Further investigations are needed to define the precise role of this tick in Lyme disease transmission. Clarification is needed on the apparent low frequency of infection, low spirochete burden, and low survival rate in *A. americanum*. *Amblyomma americanum*'s ability to transmit Lyme disease may be of extreme importance because of the extensive range of this species. Efforts to identify vectors of Lyme disease in Texas, Arkansas, and Georgia (*7*) may benefit from this new information (*14*).

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Complete Development of *Cryptosporidium* in Cell Culture

Abstract. *Protozoan parasites of the genus* Cryptosporidium *cause a short-term, flu-like, gastrointestinal illness in immunocompetent persons and severe, persistent, life-threatening diarrhea in immunodeficient individuals. No effective therapy is available for the treatment of cryptosporidiosis in the immunodeficient host. Complete development (from sporozoite to sporulated oocyst) of a human isolate of* Cryptosporidium *was achieved in cultured human fetal lung cells and primary chicken kidney and porcine kidney cells. The growth of this newly recognized zoonotic agent in cell culture now provides a means of studying its behavior, development, and metabolism, and a mechanism for evaluation of potentially useful therapeutic agents.*

Protozoans of the genus *Cryptosporidium* (Phylum Apicomplexa; Suborder Eimeriorina) are small (2 to 6 μm) coccidian parasites that inhabit the microvillous region of epithelial cells of a variety of animals, including man (*1*). Until recently, *Cryptosporidium* infections were considered rare in animals, and in man they were thought to be the result of a little-known opportunistic pathogen outside its normal host range (*2*). Our concept of cryptosporidiosis has changed within the past several years to that of an important cause of gastroenteritis and diarrhea in several animal species, especially calves, lambs, goats, and humans (*1*).

In immunocompetent humans, *Cryptosporidium* may produce a short-term, flu-like, gastrointestinal illness (*3, 4*). This contrasts sharply with the prolonged, severe diarrhea in immunodeficient individuals who contract cryptosporidiosis (*3, 5*), especially those with acquired immunodeficiency syndrome (AIDS). Recent reports of respiratory (*6*) and biliary (*7*) infections demonstrate that *Cryptosporidium* is not always confined to the gastrointestinal tract of immunodeficient persons.

Experimental infections in farm and laboratory animals have established that *Cryptosporidium* has little or no host specificity, and that it is transmitted by ingestion of oocysts that are fully sporulated and infective at the time they are passed in the feces (*1, 3, 4*). Calves and perhaps companion animals such as kittens, puppies, and rodents may serve as sources of human infection (*3, 4*), and human-to-human transmission may occur (*8*). Thus, cryptosporidiosis has joined the list of more than 150 zoonoses; those diseases caused by agents that are naturally transmitted between other vertebrate animals and man (*9*).

We report here the complete development (from sporozoite to infective oocyst) of a human isolate of *Cryptosporidium* in cell culture. Cultivation of *Cryptosporidium* in vitro will enable investigators to determine precisely the biology of this intracellular parasite in the absence of other microbes found in the host intestine.

Cryptosporidium oocysts were obtained from the feces of a patient with persistent cryptosporidiosis and the clinical and laboratory features of AIDS, or from the feces of goats experimentally infected with this human isolate (*3*). Feces containing oocysts were stored in a 2.5 percent (weight to volume) solution of $K_2Cr_2O_7$ at 4°C for up to 12 months prior to use. Oocysts were purified and concentrated by flotation in Sheather's sugar solution (*3*), washed three times by centrifugation in phosphate-buffered saline [(PBS), *p*H 7.2], and incubated in PBS containing 0.1 percent (weight to volume) glucose, penicillin (5000 IU/ml), streptomycin (5 mg/ml), and amphotericin-B (20 μg/ml), for 3 hours at 37°C to kill microbial contaminants (*10*). The oocysts were then washed three times and incubated in PBS containing 0.25 percent trypsin and 0.75 percent sodium taurocholate (each weight to volume) for 30 to 60 minutes at 37°C to obtain free sporozoites (*10*).

Excysted (free) sporozoites were washed twice in PBS and resuspended in growth medium (*11*) and approximately 150,000 sporozoites were added to each Leighton tube containing a monolayer of cells growing on a 5 cm^2 plastic cover slip. After incubation for 4 hours at 37°C in a 5 percent CO_2 atmosphere to allow attachment and penetration of sporozoites, cell monolayers were rinsed vigorously with maintenance medium (*12*). Infected cell cultures were kept in maintenance medium at 37°C with a 5 percent CO_2 atmosphere throughout the study. Cells used to study the developmental cycle of *Cryptosporidium* were human fetal lung (HFL) cells (Flow 2000). Primary chicken kidney (PCK) and porcine kidney (PK-10) cells were also examined for their ability to support the growth of *Cryptosporidium*. The cell monolayers were examined at various times after inoculation of sporozoites for developmental stages of *Cryptosporidium* by

Nomarski interference contrast microscopy (*10*).

Representative developmental stages of *Cryptosporidium* in HFL cells are shown in Fig. 1, and their time of appearance, presence, and relative numbers are presented in Table 1. After the initial 4-hour incubation period, some sporozoites floated free in the medium; however, most had attached to or had penetrated HFL cells. By 8 hours very few free sporozoites were observed, the remainder had transformed into trophozoites within parasitophorous vacuoles of HFL cells. Merogonous development proceeded rapidly with the appearance of mature type I meronts (with six or eight merozoites) by 12 hours, and mature type II meronts (with four merozoites) by 24 hours after inoculation of sporozoites. Sexual stages (macrogametes and microgametocytes) were present by 48 hours, and the first oocysts (unsporulated and sporulated) were observed within HFL cells 72 hours after inoculation of sporozoites. Sporulated oocysts, morphologically identical to those in the feces of experimentally infected calves, goats, and mice (*3*), were the only developmental stage seen in HFL on days 7, 8, 11, 18, 28, and 31 after inoculation of sporozoites. Most of these sporulated oocysts remained within parasitophorous vacuoles of the HFL cells.

Table 1. Developmental stages of *Cryptosporidium* in human fetal lung cells at selected times after inoculation of approximately 150,000 sporozoites. Symbols: 1+, 1 to 5 per field; 2+, 6 to 10 per field; and 3+, more than 10 per field (examined at ×400).

Time after inoculation (hours)	Trophozoites	Meronts		Macrogametes and microgametocytes	Oocyst
		Type I	Type II		
4	1+	0	0	0	0
8	2+	0	0	0	0
12	2+	1+	0	0	0
16	2+	2+	0	0	0
24	1+	1+	2+	0	0
48	2+	3+	2+	1+	0
72	2+	3+	2+	2+	1+
96	2+	1+	3+	2+	2+
120	2+	0	2+	3+	2+
144	1+	0	1+	2+	3+
168	0	0	0	0	3+
192	0	0	0	0	2+
11, 18*	0	0	0	0	2+
28, 31*	0	0	0	0	1+

*Days after inoculation with sporozoites.

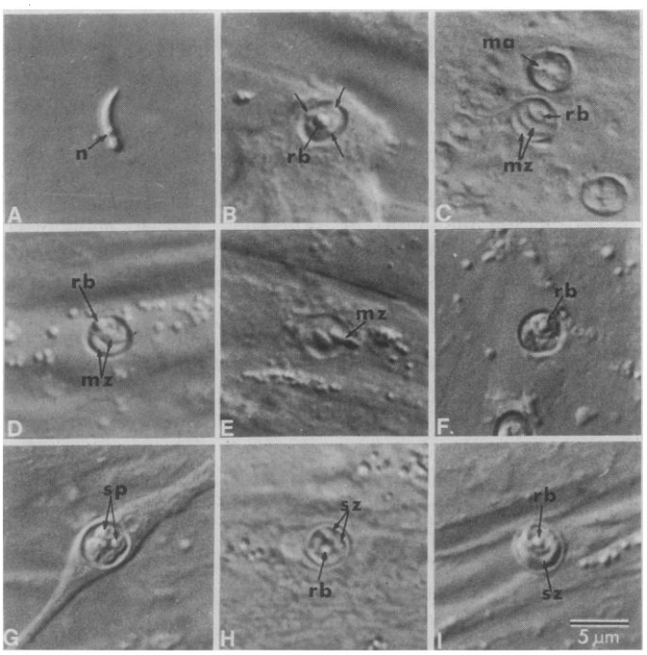

Fig. 1. Representative developmental stages of *Cryptosporidium* in human fetal lung cell cultures after inoculation of sporozoites. Nomarski interference contrast photomicrographs of living cells (×1600). (A) Sporozoite free in culture medium 4 hours after inoculation. (B) Immature type I meront with central refractile body (*rb*) and nuclei (arrows) near the periphery, 12 hours after inoculation. (C) Mature type I meront with merozoites (*mz*) and refractile body (*rb*). A macrogamete (*ma*) is also shown. (D) Mature type II meront with merozoites (*mz*) and refractile body (*rb*). (E) Type II merozoite (*mz*) in the process of leaving the parasitophorous vacuole of the host cell. Two merozoites have already escaped. (F) Macrogamete with prominent refractile body (*rb*) and granular cytoplasm. (G) Oocyst undergoing sporogony within parasitophorous vacuole of the host cell. Note that there are four sporoblasts at this stage of development. (H) Optical cross section of a fully sporulated oocyst in a host cell. Note the sporozoites (*sz*) and refractile body (*rb*). (I) Side view of fully sporulated oocyst in a host cell. Note the sporozoite (*sz*) and the refractile body.

Infectivity of cell culture–derived oocysts for suckling mice was also tested. HFL cells containing oocysts were harvested by trypsinization, incubated in 2.5 percent $K_2Cr_2O_7$ for 4 hours at 4°C to kill all developmental stages except oocysts, washed three times by centrifugation in PBS, and then inoculated into four 5-day-old Swiss-Webster mice. Five days later, the four inoculated mice and four uninoculated littermates were examined for the presence of *Cryptosporidium* infections as described previously (*3*, *10*).

All four mice inoculated with cell culture–derived oocysts had numerous developmental stages of *Cryptosporidium* in their ileum enterocytes, and numerous oocysts in their feces when examined 5 days later. No developmental stages of *Cryptosporidium* were observed in enterocytes of the uninoculated littermates, and no oocysts were found in their feces.

The development of *Cryptosporidium* in HFL cells during the first 3 days after inoculation of sporozoites was similar to that reported in suckling mice inoculated orally with oocysts isolated from humans or calves (*3*, *13*, *14*). However, on days 4 through 8 after inoculation, the numbers and types of developmental stages observed in HFL cells differed markedly from those in mice. During this 4-day period, the number of developmental stages, including meronts, increased in mice, perhaps because of the occurrence of an autoinfective cycle (*3*, *13*, *14*). In HFL cells, however, the number of parasites decreased from days 4 through 8, with type I and type II meronts no longer present by days 5 and 7, respectively (Table 1).

Some oocysts developing in enterocytes of experimentallty infected mice and in endodermal cells of the chorioallantoic membrane of chicken embryos are thin-walled (autoinfective oocysts); the remaining oocysts are the highly resistant, thick-walled forms that pass unaltered through the intestine and transmit the infection to susceptible hosts by way of fecal-oral contamination (*3*, *13*, *14*). Thin-walled oocysts were not observed in HFL cells in the present study. Most of the thick-walled oocysts that developed in HFL cells remained within parasitophorous vacuoles; only a small proportion were released into the culture medium.

Reinitiation of merogonous development by sporozoites released from autoinfective oocysts and recycling of type I meronts (*10, 13, 14*) are features of the life cycle of *Cryptosporidium* that may explain why a small number of oocysts are capable of producing overwhelming infections in succeptible animals and man (*8*). These features may also explain why immunodeficient individuals may develop persistent, life-threatening intestinal infections in the absence of repeated oral exposure to oocysts (*3*), and why very heavy *Cryptosporidium* infestations can develop in the respiratory (*6*) and biliary (*7*) tracts of such patients.

In HFL cells, it appears that sporozoites develop into mature type I meronts within 16 hours (Table 1). The presence of type I meronts through 96 hours after inoculation is most likely due to a recycling of one or both asexual stages since sporozoites were not seen beyond 8 hours and no apparent autoinfective cycle was observed. Recycling of type I meronts has been reported in experimentally infected mice (*3, 13, 14*) and chicken embryos (*10*).

Since human and calf isolates of *Cryptosporidium* exhibit little or no host specificity (*1, 3*), it was not surprising that PK-10 and PCK cells also supported complete development (from sporozoite to sporulated oocysts) of the human isolate. However, the number of parasites developing in these two cell types was smaller than those in HFL cell cultures.

We are aware of only one other coccidian species, *Eimeria tenella*, which has been reported to complete its entire developmental cycle (from sporozoites to unsporulated oocyst) in cell culture [see (*15*) for review]. Unlike *E. tenella*, however, oocysts of *Cryptosporidium* complete sporogonous development within the host cells grown in vitro. Not only were the in vitro derived oocysts of *Cryptosporidium* sporulated by morphologic criteria, but they were also infective to mice.

Cryptosporidiosis is most ominous in its effects on morbidity and its contributions to mortality in patients with AIDS (*16*). No effective therapy is available to eradicate this agent once it becomes established in an immunodeficient individual (*5*). The growth of *Cryptosporidium* in cell culture not only provides a means of studying its behavior, development, and metabolism but it also provides a mechanism for rapid evaluation of potentially useful therapeutic agents.

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Suppression of Prolactin in Pigs by *Escherichia coli* Endotoxin

Abstract. *An endotoxin produced by Escherichia coli caused a decrease in prolactin concentrations in the plasma of sows when given at low dosages 2 days postpartum. Five to tenfold increases occurred in the plasma cortisol concentrations. Piglet growth, used as an indicator of milk secretion by the sows, was significantly depressed after the endotoxin administration. Some cases of lactation failure in the periparturient sow may thus be due to endotoxins suppressing prolactin concentrations. This appears to be the first report of a bacterial endotoxin having an effect on prolactin in any species.*

Insufficient milk production by sows and the resultant malnourishment of piglets may be directly responsible for between 6 and 17 percent of all preweaning deaths in commercial pig production facilities and represents a multimillion dollar annual loss to the American pork industry (*1, 2*). Malnourishment also contributes to the neonatal susceptibility of piglets to transmissible gastroenteritis, *Escherichia coli* enteritis, and crushing by the sow, the primary causes of death in the neonatal pig (*2*). Sows with insufficient milk frequently show one or more of the following clinical signs: pyrexia, anorexia, leukopenia, lethargy, mastitis, metritis (infrequently), and blanching of the mammary glands (*3*).

Sows with agalactia frequently have clinical signs of mastitis, which in many instances is due to Gram-negative bacteria, including *E. coli* (*4*). When placed in the mammary gland or uterus of postpartum sows, *E. coli* endotoxin is readily absorbed and can be assayed in the blood (*5*). Prolactin concentrations in the plasma of agalactic animals is reduced (*6*), and treatment of sows with *E. coli* endotoxin produces signs typical of natural cases of hypogalactia (*7*).

For the experiments described here we used normal sows of Yorkshire/Lan

Table 1. Changes in mean piglet weight (grams per hour per piglet) for days 1 to 3 postpartum divided into three intervals per day (2400 to 0800 hours, 0800 to 1600 hours, and 1600 to 2400 hours). Analysis of variance for each interval on day 2 revealed no statistically significant difference between groups ($P = 0.05$). Data are expressed as means (standard error) ($N = 3$ for each group at each dose of endotoxin).

Dose of endotoxin (mg)	Day 1 postpartum			Day 2 postpartum			Day 3 postpartum		
	1	2	3	1	2	3	1	2	3
4	2.0 (1.2)	2.5 (1.2)	5.2 (0.4)	5.5 (2.3)	−1.5* (1.6)	6.3 (0.4)	5.1 (0.6)	2.5 (0.7)	9.2 (1.6)
8	4.6 (0.6)	2.2 (1.0)	5.3 (0.3)	6.2 (0.7)	−3.1* (1.0)	3.8 (0.6)	5.9 (0.7)	2.9 (0.9)	10.2 (1.1)
16	6.4 (0.6)	1.1 (0.4)	8.7 (1.9)	5.0 (1.0)	−3.2* (1.1)	7.1 (1.7)	5.3 (1.4)	2.4 (1.6)	10.2 (0.5)

*Value significantly different from corresponding period for groups 1 and 3 postpartum ($P < 0.025$, $P < 0.002$, and $P < 0.03$ for the groups receiving 4, 8, and 16 mg of endotoxin, respectively).