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Growth of *Trachipleistophora hominis* (Microsporidia: Pleistophoridae) in C_2,C_{12} mouse myoblast cells and response to treatment with albendazole

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Abstract. The microsporidium *Trachipleistophora hominis* Hollister, Canning, Weidner, Field, Kench et Marriott, 1996, originally isolated from human skeletal muscle cells, inhibited myotube formation from myoblasts when grown in a mouse myoblast cell line C_2,C_{12}. Uninfected cultures readily converted to myotubes. Albendazole, a drug with known antimicrosporidial activity, was tested against *T. hominis* in C_2,C_{12} cells. The drug was added when infection had reached 75% of C_2,C_{12} cells, a level comparable to that obtained in heavily infected muscle *in vivo*. Doses of 1 ng/ml and 10 ng/ml had no effect on merogony or sporogony. In cultures exposed to 100 ng/ml albendazole, the C_2,C_{12} cells remained in good condition while infection levels dropped to 25% over 7 weeks. Drug doses of 500 ng/ml and 1,000 ng/ml were deleterious to the host cells but some spores retained viability and were able to establish new infections once albendazole pressure was removed. *T. hominis* meronts exposed to 100 ng/ml albendazole mostly lacked the normally thick surface coat and its reticulate extensions. Meronts were not seen in cultures exposed to higher drug doses. Albendazole at a concentration of 100 ng/ml and higher had a profound effect on spore morphogenesis. There was erratic coiling of the polar tube, often involving the formation of double tubes, and chaotic disposition of membranes which could have been those of polaroplast. The *in vitro* susceptibility of *T. hominis* to albendazole was low in comparison with *in vitro* susceptibility of other microsporidia of human origin.

In 1995 an incapacitating myositis was diagnosed in an AIDS patient in Australia and a case report demonstrating the presence of microsporidia in skeletal muscle, conjunctival cells and nasopharyngeal washings was presented (Field et al. 1996). *In vitro* cultures of the microsporidium were established from a muscle biopsy and the parasite was described as a new genus and species, *Trachipleistophora hominis* Hollister, Canning, Weidner, Field, Kench et Marriott, 1996 (Hollister et al. 1996). At the time of admission to hospital the patient was immobilised by severe muscular pain and subsequent histology of a deltoid muscle biopsy revealed extensive lesions, each consisting of a central necrotic area surrounded by heavily infected myofibres. After treatment with several drugs including albendazole (400 mg twice daily) the symptoms abated and the patient was able to walk again (Field et al. 1996). Using spores produced *in vitro*, infections were established in athymic mice by inoculation intraperitoneally, and intramuscularly into the hind leg (Hollister et al. 1996). Later, infections were established in the mice by the oral route (Cheney et al. 2000). All organs of the mice except brain and eye showed signs of infection after one or other of the routes of infection. However, after intramuscular inoculation, infection spread beyond the initial site only after very heavy infections had developed in the muscle.

The therapeutic benefits of albendazole as an antimicrosporidial drug have been well documented for clinical cases (e.g., Blanshard et al. 1992, Dore et al. 1995, Molina et al. 1995, Silverstein et al. 1997). Albendazole is generally regarded as ineffective against the human intestinal microsporidium *Enterocytozoon bieneusi* but highly effective for the other microsporidia, of human and non-human origin, against which it has been tested (Kotler and Orenstein 1999).

The original cultures of *T. hominis* were established in canine, rabbit and monkey kidney cell lines (MDCK, RK13 and COS-1) and in rat and mouse myoblast cell lines (L_6,C_{10} and G7). Growth was prolific in COS-1 and RK13 but was slower in the other cultures (Hollister et al. 1996). In the present study another mouse myoblast cell line, C_2,C_{12}, was used, which readily differentiates into myotubes. Once the cells have differentiated they form a network of contracting cells, the movements of which generally cause detachment and destruction of the monolayer. Subculture before differentiation is advanced may be necessary to avoid loss of the cell line. C_2,C_{12} cells were used in the present study to determine whether infection with *T. hominis* would interfere with myotube differentiation from myoblasts and to observe the effects of albendazole on the development of *T. hominis*.

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MATERIALS AND METHODS

C_2,C_{12} cells were received as newly passaged myoblasts in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and were cultured in the same medium with 100 iu/ml penicillin, 100 µg/ml streptomycin and kanamycin and 8% foetal calf serum (FCS). Routine cultures were grown in 25 cm^2 base flasks (T25) at 34°C with 5% CO_2, and were subcultured at 4-5 day intervals. For subculture, monolayers were loosened from the surface with 0.25% trypsin EDTA (Life Technologies), the cells were washed 3 times in PBS, resuspended in DMEM and passaged into 2 or 3 flasks.

Albendazole solutions were prepared by dissolving 5 mg albendazole in 1 ml dimethylsulphoxide (DMSO). The drug in solution was diluted in DMEM to give a stock solution of 10 µg/ml. After sterilisation by filtration through 0.2 µm filters, the stock was further diluted to give concentrations of 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml and 1,000 ng/ml.

Effect of *T. hominis* on C_2,C_{12} differentiation into myotubes. A series of C_2,C_{12} cultures was set up in T25 flasks, half of which were infected with 10^7 purified spores of *T. hominis* and the other half were uninfected. Each week for eight weeks one flask from each set was subcultured at a split ratio of 1 : 2, while the remainder was maintained simply by change of medium containing 8% FCS. The unpassaged cultures and subcultures were monitored for level of infection, myotube differentiation and survival after passage. As C_2,C_{12} myoblasts infected with *T. hominis* become grossly hypertrophied without releasing spores into the medium, spores harvested from the supernatant could not be used as a measure of infection. Infection levels were monitored by eye on percentage of cultured cells infected on a five-point scale representing approximately 5%, 25%, 50%, 75% and > 95%. Assessment of infection level was made for each culture overall, as some areas had higher or lower levels due to irregular spread of infection.

Effects of albendazole on *T. hominis* in C_2,C_{12} cells. Twenty T25 flasks and one 24-well plate containing 13 mm diameter glass coverslips were seeded with C_2,C_{12} cells. After 48 h, 2.4×10^7 spores of *T. hominis* were added to each flask and 10^6 spores to each well of the plate. The medium with 8% FCS was changed initially every 10 days, then at 4-5 day intervals as necessary. Subcultures were made after 30 days from 9 of the flasks at a split ratio of 1 : 3 (38 flasks in total) and another 24-well plate was established. After a further two weeks, when 75% of cells were infected, a preliminary test was set up to establish effective doses of albendazole, using 1 ng/ml, 10 ng/ml and 100 ng/ml in three replicate flasks and wells for each dose, in comparison with cultures without drug.

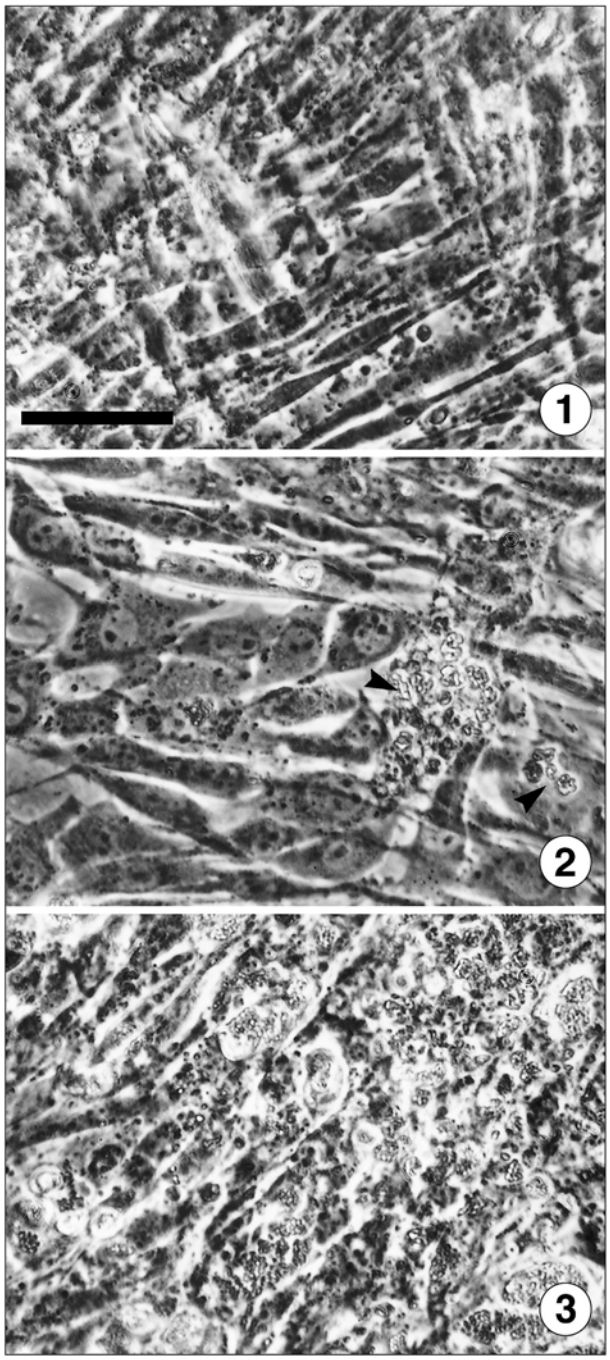
In a second test, set up after a further three weeks, some of the remaining 38 flasks (see above) and the second plate were used to test albendazole doses of 100 ng/ml, 500 ng/ml and 1,000 ng/ml. Controls for these experiments, with three replicates, were medium alone and medium containing 0.5 µl/ml DMSO (0.05%), higher than the level of 0.2 µl/ml (0.02%) used in the flasks with 1,000 ng/ml albendazole. Subcultures were performed as necessary. Albendazole was withdrawn after 50 days and the cultures were maintained with medium only, for another 50 days. At this time, spores were harvested from control cultures (medium only and medium plus DMSO) and from cultures treated with 1,000 ng/ml, 500 ng/ml and 100 ng/ml albendazole. Spores were washed and added to fresh C_2,C_{12} cultures, to test for viability. The numbers of spores applied to the new cultures were 5.5×10^6 spores recovered from cultures exposed to 1,000 ng/ml albendazole, 3.83×10^6 spores recovered from cultures exposed to 500 ng/ml albendazole, 6.98×10^6 spores recovered from cultures exposed to 100 ng/ml albendazole, 1.47×10^6 spores recovered from control cultures containing DMSO and 1.07×10^7 spores recovered from control cultures exposed to medium only. One culture of C_2,C_{12} cells which had not been infected was kept as a control for the possibility of extraneous infection.

Electron microscopy. Coverslips from the second series of albendazole experiments were removed after eight weeks into 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Postfixation was in 1% osmium tetroxide in buffer and dehydration was in an ethanol series. The coverslips were transferred to propylene oxide, then mixtures of 70 : 30 and 30 : 70 propylene oxide : Agar 100 resin (Agar Scientific, Stansted, Essex, UK) and finally into Agar 100 resin. Polymerised blocks of resin were placed over parts of each monolayer and the coverslips were incubated overnight at 65°C for polymerisation of the new resin. The newly embedded monolayers were snapped off from the coverslips and sections were cut from the exposed surfaces. Sections were stained with uranyl acetate and lead citrate and examined under an AEI EM801 electron microscope.

RESULTS

Effect of *T. hominis* infection on myotube differentiation of C_2,C_{12} cells

In the uninfected cultures that had been maintained by medium change alone, almost 100% of cells were in the form of dense mats of contracting myotubes by day 5. After confluency and overgrowth of myotubes in different directions (Fig. 1), there was regular patchy detachment of cells and new growth from myoblasts, so that full myotube layers were restored even after 7 and 8 weeks prior to subculture. Subcultures were made each week from cultures that had been maintained by medium change alone and all were successful even at week 8 when the experiment was terminated. After subculture, there was restoration of myoblast monolayers to confluency usually within a week followed by differentiation into myotubes. The percentage of myotubes was always lower in the infected cultures, once reaching 80% after the first subculture, when the infection level was 5% but thereafter did not exceed 30% myotubes and was usually 100% myoblast, even when the infection level was no higher than 25% (Fig. 2). Infection remained low until week 5 when about 50% of cells were infected and was steady at this level beyond the eighth passage, when the experiment was terminated.



Figs. 1-3. Light micrographs of C_2,C_{12} cells, uninfected and infected with *Trachipleistophora hominis*. **Fig. 1.** Uninfected C_2,C_{12} cells maintained without passage by medium change alone for 28 days. The cells have formed into dense layers of contracting myotubes but subculture was still possible by regrowth from a small proportion of myoblasts. **Fig. 2.** C_2,C_{12} cells infected with *T. hominis*. Culture maintained by medium change alone for 14 days, then passaged and maintained for a further 10 days. Myotube differentiation is inhibited, all cells remain as myoblasts although the infection (arrowheads) does not exceed 25% of cells. **Fig. 3.** C_2,C_{12} cells showing 75% of cells infected as used to test the efficacy of albendazole. Scale bar on Fig. 1 = 0.1 mm applies to all figures.

Effect of albendazole on *T. hominis* in C_2,C_{12} cell culture

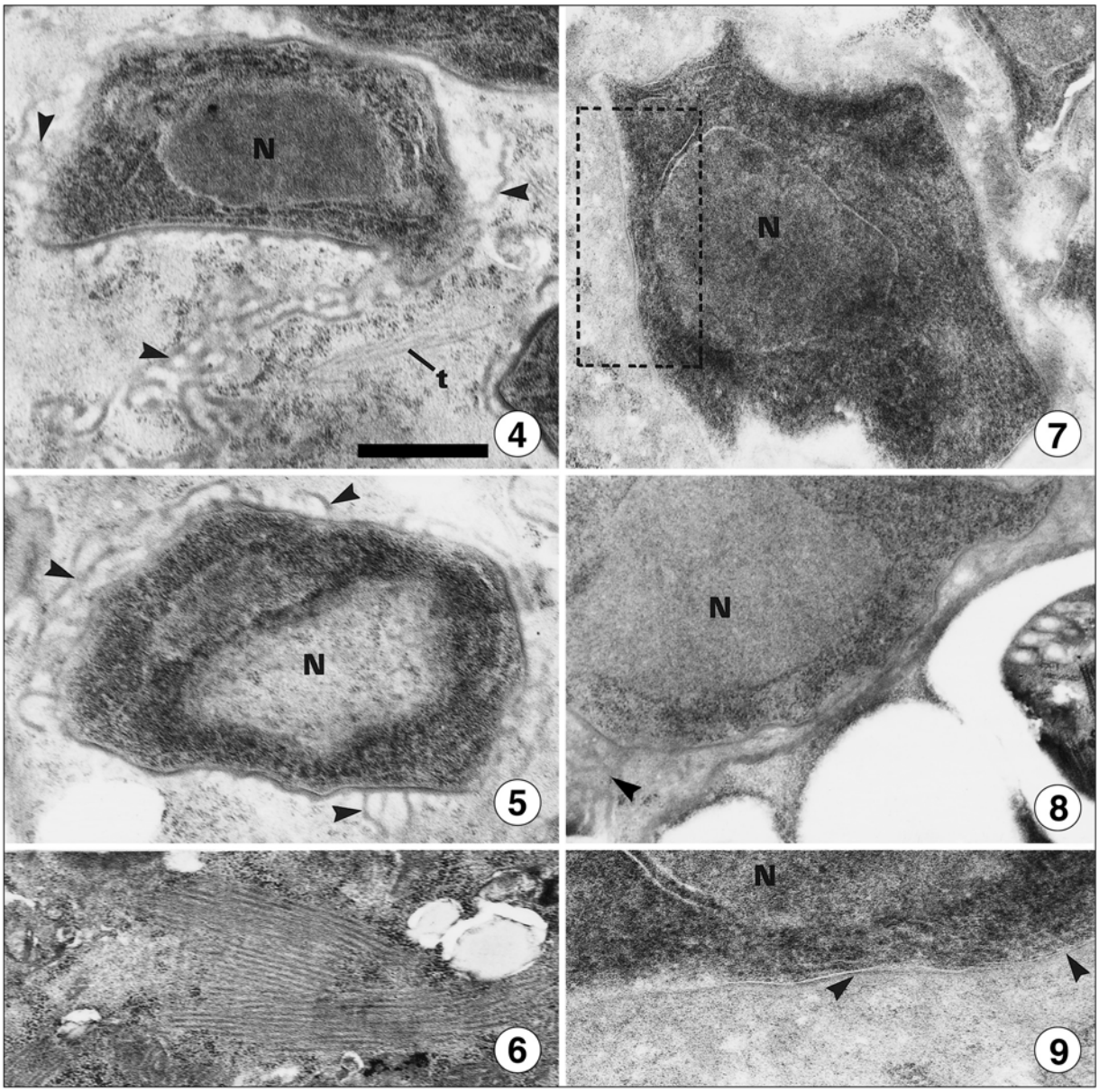
In a preliminary experiment initiated with cultures showing about 75% of cells infected (Fig. 3), it was determined that albendazole at 1 ng/ml and 10 ng/ml had no effect on multiplication of meronts, so that levels of infection did not differ from those of control cultures throughout 6 weeks of observation. In control cultures (medium without drug) and in cultures to which 100 ng/ml albendazole had been added there was an initial rise in infection to >95%. However, by the fifth week infection had dropped to 50% in the 100 ng/ml drug-treated cultures. By the sixth week infection had dropped back to 25% in the 100 ng/ml albendazole-treated cultures but remained at >95% in the controls. No myotubes were present in any cultures.

As 100 ng/ml albendazole appeared to be the minimum dose for control of *T. hominis* in C_2,C_{12} cultures, doses of 100 ng/ml, 500 ng/ml and 1,000 ng/ml were applied to cultures carrying 75% infections. After three weeks, control groups and cultures exposed to 100 ng/ml albendazole still showed 75% infected cells and almost complete cell coverage of culture surfaces was restored after subculture. At the same time, in cultures exposed to 500 ng/ml albendazole, 50% of the monolayer had been destroyed and infection remained at 75%. With 1,000 ng/ml only 15-20% of the monolayer remained, infection was still at 75% and no meronts were seen.

After a further week, control cultures remained at 75% with good monolayers. With 100 ng/ml albendazole infection had dropped to 50% but cell monolayers remained good. With 500 ng/ml and 1,000 ng/ml there was less than 20% cell coverage and 25% infection. For the remaining three weeks of the trial control cultures reached >95% infection in good monolayers, while cultures with 100 ng/ml albendazole had good monolayers, but only 25% infection. Cultures exposed to 500 ng/ml and 1,000 ng/ml were severely depleted of cells and infection was at 25% with 500 ng/ml of drug but barely detectable with 1,000 ng/ml. No myotubes were present and infection did not disappear entirely in any of the cultures.

When albendazole treatment was discontinued, there was gradual restoration of monolayers in infected cultures where the cells had been severely depleted and patchy myotube formation was initiated during weeks 3 and 4 only of the 7-week observation period, while infection was held at 25%. Infections gradually increased, reaching 50% even in cultures that had been exposed to 1,000 ng/ml albendazole, and, as infections built up, so myotube formation was halted.

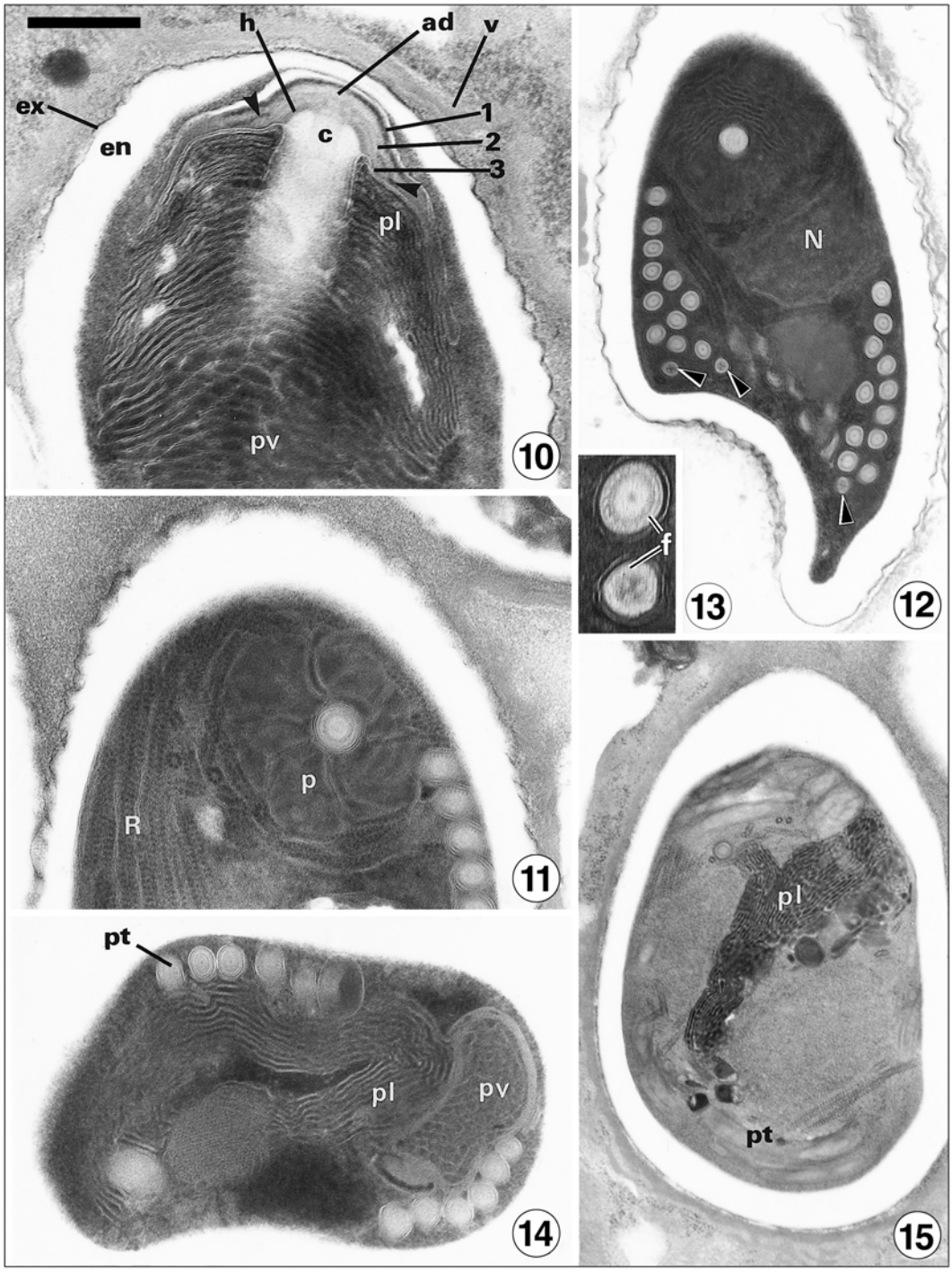
All uninfected C_2,C_{12} cultures exposed to spores, recovered from cultures treated with 1,000 ng, 500 ng, 100 ng albendazole and from cultures supported by medium only or medium plus DMSO, showed signs of infection after 12 days and reached 50% infection by 5 weeks when the experiment was terminated. The culture which received no spores remained uninfected.



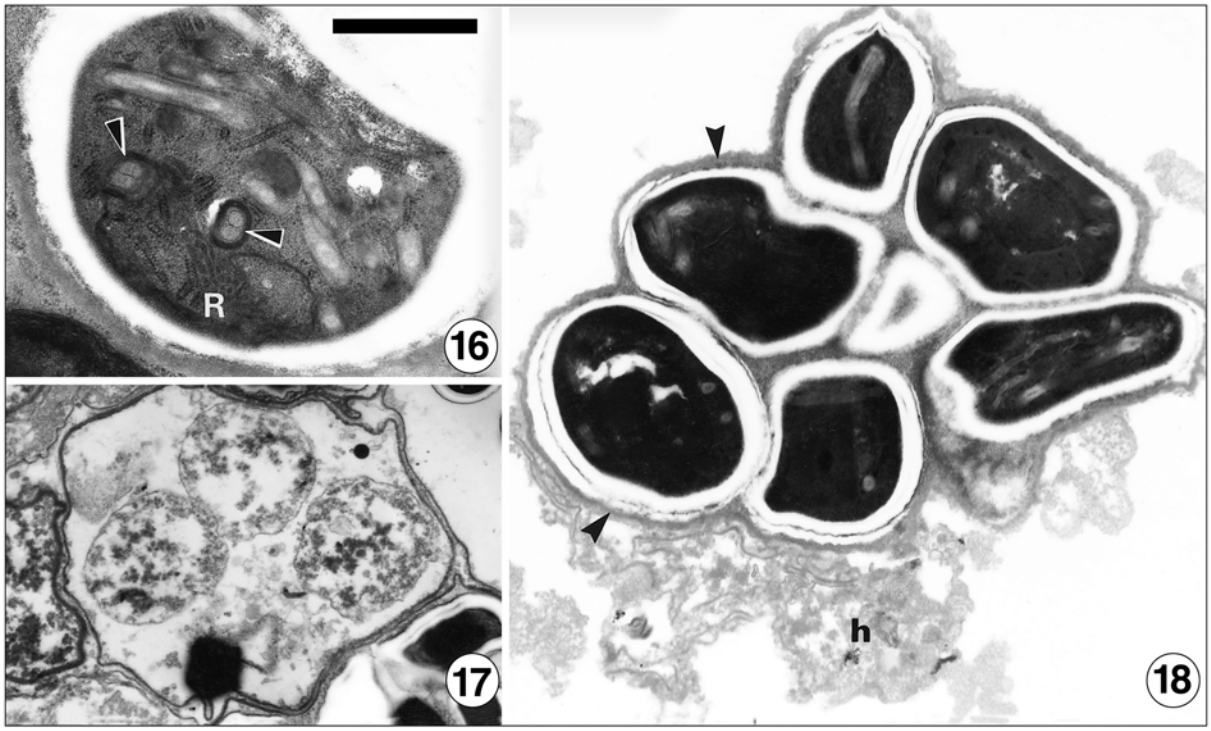
Figs. 4-9. Normal and albendazole-treated *Trachipleistophora hominis* meronts in C_2,C_{12} cell culture. **Figs. 4, 5.** Normal meronts showing surface coat extending as an extensive reticulum (arrowheads), which connect with tubules (t in Fig. 4), in the host cell cytoplasm. **Fig. 6.** Bundles of tubules in host cell cytoplasm, probably of parasite origin which are associated with the surface of *T. hominis* meronts. **Fig. 7.** Meront exposed to 100 ng/ml albendazole. The surface is devoid of the thick surface coat and reticular structures. The boxed area is enlarged in Fig. 9. **Fig. 8.** Meront exposed to 100 ng/ml albendazole showing reticulate extensions (arrowhead) from a thinner than normal surface coat. It lies close to a group of totally abnormal spores. **Fig. 9.** Enlargement of the boxed area of Fig. 7. The plasma membrane (arrowheads) is devoid of surface coat. N – nucleus; t – tubules. Scale bar on Fig. 4 applies to all figures; Figs. 4, 5 = 900 nm; Fig. 6 = 800 nm; Figs. 7-9 = 870 nm.

Ultrastructural changes in albendazole-treated *T. hominis*

All stages of *T. hominis* in untreated and DMSO controls were normal, conforming to the morphology observed in human skeletal muscle (Field et al. 1996) and in mouse skeletal muscle and RK13 and COS-1 cell culture (Hollister et al. 1996). Meronts, many of which were in division, showed 1-3 nuclei in the plane of section. The plaque matrix, consisting of surface coat and reticulate extensions into host cell cytoplasm, was present (Figs. 4, 5), as were bundles of parallel tubules in the host cell cytoplasm (Figs. 4, 6) which are known



Figs. 10-15. Normal and albendazole-treated spores of *Trachipleistophora hominis* in C_2,C_{12} cell culture. **Fig. 10.** Anterior end of normal spore lying close to the sporophorous vesicle envelope. Anchoring disc has an anterior electron-dense layer (1) and two fine granular layers (2, 3) which merge to form a single denser layer (arrowheads). **Fig. 11.** Normal spore: petaloid arrangement of polaroplast around the straight region of the polar tube. **Fig. 12.** Oblique section of normal spore showing 11 wide coils of the polar tube and 1.5 narrow coils (arrowheads). **Fig. 13.** Enlargement of part of Fig. 12 showing a wide polar tube section with central dot in a lucent ring and a narrow coil with three lucent spots in a dense core. Both types of coil show a ring of longitudinally running fibrils. **Figs. 14, 15.** Completely abnormal spores exposed to 1,000 ng/ml albendazole. The membranes may be of lamellar and vesicular polaroplast nature. ad – anchoring disc; c – core of polar tube; en – endospore; ex – exospore; f – fibrils; h – hinge; N – nucleus; p – polaroplast; pl – lamellar polaroplast; pt – polar tube; pv – vesicular polaroplast; R – ribosomes; v – sporophorous vesicle envelope. Scale bar on Fig. 10 applies to all figures; Fig. 10 = 300 nm; Fig. 11 = 340 nm; Fig. 12 = 540 nm; Fig. 13 = 150 nm; Fig. 14 = 750 nm; Fig. 15 = 400 nm.



Figs. 16-18. Sporogonic stages of *Trachipleistophora hominis* in C_2,C_{12} cell culture exposed to albendazole. **Fig. 16.** Abnormal spore exposed to 100 ng/ml albendazole showing chaotic arrangement of polar tube. Double polar tubes (arrowheads) with two core structures within four membranes were common. **Fig. 17.** Abnormal division products of sporont within sporophorous vesicle in cultures exposed to 500 ng/ml albendazole. **Fig. 18.** Sporophorous vesicle (arrowheads) containing abnormal spores. The host cell, exposed to 1,000 ng/ml is totally degenerate. h – host cell; R – ribosomes. Bar on Fig. 16 applies to all figures; Fig. 16 = 800 nm; Fig. 17 = 2.8 μm; Fig. 18 = 1.25 μm.

to connect with the meront surface structures (Weidner et al. 1997). Of the cultures treated with albendazole at 100 ng/ml, 500 ng/ml or 1,000 ng/ml, meronts were only observed in those exposed to 100 ng/ml. These were of normal size but most of them lacked the plaque matrix (Figs. 7, 9) and none was seen in division. In rare cases the reticulate extensions were present but the surface coat was thinner than normal (Fig. 8). Spore structure in control cultures was particularly clear showing the wide polar tube insertion into the flattened anchoring disc (the central region of the polar sac) and lamellar and vesicular regions of polaroplast (Fig. 10). The anchoring disc, without obvious boundary, merged into the lateral "arms" of the polar sac, which extended back over at least half of the membranous region of polaroplast. There was an anterior electron-dense layer of the anchoring disc, and two finely-granular layers in the matrix, which merged to form a single denser layer at the "shoulders" of the polar sac. The core of the polar tube at its anterior end was connected by a "hinge" to the base of the polar sac. Other features not previously observed were the petaloid arrangement of posterior polaroplast (Fig. 11) and anisofilar polar tube with 1.5 posterior coils 90 nm wide, compared with the 10-11 wider coils of 120 nm diameter (Fig. 12). The posterior coils also had a different core structure with several lucent areas in an electron-dense background, as opposed to a single electron-dense core in a lucent background. A ring of longitudinally running fibrils within the two membranes which formed the "sleeve", was present in both types of coil (Fig. 13).

Cultures exposed to albendazole concentrations of 100 ng/ml and higher showed a preponderance of totally abnormal spores with bizarre arrangements of parallel membranes and erratic coiling of the polar tube (Figs. 14-16). A common abnormality of the polar tube was an arrangement of two incomplete tubes with their concentric ring structures bound together by four membranes, equivalent to a double sleeve (Fig. 16). Sporogonic division within sporophorous vesicles had occurred, albeit with degenerate products (Fig. 17), but these divisions might have occurred before the cultures were exposed to albendazole. Host cell structure was disrupted due to the high levels of infection in all groups, including controls, but, at the higher drug levels, infected cells were virtually destroyed (Fig. 18).

DISCUSSION

Effect of *T. hominis* on myotube differentiation

In a typical progression of C_2,C_{12} myoblast cell cultures maintained in DMEM with 20% FCS, DNA synthesis and cell division proceed until confluency is reached. At this point cell division ceases and a medium change to DMEM with 2% horse serum stimulates cell fusion to form multinucleate myotubes (Chiu and Blau 1984). The myotubes synthesise muscle specific proteins and the spontaneous contractions which follow, often lead to detachment of large swathes of the confluent sheet of cells. We maintained our uninfected C_2,C_{12} cultures for up to eight weeks without subculture but subculture was always possible from the small proportion of cells which remained attached. This shows that some undifferentiated myoblasts were always present and were responsible for regrowth and differentiation into myotubes. Myoblast persistence might have been related to the serum concentration which at 8% was lower than has been used previously in myogenic manipulation (Yaffe and Saxel 1977). Differentiation into myotubes in *T. hominis*-infected cultures was inhibited in confluent cultures even when less than 25% of cells were infected. If this could be extrapolated to human muscle *in vivo*, repair of muscle from satellite cells (myoblasts) would lag behind destruction of myotubes and might be one of the causes of the loss of mobility as seen in the patient from whom *T. hominis* was isolated.

Effect of albendazole on *T. hominis* in C_2,C_{12} cells

DMSO has been shown to prevent L_8 myoblasts from entering the G_0 stage of the cell cycle, which is necessary for their differentiation into myotubes (Blau and Epstein 1979). According to their study, 65% of cells in a 4-5 day confluent monolayer had differentiated as multinucleate myotubes in the absence of DMSO. Furthermore, inhibition of differentiation by DMSO was concentration-dependent, with only about 40% of fused cells developing in the presence of 0.5% DMSO and none in the presence of 2% DMSO (Blau and Epstein 1979). DMSO was included in our drug trial because it was used as the solvent for albendazole. However, the highest concentrations (0.02% in 1,000 ng/ml albendazole, 0.05% in the control) were well below levels which would significantly inhibit differentiation. DMSO was not responsible for myotube inhibition, as shown by the absence of myotubes in infected control cultures without DMSO, and by the restriction of myotubes to transient and patchy formation in cultures with low levels of infection when albendazole was withdrawn: myotube differentiation was halted in these cultures when the infection level was restored to 25%, showing that the effect was due to infection rather than DMSO.

The patient from whom *T. hominis* was isolated recovered mobility and was free of pain after treatment with a combination of drugs which included albendazole. However before treatment there was already a debilitating myositis with extensive lesions packed with microsporidia in the skeletal muscle. In an attempt to reproduce some of the conditions under which albendazole was presumed to have enabled the patient to recover, infection levels in C_2,C_{12} cultures were allowed to reach 75% of cells infected before albendazole was added.

In all cultures the percentage of infected cells dropped immediately after subculture during the period when monolayer coverage was being restored by uninfected myoblast replication but in the medium only and medium plus DMSO controls, infection was eventually >95%. In cultures treated with 500 ng/ml and 1,000 ng/ml, the drug concentration was deleterious to the host cells so that it was impossible to separate the direct and indirect effects of the drug. At 100 ng/ml the C_2,C_{12} cells were unaffected by the drug and monolayers were quickly restored. However, in all cultures exposed to 100 ng/ml and above there was massive disruption of spore development, although reduction in the percentage of infected cells was not apparent in the cultures treated with 100 ng/ml until the fourth or fifth weeks. Drug concentrations below this did not affect parasite morphogenesis. When albendazole treatment was discontinued cell monolayers recovered in the treated groups and, as the infection remained low for three weeks, patches of myotubes began to develop. Eventually there were signs of parasite recovery in all treated cultures, showing that some parasite stages were unaffected even by the highest drug concentration. Viability of at least some spores exposed to albendazole was apparent when spores recovered from albendazole-treated cultures were able to initiate infections in new C_2,C_{12} cultures. Whether total parasite destruction could be achieved if albendazole were applied during early infections was not tested.

Since its discovery as an antimicrosporidial agent *in vivo* (Blanshard et al. 1992), albendazole has been tested against various microsporidia *in vitro* with widely different responses to varying concentrations. In the first *in vitro* study (Colbourn et al. 1994) using *Encephalitozoon cuniculi*, concentrations of 4.2 µg/ml and 2.1 µg/ml caused profound effects on the parasites developing in canine kidney cells (MDCK), especially the inhibition of nuclear and cytoplasmic division, formation of bundles of 35 nm intra-cytoplasmic tubules in meronts and abnormalities in spores, while the host cells were unaffected. When purified mature spores were incubated with albendazole for 24 h they remained viable, suggesting that spores that mature before exposure to albendazole can survive and reactivate infections when drug pressure is removed. Weiss et al. (1994) used albendazole doses of 10 µg/ml, 5 µg/ml and 2.5 µg/ml on *E. cuniculi* in rabbit kidney (RK13) cells, all of which eliminated infections but the highest dose also damaged the host cells. In the present study with

T. hominis in C_2,C_{12} cells, damage of host cells, that was already apparent at doses of 0.5 µg/ml and 1.0 µg/ml, probably reflects the different sensitivity of these cells versus RK13 cells as we had previously found that MDCK cells were tolerant of albendazole concentrations of 4.2 µg/ml (Colbourn et al. 1994). This shows that the choice of cell line can affect the outcome of *in vitro* drug tests.

In a recent survey of drugs with anti-microsporidial potential (Didier et al. 1998), albendazole was tested against *Encephalitozoon intestinalis* and *Vittaforma corneae* in RK13 cells. Our results for *T. hominis* in C_2,C_{12} cells are in general agreement with those of Didier et al. (1998) on toxicity of albendazole to host cells and inhibition of parasite development, except that they reported significant inhibition of development (66% below control values) of *E. intestinalis* (not *V. corneae*) in the presence of 0.01 µg/ml albendazole. This could be explained by the different microsporidian species or host cells or by the design of the experiments. Didier et al. (1998) applied albendazole to cultures only 3 hours after infecting them, thus providing the drug with an opportunity to act on early stages (sporoplasms) before active division occurred. Similar explanations are possible for sensitivity of *E. cuniculi* in MRC5 fibroblasts (Beauvais et al. 1994) in which 90% inhibition of development was obtained with 0.005 µg/ml albendazole. In these experiments albendazole was added 5 hours after application of spores to the MRC5 cells.

Ditrich et al. (1994) obtained 50% inhibition of *E. cuniculi* and *Encephalitozoon hellem* in monkey kidney cells (Vero), with doses of albendazole as low as 1 ng/ml and 4 ng/ml respectively and total elimination of infection with 15 ng/ml and 8 ng/ml respectively. In our study on *T. hominis* we did not succeed in eliminating infections even at 1,000 ng/ml albendazole. The sequence of the β-tubulin gene is not known for *T. hominis*. It is therefore not possible to predict whether albendazole resistance of this microsporidium is associated with the absence of Glu-198 and Phe-200. These amino acids are known to be strong predictors of benzimidazole sensitivity (Katiyar et al. 1994) and are present in *Encephalitozoon* spp. (Edlind et al. 1994). The low sensitivity of *T. hominis* found in the present study may be due to the β-tubulin amino acid sequence or to the presence of sporophorous vesicles around spores or to the design of the experiment in which there was an abundance of mature spores before the drug was applied.

Albendazole acts by preventing polymerisation of microtubules from units of α- and β-tubulin (Lacey 1990). In microsporidia it would be expected to obstruct nuclear division by preventing spindle microtubule assembly. Microtubules have rarely been observed in the cytoplasm of microsporidia but are always formed in the nuclei for chromosome alignment. In the present study, and in the majority of earlier studies, the most striking manifestation of the effect of albendazole on microsporidia has been the disarray of polar tube and polaroplast in spores. Abnormalities in meronts have been reported by Haque et al. (1993), Colbourn et al. (1994) and Silveira and Canning (1995). Colbourn et al. (1994) reported on the presence of bundles of 35 nm tubules in the cytoplasm of albendazole-treated *E. cuniculi* meronts. Similar tubules were found in *E. cuniculi* meronts and sporonts in albendazole-treated SCID mice (Koudela et al. 1994). The greater diameter of these tubules indicates that they are not microtubules and Colbourn et al. (1994) suggested that they may represent an aberrant form of the mechanism for deposition of surface coat during the transition to sporonts.

The polar tube and its anchoring disc in the polar sac originate in a system of Golgi vesicles first located near the nucleus of sporoblasts. While the polar sac and anchoring disc come to occupy an anterior position, the Golgi vesicles adopt a posterior position and are involved in the formation of the coils of the polar tube (Vinckier et al. 1971, Takvorian and Cali 1996). The coils must ultimately join up with the anchoring disc. Several polar tube proteins of differing molecular weights have been identified from microsporidia (reviewed by Keohane and Weiss 1999). None was found to react with antibodies raised to α-tubulin or β-tubulin (Keohane et al. 1996). In the absence of tubulin from polar tubes the disarray of polar tube organisation in spores exposed to albendazole is therefore not a direct result of the inhibition of microtubule polymerisation. Cytoplasmic microtubules have occasionally been reported in microsporidian meronts (Bigliardi et al. 1998, Canning et al. 1999) but never in spores. However their absence from spore cytoplasm has not been established with certainty because the density of spores would tend to obscure them. Nevertheless it is likely that albendazole is taken up by prespore stages rather than by thick-walled spores and will cause sufficient disruption of morphogenesis to disable the maturation of spores.

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