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In vitro cultivation of the human microsporidium *Vittaforma corneae*: development and effect of albendazole

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Abstract. When *in vitro* growth of *Vittaforma corneae* was tested using MDCK, MRC-5, XEN, L-929 and FHM cell lines, propagation occurred only in MDCK, MRC-5 and XEN cells. The intervals required for the various stages of the life cycle to develop were the same in all the cell lines tested. The MDCK cell line was selected to support the growth of *V. corneae in vitro* and provide the system for *in vitro* testing of drugs. The weekly output of *V. corneae* spores from the MDCK cell monolayer was monitored over a 61-week period during which there were fluctuations but no definite increase or decrease in output. Albendazole at 2.1 or 4.2 μg/ml in MEM was tested against *V. corneae* in MDCK cell monolayers and showed antimicrosporidial activity. The percentage of infected cells was reduced in the presence of the drug and there were ultrastructural abnormalities in all stages of the life cycle. The drug prevents parasite division.

The first microsporidium of mammalian origin to be cultured *in vitro* was *Encephalitozoon cuniculi*. It was established from naturally-infected rabbit choroid plexus cells (RCP) (Shadduck 1969), these cells later being found to support abundant growth of the parasite in comparison with other cells (Vávra et al. 1972, Montrey et al. 1973). After testing several cell lines, Waller (1975) found that the Madin Darby Canine Kidney (MDCK) cell line was a suitable choice for propagation of large amounts of *E. cuniculi*, as the system was simple to maintain and an equilibrium between cell destruction by the microsporidia and cell replacement was achieved.

Other microsporidia of mammalian origin that have been propagated *in vitro* have all been derived from humans, including an isolate of *E. cuniculi* from the urine of AIDS patients into MDCK (Hollister et al. 1993a, 1995) and E-6 and HLF cell lines (De Groote et al. 1995). *Nosema corneum* described from the cornea of an immunocompetent patient (Davis et al. 1990) was propagated in rabbit corneal epithelium by Shadduck et al. (1990). On the basis of ultrastructural differences from the type species *Nosema bombycis*, *N. corneum* was renamed *Vittaforma corneae* (Silveira and Canning 1995). *Encephalitozoon hellem* was isolated from human cornea into MDCK cells (Didier et al. 1991), and has also been established from nasal polyp tissue into MDCK cells (Hollister et al. 1993b) and from urine into several cell lines (Visvesvara et al. 1991). Another isolate of *Encephalitozoon* (possibly *E. hellem*) has been cultured from corneal scrapings into MRC-5 cells (Desser et al. 1992). *Encephalitozoon intestinalis*, which has been transferred from the genus *Septata* by Hartskeerl et al. (1995), has been cultured in RK cells (Van Gool et al. 1994)

Albendazole is an anthelmintic drug, which is an inhibitor of tubulin polymerisation (Lacey 1990). Some improvement of the chronic diarrhoea associated with *Enterocytozoon bieneusi* infection in AIDS patients had been noted after treatment with albendazole without elimination of infection (Blanshard et al. 1992). The same drug brought about clinical improvement in a patient with a chronic infection of *E. hellem* in the nasal sinuses and polyps. During treatment there was regression of the hypertrophic nasal tissue and improvement of his breathing difficulties continued until his death from AIDS dementia complex (Lacey et al. 1992). An infection with an *Encephalitozoon*-like species (now known to be *E. intestinalis*) completely resolved in an AIDS patient after treatment for 10 days with albendazole. The parasites disappeared from stool, urine and maxillary sinus lavage (Eeftinck Schattenkerk et al. 1993). Similarly *E. intestinalis* in an AIDS patient disappeared completely after albendazole treatment (Orenstein et al. 1993). Preliminary clinical tests, thus, indicate that albendazole might be completely effective against *E. hellem* and *E. intestinalis* but only partially effective against *E. bieneusi*.

In vitro cultures of microsporidia provide a suitable way for preliminary testing of drugs for antimicrosporidial activity. The activity of two drugs, fumagillin and albendazole, against natural infections of microsporidia has been confirmed by *in vitro* tests. Fumagillin, a recognised antimicrosporidial drug, commonly used in bee keeping, has inhibited multiplication of *E. cuniculi in vitro* (Shadduck 1980). Its antimicrosporidial activity has been confirmed *in vitro* using *N. bombycis* and *E. cuniculi* grown respectively in a *Spodoptera frugiperda* cell line (Haque et al. 1993) and in MDCK cells (Colbourn et al. 1994). It prevents nuclear division (the

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intranuclear spindle being the site of microtubule assembly) in both species.

This work examines the development of *V. corneae in vitro* and uses the system to test the effect of albendazole on the species.

MATERIALS AND METHODS

Cell cultures

Cells were cultured in 24 or 83 cm^2 base culture flasks and in 24-well tissue culture plates. All tissue culture media were supplemented with foetal calf serum (FCS) at 10 % for cell monolayer growth and 2 % for maintenance of the monolayer, and with 100 UI/ml penicillin (pen), 100 µg/ml streptomycin (strep) and 2 mM L-glutamine (L-glu). Special additives were used for each cell line as follows:

XEN (*Xenopus* fibroblasts) cells were cultured in Liebovitz medium (L-15), supplemented with 10 % tryptose phosphate broth (TPB). The cells were cultured at 27°C in air.

FHM (Fathead minnow) cells were cultured in the Glasgow modification of Minimum Essential Medium (GMEM), supplemented with 10 % TPB, 10 % tris/HCl buffer 0.16M pH 7.4 and 3.7 % sodium bicarbonate (7.5 % w/v stock solution). The cells were cultured at 27°C in air.

MDCK (Madin Darby Canine Kidney) and MRC-5 (Human Embryo Lung Fibroblast) cells were cultured in Eagle's Minimum Essential Medium with Earle's salts, supplemented with 100 µg/ml kanamycin, as well as pen and strep and 2.2 g/l sodium bicarbonate.

L-929 (mouse fibroblasts) cells were cultured in Dulbecco's modification of Minimum Essential Medium with 1 g/l glucose, 24 mM Hepes buffer and 110 mg/l sodium pyruvate supplemented with 100 µg/ml kanamycin as well as pen and strep, 3.7 g/l sodium bicarbonate and 5 % FCS. The cells were cultured in a gas mixture of 5 % CO_2 in air at 37°C.

Harvesting of spores

Vittaforma corneae was grown in MDCK cells. The medium was changed weekly, and the cells were passaged at a split ratio of 1 flask into 3 at monthly intervals. Supernatants containing spores were collected from culture flasks, centrifuged at 1200 g for 10 min, and resuspended in phosphate buffered saline (PBS). The spores were purified by centrifugation at 1200 g for 30 min through 50 % percoll in PBS, washed and resuspended in PBS. The total number of spores was estimated by counting on a haemocytometer, and taking the mean of three counts.

In vitro determination of *V. corneae* life cycle

One million spores were added to the suspensions of MDCK, MRC-5, L-929, XEN and FHM cells, each suspension containing 2×10^5 cells. The mixtures were allowed to rest for 30 min in 10 ml polystyrene tubes and were then centrifuged at 500 g, resuspended in the appropriate fresh medium and were seeded on to coverslips in 24-well tissue culture plates. During incubation three coverslips were taken every 8 h for the first 72 h and every 12 h thereafter. The coverslips were fixed in methanol for 1 min and stained for 30 min in 10 % Giemsa stain in phosphate buffer, pH 7.2. Coverslips were mounted on to slides using Euparal vert and observed at 1000× magnification. The proportion of infected cells was determined by examining 10 random places on each coverslip and taking the mean of three counts of 1000 cells. The mean number of organisms per infected cell was determined by counts in 100 infected cells.

Weekly production of *V. corneae* in MDCK cells

The output of *V. corneae* grown in MDCK cells, was assessed by counting the number of spores in the supernatant from 24 cm^2 base culture flasks. For this purpose a MDCK cell monolayer was infected with 10^7 purified spores. The supernatant from 3 flasks was collected weekly thereafter for 63 weeks. The number of spores was estimated by taking the mean of three counts using a haemocytometer.

In vitro test for antimicrosporidial properties of albendazole

A stock solution was prepared by diluting 3 mg of albendazole powder (Smithkline Beecham) in 100 µl of dimethyl sulphoxide (DMSO). This was used to generate a solution of 10 µg albendazole/ml MEM although some of the drug remained in suspension. This mixture was sterilised by filtration through a 0.22 µm millipore filter. The filtrate when analysed was found to have a drug concentration of 4.2 µg/ml. Medium with drug was used as 4.2 µg/ml or was diluted further for use at 2.1 µg/ml solution.

MDCK cells infected with *V. corneae* were grown on coverslips in wells of a 24-well tissue culture plate, the cells being set up from a suspension derived from a single flask. When the cells had grown to confluent monolayers, 3 coverslips were removed to estimate the initial level of infection. In the remaining wells, the medium was replaced with fresh medium, medium with 4.2 µg/ml or 2.1 µg/ml of drug or with medium containing solely the same concentration of the drug solvent (DMSO). Cultures were exposed to treatment for 1 or 2 weeks. After one week fresh drug solutions were prepared and the media replaced. After exposure, 3 coverslips of each treatment were removed, washed in medium, fixed in methanol and stained with 10 % Giemsa stain. The effect of the drug was assessed by estimating the percentage of infected cells on the Giemsa-stained coverslips mounted on slides. One thousand cells were counted at random points on each coverslip.

Electron microscopy

Cells were processed as monolayers attached to coverslips. Specimens were fixed in 2.5 % glutaraldehyde in 0.1M cacodylate buffer for 1 h, rinsed in buffer and post fixed in 1 % osmium tetroxide in 0.1M cacodylate buffer. They were then rinsed with 0.1M sodium acetate, transferred to 2 % aqueous uranyl acetate for 1 h, rinsed in 0.1M sodium acetate, passed trough 35 % and 50 % acetone into a solution of 1 % phosphotungstic acid, and 1 % uranyl acetate in 70 % acetone overnight. They were dehydrated in an acetone series and embedded in Spurr resin. Coverslips were placed with the cell surface face down on the top of a pre-polymerised resin block, which had been previously roughened on the surface. After polymerisation, glass coverslips were removed by lifting the edge with a razor blade. Sections were cut, mounted on copper grids and stained in 2 % uranyl acetate and 2 % lead citrate. The sections were examined using a Phillips EM300 electron microscope.

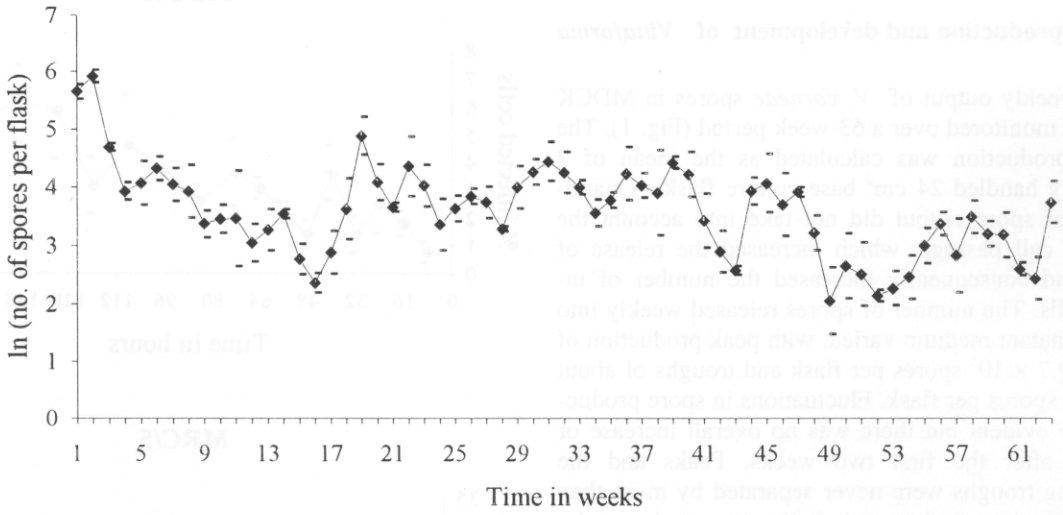
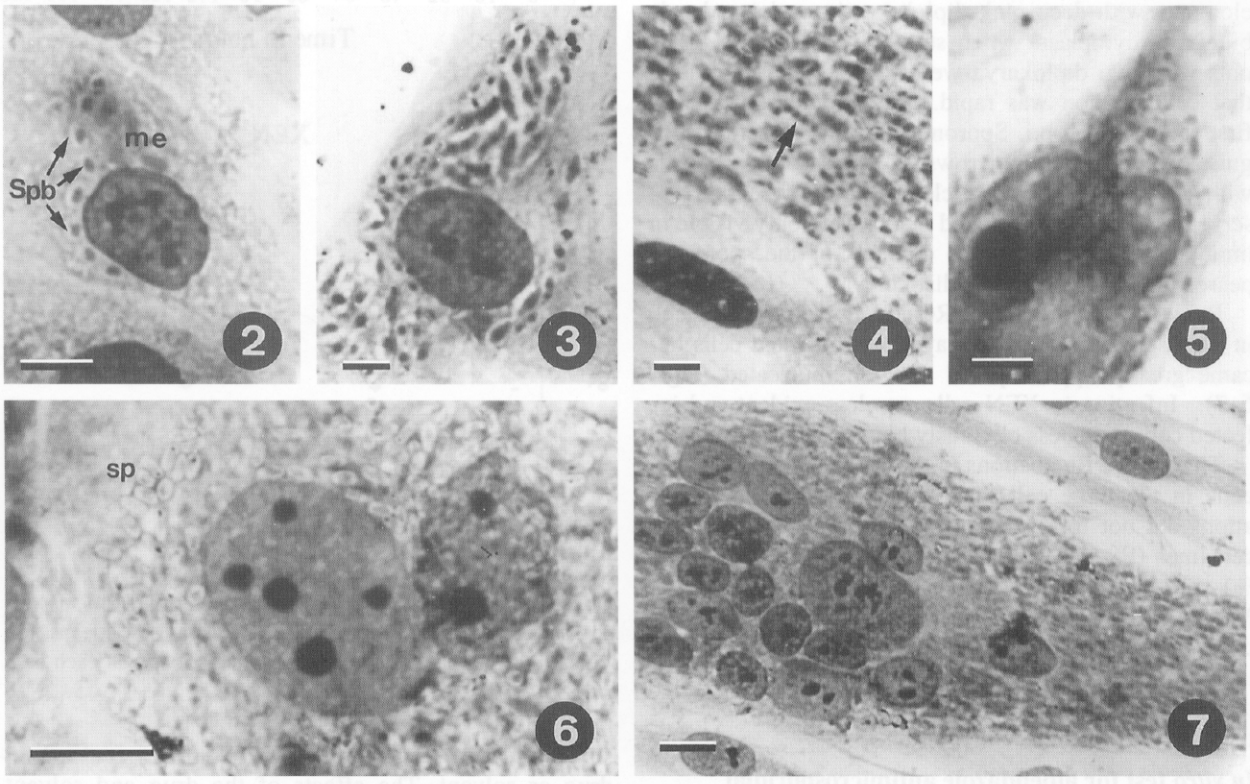


Fig. 1. Weekly output of *Vittaforma corneae* from infected MDCK cells. Cell monolayers were inoculated with 10^7 purified spores on day 0. Each value represents the arithmetic mean and standard deviation of the number of spores produced in three separate tissue culture flasks with a culturing area of 24 cm^2. The values represented are Naperian Logarithms.



Figs. 2–7. *Vittaforma corneae* infected cells, grown on glass coverslips and stained with Giemsa. **Figs. 2–3.** MDCK cells. **Fig. 4.** MRC-5 cells. **Fig. 5.** XEN cells. **Figs. 6–7.** Hypertrophic and multinucleated cells infected with *V. corneae*. **Fig. 6.** MDCK cells. **Fig. 7.** MRC-5. Meronts (me); sporoblasts (spb); spores (sp); multinucleated sporont (arrow). Bars = 5 µm (Figs. 2–5) and 10 µm (Figs. 6–7).

RESULTS

In vitro production and development of *Vittaforma corneae*

The weekly output of *V. corneae* spores in MDCK cells was monitored over a 63-week period (Fig. 1). The weekly production was calculated as the mean of 3 identically handled 24 cm^2 base culture flasks. Quantification of spore output did not take into account the effect of cell passage, which increased the release of spores and consequently increased the number of infected cells. The number of spores released weekly into the supernatant medium varied, with peak production of around 9.7×10^5 spores per flask and troughs of about 1.1×10^5 spores per flask. Fluctuations in spore production were evident but there was no overall increase or decrease after the first two weeks. Peaks and the succeeding troughs were never separated by more than 6 weeks. Even so, a clear periodicity was not observed.

Infection was not established in L-929 and FHM cells. No differences were observed in the developmental stages obtained in MDCK, MRC-5 and XEN cell lines, the intervals between the various stages of the life cycle being the same. Meronts, sporoblasts and spores were visible in all three cell lines (Figs. 2–7). Merogonic stages were first observed at 16 h p.i.. They were elongate, with deep pink diplokaryotic nuclei and pale blue-grey cytoplasm when stained with Giemsa. Meronts with two diplokarya were rarely seen, suggesting that cell division was rapid. Sporogonial stages were first seen at 40 h p.i. Sporonts were difficult to distinguish from meronts but rows of at least 4 sporoblasts with compact nuclei were clear. Spores were first observed at 48 h p.i. The paired nuclei were only visible in immature spores, when the contents of the spore appeared pale blue within the distinct wall.

Infected MDCK and MRC-5 cells were aggregated in small areas of the monolayer, and infected cells became greatly hypertrophied and multinucleated (Figs. 6–7). Infection in XEN cells was less evident and infected cells were less obviously hypertrophic.

There were fluctuations in the percentage of infected cells in the three cell lines tested, though the fluctuations did not follow the same pattern in the different cell lines (Fig. 8). The highest level of infection (24.2 %) was observed in MRC-5 cells at about 144 h. In MDCK cells there was gradual increase in infected cells, although the mean peak level was below 6 % during the period of observation. The XEN cell line was the least susceptible with a peak at 2.1 % at 32 h and thereafter a gradual decline.

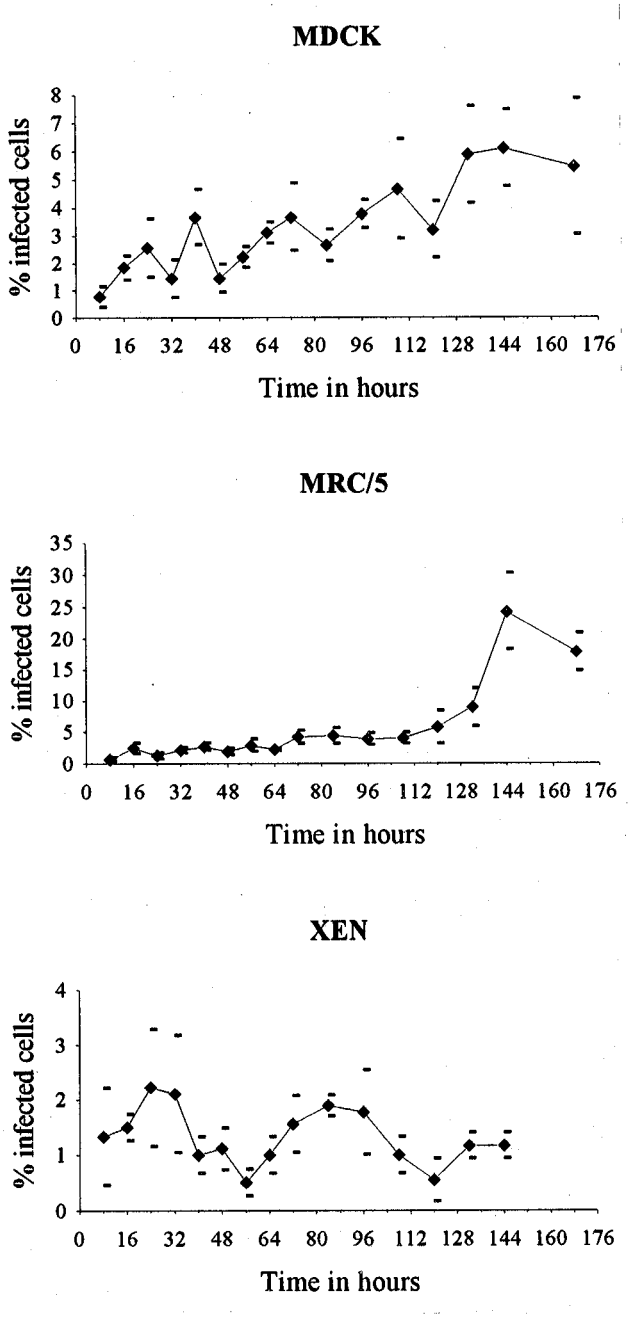


Fig. 8. Propagation of *Vittaforma corneae* in MDCK, MRC-5 and XEN cell lines. Cells and parasites were mixed in suspension and later plated on coverslips. Values are the means and standard deviations of counts of 1000 cells made on each of 3 coverslips.

In vitro test for albendazole antimicrosporidial properties

The MDCK cell line was selected as the system for *in vitro* testing of albendazole. Albendazole was tested at 2.1 and 4.2 µg/ml solution in MEM, added to *V. corneae* infected MDCK cell monolayers with an initial level of infection of 22.6 %. The effect of the drug solvent (DMSO) was also tested. Controls received no drug or solvent. The effects of the drug and solvent were analysed by estimating the percentage of infected cells on days 7 and 14 using 3 replicates for each treatment.

On day 7 the percentage of infected cells in the untreated control had risen to 28.5 %. In cultures to which DMSO had been added the level had dropped to 16.2 %

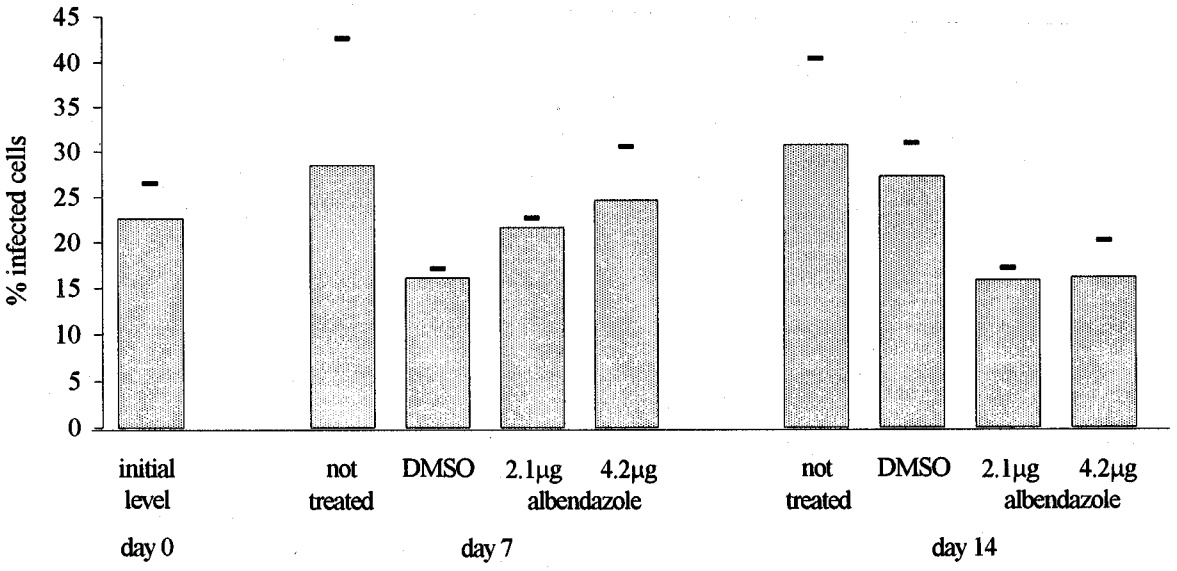


Fig. 9. Effect of albendazole on percentage of infection of *Vittaforma corneae* in MDC K cells. Horizontal bars represent standard deviations.

while the levels in cultures containing 2.1 and 4.2 µg/ml albendazole the levels were 21.6 % and 24.6 % respectively. On day 14 there was a further small increase in the percentage of infected cells in the controls to 30.7 % and the level in the DMSO control had risen above the initial level to 27.2 %. However, in the presence of albendazole the level had fallen to 15.9 % and 16.1 % for the 2.1 and 4.2 µg/ml doses respectively (Fig. 9).

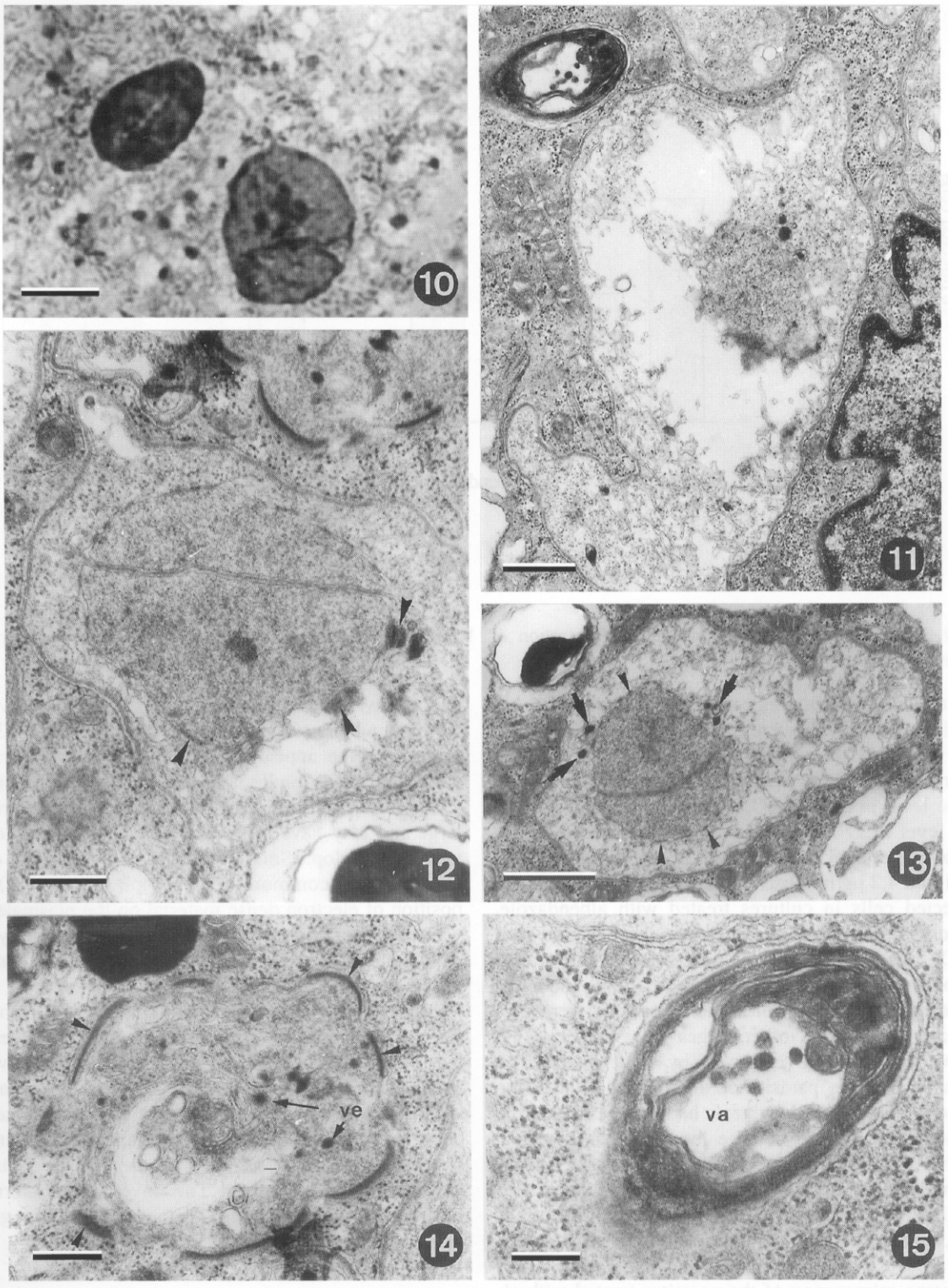
Infected cells in cultures treated with albendazole, were less crowded with parasites than those treated with DMSO only or non-treated cells. Meronts, sporonts, sporoblasts and spores were distributed throughout the cytoplasm, though sporoblasts and sporonts were predominant, even when other stages were present (Fig. 10). No effects were observed by light or electron microscopy on the parasite or on MDCK cells in the presence of DMSO. Albendazole had some effect on the cell monolayer, which became less dense as observed by light microscopy. The cells appeared bigger, vacuolated and stained lighter with Giemsa, the appearance was normal by electron microscopy with abundant ribosomes, intact mitochondria and endoplasmatic reticulum (e.r.)

Electron microscopy of the parasites treated with albendazole revealed ultrastructural abnormalities, which were consistently present and were different from those occasionally observed in the controls. Meronts were enlarged with very pale cytoplasm, in which ribosomes and large spaces (possibly representing greatly dilated e.r.) were visible (Figs. 11–13). No meronts were observed with more than one diplokaryon. Electron dense deposits within the membranes of the nuclear envelope were abundant, even in a single section (Figs. 12–13). Some of these appeared to be polar plaques normally associated with spindle poles but there were no intranuclear spindles. Other dense deposits were abnormally large and may have been situated on the outside of the nuclear envelop. Small vesicles with electron dense contents were common in the cytoplasm (Fig. 13). Deposition of electron dense material on the plasmalemma was observed indicating transition to sporogony, but development of sporont structures was highly compromised by the drug (Fig. 14). The cytoplasm of these stages was rich in rough e.r. and concentric membranes were present. Spore organisation varied considerably but normal spores were not seen (Fig. 15). They varied from totally indistinct masses of electron dense material within an incomplete wall, occasionally with some evidence of nuclei, to spores with concentric membraneous structures around a central vacuole which contained small spherical structures. The enveloping cisterna of host e.r. around all stages, characteristic of the genus, was unaffected by the drug.

DISCUSSION

In vitro cultivation of *Vittaforma corneae*

The weekly production of *V. corneae* spores released into the culture supernatant showed a prolonged series of oscillations. This differs from that of



Figs. 10–15. Abnormalities of *Vittaforma corneae* in MDCK cells treated with 4.1μg/ml albendazole for 7 days. **Fig. 10.** Light micrograph of cell monolayer stained with Giemsa. The cell cytoplasm, containing mainly sporoblasts and spores, appears vacuolated and multinucleated. Bar = 10 μm. **Figs. 11–13.** Electron micrographs of greatly enlarged and disorganised merogonic stages, each parasite with only one diplokaryon; parasites remain enveloped by a cisterna of host endoplasmatic reticulum (e.r.). Abundant electron dense deposits are seen within the membranes of the nuclear envelope in Figs. 12 and 13 (arrowheads) and vesicles with electron dense contents are visible in the cytoplasm in Fig. 13 (arrows). **Fig. 14.** Electron micrograph of an abnormal sporont showing the persistent enveloping cisterna of e.r. Vesicles with electron dense contents (ve) are probably involved in the deposition of the extramembranous coat (arrowheads). **Fig. 15.** Electron micrograph of an abnormal spore, showing concentric membranes around a central vacuole (va). Bars = 0.5 μm (Figs. 11–12, 14), 1 μm (Fig. 13) and 0.2 μm (Fig. 15).

Encephalitozoon cuniculi reported by Waller (1975), in which a few oscillations in spore production, which occurred when the cultures were first set up, were followed by a period of steady increase that reached a plateau at around 10 weeks. Cultures of these two species also differed in that MDCK cells infected with *E. cuniculi* tend to be widely scattered in the monolayer while those infected with *V. corneae* are aggregated in small foci. The difference between the patterns of spore output of *E. cuniculi* and *V. corneae* by MDCK cells probably lies in the relationship of these species with their host cells. Development of *E. cuniculi* occurs in parasitophorous vacuoles and, although *E. cuniculi*-infected cells become hypertrophic and packed with parasites, the vacuoles and the cells break down liberating the spores into the medium and thus dispersing the infection widely in the monolayer.

V. corneae develops directly in the cytoplasm of host cells. The cells become greatly enlarged and multinucleate but do not readily break down. Aggregates of infected cells probably arise as a result of division of cells at an early stage of infection or because newly produced spores germinate *in situ*, and inoculate sporoplasms into adjacent cells. Aggregation of *V. corneae*-infected cells and the high number of parasites per infected cell was probably responsible for the big fluctuations in weekly spore recovery from the supernatant because massively overcrowded cells would detach from the monolayer and degenerate. This would bring about a sudden dramatic increase in the number of spores in the supernatant. The rapid increase in the number of spores in the supernatant would be followed by infection of new cells and production of new aggregates, and, while parasites were multiplying within cells, a decline in the number of detectable spores in the supernatant.

The early growth of *V. corneae* in the different cell lines, estimated by the percentage of infected cells, did not follow the growth patterns of some other microsporidia that have been studied in culture. *E. cuniculi* cultured in RCP cells showed an initial period of rapid increase, after which a plateau of growth was reached (Shadduck and Polley 1978). The same type of growth was detected by Xie (1988) for *Cystosporogenes operophterae* in a *Spodoptera frugiperda* cell line, by Kawarabata and Ishihara (1984) for *Nosema bombycis* in a cell line of *Antheraea eucalypti*, and by Kurtti and Brooks (1977) for a *Nosema* sp. propagated in a *Heliothis zea* cell line. Although there was a gradual increase of *V. corneae* in MDCK cells, this was accompanied by strong fluctuations. In MRC-5 cells there was a lag period up to 128 h followed by a sudden increase. In XEN cells there was a gradual decline with fluctuations. Fluctuation in the percentage of infected cells might also relate to the different multiplication rates of cells and parasites.

The MRC-5 cell line supported abundant growth of the parasite in comparison with the other cell lines, but these cells were more fragile than MDCK or XEN and, after the peak of infection, the cell monolayer broke up and thereafter it was difficult to restore cells to their original state. MDCK cells were the most suitable for mass production, since they were simple to maintain and an equilibrium between cell growth and replacement was achieved. The equilibrium state was similar to that found by Waller (1975) for *E. cuniculi* in MDCK cells. Of the three cell lines that were successfully infected with *V. corneae,* XEN cells were the least productive, probably as a consequence of the temperature at which it was necessary to incubate the cells.

The intervals before the different stages of the life cycle developed were the same in MDCK, MRC-5 and XEN cell lines, suggesting that onset of different stages is independent of the type of host cell tested, and independent of temperature, since MDCK and MRC-5 cells were incubated at 37°C and XEN cells at 27°C.

MDCK and MRC-5 cells, when infected with *V. corneae,* became hypertrophic and multinucleate. Infected cells in the monolayer reach large sizes without major cell damage. This is probably a consequence of parasite stimulation of host cell growth so that a higher number of the parasites may be accommodated inside the host cell. Hypertrophy probably results from competition between the parasites and the constituents of the cytoplasm for nutrients. If multiplicative stages are able to obtain an advantage, this would be sufficient to induce atrophic changes in the cytoplasm (Weissenberg 1976). Merogonic stages were observed in most of the infected cells, even when heavily packed with parasites, suggesting that merogony continues, even if the parasite burden in the cell is high and a large number of spores is present. This can only occur if the host cell is capable of sustaining the growth of the parasites, so continuous merogonic division may induce a burst of metabolism by the host cell resulting in hypertrophy.

Microsporidia derived from invertebrates can be propagated in cell lines of vertebrate origin. For example *Nosema algerae* from mosquitoes and *Nosema eurytremae* from trematodes have been propagated in *Xenopus* and embryonic rat brain cell lines (Smith et al. 1982), and the former was also propagated in a pig kidney cell line (Undeen 1975). *N. bombycis* has been grown in primary cultures of rat, mouse, rabbit and chicken embryo cells (Ishihara 1968). However, in none of these cells did development occur at 37°C, demonstrating that body temperature is a limiting factor for these microsporida.

Transmission of invertebrate microsporidia to mammals is normally considered impossible, temperature being a limiting factor for development. This idea was strengthened by Undeen and Alger (1976) when they found that only limited development occurred of *N. algerae* in the extremities of white mice, that had been inoculated subcutaneously, and no infection occurred after intraperitoneal or intravenous inoculations. *V. corneae*, which is of human origin and thereafter presumed to be adapted to human body temperature, was able to

grow at temperatures varying from 27 to 37°C.

Microsporidia appear to be more tolerant of temperatures changes when these are below, rather than above the optimum. This is suggested by the growth of *E. cuniculi* in FHM cells at 18°C (Bedrník and Vávra 1972) and *V. corneae* in XEN cells at 27°C. Growth of *V. corneae* at temperatures below human body temperature is not by itself indicative of a non-mammalian origin of the infection, as the parasite was isolated from the cornea which has a lower temperature than the rest of the human body.

In vitro tests for the antimicrosporidial properties of albendazole

Treatment of microsporidiosis is a difficult task, since the parasite life cycle occurs entirely inside the host cells, and the spores have a thick wall, which is a barrier to drug penetration. As the drug has to enter host cells, it has to be toxic to microsporidia at levels which do not damage host cells. The target for drug action has to be the vegetative stages as drugs are unlikely to penetrate the spore wall. Destruction of the meronts should lead to elimination of the infection if applied before spores are produced but, if mature spores are already formed, infection could recur when the drug is discontinued.

Several drugs have been used to treat microsporidian infections. Fumagillin was the first drug to be used effectively in the control of bee microsporidiosis due to *Nosema apis* (Katznelson and Jamieson 1952) and has subsequently been reported to have a broad effect on several other microsporidian species in insects (Lewis and Lynch 1970, Wilson 1974, Armstrong 1976). In vertebrates fumagillin has inhibited development of *Pleistophora anguillarum* in eels, *Anguilla japonica* (Kano and Fukui 1982), prevented multiplication of *E. cuniculi in vitro* (Shadduck 1980) and was associated with marked improvement of keratoconjunctivitis caused by *E. hellem* when applied topically to the eye (Diesenhouse et al. 1993, Rosberger et al. 1993). Other drugs which have been reported to have antimicrosporidial activity, are benomyl (Hsiao and Hsiao 1973), toltrazuril (Mehlhorn et al. 1988, Schmahl and Mehlhorn 1989), buquinolate (Overstreet 1975), itraconazole (Liu and Myric 1989) and albendazole (Haque et al. 1993). However, few of the drugs used in invertebrates and lower vertebrates are licensed for use in human medicine.

Metronidazole (Eeftinck Schattenkerk et al. 1991), itraconazole (Yee et al. 1991), azithromycin (Hing et al. 1993), brolene (dibromopropamidine isethionate) (Metcalfe et al. 1992) and albendazole (Blanshard et al. 1992, Lacey et al. 1992, Dieterich et al. 1993, Eeftinck Schattenkerk et al. 1993, Orenstein et al. 1993) have been used against human microsporidian infections. Of these, albendazole is the most promising. It has been reported to achieve clinical improvement of the diarrhoea caused by *Enterocytozoon bieneusi* (Blanshard et al. 1992, Dieterich et al. 1993), without elimination of the parasite, and is highly effective against species of *Encephalitozoon* including *E. (S.) intestinalis* (Lacey et al. 1992, Eeftinck Schattenkerk et al. 1993, Orenstein et al. 1993).

Insolubility of albendazole is one of the major problems that needs to be overcome when *in vitro* drug tests are performed and it is essential to use an organic solvent before dilution in water. DMSO was used to dissolve the drug in the present study and was used at the same concentration without the drug as a control for its effect on host cells. No effects were detected of DMSO on MDCK cells, or on the parasite, except an initial retardation of growth. This was followed by a steady increase in the percentage of infected cells and both the parasites and the cells appeared normal by electron microscopy. Similar non* interference of DMSO on stages of *E. cuniculi* in MDCK cells and on *N. bombycis* in *S. frugiperda* cells have been reported (Haque et al. 1993, Colbourn et al. 1994).

In the present study some deleterious effects on MDCK cells were observed when they were cultured in the presence of albendazole. The cells looked larger and more vacuolated when stained with Giemsa but, surprisingly, no ultrastructural differences were detected. The effect on the intracellular parasites was dramatic.

Albendazole and other benzimidazoles are broad-spectrum anthelmintics that bind to tubulin preventing the self-association of subunits into growing microtubules (Lacey 1990). It appeared that its mode of action against *V. corneae* was to prevent formation of the intranuclear spindle and resulted in the inhibition of nuclear and consequently of cell division and enlargement of merogonic stages. However, large the parasites grew, there was never more than a single diplokaryon present. In merogony, two or more diplokarya were common in untreated parasites. The initiation of sporogony was not totally prevented by the drug, since incomplete deposition of electron dense material on the plasma membrane was observed. However, the cell structure was highly disorganised.

All cultures, with and without DMSO and albendazole, had penicillin and streptomycin as additives. These antibiotics do not interfere with the growth and replication of microsporidia and are routinely used in cultures to ensure sterility. It is unlikely that the antibiotics would enhance or diminish the effect of albendazole and it was considered that the inhibition of nuclear division and gross enlargement of stages was due to albendazole, especially in view of its known effect on microtubule assembly.

Prevention of nuclear division by albendazole has also been observed in *N. bombycis* (Haque et al. 1993) and *E. cuniculi* (Colbourn et al. 1994). The appearance

of the nuclei in *N. bombycis* differed in that there were aggregates of chromatin on one side of each nucleus, whereas in the other species the chromatin was not condensed.

In *E. cuniculi* it appeared that there was an additional effect of the drug, unrelated to tubulin assembly. There were bundles of 35 nm diameter tubules containing electron dense material resembling the material deposited as surface coat (Colbourn et al. 1994). These authors suggested that the tubules might represent a disruption in the normal mechanism for deposition of the sporont surface coat. The small vesicles in the meronts and sporonts of *V. corneae* also contained electron dense material and may also have been evidence of the disruption of this process.

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REFERENCES

ARMSTRONG E. 1976: Fumidil B and benomyl: chemical control of *Nosema kingi* in *Drosophila willistoni*. J. Invertebr. Pathol. 27: 363–366.

BEDRNÍK P., VÁVRA J. 1972: Further observations on the maintenance of *Encephalitozoon cuniculi* in tissue culture. J. Protozool. 19 (Suppl.): 75.

BLANSHARD C., ELLIS D. S., TOVEY D. G., DOWELL S., GAZZARD B. G. 1992: Treatment of intestinal microsporidiosis with albendazole in patients with AIDS. AIDS 6: 311–313.

COLBOURN N. I., HOLLISTER W. S., CURRY A., CANNING E. U. 1994: Activity of albendazole against *Encephalitozoon cuniculi in vitro*. Eur. J. Protistol. 30: 211–220.

DAVIS R. M., FONT R. L., KEISLER M. S., SHADDUCK J. A. 1990: Corneal microsporidiosis. A case report including ultrastructural observations. Ophthalmology 97: 953–957.

DE GROOTE M. A., VISVESVARA G., WILSON M. I., PIENIAZEK N. J., SLEMENDA S. B., DA SILVA A. J., LEITCH G., BRYAN R. T., REVES R. 1995: Polymerase chain reaction and culture confirmation of disseminated *Encephalitozoon cuniculi* in patients with AIDS: successful therapy with albendazole. J. Infect. Dis. 171: 1375–1378.

DESSER S. S., HONG H., YANG Y. J. 1992: Ultrastructure of the development of a species of *Encephalitozoon* cultured from the eye of an AIDS patient. Parasitol. Res. 78: 677–683.

DIDIER E. S., DIDIER P. J., FRIEDBERG D. N., STENSON S. M., ORENSTEIN J. M., YEE R. W., TIO F. O., DAVIS R. M., VOSSBRINCK C., MILLICHAMP N., SHADDUCK J. A. 1991: Isolation and characterization of a new human microsporidian, *Encephalitozoon hellem* (n.sp.), from three AIDS patients with keratoconjunctivitis. J. Infect. Dis. 163: 617–621.

DIESENHOUSE M. C., WILSON L. A., CORRENT G. C., VISVESVARA G. S., GROSSNIKLAUS H. E., BRYAN R. T. 1993: Treatment of microsporidial keratoconjunctivitis with topical fumagillin. Am. J. Ophthalmol. 115: 293–298.

DIETERICH D. T., LEW E. A., KOTLER D. P., POLES M. A., ORENSTEIN J. M. 1993: Divergence between clinical and histologic responses during treatment for *Enterocytozoon bieneusi* infection. AIDS 7 (suppl. 3): S43–44.

EEFTINCK SCHATTENKERK J. K. M., VAN GOOL T., VAN DEN BERGHWEERMAN M. A., SCHOT L. J., DANKERT J. 1993: Chronic rhinosinusitis in HIV-infected patients with microsporidiosis. AIDS 7 (suppl. 3): S36–37.

EEFTINCK SCHATTENKERK J. K. M., VAN GOOL. T., VAN KETEL R. J., BARTELSMAN J. F. W. M,. KUIKEN C. L., TERPSTRA W. J., REISS P. 1991: Clinical significance of small-intestinal microsporidiosis in HIV-1-infected individuals. Lancet 337: 895–898.

HAQUE A., HOLLISTER W. S., WILLCOX A., CANNING E. U. 1993: The antimicrosporidial activity of albendazole. J. Invertebr. Pathol. 62: 171–177.

HARTSKEERL R. A., VAN GOOL T., SCHUITEMA A. R. J., DIDIER E. S., TERPSTRA W. J. 1995: Genetic and immunological characterization of the microsporidian *Septata intestinalis* Cali, Kotler and Orenstein, 1993: reclassification to *Encephalitozoon intestinalis*. Parasitology 110: 277–285.

HING M. C., MARRIOT D. J., VERRE J., FIELD A. S. 1993: Enteric microsporidiosis: response to azithromycin therapy. AIDS, 7 (suppl. 3): S45–46.

HOLLISTER W. S., CANNING E. U., COLBOURN N. I. 1993a: A species of *Encephalitozoon* isolated from an AIDS patient: criteria for species differentiation. Folia Parasitol. 40: 293–295.

HOLLISTER W. S., CANNING E. U., COLBOURN N. I., AARONS E. J. 1995: *Encephalitozoon cuniculi* isolated from the urine of an AIDS patient, which differs from canine and murine isolates. J. Euk. Microbiol. 42: 367–372.

HOLLISTER W. S., CANNING E. U., COLBOURN N. I., CURRY A., LACEY C. J. N. 1993b: Characterisation of *Encephalitozoon hellem* (Microspora) isolated from the nasal mucosa of a patient with AIDS. Parasitology 107: 351–358.

HSIAO T. H., HSIAO C. 1973: Benomyl: a novel drug for controlling a microsporidan disease of the alfalfa weevil. J. Invertebr. Pathol. 22: 303–304.

ISHIHARA R. 1968: Growth of *Nosema bombycis* in primary cell cultures of mammalian and chicken embryos. J. Invertebr. Pathol. 11: 328–329.

KANO T., FUKUI H. 1982: Studies on *Pleistophora* infection in eel, *Anguilla japonica*. I. Experimental induction of microsporidiosis and fumagillin efficacy. Fish Pathol. 16: 193–200.

KATZNELSON H., JAMIESON C. A. 1952: Control *Nosema* disease of honeybees with fumagillin. Science 115: 70–71.

KAWARABATA T., ISHIHARA R. 1984: Infection and development of *Nosema bombycis* (Microsporida: Protozoa) in a cell line of *Antheraea eucalypti*. J. Invertebr. Pathol. 44: 52–62.

KURTTI T. J., BROOKS M. A. 1977: The rate of development of a microsporidan in moth cell culture. J. Invertebr. Pathol. 29: 126–132.

LACEY C. J. N., CLARKE A. M.T., FRASER P., METCALFE T., BONSOR G., CURRY A. 1992: Chronic microsporidian infection of the nasal mucosae, sinuses and conjunctivae in HIV disease. Genitourin. Med. 68: 179–181.

LACEY E. 1990: Mode of action of benzimidazoles. Parasitol. Today 6: 112–115.

LEWIS L. C., LYNCH R. E. 1970: Treatment of *Ostrinia nubilalis* larvae with fumidil B to control infections caused by *Perezia pyraustae*. J. Invertebr. Pathol. 15: 43–48.

LIU T. P., MYRICK G. R. 1989: Deformities in the spore of *Nosema apis* as induced by itraconazole. Parasitol. Res. 75: 498–502.

MEHLHORN H., SCHMAHL G., HABERKORN A. 1988: Toltrazuril effective against a broad spectrum of protozoan parasites. Parasitol Res. 75: 64–66.

METCALFE T., DORAN R., ROWLAND P., CURRY A., LACEY C. J. N. 1992: Microsporidial keratoconjunctivitis in a British patient with AIDS. Br. J. Ophthalmol. 76: 177–178.

MONTREY R. D., SHADDUCK J. A., PAKES S. P. 1973: *In vitro* study of host range of three isolates of *Encephalitozoon* (*Nosema*). J. Infect. Dis. 127: 450–454.

ORENSTEIN J. M., DIETERICH D. T., KOTLER D. P. 1993: Albendazole as a treatment for intestinal and disseminated microsporidiosis due to *Septata intestinalis* in AIDS patients: a report of four patients. AIDS 7 (suppl. 3): S40–42.

OVERSTREET R. M. 1975: Buquinolate as a preventive drug to control microsporidiosis in the blue crab. J. Invertebr. Pathol. 26: 213–216.

ROSBERGER D. F., SERDAREVIC O. N., ERLANDSON R. A., BRYAN R. T., SCHWARTZ D. A., VISVESVARA G. S., KEENAN P. C 1993: Successful treatment of microsporidia keratoconjunctivitis with topical fumagillin in a patient with AIDS. Cornea 12: 261–265.

SCHMAHL G., MEHLHORN H. 1989: Treatment of fish parasites. 6. Effects of Sym. Triazinone (Toltrazuril) on development stages of *Glugea anomala* Moniez, 1887 (Microsporidia): a light and electron microscopic study. Eur. J. Protistol. 24: 252–259.

SHADDUCK J. A. 1969: *Nosema cuniculi*: *in vitro* isolation. Science 166: 516–517.

SHADDUCK J. A. 1980: Effect of fumagillin on *in vitro* multiplication of *Encephalitozoon cuniculi*. J. Protozool. 27: 202–208.

SHADDUCK J. A., MECCOLI R. A., DAVIS R., FONT R. L. 1990: Isolation of a microsporidian from a human patient. J. Infect. Dis. 162: 773–776.

SHADDUCK J. A., POLLEY M. B. 1978: Some factors influencing the *in vitro* infectivity and replication of *Encephalitozoon cuniculi*. J. Protozool. 25: 491–496.

SILVEIRA H., CANNING E. U. 1995: *Vittaforma corneae* n. comb. for the human microsporidian *Nosema corneum* Shadduck, Meccoli, Davis et Font, 1990 based on its ultrastructure in the liver of experimentally infected athymic mice. J. Euk. Microbiol. 42: 158–165.

SMITH J. E., BARKER R. J., LAI P. F. 1982: Culture of microsporidia from invertebrates in vertebrate cells. Parasitology 85: 427–435.

UNDEEN A. H. 1975: Growth of *Nosema algerae* in pig kidney cell cultures. J. Protozool. 22: 107–110.

UNDEEN A. H., ALGER N. E. 1976: *Nosema algerae*: infection of the white mouse by a mosquito parasite. Exp. Parasitol. 40: 86–88.

VAN GOOL T., CANNING E. U., GILIS H., VAN DEN BERGH WEERMAN M. A., EEFTINCK SCHATTENKERK J. K. M., DANKERT J. 1994: *Septata intestinalis* frequentely isolated from stool of AIDS patients with a new cultivation method. Parasitology 109: 281–289.

VÁVRA J., BEDRNÍK P., ČINÁTL J. 1972: Isolation and *in vitro* cultivation of the mammalian microsporidian *Encephalitozoon cuniculi*. Folia Parasitol. 19: 349–354.

VISVESVARA G. S., LEITCH G. J., MOURA H., WALLACE S., WEBER R., BRYAN R. T. 1991: Culture, electron microscopy, and immunoblot studies on a microsporidian parasite isolated from the urine of a patient with AIDS. J. Protozool. 38: 105s–111s.

WALLER T. 1975: Growth of *Nosema cuniculi* in established cell lines. Lab. Anim. 9: 61–68.

WEISSENBERG R. 1976: Microsporidia interaction with host cells. In: L. A. Bulla and T. C. Cheng (Eds.), Comparative Pathobiology. Biology of the Microsporidia, Vol. 1. Plenum, New York, pp. 239–245.

WILSON G. G. 1974: The use of fumidil B to suppress the microsporidan *Nosema fumiferanae* in stock cultures of the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). Can. Entomol. 106: 995–996.

XIE W.-D. 1988: Propagation of *Cystosporogenes operophterae* (Microspora), a microsporidian parasite of the winter moth, *Operophtera brumata*, in a *Spodoptera frugiperda* cell line. Parasitology 97: 229–237.

YEE R. W., TIO F. O., MARTINEZ J. A., HELD K. S., SHADDUCK J. A., DIDIER E. S. 1991: Resolution of microsporidial epithelial keratopathy in a patient with AIDS. Ophthalmology 98: 196–201.

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