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Pathogenesis of *Helicosporidium* sp. (Chlorophyta: Trebouxiophyceae) in susceptible noctuid larvae

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Abstract

Helicosporidium sp. is a unique, achlorophyllous green alga that has been reported to infect various insect orders, including Lepidoptera, Diptera, and Coleoptera. The infectious cyst stage is ingested by the host, ruptures in the midgut lumen, and releases a filamentous cell. Histopathological examinations using larvae of a susceptible noctuid host, *Spodoptera exigua*, showed both cysts and filamentous cells affiliated with the microvillar lining of the midgut epithelium. A considerable proportion of the ingested cysts (22–39%) were recovered in feces collected 24 h after ingestion. A small number of filamentous cells passed the midgut epithelium and entered the hemocoel within 4–24 h after cyst ingestion. After 48 h, vegetative cell stages were detected in the hemolymph, followed by a 4- to 5-day period of increasing multiplication. Cyst differentiation in the colonized hemolymph began 6–7 days after the treatment.

Keywords: *Helicosporidium*; Entomopathogen; *Spodoptera exigua*; Midgut passage; Hemolymph colonization; Time course of infection; Histopathology

1. Introduction

Since its first description by Keilin (1921), the invertebrate pathogen *Helicosporidium* sp. has been detected in diverse groups of arthropods, including several orders of insects, as well as mites, crustaceans, and trematodes (Avery and Undeen, 1987; Pekkarinen, 1993; Purrini, 1984; Sayre and Clark, 1978). Until recently, however, its taxonomic position has remained unclear. Morphological and molecular studies on a *Helicosporidium* sp. isolate from the black fly *Simulium jonesi* Stone & Snoddy (Diptera, Simuliidae) have only recently identified this protist as a non-photosynthetic green alga, which is closely related to the genus *Prototheca* (Chlorophyta: Trebouxiophyceae) (Boucias et al., 2001; Tartar and Boucias, 2004; Tartar et al., 2002; Tartar et al., 2003). All susceptible arthropod species have been reported to support the growth and development of *Helicosporidium* sp. in their hemolymph (Bläske and Boucias, 2004). The life cycle of this pathogen begins with an orally transmitted cyst stage that encloses three ovoid cells and a single elongate filamentous cell within a pellicle. Upon activation in the midgut lumen, the pellicle ruptures and releases the filamentous cell and the three ovoid cells. Potentially, the filamentous cell penetrates the midgut barriers and enters the host's hemocoel. Here, vegetative cells develop, undergo several cycles of replication, and eventually form mature cysts (Boucias et al., 2001). Of the few histological studies that have investigated different stages of helicosporidial infection in invertebrate hosts (Boucias et al., 2001; Keilin, 1921; Kellen and Lindegren, 1974; Lindegren and Hoffmann, 1976; Pekkarinen, 1993; Purrini, 1984; Weiser, 1970), only two have specifically described the early events of hemolymph colonization by the pathogen.

The first, by Kellen and Lindegren (1974), documented the early phase of infection in the navel orangeworm *Paramyelois transitella* (Walker) (Lepidoptera: Pyralidae) after oral treatment in a diet-incorporation assay. Light microscopic observations of hemolymph samples and midgut

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smears 1, 3, 24, 48, and 72 h after the treatment showed the following: cysts, pellicles, filamentous cells and ovoid cells in the midgut smears at 1 h; ovoid cells in the midgut smears at 3 h; elongated ovoid cells in midgut smears and in hemolymph samples at 24 h; cell division of elongated ovoid cells within a pellicle at 48 h; spherical cells (vegetative cells) in midgut smears and hemolymph samples undergoing one to three cell divisions within a pellicle at 72 h; between 3 and 6 days, massive multiplication occurred in the hemolymph; after 6–12 days, cyst development was detected in the hemolymph.

The second, by Boucias et al. (2001), described early and late events of infection in different lepidopteran and dipteran hosts after oral challenge with infectious *Helicosporidium* sp. cysts that were originally isolated from an infected black fly larva, *S. jonesi*. In addition, they established in vitro cultures of helicosporidia and gave a detailed histological description of the different life stages during the helicosporidial life cycle by utilizing scanning and transmission electron microscopy. A scanning electron micrograph of the midgut lumen of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) showed filamentous cells associated with the brush border of midgut epidermal cells 4 h after cyst ingestion. Based on unpublished light microscopic observations, the authors hypothesized that ingested cysts bind to the peritrophic matrix, dehisce within 2 h, and release filamentous cells that penetrate the peritrophic matrix.

The objective of this histopathological study was to elucidate early events in the pathogenesis of helicosporidial infection in a susceptible host. Specifically, we determined the fate of cysts following ingestion, examined the passage of the pathogen through the midgut epithelium, and observed the invasion and colonization of the hemocoel over time.

2. Materials and methods

2.1. Insects and helicosporidial preparations

Eggs of *Helicoverpa zea* Boddie and *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) were purchased from the USDA, ARS SIMRU, Stoneville, MS. Neonates and larvae were provided with a wheat-germ-based, semi-synthetic insect diet containing antimicrobial agents and preservatives (Shorey and Hale, 1965). All insects were maintained at constant conditions (26 ± 1 °C, 70 ± 5% RH, 12-h photoperiod).

The *Helicosporidium* sp. isolate used in this study originated from infected black fly larvae, *S. jonesi*, collected from Hatchet Creek, Alachua County, FL. On a six-week basis, cysts were propagated in injected *H. zea* or *S. exigua* larvae and purified from homogenates of pupae by high-speed centrifugation (16,000*g*, 45 min) on a 5–60% linear gradient of Ludox HS40 (DuPont Chemical, Boston, MA). The band containing the cysts was collected, diluted in sterile water, and subjected to several cycles of low-speed centrifugation (4000*g*, 10 min) to remove residual gradient material. After suspension in water, cysts were counted using a hemacytometer and stored at 4 °C. Prior to each experiment, the dehiscence capability in the original cyst suspension was determined by a treatment with digestive fluid that was harvested from *S. exigua* 5th-instars, filtered, and stored at −70 °C. A total of 10^6 cysts were suspended in 100 µl of digestive fluid and incubated for 1 h at room temperature. Following two centrifugation cycles in distilled water, the pellet was suspended in 100 µl of distilled water and the number of cysts and of filamentous cells was counted with a hemacytometer. Only cyst suspensions with a dehiscence capacity of at least 70% were used for the experiments.

All experiments were conducted at constant conditions (26 ± 1 °C, 70 ± 5% RH, 12-h photoperiod). Prior to each experimental treatment, late 2nd-instar *S. exigua* larvae were starved for 20 h in empty wells of 96-well culture dishes sealed with parafilm and covered with Plexiglas. During the starvation period, larvae molted to the next instar. Early, 3rd-instar larvae were then fed 0.5-µl droplets of cyst suspension in a 5% sucrose solution at a dosage of 10^5 cysts per larva. Droplet uptake from the presented pipette tip was observed under a stereomicroscope. Treated larvae were placed in single wells of 24-well tissue culture plates and incubated at constant conditions. For routine diagnostics, hemolymph samples were withdrawn from larvae by needle puncture of a proleg. Pupae were punctured in the dorsal prothorax region. The presence of helicosporidial life stages in the hemolymph was recorded using light microscopy with differential interference contrast (DIC) optics (Leica DM R, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

2.2. Cyst ingestion and timing of hemolymph colonization

To examine the fate of ingested *Helicosporidium* sp. cysts and the onset and course of helicosporidial development in the host hemolymph, a total of 16 or 14 larvae were challenged as described above and immediately provided with a 5 × 5 × 5-mm cube of diet (fed group) or starved for an additional 24 h before provided with diet (starved group), respectively. Control larvae were treated with 5% sucrose solution. After a 24-h feeding period, larvae were transferred to new dishes, provided with diet, and incubated. Feces from the original wells were collected from each well into single microcentrifuge tubes containing 200 µl water. Samples were homogenized, mixed thoroughly for 30 s, and the numbers of recovered cysts and filamentous cells were counted using a hemacytometer.

Forty-eight hours after the treatment, four to six larvae per group were weighed and needle-punctured for hemolymph diagnostics to determine the numbers of early helicosporidial cells, hemocytes containing helicosporidia, and uninfected hemocytes per microliter of hemolymph. Under a dissecting microscope, 1 µl of hemolymph was immediately transferred into 1 µl of anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, and 41 mM citric

acid, pH 4.5) (Mead et al., 1986), and helicosporidial cells and hemocytes were identified and counted from a 1-μl droplet using DIC microscopic optics. After 7 days, six larvae per group were weighed and subjected to hemolymph diagnostics. One microliter of hemolymph was diluted in 99 μl anticoagulant buffer and the numbers of vegetative cells, cysts, hemocytes containing helicosporidia, and uninfected hemocytes were recorded using a hemacytometer. After 10 days, all surviving insects were weighed and diagnosed again and cell numbers recorded. For data analyses, all cell numbers were calculated as cells per microliter of hemolymph.

2.3. Statistical analyses

All statistical analyses were conducted using SAS System for Windows (SAS, 1999). For each experiment, numbers of recovered cysts and numbers of helicosporidial cells and hemocytes recorded per microliter of hemolymph were subjected to analysis of variance by using the procedure for general linear models (glm) (Neter et al., 1990; Rao, 1998; Younger, 1998). Means were separated using the least square means (lsmeans) statement of SAS. Throughout the article, mean values are accompanied by the standard deviation.

2.4. Pathway of infection

For histological examinations, cohorts of starved *S. exigua* 3rd-instars were treated as described above. Beginning 24 h after the treatment, larvae were provided with diet ad libitum. Digestive tracts were dissected 4, 8, 12, 24, 48, and 72 h after the treatment. Prior to dissection, larvae were submerged in 2% paraformaldehyde + 2.5% glutaraldehyde (in 0.1 M cacodylate buffer with 100 mM sucrose) for five minutes. Using forceps, the dorsal cuticle and underlying epithelium were opened and removed from the entire gut. Intact guts were rinsed five times in fresh fixative, and each gut, including the head, was transferred to a 1-ml well of a porcelain dish containing 700 μl of fresh fixative. Guts were fixed at 4 °C for 24–48 h, washed three times in 0.1 M cacodylate buffer (15 min each), and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer with 75 mM sucrose for 2 h. Following three washes in distilled water (15 min each), guts were dehydrated in a graded ethanol series (25, 50, 75, 95, 100, 100, 100%; 15 min each). After critical point drying, they were mounted to support stubs, sputter-coated, and visualized with a Hitachi S-4000 FE scanning electron microscope (SEM). After scanning of the intact organs, the guts were carefully opened and sputter-coated again to view the food bolus and the lumen side of the midgut epithelium. Three to six specimens were examined per time frame.

In addition, hemolymph samples from live insects of the same treatment-cohort were visualized under DIC optics 8, 12, and 24 h after treatment and thereafter daily for 11 days. At each time interval, four to nine insects were examined, and images were taken using Spot Advanced Software (Diagnostic Instruments, Sterling Heights, MI) to document the presence of helicosporidial life stages.

Table 1
Helicosporidia counted in feces of *Spodoptera exigua* 3rd-instar larvae after oral challenge with 10^5 cysts per larva

Group[a]	*N*	Cysts	Filamentous cells	Total cell recovery (%)
Fed	16	33,938 ± 35,744	5000 ± 5704	39 ± 38
Starved	14	17,286 ± 18,935	5000 ± 6064	22 ± 21

[a] Following oral cyst uptake, larvae were immediately transferred to diet (fed group) or starved for an additional 24 h (starved group). Feces were collected after feeding periods of 24 h.

3. Results

3.1. Cyst ingestion and timing of hemolymph colonization

In this experiment, the dynamics of cyst ingestion and defecation were investigated in combination with the events of hemolymph colonization by the pathogen. In both immediately fed and additionally starved *S. exigua* larvae, the ingestion of 10^5 cysts resulted in 100% infection (30/30) as diagnosed 7 and 10 days post-challenge. The numbers of helicosporidial cells (cysts and filamentous cells) recovered in feces varied greatly as indicated by the high standard deviations in Table 1. On average, immediately fed insects defecated twice as many ingested cysts (34 ± 36%) as additionally starved larvae (17 ± 19%). However, these differences were not statistically significant ($P = 0.13$; $df = 1$; $F = 2.43$). The number of recovered filamentous cells was the same in both groups.

Treated and control insects that were additionally starved were delayed in their development. Ten days after oral challenge, eight out of nine treated insects from the starved group were 5th-instars; one out of nine was a 4th-instar; all died before pupation. All treated insects from the fed group diagnosed at 10 days had reached the pupal stage. In the control insects, a developmental delay of additionally starved larvae was apparent after 2 days, whereas after 7 days, both starved and fed control larvae had reached the 4th- and 5th-instar at the same ratios. After 10 days, all fed control insects and 8 out of 10 starved control insects were pupae; 2 out of 10 starved control insects were 5th-instars and pupated two days later.

The number of helicosporidial cells recorded per microliter of hemolymph at 2, 7, and 10 days after cyst ingestion was influenced by the post-treatment starvation treatment (Table 2). After 2 days, the hemolymph of starved insects contained a considerable number of early vegetative cells (64 ± 26 per microliter), whereas in fed insects, low numbers (0–2) of vegetative cells per microliter of hemolymph were recorded. At 7 days post-exposure, massive numbers of vegetative cells ($4.6 \pm 4.3 \times 10^5$ per microliter) and cysts ($1.0 \pm 1.1 \times 10^5$ per microliter) were counted in hemolymph of additionally starved insects, whereas the hemolymph of immediately fed insects was colonized by high numbers of

Table 2
Numbers of helicosporidia and hemocytes in the hemolymph after oral treatment of *Spodoptera exigua* 3rd-instar larvae with 10^5 cysts per larva

Time (days)	Treatment (N)[a]	Larval weight (mg)	Helicosporidia ($\times 10^3$)/μl		Hemocytes ($\times 10^3$)/μl	
			Vegetative cells	Cysts	Containing helicosporidia	Not containing helicosporidia[b]
2	Treated fed (6)	20 ± 7	0.001 ± 0.001	0	0.0003 ± 0.0005	11.6 ± 5.3abc
	Treated starved (4)	3 ± 1	0.064 ± 0.026	0	0	1.0 ± 0.2d
	Control fed (6)	14 ± 3	NA	NA	NA	13.2 ± 7.8a
	Control starved (6)	4 ± 1	NA	NA	NA	1.7 ± 0.9d
7	Treated fed (6)	196 ± 98	11.6 ± 12.2	0	0.083 ± 0.204	12.9 ± 4.7ab
	Treated starved (6)	85 ± 59	461.9 ± 425.6	102.8 ± 112.5	1.250 ± 3.062	5.1 ± 4.2cd
	Control fed (6)	230 ± 69	NA	NA	NA	16.3 ± 8.6a
	Control starved (6)	190 ± 41	NA	NA	NA	11.5 ± 4.7abc
10	Treated fed (10)	119 ± 22	329.8 ± 338.1	105.6 ± 148.4	0.100 ± 0.316	11.6 ± 7.9ab
	Treated starved (9)	238 ± 113	617.8 ± 274.8	397.2 ± 387.5	0.333 ± 0.707	6.7 ± 3.6bd
	Control fed (9)	125 ± 25	NA	NA	NA	15.9 ± 9.3a
	Control starved (10)	163 ± 52	NA	NA	NA	15.1 ± 4.4a

NA, not applicable.
[a] Following oral cyst uptake, larvae were immediately transferred to diet (fed) or starved for an additional 24 h (starved).
[b] Means followed by a different letter are significantly different ($P \leqslant 0.05$; SAS glm procedure and lsmeans statement).

vegetative cells ($1.2 \pm 1.2 \times 10^4$ per microliter) without any cyst formation. After 10 days, the hemolymph of insects from both groups was filled with both helicosporidial cell types. Using 50% of body weight (mg) as an estimate for the total volume of hemolymph (μl) per insect, total numbers of helicosporidia and hemocytes in an infected insect were calculated. Within 10 days after oral challenge, total numbers of helicosporidial cells were $9.7 \pm 6.8 \times 10^7$ per starved insect and $2.5 \pm 3.2 \times 10^7$ per fed insect.

There were significant differences in the numbers of uninfected hemocytes (cells not containing helicosporidia) per microliter of hemolymph between the different groups of insects after different time intervals ($P < 0.0001$; df = 11; F = 4.66). After 2 days, treated and control insects of the starved groups contained approximately 1/10 the number of hemocytes recorded for the treated and control insects of the fed groups (Table 2). After 7 days, treated starved insects had significantly fewer hemocytes than treated and control fed insects; hemocyte numbers in control groups did not differ from each other or from treated fed insects at this time. After 10 days, hemocyte numbers were significantly lower in treated starved insects compared with both control groups; hemocyte numbers in treated fed insects were intermediate and did not significantly differ from the numbers counted in any of the other groups (Table 2). In *Helicosporidium* sp.-challenged insects, numbers of uninfected hemocytes per microliter hemolymph increased over time in the starved group (i.e., from 3rd- to 5th-instar), whereas they did not change in the treated, fed group (i.e., from 3rd-instar to pupa) (Table 2). Control insects showed a similar pattern of increasing and consistent numbers of hemocytes per microliter of hemolymph over time in the additionally starved and the immediately fed group, respectively.

3.2. Pathway of infection

At different time intervals following oral challenge of 3rd-instar *S. exigua* larvae with *Helicosporidium* sp. cysts, guts were dissected in fixative and prepared for SEM examination. In addition, hemolymph samples from live insects of the same cohort were subjected to routine DIC diagnostics to document the colonization of the hemolymph by helicosporidial life stages in daily intervals over time.

Up to 8 h after cyst ingestion, many cysts and filamentous cells were detected in the food bolus over the entire length of the midgut (Figs. 1A and B). Where the peritrophic matrix could be observed, it appeared discontinuous and patchy (not shown). Cysts were found embedded in the microvilli of midgut cells, some of them in the process of dehiscing (Fig. 1C). Filamentous cells penetrating the microvillar lining of the apical midgut epithelium showed their characteristic barbs mostly pointing towards the midgut lumen (Fig. 1D). Four, eight, and twenty-four hours after cyst ingestion, small numbers of filamentous cells (2–39 per midgut) were detected exiting the basal side of the midgut epithelium and invading the body cavity. In addition, considerable numbers of "free" filamentous cells were seen on the basal midgut surface. Barbs on filamentous cells invading the hemocoel but still affiliated with the midgut epithelium always pointed towards the hemocoel (Fig. 2). No helicosporidia were detected on the luminal and hemolymph surfaces of either the foregut or hindgut. There was no apparent affiliation between helicosporidia and Malpighian tubules. Occasionally, phagocytic hemocytes were observed to bind to invading filamentous cells. After 48 and 72 h, no filamentous cells were seen on the basal side of the midgut.

No helicosporidia were detected in hemolymph samples 8, 12, and 24 h after cyst ingestion. At 48 h, single, early vegetative stages were detected in all examined larvae ($n = 8$). Free bean-shaped and round vegetative cells, as well as phagocytosed cells of both types (Figs. 3A and B), were seen among a majority of uninfected hemocytes. In two individuals, the remnant of a phagocytosed filamentous cell was detected (Fig. 3C), indicating that filamentous cells fully pass through the midgut epithelium, enter the hemo-

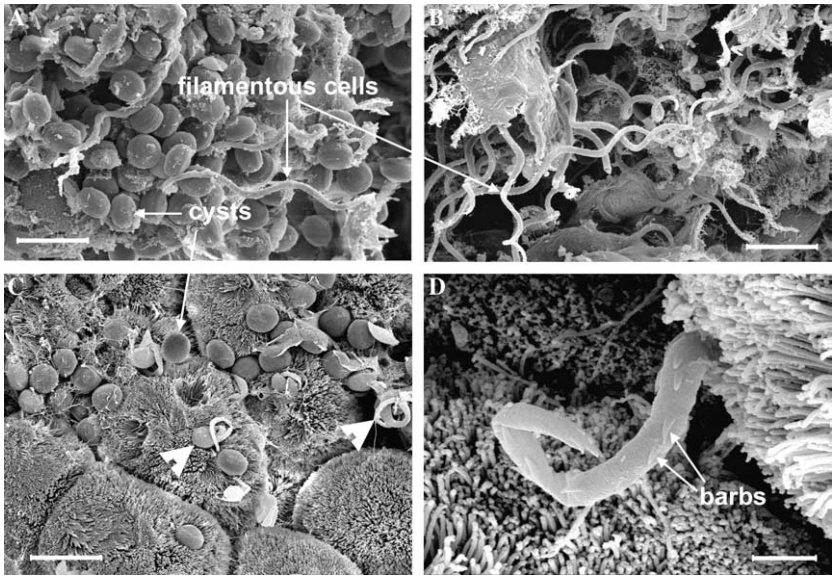

Fig. 1. Scanning electron micrographs of the midgut lumen of *Spodoptera exigua* larvae after oral challenge with *Helicosporidium* sp. cysts. (A) and (B) Numerous cysts and filamentous cells in the food bolus 8 and 4 h after cyst ingestion, respectively. (C) Intact and dehiscing (arrowheads) cysts in association with the microvillar lining of the midgut epithelium 4 h after cyst ingestion. (D) A filamentous cell entering the midgut epithelium 24 h after cyst ingestion demonstrating the orientation of the characteristic barbs, which point towards the midgut lumen. Scale bars: 10 μm (A–C) and 1 μm (D).

coel, and are phagocytosed. However, no freely circulating filamentous cells were found in the hemolymph samples at any time following the oral treatment. After 72 and 96 h, considerable numbers of vegetative cells were released from phagocytes (Figs. 3D–G). Vegetative cell replications occurred intra- and extracellularly in the host hemolymph. Hemocytes containing vegetative cells were observed to form multicellular aggregates (Figs. 3C and G). Between 48 and 120 h, the numbers of freely circulating vegetative cells increased notably. Cyst differentiation began 6–7 days after the oral treatment, when larvae had reached the 4th or 5th-instar (Fig. 3H). Cysts were found to freely circulate in the hemolymph as well as being contained in phagocytes. Numbers of cysts and vegetative cells increased during the following days (Fig. 3I). At the end of the observation period (12 days after cyst ingestion), all insects had been subjected to hemolymph diagnostics at least once, and all of them were infected (40/40).

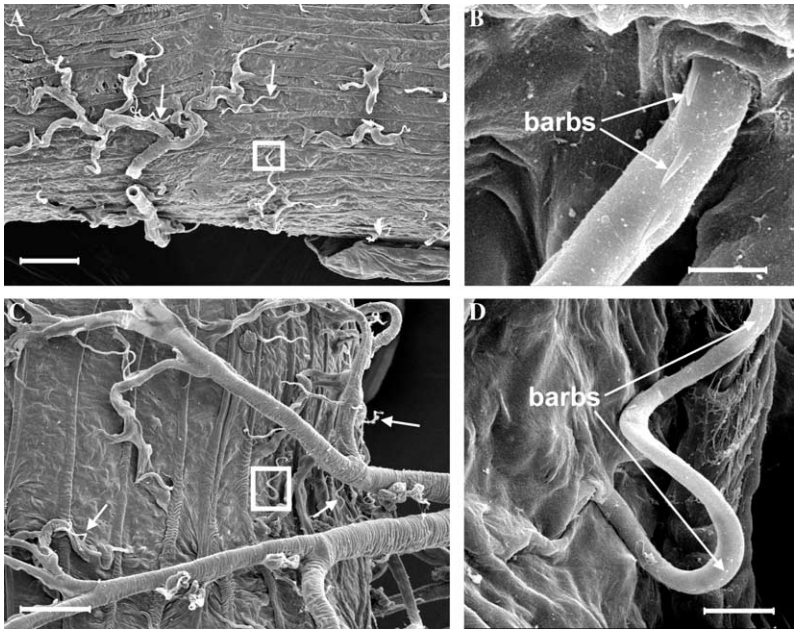

Fig. 2. Scanning electron micrographs of the basal midgut surface of *Spodoptera exigua* larvae showing invasive filamentous cells (arrows in A and C) gaining ingress into the hemocoel 8 h (A and B) and 24 h (C and D) after ingestion of *Helicosporidium* sp. cysts. The characteristic barbs seen at higher magnifications (B and D) are oriented towards the hemocoel. Scale bars: 30 μm (A and C), 3 μm (D), and 1 μm (B).

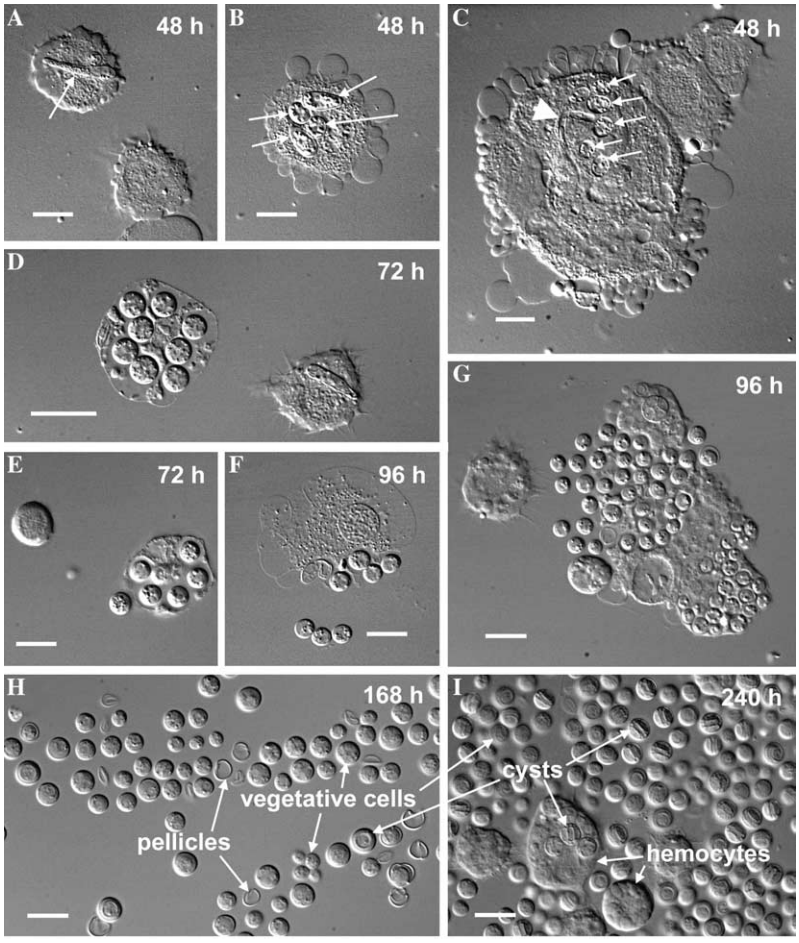

Fig. 3. Light micrographs of hemolymph sampled from *Helicosporidium* sp.-infected *Spodoptera exigua* larvae between 48 and 240 h after cysts ingestion. (A) Two hemocytes, one containing a bean-shaped vegetative cell (arrow). (B) A quartet of early vegetative cells (arrows) within a hemocyte. (C) Clustered hemocytes containing vegetative cells (arrows) and the remnants of a filamentous cell (arrowhead). (D) Two hemocytes, one containing an octet of vegetative cells and displaying cytopathic effects, the other containing a bean-shaped vegetative cell. (E) and (F) Hemocytes releasing vegetative cells. (G) Multiple vegetative cells being released from a cluster of hemocytes. (H) Several freely circulating vegetative cells, pellicles, and few early cysts. (I) Massive numbers of freely circulating cysts and vegetative cells. Note that cyst development also occurs within hemocytes. Scale bars, 10 μm.

4. Discussion

An infectious *Helicosporidium* sp. cyst must have the ability to dehisce in the midgut of the host and release the invasive filamentous cell. This dehiscence event can be triggered by the addition of digestive fluid harvested from different noctuid species, and the percentage of released filamentous cells varies according to the preparation and age of the cyst suspension used. In the experiments reported herein, filamentous cells were shown to fully pass through the midgut epithelium of *S. exigua* larvae and to initiate the colonization of the host hemolymph, resulting in a massive, vegetative replication of the pathogen.

The dynamics of cyst ingestion were investigated in combination with the events of hemolymph colonization by the pathogen. Insects that underwent an additional starvation period after cyst ingestion defecated about half the number of cysts than did insects that immediately began feeding. In addition, the onset of hemolymph colonization was earlier in starved insects than in fed insects. In starved insects, a considerable number of vegetative cells could be detected in the hemolymph 48 h after cyst ingestion, whereas in fed insects, no cells or only few cells could be observed. The food bolus, continuously passing through the digestive tract, potentially facilitated a rapid transport and subsequent defecation of cysts. Alternatively, diet ingredients (e.g., antimicrobial and/or other components) might have reduced the viability and dehiscence capability of ingested cysts or interfered with the midgut invasive process, explaining the delay in the initial hemolymph colonization (Zieler et al., 1999).

The transit of *Helicosporidium* sp. from the midgut lumen to the hemocoel, the target site for multiplication, began within a few hours after cyst ingestion. Dehiscence occurred in the midgut lumen, most likely initiated by enzymatic activity of digestive fluid. However, the massive numbers of cysts in association with the microvilli of midgut cells (Fig. 1C) indicated a breakdown in the peritrophic matrix barrier. It is possible that mechanical penetration by filamentous cells (maximum diameter 1 μm) disrupted the peritrophic matrix so that the larger cysts (diameter 5 μm) could easily access the ectoperitrophic space. Another possibility is that enzymes

released from cysts and/or filamentous cells degraded the peritrophic matrix (Vinetz et al., 2000). The transit through the midgut epithelium by the invasive filamentous cells of *Helicosporidium* sp. could be partially elucidated in this study: barbs on filamentous cells entering the midgut epithelium on the apical side were mostly orientated towards the midgut lumen, which has been shown in another lepidopteran host (Boucias et al., 2001); barbs on filamentous cells exiting the midgut on the basal side, however, always pointed towards the hemocoel, suggesting that filamentous cells changed their orientation during the passage. The question remains whether the interaction between filamentous cells and midgut epithelial cells occurs intra- or intercellularly. Examination of both the digestive tract and hemolymph indicated that filamentous cells fully pass through the midgut epithelium and enter the hemocoel. However, at no time were freely circulating filamentous cells observed in hemolymph samples. Potentially, the filamentous cells entering the hemocoel were phagocytosed by hemocytes (Fig. 3C). If so, these cells survived the cellular phagocytic reaction and began the vegetative cell cycle intracellularly, still contained within the host hemocytes. Multiplying vegetative cells appeared to induce the degradation of the phagocytic cells (Figs. 3E–G). Once released, the vegetative cells were no longer recognized as non-self and underwent numerous cycles of cell divisions. It is very likely that the pellicles of these vegetative stages mimic host surface epitopes. A similar scenario has been found with various other hemolymph-borne entomopathogens. For example, the mycopathogen, *Nomuraea rileyi*, has an outer surface that mimics the insect basement membrane (Pendland and Boucias, 1998).

The timing of hemolymph colonization and onset of cyst formation by *Helicosporidium* sp. in *S. exigua* conform to observations made in other susceptible lepidopteran hosts. In hemolymph samples of the sphingid *M. sexta*, Boucias et al. (2001) detected a few vegetative cells undergoing cell division as early as 2 days after cyst ingestion; increased numbers of vegetative cells, pellicles, and cysts were seen after 6 days, and massive numbers of cysts and vegetative cells recorded after 10 days. In hemolymph samples of the pyralid *P. transitella*, Kellen and Lindegren (1974) found elongated helicosporidial cells 24 h after cyst ingestion; elongated and spherical cells undergoing cell divisions within a pellicle at 48 and 72 h; massive multiplication of vegetative cells between 3 and 6 days; and finally cyst formation at 6–12 days. The factors responsible for cyst differentiation in the host hemolymph remain to be determined. From the results of the present study, factors associated with pupation and metamorphosis can be excluded, since massive numbers of cysts were formed in larvae as well as in prepupae and pupae. Adult noctuids infected with *Helicosporidium* sp. during the larval stages also have been reported to contain massive numbers of cysts and vegetative cells in their hemolymph (Bläske and Boucias, 2004). The onset of cyst differentiation in the insect is most likely a time- or density-dependent event. Vegetative cell development and multiplication and cyst differentiation occurred both intra- and extracellularly in the host hemocoel. To date, cyst formation has not been initiated in vitro with vegetative cell cultures of *Helicosporidium* sp. (Bläske-Lietze et al., unpublished).

Numbers of circulating hemocytes have been found to decrease as well as increase in response to hemolymph-borne entomopathogens (Gardiner and Strand, 2000; Gillespie et al., 2000; Teramoto and Tanaka, 2004). Despite the enormous numbers of helicosporidia in the hemolymph of infected *S. exigua*, total hemocyte counts revealed that numbers of hemocytes per microliter hemolymph did not change over time in fed insects and did not differ between treated and control larvae. The majority of the larvae in the fed groups were 4th-instars at 2 days, 5th-instars at 7 days, and all were pupae at 10 days of the experiment. However, counts at 2 days showed a significantly lower density of hemocytes in both treated and control larvae of additionally starved insects compared with immediately fed insects. The majority of the starved larvae, both treated and control, were 3rd-instars at this time, which suggests a significant, 10-fold increase in hemocyte numbers during the moult from 3rd- to 4th-instar. A significant (sixfold) increase of hemocytes densities from early 3rd- to late 4th-instars has also been observed in larvae of the noctuid, *Pseudoplusia includens* (Gardiner and Strand, 2000).

In summary, despite the capability of *Helicosporidium* sp. cysts to pass the peritrophic matrix, the filamentous cell was shown to be the invasive life stage of this pathogen. This cell type was able to fully penetrate the midgut epithelium and enter the hemocoel of the host insect. It remains to be discovered whether the interaction between filamentous cells and midgut epithelium occurs intra- or intercellularly. The mechanisms of cyst differentiation after massive vegetative cell replication in the hemolymph appear to be based on time-dependent or density-dependent factors and not associated with host metamorphosis.

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References

Avery, S.W., Undeen, A.H., 1987. Some characteristics of a new isolate of *Helicosporidium* and its effect upon mosquitoes. J. Invertebr. Pathol. 49, 246–251.

Bläske, V.-U., Boucias, D.G., 2004. Influence of *Helicosporidium* spp. (Chlorophyta: Trebouxiophyceae) infection on the development and survival of three noctuid species. Environ. Entomol. 33, 54–61.

Boucias, D.G., Becnel, J.J., White, S.E., Bott, M., 2001. In vivo and in vitro development of the protist *Helicosporidium* sp.. J. Eukaryot. Microbiol. 48, 460–470.

Gardiner, E.M.M., Strand, M.R., 2000. Hematopoiesis in larval *Pseudoplusia includens* and *Spodoptera frugiperda*. Arch. Insect Biochem. Physiol. 43, 147–164.

Gillespie, J.P., Burnett, C., Charnley, A.K., 2000. The immune response of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae* var *acridum*. J. Insect Physiol. 46, 429–437.

Keilin, D., 1921. On the life-history of *Helicosporidium parasiticum*, n.g., n.sp., a new type of protist parasitic in the larva of *Dasyhelea obscura* Winn. (Diptera, Ceratopogonidae) and in some other arthropods. Parasitology 13, 97–113.

Kellen, W.R., Lindegren, J.E., 1974. Life cycle of *Helicosporidium parasiticum* in the navel orangeworm, *Paramyelosis transitella*. J. Invertebr. Pathol. 23, 202–208.

Lindegren, J.E., Hoffmann, D.F., 1976. Ultrastructure of some developmental stages of *Helicosporidium* sp. in the navel orangeworm, *Paramyelosis transitella*. J. Invertebr. Pathol. 27, 105–113.

Mead, G.P., Ratcliffe, N.A., Renwrantz, L.R., 1986. The separation of insect haemocyte types on Percoll gradients; methodology and problems. J. Insect Physiol. 32, 167–177.

Neter, J., Wasserman, W., Kutner, M.H., 1990. Applied Linear Statistical Models: Regression, Analysis of Variance, and Experimental Designs. Richard Irwin, Illinois.

Pekkarinen, M., 1993. Bucephalid trematode sporocysts in brackish-water *Mytilus edulis*, new host of a *Helicosporidium* sp. (Protozoa: Helicosporidia). J. Invertebr. Pathol. 61, 214–216.

Pendland, J.C., Boucias, D.G., 1998. Characterization of monoclonal antibodies against cell wall epitopes of the insect pathogenic fungus, *Nomuraea rileyi*: differential binding to fungal surfaces and cross-reactivity with host hemocytes and basement membrane components. Eur. J. Cell Biol. 75, 118–127.

Purrini, K., 1984. Light and electron microscope studies on *Helicosporidium* sp. parasitizing oribatid mites (Oribatei, Acarina) and collembola (Apterygota, Insecta) in forest soils. J. Invertebr. Pathol. 44, 18–27.

Rao, P.V., 1998. Statistical Research Methods in the Life Sciences. Duxbury Press, Pacific Grove.

SAS., 1999. User's Guide, version 8.0. SAS Institute, Cary, NC.

Sayre, R.M., Clark, T.B., 1978. *Daphnia magna* (Cladocera: Chydoroidea) a new host of a *Helicosporidium* sp. (Protozoa: Helicosporidia). J. Invertebr. Pathol. 31, 260–261.

Shorey, H.H., Hale, R.C., 1965. Mass rearing of the larvae of nine noctuid species on a simple artificial medium. J. Econ. Entomol. 58, 522–524.

Tartar, A., Boucias, D.G., 2004. The non-photosynthetic, pathogenic green alga *Helicosporidium* sp. has retained a modified, functional plastid genome. FEMS Microbiol. Lett. 233, 153–157.

Tartar, A., Boucias, D.G., Adams, B.J., Becnel, J.J., 2002. Phylogenetic analysis identifies the invertebrate pathogen *Helicosporidium* sp. as a green alga (Chlorophyta). Int. J. Syst. Evol. Microbiol. 52, 273–279.

Tartar, A., Boucias, D.G., Becnel, J.J., Adams, B.J., 2003. Comparison of plastid 16S rRNA (rrn16) genes from *Helicosporidium* spp.: evidence supporting the reclassification of Helicosporidia as green algae (Chlorophyta). Int. J. Syst. Evol. Microbiol. 53, 1719–1723.

Teramoto, T., Tanaka, T., 2004. Mechanism of reduction in the number of the circulating hemocytes in the *Pseudaletia separata* host parasitized by *Cotesia kariyai*. J. Insect Physiol. 50, 1103–1111.

Vinetz, J.M., Valenzuela, J.G., Specht, C.A., Aravind, L., Langer, R.C., Ribeiro, J.M.C., Kaslow, D.C., 2000. Chitinases of the avian malaria parasite *Plasmodium gallinaceum*, a class of enzymes necessary for parasite invation of the mosquito midgut. J. Biol. Chem. 275, 10331–10341.

Weiser, J., 1970. *Helicosporidium parasiticum* Keilin infection in the caterpillar of a hepialid moth in Argentina. J. Protozool. 17, 440–445.

Younger, M.S., 1998. SAS® Companion for P.V. Rao's Statistical Research Methods in the Life Sciences. Duxbury Press, Pacific Grove.

Zieler, H., Nawrocki, J.P., Shahabuddin, M., 1999. *Plasmodium gallinaceum* ookinetes adhere specifically to the midgut epithelium of *Aedes aegypti* by interaction with a carbohydrate ligand. J. Exp. Biol. 202, 485–495.