

The early events of *Brachiola algerae* (Microsporidia) infection: spore germination, sporoplasm structure, and development within host cells

Peter M. Takvorian^{1,2}, Louis M. Weiss^{2,3} and Ann Cali¹

¹Department of Biologic Sciences, Rutgers University, Newark, New Jersey 07102, USA;

²Department of Pathology, ³ Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461, USA

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Abstract. *Brachiola algerae* (Vavra et Undeen, 1970) Lowman, Takvorian et Cali, 2000, originally isolated from a mosquito, has been maintained in rabbit kidney cells at 29°C in our laboratory. This culture system has made it possible to study detailed aspects of its development, including spore activation, polar tube extrusion, and the transfer of the infective sporoplasm. Employing techniques to ultrastructurally process and observe parasite activity *in situ* without disturbance of the cultures has provided details of the early developmental activities of *B. algerae* during timed intervals ranging from 5 min to 48 h. Activated and non-activated spores could be differentiated by morphological changes including the position and arrangement of the polar filament and its internal structure. The majority of spores extruded polar tubes and associated sporoplasms within 5 min post inoculation (p.i.). The multilayered interlaced network (MIN) was present in extracellular sporoplasms and appeared morphologically similar to those observed in germination buffer. Sporoplasms, observed inside host cells were ovoid, contained diplokaryotic nuclei, vesicles reminiscent of the MIN remnants, and their plasmalemma was already electron-dense with the “blister-like” structures, typical of *B. algerae*. By 15 min p.i., the first indication of parasite cell commitment to division was the presence of chromatin condensation within the diplokaryotic nuclei, cytoplasmic vesicular remnants of the MIN were still present in some parasites, and early signs of appendage formation were present. At 30 min p.i., cell division was observed, appendages became more apparent, and some MIN remnants were still present. By two hours p.i., the appendages became more elaborate and branching, and often connected parasite cells to each other. In addition to multiplication of the organisms, changes in parasite morphology from small oval cells to larger elongated “more typical” parasite cells were observed from 5 h through 36 h p.i. Multiplication of proliferative organisms continued and sporogony was well underway by 48 h p.i., producing sporonts and sporoblasts, but not spores. The observation of early or new infections in cell cultures 12–48 h p.i., suggests that there may also exist a population of spores that do not immediately discharge, but remain viable for some period of time. In addition, phagocytized spores were observed with extruded polar tubes in both the host cytoplasm and the extracellular space, suggesting another means of sporoplasm survival. Finally, extracellular discharged sporoplasms tightly abutted to the host plasmalemma, appeared to be in the process of being incorporated into the host cytoplasm by phagocytosis and/or endocytosis. These observations support the possibility of additional methods of microsporidian entry into host cells and will be discussed.

Microsporidian infections have been identified and studied in economically important fish and insects for over 150 years (Wittner 1999). Recently their identification as emerging pathogens of humans has renewed and intensified interest in these parasites. During the last twenty years, organisms from several microsporidian genera have been identified as being capable of infecting humans, especially individuals with compromised immune systems (Wittner 1999, Cali et al. 2003, 2004, Coyle et al. 2004). Immunocompetent hosts may also unknowingly harbour subclinical infections.

The microsporidian life cycle can be separated into three phases; proliferative, sporogonic, and infective (Cali and Takvorian 1999). Proliferation occurs within the host cell cytoplasm after introduction of the sporoplasm, following proliferation, they undergo sporogony, terminating in the formation of resistant spores. Each spore contains a coiled polar filament, its attachment apparatus and a sporoplasm. Spores are environmentally

resistant and may transmit infection to the next host. Once inside this host, spores are triggered to evert their coiled polar filament, extruded with sufficient force to penetrate a nearby host cell. This usually results in the transmission of the parasite’s infective sporoplasm into a host cell initiating a new cycle of infection.

Much attention has been devoted to the study of the unique infective process of the microsporidia. In particular, research has focused on several important aspects of germination: polar filament extrusion, the external and internal factors related to the triggering of the polar filament, the composition of the polar tube before and after germination and the biophysics of extrusion (reviewed in Keohane and Weiss 1999).

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Address for correspondence: P.M. Takvorian, Department of Biologic Sciences, 101 Warren St., Smith Hall, Rutgers University, Newark, NJ 07102, USA. Phone: ++1 973 353 5364; Fax: ++1 973 353 1007; E-mail: petetak@andromeda.rutgers.edu

In vitro studies of spore germination indicate that a variety of external factors may trigger activation of the extrusion apparatus. Many species will undergo germination when the external environmental pH is shifted, some require the addition of salts, and others require calcium in the extrusion process (Leitch et al. 1993, He et al. 1996, Keohane and Weiss 1999, Undeen and Vander Meer 1999). Undeen and Frixione (1990), studying *B. algerae* spores, proposed that an increase in metabolites, from a conversion of trehalose to glucose, within the spore provides for the creation of a massive osmotic gradient, resulting in the influx of water into the spore, followed by the rupturing of the apical polar cap, and extrusion of the coiled polar filament. Frixione et al. (1997) suggested the possibility that aquaporin proteins located in the plasma membrane may facilitate the influx of water into the spore.

Due to its relatively large spore size, ease of cultivation, and ability to be activated, *B. algerae* has been extensively used for extrusion studies (Keohane and Weiss 1999). A recent study of *B. algerae* spores (Cali et al. 2002) provides details of the changes of the internal spore morphology, polar tube location in association with membranes, translocation, eversion, and the release of the sporoplasm during the activation process. Additionally, a number of *in vivo* experimental studies of the infective process of *B. algerae*, in a variety of host organisms, support the *in vitro* observations (Vavra and Undeen 1970, Canning and Sinden 1973, Undeen and Alger 1976, Avery and Anthony 1983, Trammer et al. 1999, Koudela et al. 2001).

Since *B. algerae* has the ability to infect and successfully develop in a variety of cell culture systems, a number of studies have been conducted on its growth in culture (Undeen 1975, Moura et al. 1999, Trammer et al. 1999, Lowman et al. 2000). At least two of these studies have described development of *B. algerae* during a timed progression from initial infection through sporogony: one, using its mosquito host (Avery and Anthony 1983), and another using cell culture at varying temperatures (Lowman et al. 2000). These studies provided information about the sequence of infection and development during various times p.i., starting at one hour in the mosquito and two hours in cell culture.

In light of the recent report of *B. algerae* as the cause of human myositis (Coyle et al. 2004), we believed it important to more closely re-examine the infection process of this pathogen. The following report documents events from the activation of the spore, to sporoplasm transfer, and initiation of infection starting 5 min p.i. in a cell culture system that provides for the subsequent *in situ* observations of the events that follow over a 48-h period, including delayed infection and alternative modes of infection.

MATERIALS AND METHODS

Cell culture. Rabbit kidney (RK 13) cells (American Type Culture Collection) were maintained in a culture medium of Minimal Essential Media (Gibco) containing 7% foetal calf serum (Hyclone), and 1% penicillin/streptomycin/amphotericin B (Invitrogen) and grown at 29°C in 25-cm² cell culture flasks.

Spore propagation. The *Brachiola algerae* (Vavra et Undeen, 1970), Lowman, Takvorian et Cali, 2000 organism used in these experiments was provided by Dr. A. Undeen from his original mosquito (*Anopheles stephensi*) isolate material, and will be referred to as the *B. algerae* reference isolate (RBA) to distinguish it from other *B. algerae* isolates. RBA spores were propagated in RK 13 cell culture as previously described (Lowman et al. 2000). Briefly, spores were collected from infected cell culture media weekly. Collected culture medium was centrifuged at 10,000 g for 5 min, the supernatant was removed, and the pelleted spores were resuspended in sterile distilled water and stored at 4°C.

Inoculation of RK 13 cells with *Brachiola algerae*. Cells were grown to 80–90% confluence at 29°C in 50-mm containers, using the previously described culture medium. The culture medium was removed and 100 µl of 1×10^7 /ml *B. algerae* spores were added to each of the cell cultures and incubated for 5 min. After this inoculation, the medium was replaced with fresh culture medium as needed. Fourteen cultures were designated 5, 15, 30, 45 min and 1–6, 12, 18, 24, 48 h p.i. for the period of time that each was allowed to develop before the removal of medium and the addition of fixative to the cultures.

Transmission electron microscopy. The cultures were fixed with 0.1 M cacodylate buffered 2.5% glutaraldehyde, post-fixed in buffered 1% osmium tetroxide, stained *en-bloc* with uranyl acetate, dehydrated in graded ethanols, and embedded *in situ*, in Epon, to minimize repositioning of the culture contents. Thin sections were stained with uranyl acetate and lead citrate, and then observed and photographed with a Phillips/FEI Tecnai 12 transmission electron microscope at the Rutgers Newark Electron Microscopy Facility.

RESULTS

Five minutes post inoculation

Examination of the cultures by electron microscopy indicated that by 5 min p.i., several extracellular areas contained spores which appeared as typical inactive *B. algerae* spores containing a single row of polar filament coil cross-sections, a centrally located diplokaryon, and an anterior anchoring disc connected to the straight portion of the polar filament. In addition, a number of spores had already discharged their polar tubes and appeared as empty spore cases. Other spores were in an early phase of activation, indicated by the translocation of the polar filament coil from a single row into multiple rows (Fig. 1). The reposition of the polar filament was accompanied by the presence of open space surrounded

by extensive membrane complexes in the immediate vicinity of the filament cross-sections. In addition, a structural change of the internal organisation of the polar filament from a series of concentric rings of dense and light material, to a single dense outer ring that encompassed several cylinder-like structures, each separated by dense material, became apparent during activation. The alternating cylinders and dense material formed a ring around a central dense core, and resembled a sprocket (Fig. 1). During the early phase of activation, the anterior of the spore may elongate but not rupture. In more advanced spore activation (Fig. 2), the polar filament was often observed in various stages of exiting the anterior spore eversion complex or aperture. The exiting filament passed through a medium-dense collar-like anterior aperture structure. The open spaces inside the spore increased, as the membrane repositioning around the filament coil became more pronounced. When the membranous and tubular polaroplast diminished, the diplokaryon became repositioned to a more posterior part of the spore (Fig. 2). The number of filament coil cross-sections and the quantity of sporoplasm inside the spore vary with the state of germination. In some spores, membranes, membrane channels, and dense material remain inside the discharged spore coat, which may still have its everted polar tube attached at the anterior aperture (Fig. 3).

In addition to numerous inactive spores, activated spores in various stages of discharge, and portions of polar tubes, the extracellular space also contained discharged sporoplasms. The ovoid or spherical sporoplasm cells were approximately 1.5–2.0 μm in diameter (Fig. 4). Its outer limiting membrane was a typical “thin” plasmalemma enclosing the cytoplasm that contained a more-dense vesicular network identified as the multilayered interlaced network (MIN). This MIN is located just below the plasmalemma and is usually associated with the polar tube (Fig. 5). Some of these extracellular sporoplasms were in very close proximity to the host cell plasmalemma (Fig. 6), and some appeared to be in the process of being phagocytized by the RK 13 cells (Fig. 7). In addition, some host cells had portions of polar tubes protruding from their plasmalemma (Fig.

8), others had polar tubes embedded in their cytoplasm (Fig. 9), and in a few cells, the polar tubes were observed in the cytoplasm in the immediate vicinity of infective intracellular sporoplasms (Fig. 10). These intracellular sporoplasms were embedded directly in the host cell cytoplasm and their plasmalemma was thicker, denser, and covered with numerous short vesicular appendages producing a blister-like appearance (Fig. 11). The MIN was no longer present, but some vesicular remnants of it were observed in the sporoplasm (Fig. 12) and the diplokarya inside the sporoplasms were usually difficult to see (Fig. 11). The most significant change observed in 15 min p.i. samples was condensation of heterochromatin in some sporoplasms.

Thirty minutes post inoculation

By 30 min p.i., some of the intracellular organisms had divided and started to produce appendages that extended deeper into the host cytoplasm. Their vesiculotubular surface coat was more prominent, and only scant remnants of the MIN were present as vesicles inside their cytoplasm (Fig. 13). These features indicated a transition of the sporoplasm to the proliferative form of development, confirmed by observation of the first division of the parasite (Fig. 14). Additionally, phagocytosis of extracellular spores by the RK 13 cells was now evident (Fig. 15).

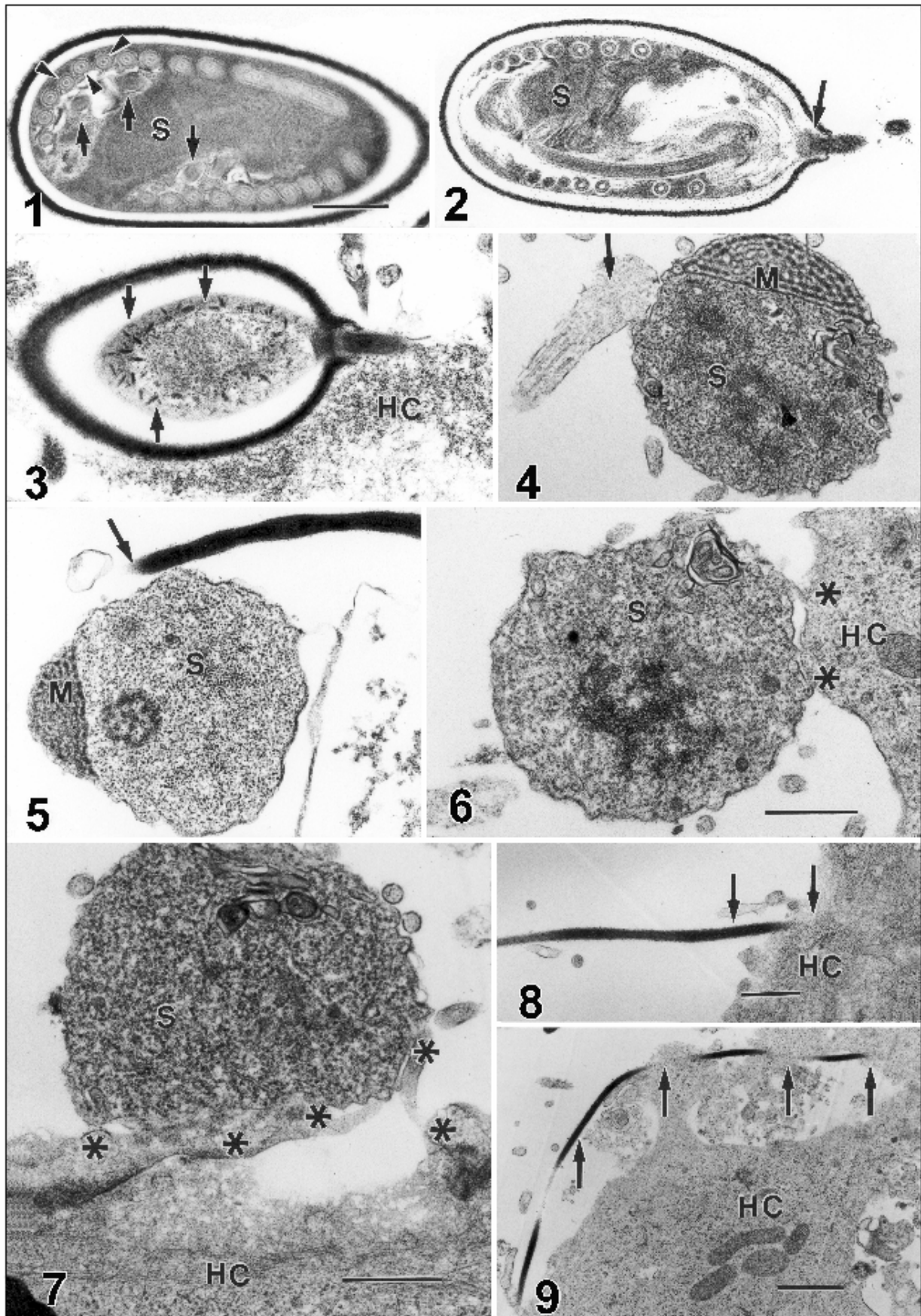
Two hours post inoculation

The early proliferative forms, in 2 h p.i. cultures, continued to divide and produced more extensive appendages. Some of these appendages appeared to remain attached to the recently divided proliferative organisms (Fig. 16). Examination of the RK 13 cells that phagocytized spores in the cultures, revealed the presence of intact spores, partially and fully discharged spores, and portions of polar tubes in both the host cell cytoplasm and extracellular space (Fig. 17).

Three hours post inoculation

The most notable change was a continued increase in complexity and elongation of the parasite appendages (Fig. 18). Similar developmental features were noted during hour 4.

Figs. 1–9. Electron micrographs of *Brachiola algerae* spores, extruded polar tubes, and sporoplasms, in the extracellular space. **Fig. 1.** An activated spore whose polar filament has started the process of translocation. The cross-sections of the filament have moved from a single uniform row, to pairs or small clusters (arrows) that are in membrane-bounded spaces. The organisation of the filament's internal structure is now a series of lucent cylinders interspersed with dense particulate material arranged around a central dense core (arrowheads). The sporoplasm (S) is surrounded by the filament coils. **Fig. 2.** An activated spore still containing some portion of its coiled polar filament while the rest of the filament is in the process of extruding. A portion of the polar tube has passed through the apical aperture (arrow) and exited the spore. Note the increase of membranes in the spore and the change of the polar filament core from solid to hollow. The sporoplasm (S) has not exited the spore at this time. **Fig. 3.** An activated spore that still is attached to a fully extruded polar tube. The remnants of membranes, and channels (arrows), still remain in the empty spore. Note the spore is tightly abutted to the cell cytoplasm and the polar tube has entered the host cell (HC). **Fig. 4.** An extruded sporoplasm (S) containing a well-defined MIN (M) with a portion of the polar tube (arrow) still attached. **Fig. 5.** A sporoplasm (S) containing a prominent MIN (M) has a portion of polar tube (arrow) remaining in close proximity to it in the extracellular space. Note the surface of the sporoplasm is limited by a thin plasmalemma. **Fig. 6.** A sporoplasm (S) tightly abutted to a host cell (HC) plasmalemma (*) in at least two places. **Fig. 7.** A sporoplasm (S) abutted to a host cell (HC). The host cell protrusions (*) appear to be in the process of phagocytizing the sporoplasm. **Fig. 8.** A portion of extruded polar tube (arrow) has pierced the host cell (HC) cytoplasm. **Fig. 9.** A curved polar tube pierces the host cytoplasm in several places (arrows). Scale bars: Figs. 1–5 (same magnification), 6–8 = 0.5 μm ; Fig. 9 = 1.0 μm .



Five hours post inoculation

By 5 h p.i. the developing stages became more elongated, produced very elaborate vesiculotubular appendages, and appeared more like “typical” proliferative cells (Fig. 19).

Eighteen hours post inoculation

No major morphological changes were observed until 18 h, when the elongated parasite cells appeared to contain more endoplasmic reticulum around the diplokaryon, and the cell surface was heavily ornamented, all features typical of *B. algerae* proliferative phase morphology (Fig. 20). Parasite cell nuclei containing condensed chromatin and spindle plaques, indicative of proliferation, were also more commonly observed in these samples (Fig. 21).

Twenty-four hours post inoculation

During this next six-hour interval, some of the proliferative cell surfaces became more uniformly dense and smooth. The appendages and blister-like surface ornamentation was no longer present on areas of the plasmalemma, but some “ornamentation” material was observed in the host cytoplasm (Fig. 22). Examination of 36 h p.i. samples indicated that little if any notable changes in parasite morphology had occurred.

Forty-eight hours post inoculation

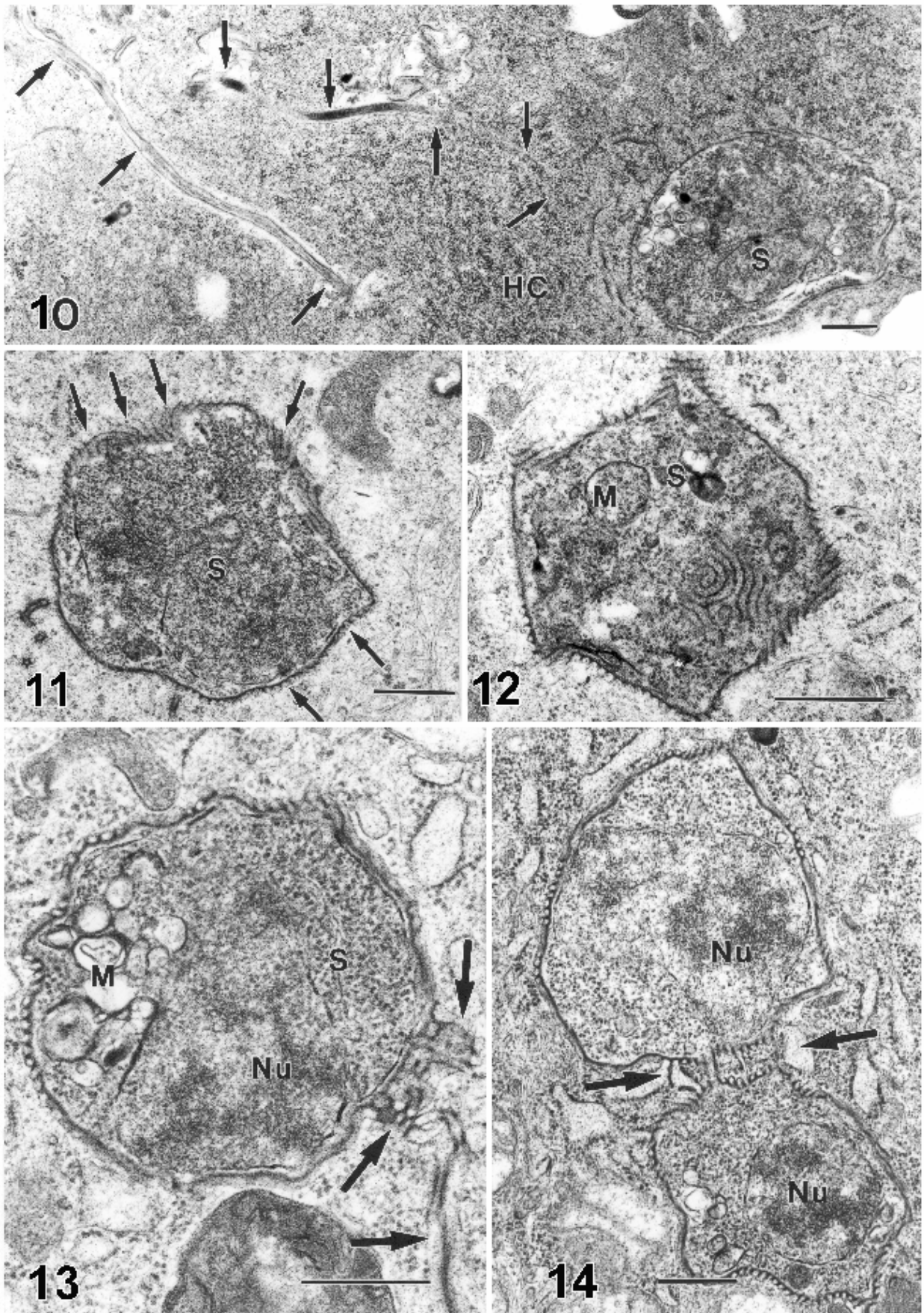
A number of sporonts and sporoblasts were present in the host cell cytoplasm by 48 h p.i. The predominant cell forms were in the sporogonic phase. Sporonts were identified by their condensed size, dense cytoplasm and uniformly thick surface coat devoid of most ornamentation and appendages. A clear space (possibly a fixation artifact) around the sporont gave one the impression of development inside a cytoplasmic vacuole (Figs. 23, 24). Sporoblasts were more condensed, had a very dense cytoplasm, were irregular in shape, and usually contained varied stages of developing polar filaments. Both sporonts and sporoblasts were surrounded by quantities of dense material and remnants of the vesiculotubular appendages scattered inside the host cytoplasm (Fig. 24). While no spores were observed in any of the 48-h material, several proliferative stages were intermixed with the sporonts and sporoblasts, indicating the asynchronous developmental nature of *B. algerae*.

DISCUSSION

The sequential *in situ* study of *Brachiola algerae* in RK 13 cells provided information as to the period of time post inoculation that was necessary to establish infection in a host cell, the time necessary for the onset of various stages of development, and to visualise the means of infective sporoplasm entry into host cells. Additionally, since the infection did not produce spores in the time sequence studied, new infections several hours p.i. can only be attributed to delayed spore germination, an important finding, which will be discussed.

The inactivate RBa spores possess a thick electron-dense exospore coat over a wider electron-lucent endospore which encloses the spore cytoplasm. The spores contained 8–11 polar filament coils arranged in a single row surrounding the diplokaryon. This is typical of the microsporidia and has been observed and described in many spores of various genera (Vávra and Larsson 1999). These features are also consistent with the numerous *B. algerae* spore descriptions in the literature (Vávra and Undeen 1970, Canning and Sinden 1973, Avery and Anthony 1983, Cali et al. 2002). The initial spore activation was indicated by a series of subtle morphological changes to the contents of the spore, which continue into very obvious internal changes. The polar filament of activated spores translocated from a single row into a second row or more commonly into a group or cluster of polar filament coil cross-sections. Repositioning of the filament was accompanied by the formation of electron-lucent “open” spaces surrounded by layers of membrane. In addition to the movement of the filament, a structural change of the internal organisation of the filament also became obvious during activation. Cross-sectional views of non-activated filaments revealed a series of alternating concentric rings of dense and light material surrounding a central dense core. During activation, the outer ring of the filament encloses 14–16 electron-lucent cylinder-like structures, each separated by dense particulate material which projects from the dense central core toward the periphery of the filament. These observations of polar filament translocation, membrane-bounded spaces around the filament, and changes to its internal structure, are consistent with a recent detailed study of *B. algerae* spores activated in germination buffer (Cali et al. 2002).

Figs. 10–14. The intracellular development of *Brachiola algerae* in RK 13 cells from 5 through 30 minutes, post inoculation (p.i.). **Fig. 10.** Portions of polar tubes (arrows) embedded in the host cell (HC) cytoplasm. A polar tube ending in the vicinity of a sporoplasm (S) inside the host cell, 5 min p.i. **Fig. 11.** A sporoplasm (S) in the host cell cytoplasm, 5 min p.i. The sporoplasm has a thickened plasmalemma decorated with vesiculotubular appendages and blister-like structures (arrows). The parasite is in direct contact with the host cell cytoplasm, no vacuole or space separates them. **Fig. 12.** Five min p.i., a sporoplasm (S) whose surface is almost completely covered by the blister-like appendages, observed on proliferative forms. The cytoplasm contains some vesicles that are remnants of the MIN (M). **Fig. 13.** A proliferative form/sporoplasm (S), 30 min p.i., with appendages (arrows) extending away from the parasite and into the host cytoplasm. There are several MIN remnants (M) and an obvious nucleus (Nu). **Fig. 14.** Two early proliferative cells, which appear to have divided, are still attached by shared appendages (arrows). This division clearly demonstrates the transition of the parasite from an invasive sporoplasm stage to a proliferative cell, by 30 min p.i. Scale bars = 0.5 μ m.



Late stages of spore activation are much more obvious and are usually indicated by the presence of an anterior aperture through which the polar filament exits the spore, becoming the hollow polar tube. Spores extruding their polar tube, spores containing some cytoplasmic material but no polar tube, and completely empty spores, were observed in virtually every sample examined ranging from 5 min to 48 h p.i. The largest number of extruding spores was observed in the earlier time sequences, ranging from 5 min through 3 h p.i. Avery and Anthony (1983) reported the presence of *B. algerae* germinated spores and discharged sporoplasms in the midgut of *Anopheles albimanus* larvae, one hour after inoculation of spores. Lowman et al. (2000) identified *B. algerae* sporoplasms in RK 13 cells 2 h p.i. at 29°C. A recent study (Cali et al. 2002) indicated that some *B. algerae* spores became activated and discharged their sporoplasms 30 s after placement in germination buffer and that 70% of the spores germinated within 5 min in this buffer.

In the present study, sporoplasms were observed inside host cells 5 min p.i. The intracellular sporoplasms appeared as ovoid or spherical cells that were approximately 1.5–2.0 µm in diameter and contained a diplokaryon, and whorled vesicular structures that were probably remnants of the multilayered interlaced network (MIN), which was described in detail by Cali et al. (2002). The sporoplasms had a blister-like ornamentation protruding from their limiting membrane and were in direct contact with the host cell cytoplasm. As the proliferative organisms continued their development over time, their surface appendages became more complex and extended into the host cytoplasm. Some sporoplasms observed 30 min p.i., appeared to have just divided. After cytoplasmic division, their appendages were sometimes still attached. The division of sporoplasms indicated the parasite's transition from an initial infective to proliferative form, in less than 30 min.

By 5 min p.i., a number of host cells had portions of polar tubes protruding from their cytoplasm. In some cells, as demonstrated by Fig. 10, portions of the polar tube could be observed inside the cytoplasm and ending in the vicinity of a sporoplasm. The traditional hypothesis of how microsporidia infect their host cell (reviewed in Wittner 1999) is sporoplasm transfer via the extruded polar tube from spores outside the host cell depositing sporoplasms into the host cell, as suggested by our Figs. 8–10. A few reports (Couzinet et al. 2000, Nasonova et

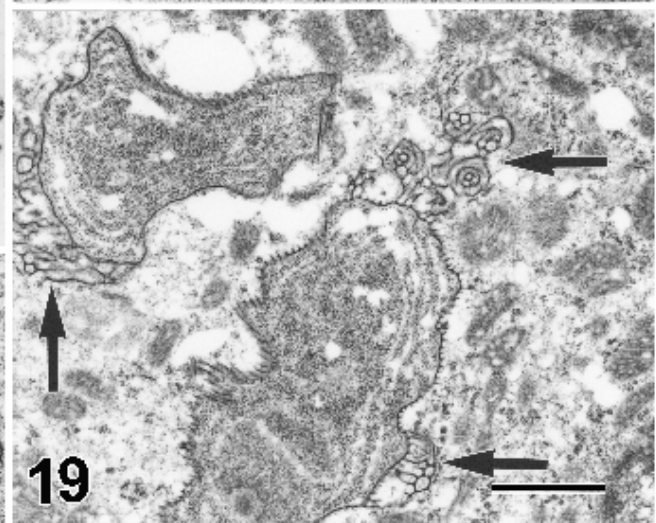
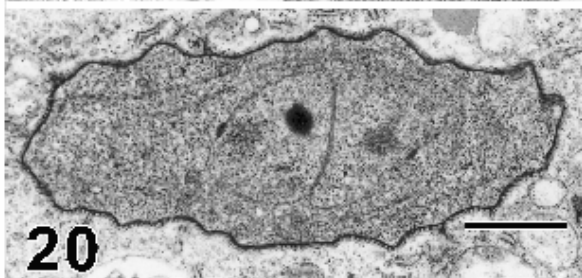
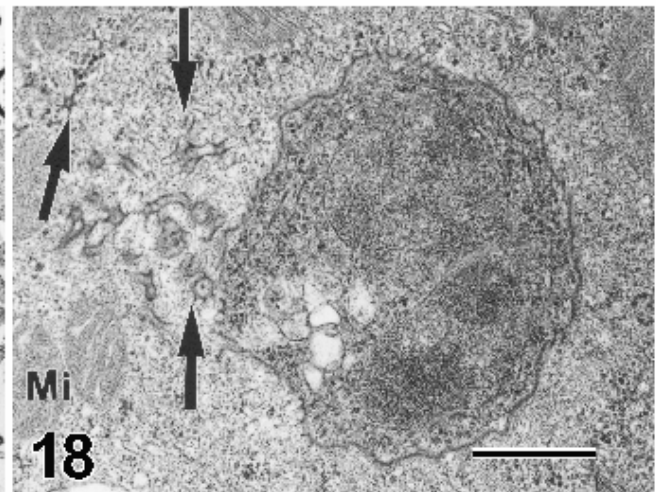
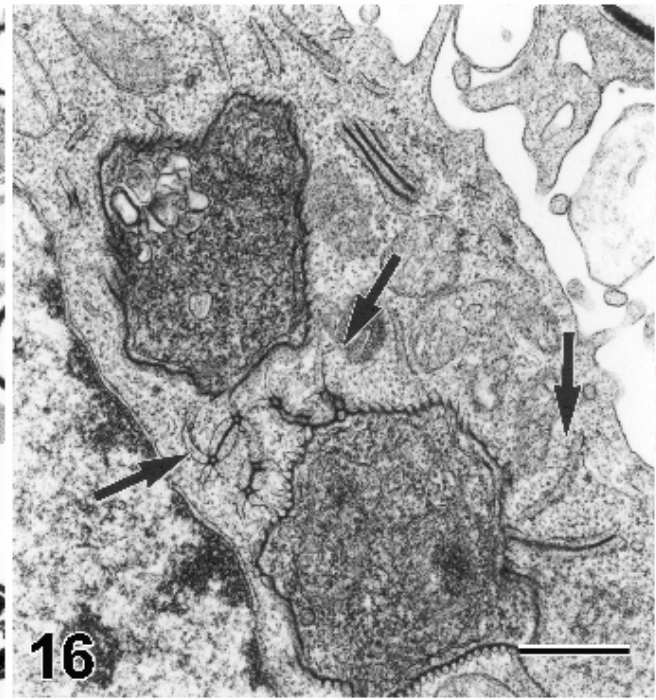
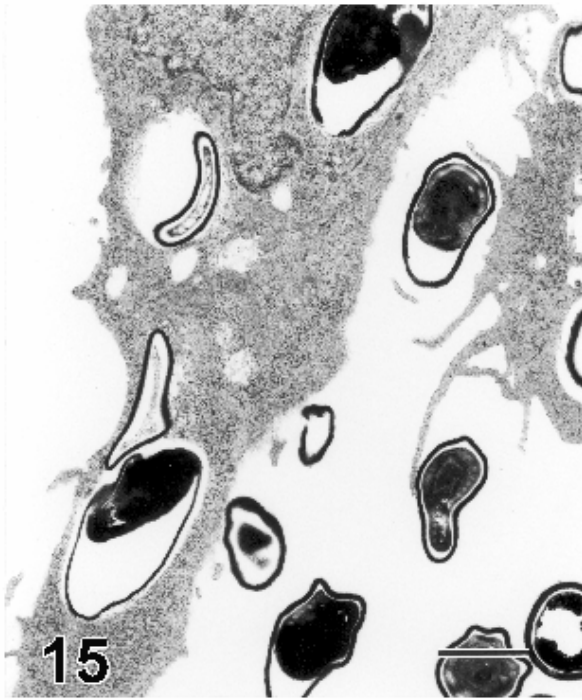
al. 2001) and a recent review by Franzen (2004), suggest that an additional mode of transmission of infection may be that following phagocytosis spores germinate and the polar tube penetrates the phagosome vacuole enabling the sporoplasm to escape the phagosome and enter the host cell cytoplasm. In the current study, phagocytosis of spores was first observed within 30 min p.i., but there was no evidence of spore activation or polar tube extrusion. However, by 2 h p.i., large numbers of phagocytized intact and empty spores were present in some RK 13 cells and portions of extruded polar tubes were also present within the host cytoplasm and the extracellular space. Phagocytosis of spores by host cells in both cell culture and in animal hosts appears to be a fairly common observation (Couzinet et al. 2000, Nasonova et al. 2001). The suggestion that phagocytosis of spores is an alternative mode of infection is a relatively new (Franzen 2004) hypothesis that is supported by Fig. 17.

By 3 h p.i., the parasites developed extensive appendages, continued to undergo additional divisions, and by 5 h p.i., appeared to be well into transition to more typical appearing proliferative forms. The initial round or ovoid shape of the sporoplasm and early proliferative cells transform into more easily recognizable elongated "typical" *B. algerae* proliferative cells containing well-defined cytoplasm with endoplasmic reticulum, diplokarya, and a plasmalemma covered with extensive vesiculotubular appendages which produce a blister-like appearance by 18 h p.i. These developmental changes agree with those observed in mosquito larvae infected with *B. algerae* (Avery and Anthony 1983) and in RK 13 cells (Lowman et al. 2000).

Between 18 and 36 h p.i., the parasite cells continued to proliferate and completed multiple cell divisions. Although parasite cytokinesis was not observed, the onset of karyokinesis was well illustrated by the presence of nuclear membrane indentation, accumulation of dense heterochromatin in the nucleus, and the presence of spindle plaques. This relatively extended period of proliferation with no indication of entry into sporogony is supported by other reports (Avery and Anthony 1983, Lowman et al. 2000).

Both proliferative and sporogonic phases of development were present by 48 h p.i. Sporogony was indicated by the presence of sporonts which tend to have a smaller cell size, greater cytoplasmic density, a uniformly thickened plasmalemma covering, the detach-

Figs. 15–20. Intracellular events and development of *Brachiola algerae* from 30 minutes through 18 hours p.i. **Fig. 15.** Phagocytosis of spores by cells occurs within the first 30 min p.i. and there are no signs of germination or polar tube extrusion. **Fig. 16.** Two recently divided organisms still connected by an extensive network of vesiculotubular appendages (arrows), 2 h p.i. **Fig. 17.** Phagocytized spores 2 h p.i. Note the spore shells are devoid of contents and the host cell cytoplasm now has numerous extruded polar tubes (arrows) embedded in it. **Fig. 18.** Proliferative stage of development, 3 h p.i., with extensive vesiculotubular appendages (arrows) infiltrating the host cytoplasm. Note that the appendages are in close proximity to the host mitochondria (Mi). **Fig. 19.** Two proliferating organisms with extensive vesiculotubular appendages protruding from their surfaces, 5 h p.i. These cells are elongated, differentiating them from the earlier stages. **Fig. 20.** "Typical" elongated proliferative form with a blister-like surface coat and vesiculotubular appendages, 18 h p.i. Scale bars: Figs. 15, 17, 19, 20 = 1.0 µm; Figs. 16, 18 = 0.5 µm.



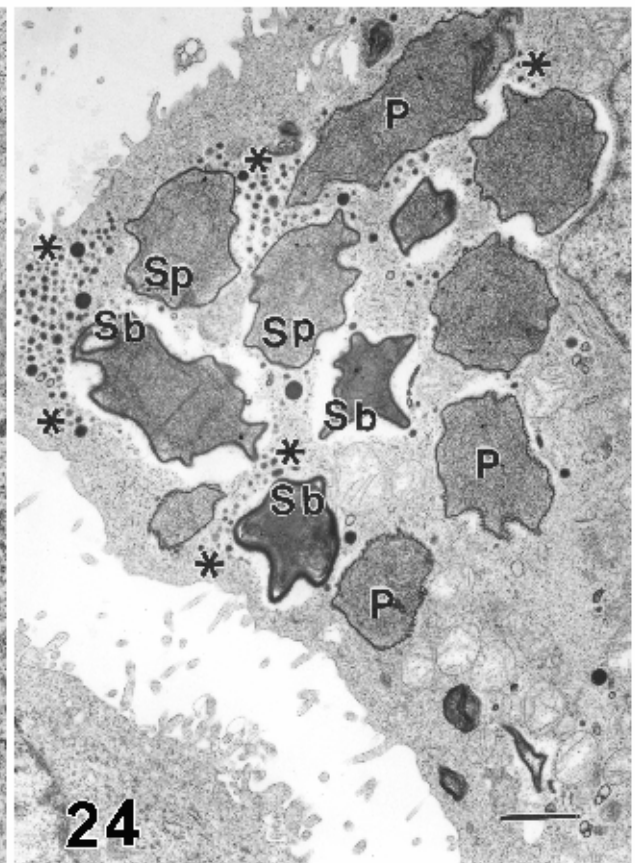
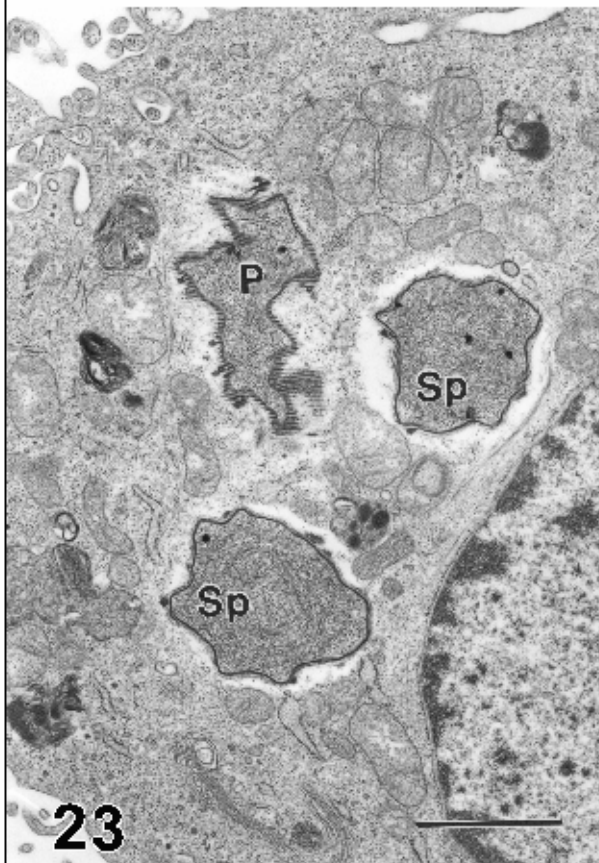
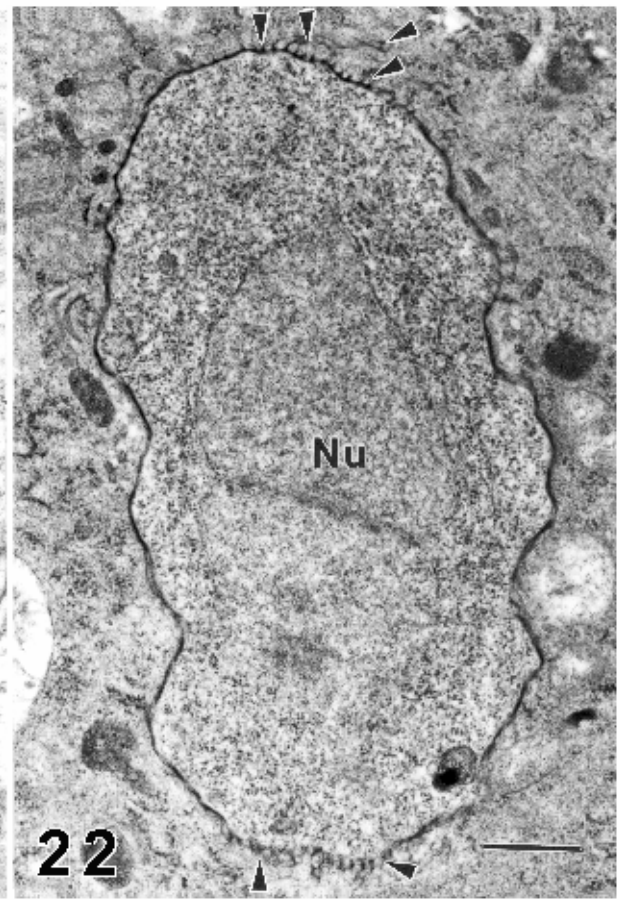
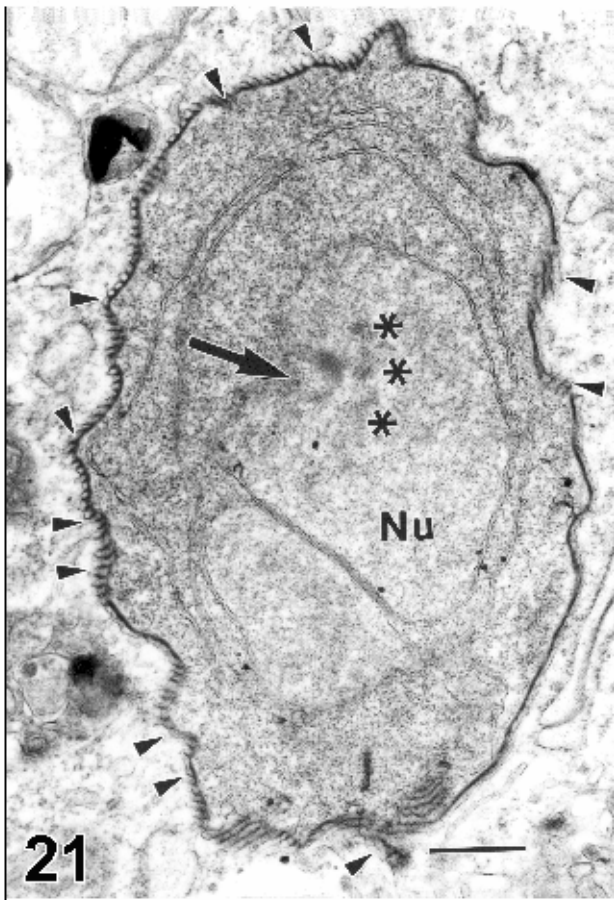
ment of appendages, and the formation of a vacuole-like space (possibly a fixation artifact) in the host cytoplasm around the sporont, which are features typical of the genus *Brachiola* (Cali et al. 1998). In addition to sporonts, there were numerous sporoblasts containing developing polar filaments, and some proliferative cells, indicating the asynchronous developmental nature of this organism and other species in the genus *Brachiola* (Cali et al. 1998, Lowman et al. 2000). Despite the presence of some late sporoblasts, no new spores were observed in any of the cells through 48 h p.i. This differed from Avery and Anthony (1983), who observed the presence of immature spores by 48 h p.i. in mosquitoes, but coincided with the study by Lowman et al. (2000) who reported that mature spores required 72 h p.i. to develop in RK 13 cells at 29°C.

Since the current study ended at 48 h p.i. and sporoblasts were the latest developmental stage observed, it is reasonable to conclude that no new second-generation spores were formed during this time period. Despite the absence of “new” spores, several RK 13 cells had sporoplasms and very early infections present in their cytoplasm 12 through 48 h p.i. These observations suggest that, although inoculum was removed after the initial exposure and fresh medium was added to the cells, many potentially infective spores remained in the extracellular environment of the culture vessels, probably bound to cell surfaces. These “new” early infections were obviously produced by spores from the original inoculum that discharged much later after initial inoculation, demonstrating that some spores can be activated over a long period of time. A recent study of spore germination reports that approximately 70% of *B. algerae* spores discharged within 5 min in germination buffer, leaving about 30% of the spores intact (Cali et al. 2002). The observation of early or new infections in cell cultures 12–48 h p.i. suggests that there may also exist a population of spores that do not immediately discharge. Avery and Anthony (1983) reported that, “virtually all tissues in the thorax and the first three abdominal segments” of *A. albimanus* larvae were infected with *B. algerae*. Their report and the current observations suggest that some spores become activated at different times as they travel through the digestive tract. This delayed activation may be an adaptation that enables some spores to travel further away from the initial site

of activation. While this may reduce the number of sporoplasms entering an individual cell or tissue region, it may serve to increase the total number of cells that become infected in a given area. It may also foster the spread of infection to other areas within the host, establishing secondary and/or disseminated infection sites, rather than immediately discharging in one area. The current study further indicates that there is a population of *B. algerae* spores that become activated, discharge their polar tubes, and transfer their sporoplasm almost immediately upon entry into a receptive environment and a second, probably smaller, population which has a delayed germination, possibly in excess of 48 h p.i.

One of the most interesting observations from the current study was the presence of numerous sporoplasms, in the extracellular spaces. These extracellular sporoplasms had a slightly different appearance than those inside the host cell. Their outer limiting membrane was thin and smooth, devoid of appendages and the blister-like surface projections observed on sporoplasms inside the host cell cytoplasm. The cytoplasm of the extracellular sporoplasm contained an obvious MIN, which was often associated with the bulbous end of the extruded polar tube. The morphology of these sporoplasms was consistent with those released in germination buffer as described by Cali et al. (2002). The close interaction between the surfaces of extracellular sporoplasms and host cells was both unexpected and interesting. Some sporoplasms appeared to tightly abut the culture cell plasmalemma as demonstrated in Fig. 6. In addition to this close abutment, some sporoplasms appeared to be in the process of being phagocytized by the RK 13 cells as illustrated by Fig. 7. It is possible that the polar tube in addition to puncturing the cell plasmalemma, may function as a mechanism to deliver the sporoplasm into an invagination in the host cell (e.g. a microenvironment) similar to the process described by Schottelius et al. (2000). The final penetration of the host cell by a sporoplasm may, in some microsporidia, be a consequence of the interaction of the host cell and the extruded sporoplasm. Since several sporoplasms were observed in the extracellular environment, it may also be possible that the host cells actively phagocytize them. The possibility that extracellular sporoplasms may be capable of infecting host cells as suggested by Figs. 6 and 7 is intriguing.

Figs. 21–24. Intracellular development of *Brachiola algerae* from 18 hours through 48 h p.i. **Fig. 21.** An elongated proliferative cell with a blister-like surface (arrowheads) observed at 18 h p.i. The nuclear (Nu) spindle plaque (arrow) and dense chromatin (*) are indicative of karyokinesis. **Fig. 22.** An elongated parasite that has blister-like ridges (arrowheads) on some surface areas and a relatively uniformly thick smooth surface coat on others. This loss of surface ornamentation indicates the transition of the parasite into the sporogonic phase of development by 24 h p.i. **Fig. 23.** By 48 h p.i. sporogony is underway and host cells containing both sporonts (Sp) and proliferative (P) cells are apparent. Sporonts are small, dense, more rounded cells that have condensed their cytoplasm. They are limited by a uniformly dense surface covering with scant ornamentation and are surrounded by a clear area in the host cytoplasm. These features differentiate sporonts from the heavily ornamented and more elongated proliferative cells. **Fig. 24.** In most host cells, sporogony is well underway by 48 h, as indicated by the presence of sporoblasts (Sb) in addition to sporonts (Sp) and a few proliferative (P) cells. The host cell cytoplasm in the immediate vicinity of the sporogonic stages is filled with dense tubular and spherical material (*). This material is the remnant of the vesiculotubular appendages that were present on the earlier developmental stages. Scale bars: Figs. 21, 22 = 0.5 µm; Figs. 23, 24 = 1.0 µm.



The study of both the initial infection and subsequent developmental stages during a progression of time was possible by the utilisation of an *in situ* fixation and embedding method. These methods provide for minimal alteration of the spatial relationship of the infective forms maintaining the relative positions of the spores, polar tubes, sporoplasms, and the host cells. As a result, this report demonstrates that some infection is established within five minutes of spore introduction to cell cultures, that the extruded polar tubes can penetrate deep into the host cytoplasm depositing a sporoplasm, that phagocytized spores are also able to germinate and release their sporoplasm from the phagosome, and that

extracellular sporoplasms often tightly abut the host cell plasmalemma, and may become internalised by phagocytosis or a form of endocytosis.

The observations described in this report suggest that microsporidian entry into host cells may be much more diverse than initially thought and that additional study of the infection process is warranted.

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