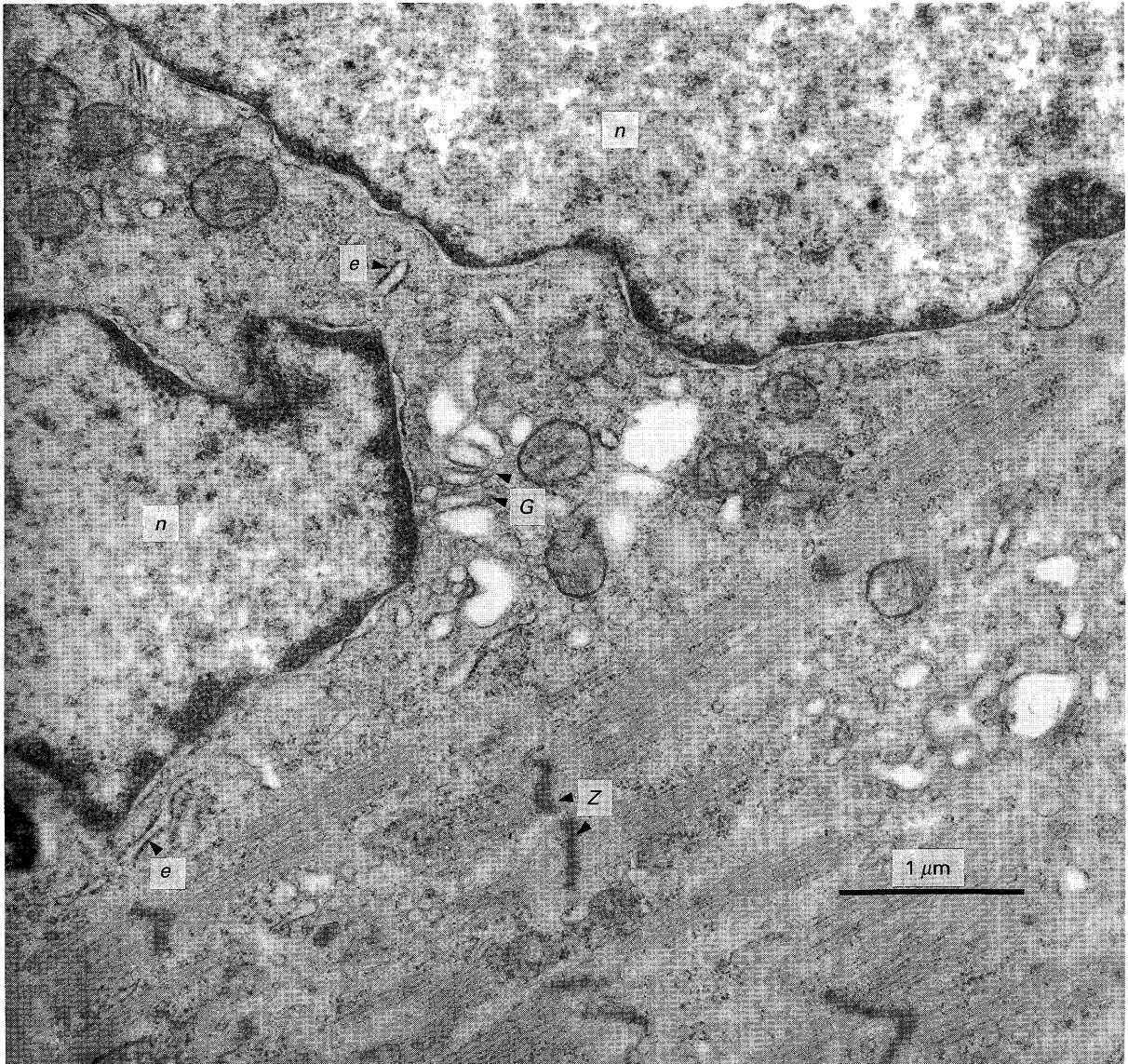
Fig. 4. Electron micrograph of a longitudinal section through thigh muscle of a mouse injected every other day for 14 days with ES products of *Trichinella spiralis*. Note the presence of two hypertrophic nuclei (*n*) in a row, the dispersed muscle myofibrils with Z bands (*Z*), rough endoplasmic reticulum (*e*) and Golgi complex (*G*) in sarcoplasm.

increasing disorganization of the sarcoplasmic reticulum and an increase in rough endoplasmic reticulum within the sarcoplasm as well as in numbers of Golgi complexes. Small cells were observed partly embedded in altered muscle fibres (Fig. 3). Numerous lymphocytes occurred in the interfibre regions, especially adjacent to altered fibres. Neutrophils, eosinophils, plasmacytes and macrophages were also abundant.

In the 14-day treatment, as compared to day 5, more muscle fibres near the site of injections were reorganized and contained prominent hypertrophic nuclei which were more numerous (at least 50 in some fibres). They varied from round to oval in shape (mean size = $10 \times 7 \mu$m, $n = 30$), with 1–2 compact nucleoli. Some hypertrophic nuclei were in the form of a chain within the fibre. Various phases of mitosis were observed in some cells of unknown

origin in the interfibre region and in muscle bundles. The muscle bundles were distinctly separated from each other. In cross-sections, they resembled nurse cells in parasitized tissues. One to two small cells could be seen entering some reorganized bundles from the interfibre region. Cellular infiltrations in the interfibre region were less extensive than those observed previously and they were dominated by mononuclear leucocytes. An increase in vascularization occurred around muscle bundles.

Ultrastructural alterations of muscle fibres were basically similar to those described from day 5. However, in some fibres, the bundles of myofibrils had become more loosely packed within the sarcoplasm but the sarcomeres, the sarcoplasmic reticulum and the triads were still well organized. More pinocytotic vesicles were present at the surface of the fibre than those in control sections. In other fibres,
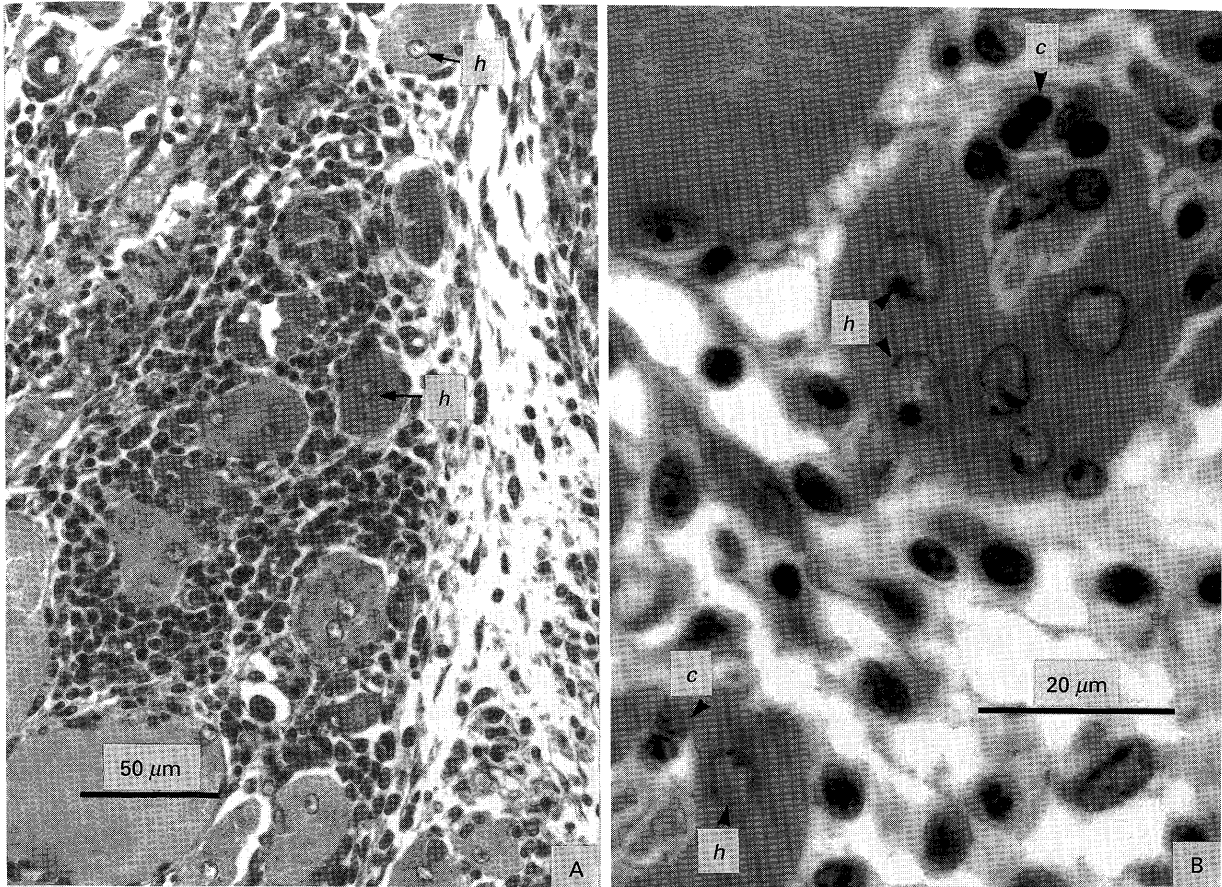
Fig. 5. Photographs of cross-sections of thigh muscle from a mouse injected every other day for 14 days with ES products of *Trichinella spiralis* and killed 3 days later. (A) Presence of hypertrophic nuclei (*h*) in many muscle bundles which are surrounded by numerous mononuclear leucocytes. (B) Presence of small cells (*c*) in a cluster and hypertrophic nuclei (*h*) in two muscle bundles.

the bundles of myofibrils were less well aligned and the sarcomeres were still recognizable. The Z bands were easily recognized but the I and A bands were less distinct than in controls. There was an increase in endoplasmic reticulum. The sarcoplasmic reticulum had become disorganized and Golgi complexes were more frequently seen in the sarcoplasm close to the enlarged nuclei.

Some fibres exhibited even more marked changes where the bundles of myofibrils were less compact and were widely separated within the sarcoplasm. The typical sarcomere appearance of Z, I and A bands had almost disappeared although the filaments of actin and myosin were still recognizable (Fig. 4). The sarcoplasmic reticulum was more widely distributed within the sarcoplasm and there was more rough endoplasmic reticulum. The mitochondria, which were scattered throughout the sarcoplasm, were less numerous than in control muscle. Golgi complexes were frequently observed adjacent to the nuclei. Pinocytotic vesicles were more abundant on and beneath the sarcolemma. Glycogen was less abundant as compared to those in fibres with limited changes and from controls. Numerous fibres of collagen were located in interfibre regions.

In the 17, 20 and 40-day treatment-groups mice were given injections every other day for 14 days and were killed on day 17, 20 and 40. In animals killed on day 17, infiltrations of mononuclear leucocytes were seen in interfibre regions (Fig. 5A). Numerous blood vessels were located near reorganized muscles. Many reorganized muscles contained hypertrophic nuclei and small cells, some of which were observed entering the bundles in groups (Fig. 5B). The origin of the cells was unknown but some resembled small lymphocytes as they showed darkly stained nuclei. The mean size of the hypertrophic nuclei was $10 \cdot 6 \times 7 \cdot 2$ μm ($n = 30$). The myofibrils in the multi-nucleated complex were usually indistinct.

Muscles from animals killed on day 20 still showed evidence of reorganization, bearing centrally located hypertrophic nuclei whose sizes were similar to those seen on day 17. There was little cellular infiltration in the interfibre region. Muscles from animals killed on day 40 also showed centrally located nuclei but they were generally smaller (mean = $4 \cdot 8 \times 7 \cdot 2$ μm) and some were pyknotic.

Changes induced by implanted osmotic pump. Muscle samples from the rat implanted with a subcutaneous
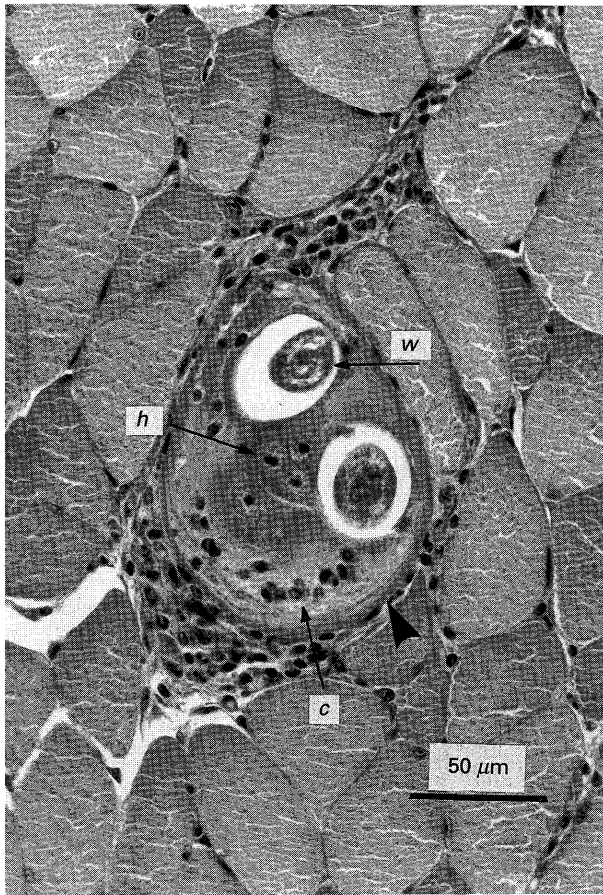
Fig. 6. Photograph of cross-section of thigh muscle from a mouse injected subcutaneously with infective-stage larvae of *Trichinella spiralis* 30 days after injection. Note the presence of a well-developed nurse cell containing hypertrophic nuclei (*h*), small cells (*c*) and worm (*w*). The nurse cell is encapsulated by collagenous fibres (arrow).

pump exhibited the same type of reorganization as those from injected mice. The lesions were localized in tissues around the tip of the cannula (leading from the pump). However, in these samples, cellular infiltrations in the interfibre region were more intensive than those from animals given intramuscular injections.

The implantation of an intraperitoneal pump failed to elicit any lesion in the diaphragm or abdominal muscles.

Nurse cell formation by infective-stage larvae. Fully developed infective-stage larvae could induce nurse cell formation when injected either subcutaneously or intramuscularly into mice. The subcutaneous injection was a better method because markedly less cellular reaction was elicited. Moreover, when the intramuscular method was used, many degenerating worms were noted. They were probably killed by the high pressure generated during such injections.

Five days after subcutaneous injection, most worms were still located in subcutaneous tissues. A few worms were in the interfibre region of sub-

cutaneous muscles where there was a heavy infiltration of polymorphs and mononuclear leucocytes. Slightly hypertrophic nuclei (mean size = 5 × 7·4 μm) were present in a few muscle bundles; the mean number of nucleoli per bundle was 4. Some muscle bundles showed signs of degeneration and reorganization, with the invasion of small mononuclear cells of unknown origin. The myofibrils in these bundles were poorly differentiated and the degree of reorganization was remarkably similar to that observed on days 14 and 17 after injection of ES.

Fourteen days after subcutaneous injection, many nurse cells containing worms were already well formed in muscle bundles where the myofilaments had disappeared. These nurse cells were randomly distributed in muscles. Large hypertrophic nuclei (mean size = 10·6 × 14·4 μm), each with 1–2 distinct nucleoli, were located inside nurse cells. Some nuclei were stained darker than others. Small nuclei were also present in the complex. Mitosis was observed in some small cells located inside the complex and in the interfibre region. Thirty days after injection, the nurse cell contained a larger number of hypertrophic nuclei and small cells. The layer of collagenous fibres encapsulating the complex was also prominent (Fig. 6).

Nurse cell formation by newborn larvae. Five days after injection, larvae were located inside cavities in muscle bundles. Three to four hypertrophic nuclei, each with 1–2 nucleoli, were observed per infected bundle. The nuclei were generally located in the periphery of the bundle but some had migrated to the central region and appeared in a chain form. The mean size of the hypertrophic nuclei was 9·2 × 12·7 μm. Cellular infiltrations were not observed.

Fourteen and 20 days after injection, the degree of reorganization in muscles was similar to that observed in the injection of infective-stage larvae for a similar period.

IFAT and peroxidase labelling. Immunodominant epitopes of 45–53 kDa glycoproteins present in *T. spiralis* ES products could not be detected in hypertrophic nuclei formed in muscles by injecting ES products or implanting mini-osmotic pumps. However, hypertrophic nuclei in nurse cells formed by infective-stage or newborn larvae 14 days after infection reacted positively with monoclonal and polyclonal antibodies against ES epitopes when tested by the IFAT and peroxidase methods. But hypertrophic nuclei of nurse cells obtained 5 days after infection were negatively stained.

Test for mitogenicity. The stimulation of unsensitized human lymphocytes in RPMI 1640 culture medium was studied using ES products from *T. spiralis* and PHA (Table 1). As compared to the PHA data, the

Table 1. *Comparison of the stimulation of unsensitized human lymphocytes in RPMI 1640 culture medium by excretory/secretory (ES) products of* Trichinella spiralis *and phytohaemagglutinin (PHA)*

(Culture volume: 200 μl/well. Final cell concentration: 500 cells/well. PHA-stimulated cultures contained 20% normal human AB serum. ES-stimulated cultures contained 5% normal human AB serum. Culture period: 72 h. [^{3}H]thymidine concentration: 1 μCi/ml.)

Mitogen concentration (/ml)	Mean count (/min)	Stimulation/10^6 cells
BKGD*	64	—
PHA		
3·13 μg	14019	279100
6·25 μg	13971	278147
12·50 μg	16237	323450
ES		
2·00 mg	64	—
1·00 mg	58	—
0·50 mg	79	300
0·25 mg	94	600
6·25 μg	48	—
12·5 μg	96	640

* BKGD: background (5% AB serum).

counts per minute (cpm) for the ES products were extremely low, slightly higher than those of the background. No stimulation was observed when the concentration of ES was at 1–2 mg/ml. The stimulation was highest when the concentration of ES was at 12·5 μg/ml.

T. pseudospiralis *experiments*

The experimental results were basically similar to those of *T. spiralis*. In injected muscles, there was an increase in lipids but a decrease in glycogen.

DISCUSSION

There is good evidence to suggest that the ES products of trichinellids may be pyrogenic. Intensive inflammatory reactions were invariably observed in muscles after intramuscular injections of ES from *T. spiralis* or implantation of a mini-osmotic pump containing ES. The products could mobilize a wide range of mononuclear and polymorphonuclear cells. The dissolution of myofibres in many muscle bundles was extensive. A similar level of reactions was not observed in control animals injected either with RPMI medium or 0·85% saline or BSA or egg albumin. Injections of ES also elicited a marked increase in vascularization around the site of injection. Wright *et al.* (1989) and Baruch & Despommier (1991) also observed that the nurse cells of *T. spiralis* were surrounded by an elaborate network of blood vessels. Baruch & Despommier (1991)

suggested that the worm may induce angiogenesis directly through the secretion of unique products or indirectly through the production of host-derived angiogenic factors. However, our results indicate that the increase in vascularization is probably directly associated with the nature of the inflammatory and repair process which is triggered by an extensive tissue injury.

The effects produced by injecting ES products of *T. spiralis* and *T. pseudospiralis* into muscles are similar. The prominent lesions are as follows: an increase in number and size of muscle nuclei, the migration of nuclei to the central region of muscle bundles (myotube formation) invasion of bundles by mononuclear cells and mitosis. These lesions are likely to be part of the normal degenerative and regenerative response of muscle to injury because similar changes could also be induced by injecting BSA, egg albumin or saline although changes were markedly weaker and were only localized to a few muscle bundles. The injection of RPMI elicited little reorganization, indicating that RPMI causes the least amount of muscle damage as compared to other injections. The reason for this is not known.

Hyaline degeneration, immigration of phagocytes of extramuscular origin, the presence of two large nucleoli in hypertrophic myonuclei and the packing of myonuclei in the form of a syncytium were also observed by Forbus (1926) and Millar (1934) as a result of injecting vital stains or by crushing muscles. Each successive injection of ES apparently initiated its own cycle of destruction, followed by regeneration. This was also noted by Benoit & Belt (1970) who injected muscles with bupivacaine. The decrease in glycogen observed in injected muscles may be a general phenomenon of muscle injury which was reviewed by Carlson & Faulkner (1983).

In the present study, injection of ES products or other solutions induced mitosis in two types of cells. One type, which was located in the interfibre region, was probably a phagocyte because of its general morphology. The second type was found inside muscle bundles. Although the identity of the latter cell could not be determined, it is probably the satellite cell which is a stem cell normally located in the subsarcolemmal region. Such cells are responsible for regenerative myogenesis (Church, Noronha & Allbrook, 1966; Allbrook, Han & Hellmuth, 1971; Carlson & Faulkner, 1983). The nucleus of the satellite cell cannot be distinguished from myonucleus in paraffin or semi-thin sections (Moss & Leblond, 1971). Bischoff (1986) suggested that in crushed muscles, a high molecular weight polypeptide would be released as a mitogen, resulting in the proliferation and differentiation of satellite cells which then migrate to the site of injury.

The data of the lymphocyte transformation test show that the cpm for the ES products is substantially lower than that of PHA which is a common

mitogen. Three different interpretations of this finding are possible. The ES product may not contain a mitogen or its mitogenic action may be different from that of PHA. Alternatively, proteases and other substances which are present in the highly concentrated crude ES (2 mg–12·5 μg/ml) used in the test, might have actually lysed the lymphocytes.

The migration of mononuclear cells from the interfibre region to muscle bundles was observed both in animals injected with various preparations or with infective-stage larvae. Some cells appear to be small lymphocytes. The most unusual feature is that these cells entered muscle bundles in a pack (Fig. 5B). However, the migration is presumably a regenerative response and this may explain the origin of small cells which are often found inside nurse cells.

Epitopes which are specific to ES antigens of *T. spiralis* could not be detected in hypertrophic nuclei of injected muscles although they were present in hypertrophic nuclei of nurse cells formed by worms as early as 14 days post-infection. The latter phenomenon was also observed in a previous study (Lee *et al.* 1991). One possible explanation of the result is that, unlike the parasitized condition (where ES materials are continuously produced), the nuclei in the injected muscles were only exposed to the ES products for a brief period. Since only crude ES products were used for the injections, the concentration of effector molecules in the inoculum may be too low to allow them to enter myonuclei and to be detectable. Another possibility is that the fluorescent or peroxidase methods used in the immunocytolocalization are not sufficiently sensitive. An *in vitro* assay using labelled purified ES antigens and muscle cell culture will provide a better means of monitoring the entrance of parasitic molecules into host nuclei.

Although the present injection experiments have not clearly demonstrated that muscle reorganization is induced by effector molecules secreted by *T. spiralis* or *T. pseudospiralis*, we still believe this is the method adopted by trichinellids to form nurse cells, as postulated in our previous study (Lee *et al.* 1991). This idea is further reinforced by a recent finding of Vassilatis *et al.* (1992) who cloned a cDNA encoding the *T. spiralis*-derived 43 kDa secreted glycoprotein. Antibodies raised against this fusion protein were used in an immunocytolocalization study. The authors suggested that either this protein, or a closely related family member, is secreted into muscles and then translocated to myonuclei.

Therefore, in view of the above observations, we postulate 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 genomic expression of myonuclei. The two events are mediated by different types of specific effector molecules which initiate the in-fectious process and modulate the host environment. One event leads to the dissolution of myofibres, myotube formation, mitosis, migration of cells into muscle bundles and angiogenesis. The other involves the translocation of molecules through the nuclear membrane into hypertrophic nuclei to alter and regulate genomic expression.

The composition of ES products of trichinellids is poorly known and different molecules may be secreted during various phases of worm development in muscles. Ko & Wong (1992) found that ES antigens from pre-encysted and encysted larvae of *T. spiralis* showed differences in SDS–PAGE profiles and in cross-reactivities with heterologous antisera. The former ES products are dominated by low molecular mass proteins and the antigenic profile resembles that of adult worms (Ko & Yeung, 1989).

Experiments in progress have shown that the ES products of infective larvae of *T. spiralis*, among other substances, actually contain proteases and heat shock proteins (details to be published elsewhere). Zenka, Hulinska & Jeorov, (1980) reported that a large quantity of *n*-butylamine was secreted by *T. pseudospiralis*. Injection of this amine into the gluteal muscle of mice resulted in degenerative and regenerative changes similar to those seen in *T. pseudospiralis* infection. Thus, *n*-butylamine amine was implicated as one of the factors which could induce muscle reorganization.

The present study shows that the infective-stage larva retains the ability to induce nurse cell formation. This may be an important adaptation which would enable the parasite to establish in other muscles if its original nurse cell is destroyed or ceases to function properly. Although this transposing activity is a speculation, the ability of infective-stage larvae to form nurse cells clearly indicates that they can infect research workers via skin abrasions or open wounds. Therefore, precaution must be taken in handling these worms.

REFERENCES

ALLBROOK, D. B., HAN, M. F. & HELLMUTH, A. E. (1971). Population of muscle satellite cells in relation to age and mitotic activity. *Pathology* **3**, 233–43.

BARUCH, A. M. & DESPOMMIER, D. D. (1991). Blood vessels in *Trichinella spiralis* infections: a study using vascular casts. *Journal of Parasitology* **77**, 99–103.

BENOIT, P. W. & BELT, D. (1970). Destruction and regeneration of skeletal muscle after treatment with a local anaesthetic, bupivacaine (Marcaine). *Journal of Anatomy* **107**, 547–56.

BISCHOFF, R. (1986). A satellite cell mitogen from crushed adult muscle. *Developmental Biology* **115**, 140–7.

CARLSON, B. M. & FAULKNER, J. A. (1983). The regeneration of skeletal muscle fibres following injury: a review. *Medicine and Science in Sports and Exercise* **15**, 187–98.

CHURCH, J. C. T., NORONHA, R. F. X. & ALLBROOK, D. B. (1966). Satellite cells and skeletal muscle regeneration. *British Journal of Surgery* **53**, 638–42.

DESPOMMIER, D. D. (1983). Biology. In *Trichinella and Trichinosis* (ed. Campbell, W. C.), pp. 31–73. New York: Plenum Press.

DESPOMMIER, D. D. (1990). *Trichinella spiralis*: the worm that would be a virus. *Parasitology Today* **6**, 193–6.

DESPOMMIER, D. D., GOLD, A. M., BUCK, S. W., CAPO, V. & SILBERSTEIN, D. (1990). *Trichinella spiralis*: secreted antigen of infective larvae localizes to the cytoplasm and nucleoplasm of infected host cells. *Experimental Parasitology* **71**, 27–38.

FORBUS, W. D. (1926). Pathological changes in voluntary muscle. II. Experimental study of degeneration and regeneration of striated muscle with vital stains. *Archives of Pathology and Laboratory Medicine* **2**, 486–99.

GAMBLE, H. R. & GRAHAM, C. E. (1984). Monoclonal antibody-purified antigen for the immunodiagnosis of trichinosis. *American Journal of Veterinary Research* **45**, 67–74.

GAMBLE, H. R., ANDERSON, W. R., GRAHAM, C. E. & MURRELL, K. D. (1983). Diagnosis of swine trichinosis by enzyme-linked immunosorbent assays for the diagnosis of swine trichinosis by enzyme-linked immunosorbent assay (ELISA) using an excretory-secretory antigen. *Veterinary Parasitology* **13**, 349–61.

HULINSKA, D., GRIM, M. & MACKINNON, B. M. (1985). Alterations of muscle fibres of mice experimentally infected with *Trichinella pseudospiralis*, *T. spiralis* and *T. nativa*. In *Trichinellosis* (ed. Kim, C. W.), pp. 152–162. Albany, New York: The State University of New York Press.

KO, R. C., FAN, L. & LEE, D. L. (1992). Experimental reorganization of host muscle cells by excretory/secretory products of infective *Trichinella spiralis* larvae. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86**, 77–8.

KO, R. C. & WONG, T. B. (1992). *Trichinella spiralis*: specificity of ES antigens from pre-encysted larvae. *Journal of Helminthology* **66**, 38–44.

KO, R. C. & YEUNG, M. H. (1989). Specificity of ES antigens in detection of *Trichinella spiralis* antibodies in Chinese pigs. *Tropical Biomedicine* **6**, 99–111.

LEE, D. L. & SHIVERS, R. R. (1987). A freeze-fracture study of muscle fibres infected with *Trichinella spiralis*. *Tissues and Cell* **19**, 665–71.

LEE, D. L., KO, R. C., YI, X. Y. & YEUNG, M. H. F. (1991). *Trichinella spiralis*: antigenic epitopes from the stichocytes detected in the hypertrophic nuclei and cytoplasm of the parasitized muscle fibre (nurse cell) of the host. *Parasitology* **102**, 117–23.

MILLAR, W. G. (1934). Regeneration of skeletal muscle in young rabbits. *Journal of Pathology* **38**, 145–51.

MOSS, F. P. & LEBLOND, C. P. (1971). Satellite cells as the source of nuclei in muscles of growing rats. *Anatomy Records* **170**, 421–34.

VASSILATIS, D. K., DESPOMMIER, D. D., MISEK, D. E., POLVERE, R. I., GOLD, A. M. & VAN DER PLOEG, L. H. T. (1992). Analysis of a 43 kDa glycoprotein from the intracellular parasitic nematode, *Trichinella spiralis*. *Journal of Biological Chemistry* **267**, 18459–65.

WRIGHT, K. A., MATTA, I., HONG, H. P. & FLOOD, N. (1989). *Trichinella* larvae and the vasculature of the murine diaphragm. In *Trichinellosis* (ed. Tanner, C. E., Martinez Fernandez, C. E. & Bolas Fernandez, F.), pp. 70–75. Madrid: Consejo Superior de Investigaciones Científicas Press.

ZENKA, J., HULINSKA, D. & JEOROV, A. (1980). An analysis of *Trichinella pseudospiralis* excretions and study of their effect on striated muscles of mouse. *Folia Parasitologica* **36**, 177–83.