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DEVELOPMENTAL BIOLOGY OF *CYSTOISOSPORA* (APICOMPLEXA: SARCOCYSTIDAE) MONOZOIC TISSUE CYSTS

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ABSTRACT: Tissue cyst stages are an intriguing aspect of the developmental cycle and transmission of species of Sarcocystidae. Tissue-cyst stages of *Toxoplasma*, *Hammondia*, *Neospora*, *Besnoitia*, and *Sarcocystis* contain many infectious stages (bradyzoites). The tissue cyst stage of *Cystoisospora* (syn. *Isospora*) possesses only 1 infectious stage (zoite), and is therefore referred to as a monozoic tissue cyst (MZTC). No tissue cyst stages are presently known for members of *Nephroisospora*. The present report examines the developmental biology of MZTC stages of *Cystoisospora* Frenkel, 1977. These parasites cause intestinal coccidiosis in cats, dogs, pigs, and humans. The MZTC stages of *C. belli* are believed to be associated with reoccurrence of clinical disease in humans.

Frenkel (1977) erected the genus *Cystoisospora* for mammalian coccidia in the genus *Isospora* with characteristics that include oocysts containing 2 sporocysts with 4 sporozoites each, no Stieda bodies in sporocysts, and the ability to produce monozoic tissue cysts (MZTC) in intermediate or paratenic hosts. He designated *Cystoisospora felis* as the type species. Molecular studies support the placement of mammalian *Cystoisospora* species in Sarcocystidae (Carreno et al., 1998; Franzen et al., 2000; Barta et al., 2001, 2005).

Frenkel (1977) came to this conclusion because 2 yr after the life cycle of *Toxoplasma gondii* in cats was described (Frenkel et al., 1970), he and Dubey (Frenkel and Dubey, 1972) demonstrated the occurrence of tissue cyst stages of 2 other intestinal coccidia of cats, *C. felis* and *Cystoisospora rivolta*, in rodent paratenic hosts. The production of oocysts by cats fed rodent tissues demonstrated that these feline coccidia also could have different hosts in their developmental cycles and that they were not strictly monoxenous. The tissues of mice, rats, and hamsters fed sporulated oocysts of these parasites contained MZTC stages that were able to produce patent infections in kittens fed infected rodent tissues (Figs. 1, 2). That same year, Dubey and Frenkel (1972) demonstrated that extraintestinal (EIN) stages would occur in the tissues of cats (true definitive hosts) fed oocysts of *C. felis* or *C. rivolta* oocysts (Fig. 2). These EIN stages (Fig. 2C, D) were structurally similar to the asexual stages present in the intestines of cats and they could be found in groups of 2–15 zoites (Dubey and Frenkel, 1972). Shortly after these findings in cats, it was demonstrated that intestinal *Cystoisospora* species of dogs could also produce MZTC stages in rodents (Dubey, 1975, 1978a, 1978b; Dubey and Mehlhorn, 1978; Rommel and Zielasko, 1981) and that patent infections would occur in dogs fed the tissues of infected rodents.

In 1987, MZTC stages (Fig. 3) were reported in the mesenteric and tracheo-bronchial lymph nodes of a human patient with intestinal *Cystoisospora belli* infection who had developed acquired immune deficiency syndrome (Restrepo et al., 1987). These MZTC stages were similar to MZTC stages of feline and canine *Cystoisospora* that occur in paratenic hosts. These MZTC are numerous in AIDS patients infected with *C. belli* and only 1 zoite is found in each MZTC. Mitchell et al. (2009) subsequently described the production of MZTC stages of *Cystoisospora canis* in cell culture (Fig. 4) and demonstrated that they were similar in ultrastructure to stages that develop in rodent hosts. The present report summarizes our current understanding of the biology of the MZTC stages in species of *Cystoisospora*.

MONOZOIC TISSUE CYST STAGES IN PARATENIC HOSTS

Development

In the original description of rodents as vectors for feline *Cystoisospora* species division by endodyogeny was reportedly observed (see fig. 1E, Frenkel and Dubey, 1972). If this is the true situation, it appears to be an infrequent occurrence because division by endodyogeny has not been reported since then for *C. felis* or *C. rivolta*. It also has not been reported to occur in the canine *Cystoisospora* MZTC stages examined to date in mouse tissues (Frenkel and Smith, 2003). The formation of MZTC stages is a defining characteristic in species of *Cystoisospora* (Frenkel, 1977). No division has been observed in a detailed study of *C. rivolta* MZTC stage development in mice (Dubey, 1979). We now believe that the 2 zoites likely entered the same host cell, rather than division of 1 zoite, mistakenly interpreted as endodyogeny by Frenkel and Dubey (1972).

Dubey (1979) demonstrated that sporulated oocysts of *C. rivolta* would excyst and the liberated sporozoites most frequently invaded the mesenteric lymph nodes (MLN) of mice, which remained infected for at least 23 mo. He also demonstrated that organisms in MZTC increased in size for the first 31 days and MZTC could be found in the spleen, liver, and skeletal muscle of mice (Dubey, 1979). Dubey (1979) was not able to transmit *C. rivolta* MZTC from mouse to mouse orally or by intraperitoneal inoculation of infected tissues. This is apparently the only attempt to demonstrate oral infectivity of MZTC of *Cystoisospora* species for paratenic hosts.

Dubey (1975) used bioassay to demonstrate that mice were a paratenic host for *C. canis*. Dogs fed 1×10^5 *C. canis* oocysts or MZTC stages in the tissues from mice fed 1×10^5 oocysts produced similar numbers of oocysts and had a similar prepatent period. Cats were shown to develop tissue infection with *C. canis* with the use of bioassay of their tissues (Dubey, 1975). The structure of these orally infective stages of *C. canis* in cat tissue is unknown.

The appearance of MZTC stages of *C. felis*, *C. rivolta*, *C. canis*, and *Cystoisospora ohioensis* in experimentally infected rodents are similar (Mehlhorn and Markus, 1976; Dubey and Mehlhorn, 1978; Boch et al., 1981; Markus, 1983). A MZTC contains a single organism (Fig. 1) that resembles a sporozoite (Roberts et al., 1972) with a nucleus, 1 or occasionally 2 crystalloid bodies,

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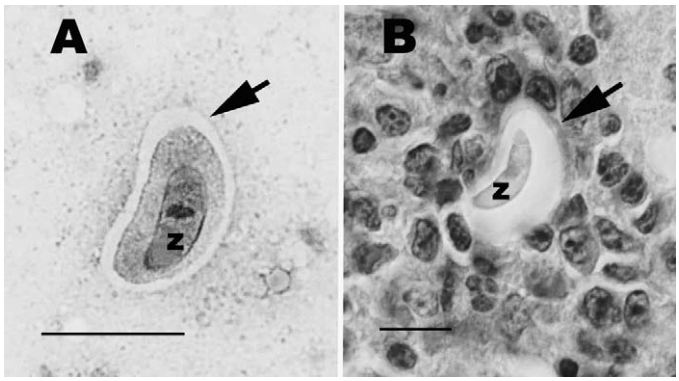

FIGURE 1. Developmental stages of *Cystoisospora felis* in the mesenteric lymph node of a mouse infected 8 wk previously with oocysts. (**A**) Giemsa-stained impression smear of a monozoic tissue cyst demonstrating a nonstaining wall (arrow), the parasitophorous vacuole, and the zoite (z). Bar = 10 μm. (**B**) Hematoxylin and eosin–stained tissue section demonstrating a MZTC of *C. felis*. The arrow points to the tissue cyst wall that encloses the zoite (z). Bar = 10 μm.

amylopectin granules, micronemes, dense bodies, rhoptries, and a conoid. Frenkel and Dubey (1972) used the term *zoite* to describe this stage. Each zoite is in a parasitophorous vacuole (PV) that is composed of a PV membrane lined with various amounts of granular material making up the tissue cyst wall (Fig. 1). Monozoic tissue cysts of *C. felis*, *C. rivolta*, and *C. ohioensis* increase in size over time, but division does not occur (Frenkel and Dubey, 1972; Dubey and Mehlhorn, 1978).

Differences in the light microscopic appearance of MZTC stages present in paratenic hosts may vary depending upon the preparation being examined. In impression smears fixed in methanol and stained with Giemsa, the tissue cyst wall appears unstained, whereas the contents of the parasitophorous vacuole and the zoite acquire the stain (Fig. 1A). In hematoxylin and eosin–stained sections, the tissue cyst wall are eosinophilic, as is the zoite, whereas the contents of the PV do not stain. Occasionally, a clear area will appear to enclose the MZTC, but this is likely an artifact of tissue fixation and histological processing (Fig. 2E). A tissue cyst wall surrounded only by host-cell cytoplasm and enclosing a single zoite is visible in MZTC stages grown in cell culture and examined nonfixed, with the use of differential contrast optics (Fig. 4A).

Bioassay studies demonstrating transmission of MZTC

Much of the data about transmission of *Cystoisospora* species in nonrodent paratenic or potential intermediate hosts are based on tissue-feeding studies. Most of these studies do not attempt to demonstrate or fail to demonstrate developmental stages in the source tissues fed to cats or dogs. Unfortunately, it is not possible to determine if MZTC or some other developmental stages were present and produced the patent infection in the definitive host.

Bioassays in cats have demonstrated that orally infectious stages of *C. felis* and *C. rivolta* can form in gerbils (*Meriones unguiculatus*), guinea pigs (*Cavia porcellus*), rabbits (*Orytolagus cuniculus*), and chickens (*Gallus gallus domesticus*) fed oocysts (see De Oliveira et al., 2007). Stages present in gerbil tissue survived exposure to acid–pepsin solution, demonstrating a similarity to tissue cysts of *T. gondii* (De Oliveira et al., 2007). Unfortunately, the authors only described the stages that had survived the acid–pepsin digestion treatment (De Oliveira et al., 2007) and it is unclear if they were MZTC or some other developmental stage. A rabbit fed oocysts of *C. ohioensis* had stages in its MLN 4 days after infection (Hilali et al., 1992); however, the description of the stage present in the rabbit MLN was vague and indicated that it was oval in shape and measured 10.5 by 6.7 μm. Studies in swine (*Sus scrofa*) and cattle (*Bos taurus*) indicate that these animals can be infected and harbor *C. felis* stages that are infectious for cats (Fayer and Frenkel, 1979; Wolters et al., 1980; Melo et al., 2003). Measurements of stages in swine tissues that were digested in acid–pepsin solution were presented in the study of Melo et al. (2003), but no description of the stages present in the predigested tissue was provided, making it impossible to determine if MZTC were present or if other asexual stages were responsible for initiating infections in the definitive host. Bioassay of naturally exposed camel (*Camelus dromedaries*) meat produced patent *C. canis* infections in dogs and bioassay of sheep (*Ovis aries*) meat resulted in *C. canis* and *C. ohioensis* infections in dogs (Hilali et al., 1992). These authors did not determine if MZTC were present in the tissues fed to dogs (Hilali et al., 1992).

Failure to demonstrate MZTC in paratenic hosts of *Cystoisospora suis* and *C. belli*

Neonatal coccidiosis caused by *C. suis* is an important disease of young nursing pigs born in confinement (Stuart et al., 1980). The structure of the oocyst stage (Lindsay et al., 1980), excystation (Pinckney et al., 1993), and endogenous life cycle stages (Lindsay et al., 1980) are consistent with those reported for species of *Cystoisospora*. Studies designed to determine if MZTC stages of *C. suis* are present in mice fed sporulated oocysts have all been negative (Stuart et al., 1982; Pinckney et al., 1993). The results of feeding tissues from mice orally fed *C. suis* to pigs were inconclusive, and MZTC stages were not observed in the tissues of mice fed *C. suis* oocysts (Stuart et al., 1982). Pinckney et al. (1993) were unable to demonstrate MZTC stages in outbred Swiss-Webster or inbred BALB/c mice. They attempted to immunosuppress outbred Swiss-Webster and inbred BALB/c mice with methylprednisolone acetate prior to exposure and were still not able to demonstrate infections (Pinckney et al., 1993).

Frenkel et al. (2003) were not able to demonstrate MZTC stages of *C. belli* in the MLN of a 28-day-old 50-kg calf fed 1×10^6 *C. belli* oocysts or in tissues of 3, 2-day-old chickens fed 3×10^4 *C. belli* oocysts. The *C. belli* oocysts were obtained from a 26-yr-old patient who died because of complications of AIDS. Examination of lymph nodes obtained at necropsy indicated that MZTC stages containing a single zoite were present in the patient and developmental stages of *C. belli* were observed in the bile duct epithelium (Frenkel et al., 2003). The previous authors suggested that humans may become infected by ingesting MZTC in undercooked tissue from paratenic hosts, and that this mode might be important in areas with good sanitation, preventing fecal–oral transmission.

OOCYST-INDUCED INFECTIONS COMPARED TO RODENT TISSUE CYST INDUCED INFECTIONS IN THE DEFINITIVE HOST

Dubey and Streitel (1976) compared the feeding of sporulated oocysts of *C. felis* and *C. rivolta* to cats by feeding the tissues

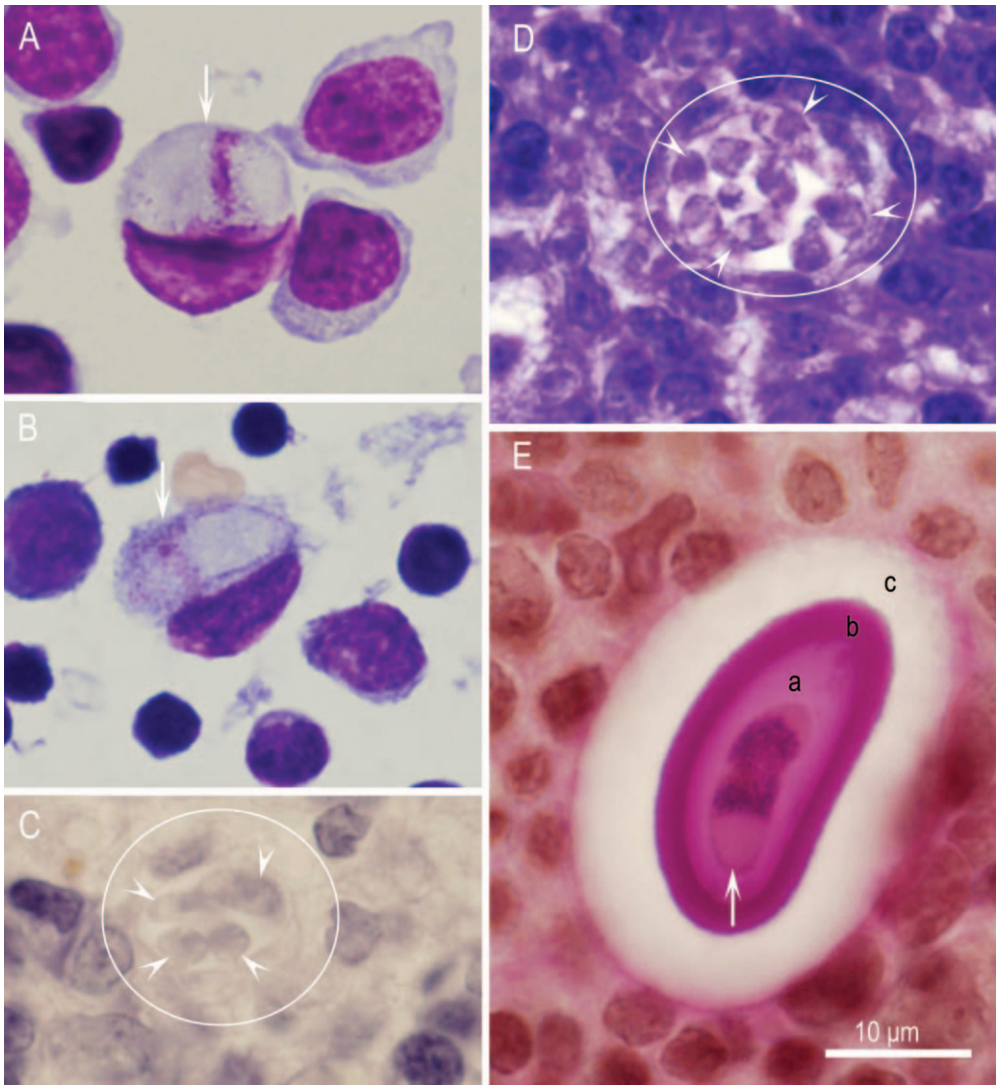

FIGURE 2. *Cystoisospora felis* stages in mesenteric lymph nodes of cats (**A–D**), and mouse (**E**) fed oocysts. A, B, and D stained with Giemsa, C and E periodic acid Schiff reaction counter stained with hematoxylin (PASH). Photos A and B touch smears; C, D, and E, histologic sections. A, B—day 2 postinoculation (PI); C, D—day 4 PI; E—23 mo PI. Bar applies to all figures. (A, B) Arrows point to zoites in lymphocytes. (C, D) Groups of zoites (arrowheads) in circled area. These organisms stained lighter than the host cells and sections had to be overstained to reveal these stages. (E) Monozoic tissue cyst. Arrow points to a sporozoite. Note bands of amylopectin stained red with PASH. The sporozoite is surrounded by lightly stained space (a), an intensely stained cyst wall (b), and a hollow space (c) surrounding the tissue cyst, perhaps an artifact of staining. However, these morphological features are constant. These preparations were made in 1971 by 1 of us (JPD) and photographed 42 yr later.

(containing MZTC stages) of mice fed similar numbers of oocysts of these parasites. They found that the prepatent period of *C. felis* was slightly shorter, but the prepatent period of *C. rivolta* was the same (Dubey and Streitel, 1976). They also found that the numbers of oocysts excreted by cats was similar regardless of the inoculum. Dubey (1975) found that infections produced by *C. canis* oocysts or tissues from *C. canis*–infected mice produced similar infections in dogs. These studies suggest that the efficacy of MZTC stages at producing intestinal infection is equivalent to oocysts at producing intestinal infection in the definitive host.

Studies using *C. ohioensis*–infected mice demonstrated that MZTC stages in paratenic hosts are potentially pathogenic for susceptible dogs (Dubey, 1978b). Four 17-day-old dogs were fed homogenates of MLN and spleen from mice fed *C. ohioensis* oocysts. Clinical signs and *C. ohioensis*–induced lesions were not observed in dogs examined 24 or 48 hr after infection. Diarrhea was observed in the remaining 2 dogs 3 days after they were fed infected mouse tissues (Dubey, 1978b). Microscopic lesions consisting of villous atrophy and necrosis of the intestinal epithelium were observed in the intestinal tissue sections of the dogs examined 72 and 96 hr after oral infection with mouse tissues containing MZTC stages. No stages were present in the MLN or spleen from these 4 dogs.

However, little is known about the MZTC stages of *Cystoisospora burrowsi* from dogs. Rommel and Zielasko (1981) reported that the prepatent period of *C. burrowsi* was similar in dogs fed oocysts (6–9 days) or tissues (7–11 days) from experimentally infected mice or rats.

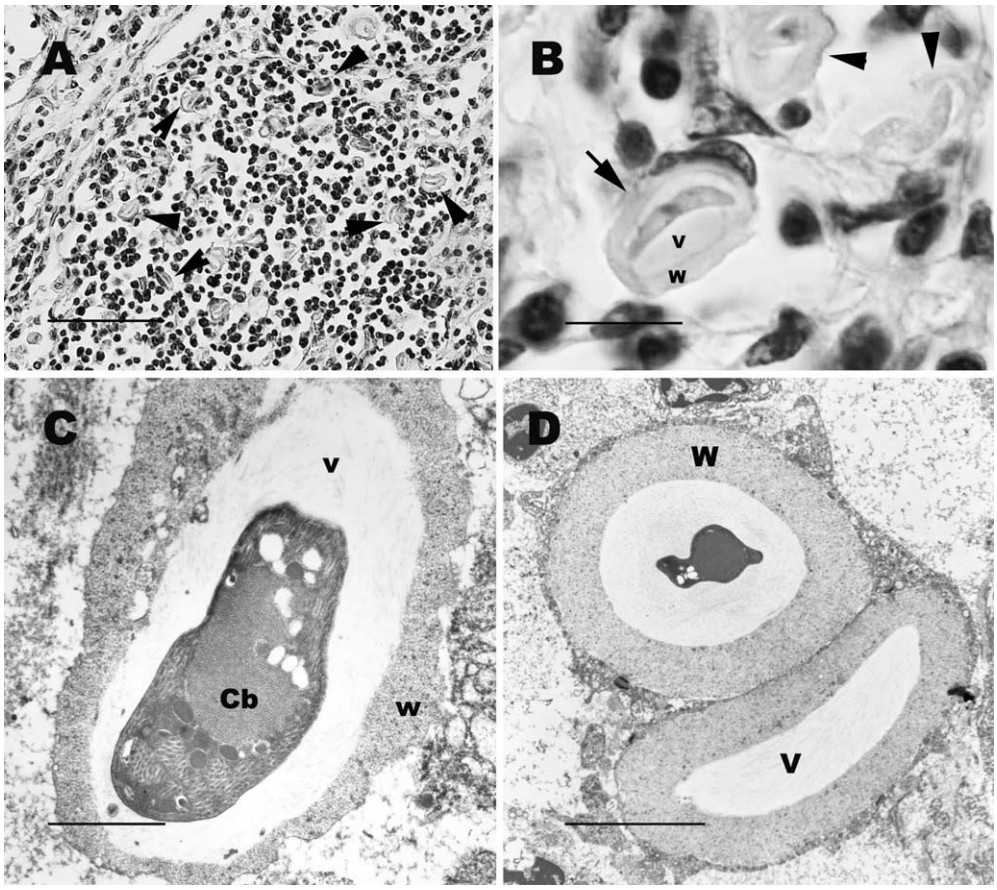

FIGURE 3. Light (**A, B**) and transmission electron (**C, D**) micrographs of monozoic tissue cysts (MZTC) of *Cystoisospora belli* in the mesenteric lymph node of a patient with AIDS. (A) Many MZTC (arrowheads) are present in this section. Hematoxylin and eosin stain. Bar = 50 μm. (B) A MZTC (arrow) enclosed in a tissue cyst wall (w). The zoite is in the parasitophorous vacuole (V). Portions of 2 other MZTC (arrowheads) are present. H&E stain. Bar = 10 μm. (C) Transmission electron micrograph of a MZTC containing zoite. The crystalloid body (Cb) of the zoite is visible. The MZTC wall (w) surrounds the single organism within the parasitophorous vacuole (v). Bar = 5 μm. (D) Transmission electron micrograph demonstrating 2 MZTC that are apparently in the same cell. A zoite can be seen surrounded by a tissue cyst wall (W) while a MZTC with no zoite in the vacuole (V) is adjacent to it. Bar = 5 μm.

DEVELOPMENT OF EXTRAINTESTINAL STAGES IN THE DEFINITIVE HOST

Dubey and Frenkel (1972) demonstrated that following oral inoculation of sporulated oocysts of *C. felis* that kittens would develop EIN infection (Fig. 2C, D) and have orally infectious asexual stages in the MLN (4 of 4 trials), liver and spleen mixture (3 of 5 trials), brain and muscle mixture (1 of 5 trials), and lungs (1 of 5 trials) based on bioassay. The prepatent period was shortened from 7–11 days after oral feeding of oocysts to 4–8 days after feeding of kitten tissues (Dubey and Frenkel, 1972). They found zoites in the intestine singly and in groups of 2–15 organisms in the MLN of 2 kittens infected for 2 or 4 days (Dubey and Frenkel, 1972). Development of *C. felis* in these locations was either by endodyogeny or longitudinal binary fission. The presence of MZTC of *C. felis* in cats was not demonstrated microscopically. Dubey (1975) demonstrated with the use of bioassay that oocysts of *C. felis* would produce infection in the tissues of dogs. He fed mixtures of MLN, liver, lung, and spleen from dogs infected for 111 days to cats, which produced *C. felis* oocysts with a prepatent period of 8 days (Dubey, 1975). Brain and skeletal muscle from *C. felis*–infected dogs were not infectious for cats. No MZTC or EIN stages were demonstrated in the tissues of these dogs.

Oral inoculation of sporulated oocysts of *C. rivolta* to kittens resulted in EIN infection in kittens based on positive bioassay. Orally infectious stages were in the MLN (1 of 5 trials), liver and spleen mixture (3 of 5 trials), brain, muscle, and lung mixture (0 of 5 trials). There was no difference in the prepatent period in kittens fed *C. rivolta* oocysts or those fed *C. rivolta* infected tissues. Zoites were observed singly or in pairs in the MLN of 3 of 10 kittens (Dubey and Frenkel, 1972). It is not known if MZTC or EIN are the stages that are orally infectious in these kittens.

Dubey (1978a) was not able to demonstrate MZTC or EIN stages of *C. ohioensis* in sections of MLN, spleen, lung, liver, heart, skeletal muscle, and brain from dogs fed oocysts. Bioassay of MLN and spleen in dogs indicated that infective EIN or MZTC stages were present in these locations.

These studies indicate that MZTC and EIN asexual stages can occur in feline or canine hosts after ingestion of *Cystoisospora* species oocysts for which they are a true definitive host. The importance of EIN asexual stages is not as readily apparent as is the value of producing MZTC in a paratenic host. The presence of EIN asexual stages in the definitive host may just represent stages

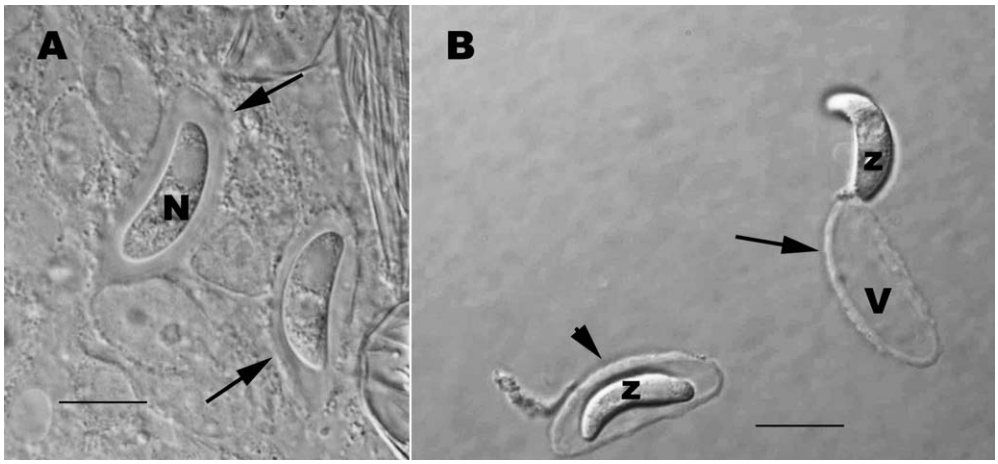

FIGURE 4. Monozoic tissue cysts of *Cystoisospora canis* 10 days postinfection in human-pigmented retinal epithelial cells viewed with differential contrast optics. (**A**) The MZTC consists of a zoite that is surrounded by a thick tissue cyst wall (arrows). The nucleus (N) of a zoite is labeled. Bar = 5 μm. (**B**) Isolated MZTC (arrowhead) containing a zoite (z) and a MZTC (arrow) that consists of a vacuole (V) surrounded by a tissue cyst wall and a zoite (z) that is undergoing egress. Bar = 5 μm.

that accidentally migrated out of the intestine and underwent subsequent development. This appears to be the case for some *Eimeria* species that can produce EIN asexual stages, but these stages are not orally infectious for a true definitive host (Lindsay et al., 1990).

In his original description of the genus *Cystoisospora*, Frenkel (1977) commented on the presence of an uncharacterized chronic stage in the intestine of the final host, leading to recurrent oocyst shedding. No new information on this developmental stage has been presented since 1977. The occurrence of massive numbers of MZTC in the MLN of AIDS patients (see below) indicates that this stage may be responsible for producing stages that eventually produce MZTC and are capable of returning to the intestinal epithelium and inducing a patent *C. belli* infection.

TISSUE CYSTS OF *C. BELLI* IN THE HUMAN HOST

Restrepo et al. (1987) provided the original description of disseminated *C. belli* infection due to MZTC in an AIDS patient. All additional cases of disseminated *C. belli* infection have been observed from individuals suffering from AIDS (Comin and Santucci, 1994; Michiels et al., 1994; Lindsay et al., 1997; Velásquez et al., 2001, 2011; Frenkel et al., 2003). Monozoic tissue cysts of *C. belli* in histological sections are thick walled and measure 12–22 × 8–10 μm in size, with each containing a single zoite. Ultrastructurally, a *C. belli* tissue cyst consists of a single centrally located zoite surrounded by a granular tissue cyst wall (Fig. 3) within a PV. Zoites contain all the cellular organelles characteristic for infective asexual stages (sporozoites, merozoites, tachyzoites, bradyzoites) of coccidial parasites (Lindsay et al., 1997; Mitchell et al., 2009). The tissue cyst wall is next to the limiting membrane of the PV. Recurrent clinical disease is common in both immunocompetent and immunosuppressed patients infected with *C. belli* and is believed to be due to reactivation of the zoites present in the MZTC and migration to the human intestinal tract (Lindsay et al., 1997; Velásquez et al., 2001). It is common for immunosuppressed patients to relapse with clinical disease once *C. belli* treatment has been stopped (Boyles et al., 2012).

Velásquez et al. (2011) reported that MZTC stages were present in the lamina propria of 2 of 7 AIDS patients that had *C. belli* developmental stages in their intestinal epithelial cells in biopsy material obtained from the distal duodenum. These authors used isolated DNA from the samples to amplify 2 fragments of the SSU-rRNA gene and the ITS-1 region. Following sequencing of the resulting amplicons, *C. belli* was identified in all cases regardless of the presence or absence of MZTC stages in the lamina propria (Velásquez et al., 2011).

The extremely large number of MZTC present in AIDS patients (Fig. 3A) is puzzling. It is unlikely that these stages are sporozoites because of the thousands of MZTC that can be observed in AIDS patients. The presence of crystalloid bodies in MZTC is a characteristic of *Cystoisospora* sp. sporozoites (Roberts et al., 1972). The merozoites of *C. suis* grown in cell culture retain their crystalloid bodies (Lindsay et al., 1991), suggesting that some merozoites of *Cystoisospora* sp. can retain crystalloid bodies. This is a reason for the authors continuing to use the term *zoite* for the stage in MZTC. It is likely that these MZTC are produced by asexual stages in the intestine that migrate to extraintestinal locations as originally suggested by Frenkel (1977). These stages of *C. belli* may be similar to those seen in cats experimentally infected with *C. felis* (see Fig. 2C, D).

TISSUE CYST STAGES OF *C. CANIS* AND DEVELOPMENT OF OTHER *CYSTOISOSPORA* IN CELL CULTURE

Mitchell (2009) demonstrated that an isolate of *C. canis* obtained from dogs from Virginia (Mitchell et al., 2007) would produce MZTC stages in bovine turbinate (BT) and African Green monkey kidney (CV-1) cell cultures. They observed infected cell cultures for 15 (BT cell) and 17 (CV-1 cells) days and found that no multiplication occurred in either host cell type. Intestinal coccidial parasites undergo more asexual generations and occasionally sexual development in host cells from the normal definitive host (Doran, 1982). Houk and Lindsay (2013) reported that an isolate of *C. canis* obtained from dogs from Brazil (Houk et al., 2013) produced MZTC stages in canine, human (Fig. 4A), monkey, and bovine cell lines. These findings are different from

development by endodyogeny that was reported to occur in the original study of in vitro development of *C. canis* by Fayer and Mahrt (1972). Madin-Darby canine kidney (MDCK) cells were used by both Fayer and Mahrt (1972) and Houk and Lindsay (2013). In the study by Fayer and Mahrt (1972), *C. canis* developed by endodyogeny in MDCK cells, whereas only MZTC stages were seen in MDCK cells in the study of Houk and Lindsay (2013).

Houk and Lindsay (2013) found that MZTC stages of *C. canis* would persist in MDCK cells for 2 mo and in human pigmented retinal epithelial (HRE) cells for 127 days. They determined that the zoites within MZTC stages could be stimulated with bile–trypsin solution to undergo egress (Fig. 4B) and that the zoites were able to penetrate additional host cells and produce new MZTC stages (Houk and Lindsay, 2013). They also demonstrated that the MZTC stages were resistant to acid–pepsin solution, suggesting that the tissue cysts were similar to those of *T. gondii*. These findings suggest that the MZTC stages would be orally infectious for dogs. Additional studies in dogs fed cell-culture produced MZTC stages are needed to confirm this speculation.

Monozoic tissue cysts were not observed in cell-culture studies conducted with sporozoites obtained from oocysts of *C. belli* (Oliveira-Silva et al., 2006), *C. felis* (Fayer and Thompson, 1974), *C. rivolta* (Fayer, 1972), or *C. suis* (Fayer et al., 1984; Lindsay and Blagburn, 1987; Lindsay et al., 1991, 1998; Worliczek et al., 2013). *Cystoisospora suis* has been manipulated with the use of selection of appropriate host cell lines to undergo complete development in swine testicle cells (Lindsay et al., 1998) and porcine intestinal epithelial cells (Worliczek et al., 2013). It is clear that the development of mammalian *Cystoisospora* species in cell culture needs to be re-examined to answer the questions raised by the differences observed in these in vitro studies.

FURTHER RESEARCH

Much additional research needs to be conducted before we can unravel the complex life cycle and epidemiology of this group of coccidial parasites. The developmental cycle in the definitive host needs to be critically re-evaluated to determine the importance of EIN stages and to search for MZTC tissue cysts in the definitive host. The developmental cycles were originally described in immune-competent hosts, and it is not known how immune suppression effects development in the definitive host. It is possible that developmental stages previously overlooked because of being present in low numbers in immune-competent definitive hosts may be more numerous and apparent in immune-suppressed definitive hosts. These types of studies may lead us to better understand *C. belli* infection in AIDS patients and the mechanism behind reoccurrence of disease.

The development of *Cystoisospora* species in cell culture needs to be further evaluated. Most early reports on the development of feline, canine, and porcine coccidia in cell culture indicated that development was limited to division by endodyogeny. However, *C. suis* has been shown to complete its entire life cycle in cell culture if the appropriate host cells are employed (Lindsay et al., 1998; Worliczek et al., 2013). It is also unclear why the original description of development of *C. canis* in cell culture was limited to asexual division by endodyogeny (Fayer and Thompson, 1974), although studies with 2 different isolates of *C. canis* have not demonstrated endodyogeny but rather the production of MZTC (Mitchell et al., 2009; Houk and Lindsay, 2013).

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