Examination of Extraintestinal Tissue Cysts of Isospora belli
Author(s): David S. Lindsay, J. P. Dubey, M. A. Toivio-Kinnucan, J. F. Michiels and Byron L. Blagburn
Published by: The American Society of Parasitologists
Stable URL: http://www.jstor.org/stable/3284235
Accessed: 19/06/2014 10:41
EXAMINATION OF EXTRATEXTINAL TISSUE CYSTS OF *ISOSPORA BELLI*

David S. Lindsay*, J. P. Dubey†, M. A. Toivio-Kinnucan, J. F. Michiels, and Byron L. Blagburn

Department of Pathobiology, College of Veterinary Medicine, 166 Greene Hall, Auburn University, Auburn, Alabama 36849-5519

ABSTRACT: Relapse is common in immunocompetent and immunosuppressed humans infected with *Isospora belli* and is believed to be associated with the presence of extraintestinal stages. In the present study, we examined this important stage in an AIDS patient using histological, immunohistological, histochemical, and ultrastructural methods to better understand the development and structure of this stage and to develop better means of detecting infections. Antiserum made in rabbits to *Isospora suis*, *Toxoplasma gondii*, *Hammondia hammondi*, *Sarcocystis neurona*, *Neospora caninum*, and *Caryospora bigenetica* were tested against *I. belli* tissue cysts in the avidin–biotin peroxidase complex (ABC) immunohistological test. Most antiserum reacted positively in the ABC test at dilutions of 1:1,000 but not at dilutions of 1:250. Some antiserum to *N. caninum* and *H. hammondi* reacted positively at dilutions of 1:1,000 in the ABC test. Most reactive antiserum stained the tissue cyst wall and not the enclosed zoite. Eight histochemical tests were examined and most were nonreactive with *I. belli* zoites or tissue cysts. Transmission electron microscopy revealed that the tissue cyst wall was composed of a membrane and was directly beneath the parasitophorous vacuole membrane. Zoites were in the center of the tissue cysts and were surrounded by fibrillar material that appeared to originate from the zoite surface. Tubulelike structures were present in the granular tissue cyst wall and in the fibrillar material that surrounded the zoite. Zoites contained a crystallloid body. New findings in the present study consisted of identifying what are probably early tissue cysts that lack a developed tissue cyst wall, demonstrating that more than 1 tissue cyst can occupy a host cell, describing the distribution of micronemes and the shedding of zoite membranes, and identifying tubular structures in the inner tissue cyst wall and inner compartment.

*Isospora belli* is a coccidial parasite that causes serious and sometimes fatal disease in humans. Infections are essentially cosmopolitan in distribution but are more common in tropical and subtropical regions, especially Haiti, Mexico, Brazil, El Salvador, tropical Africa, the Middle East, and Southeast Asia (see Lindsay et al., 1997). Symptoms of *I. belli* infection in immunocompetent patients include diarrhea, steatorrhea, headache, fever, malaise, abdominal pain, vomiting, dehydration, and weight loss, whereas infections in AIDS patients are similar but the diarrhea is very fluid and secretorylike and may lead to dehydration requiring hospitalization. Blood is not usually present in the feces. The disease is often chronic, with parasites present in the feces or biopsies for several months to years. Relapses of *I. belli* infection are common in both immunocompetent and AIDS patients.

Two reports of disseminated extraintestinal isosporiasis have been reported in patients with AIDS (Restrepo et al., 1987; Michiels et al., 1994). Tissue cysts were observed in sections of lymph nodes, spleen, and liver from these patients. These extraintestinal tissue cysts may represent a reservoir of developmental stages that can recolonize the intestine and cause recrudescence of clinical disease. The present study was done to examine tissue cysts from 1 of these patients using histological, immunohistological, histochemical, and ultrastructural methods in order to better define the structure and development of the cysts and to potentially identify improved methods of demonstrating this important stage in tissue sections.

MATERIALS AND METHODS

Material from the case reported by Michiels et al. (1994) was obtained and used in the present study.

Received 4 December 1996; revised 25 February 1997; accepted 25 February 1997.
* Present address: Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional, College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia 24061.
† U.S. Department of Agriculture, Agricultural Research Service, Parasite Biology and Epidemiology Laboratory, BARC-East, Beltsville, Maryland 20705-2350.
§ Laboratoire d’Anatomie Pathologique, Centre Hospitalier, Universitaire de Nice, France.

Histological examination

A portion of spleen embedded in paraffin was used as the source of histological sections for histological, immunohistological, and histochemical examinations. The lengths and widths of 50 zoites and widths of 50 tissue cyst walls in hematoxylin and eosin (H&E)-stained sections were determined using oil immersion and a calibrated ocular micrometer. One thousand tissue cysts were examined under oil immersion, and the number of zoites present in each tissue cyst was determined. Paraffin-embedded sections stained only with hematoxylin were obtained as a result of procedural controls for immunohistological testing (see below). Toluidine blue-stained plastic-embedded sections obtained from tissue embedded for transmission electron microscopy (TEM) were also examined.

Immunohistological examination

The avidin–biotin peroxidase immunoperoxidase complex (ABC) test was used to localize bound antibodies in tissue sections. The test was done as previously described (Lindsay and Dubey, 1989) except that tissues were exposed to 0.1% (w/v) trypsin in calcium and magnesium-free Hank’s balanced salt solution for 15 min to help unmask epitopes. Antiserum were incubated for 1 hr at room temperature with tissue sections in a humidified box. Tissue sections were counterstained with hematoxylin.

Procedural controls were omission of antiserum, omission of the biotinylated secondary antibody, and omission of the ABC. Tissue sections containing parasites of the appropriate species were used as positive controls for antiserum staining. Preimmune serum at a 1:100 dilution was also examined when available.

Rabbits were immunized with sporozoites/oocysts of *Isospora suis* (1 rabbit), oocysts/sporozoites of *Caryospora bigenetica* (1 rabbit), *mero*zoites of *Sarcocystis neurona* (1 rabbit), oocysts/sporozoites of *Toxoplasma gondii* (2 rabbits), oocysts/sporozoites of *Hammondia hammondi* (2 rabbits), and tachyzoites of *Neospora caninum* (4 rabbits) (see Dubey et al., 1990, 1996; Pinckney et al., 1993).

Histochemical examination

Histochemical tests used were Gridley’s fungal stain, silver stains (Bodin’s, Wilder’s, Grocott’s methamine), Ziehl-Neelsen’s acid-fast stain, Brown and Hopp’s Gram stain, periodic acid–Schiff’s (PAS) reaction, and Gomori’s 1 step trichrome stain. The tests were done using routine histochemical procedures (Luna, 1992). Appropriate control tissue sections were used as positive controls. *Toxoplasma gondii* tissue cysts were also used as controls for silver stains and the PAS reaction.

Ultrastructural examination

A portion of mesenteric lymph node fixed in 2.5% buffered glutaraldehyde and processed routinely for TEM was used. Thin sections were
stained with uranyl acetate and lead citrate and examined in a Philips 301 transmission electron microscope operating at 60 kV.

RESULTS

Histological findings

Numerous tissue cysts were present in the spleen (Fig. 1), and infected host cells appeared to be macrophages. Most infected cells contained 1 tissue cyst, but cells with up to 4 tissue cysts were observed occasionally. No multinucleate stages were observed. No sexual stages or oocysts were observed. Fifty zoites were 10.0–14.0 × 1.5–4.0 μm (mean ± SD, 12.2 ± 0.9 × 2.5 ± 0.5 μm). Fifty tissue cyst walls were 1.5–4.0 μm thick (2.1 ± 0.4 μm). Most zoites were slightly crescent-shaped. The surface of zoites occasionally appeared to contain grooves or small projections. These features were most evident when the zoites were optically sectioned. Zoites were located in the center of the tissue cyst, and most contained a homogenous, pale eosinophilic area of cytoplasm posterior to the nucleus in H&E-stained sections. A similar area of pale blue staining was observed in the cytoplasm of zoites in sections stained only with hematoxylin or toluidine blue (Fig. 2). The nucleus of most zoites was condensed, was located posteriorly or centrally in the zoite, and was more apparent in sections stained only with hematoxylin. The tissue cyst wall was lightly eosinophilic in H&E and pale blue in hematoxylin or toluidine blue stained sections. Single zoites were observed in 994 (99.4%) of the 1,000 tissue cysts. In the remaining 6 (0.6%) tissue cysts, it was not possible to determine if 1 or 2 zoites were present.

Immunohistological findings

Procedural controls demonstrated that none of the nonserum reagents caused positive reactivity in the ABC test. Preimmune sera to I. suis, C. bigeneta, T. gondii (rabbit no. 1), and N. caninum (rabbit no. 4) did not react in the ABC test. No other preimmune serum was examined. All antisera reacted positively with their appropriate parasite control sections. Rabbit anti-C. bigeneta and rabbit anti-N. caninum (rabbit no. 4) sera were not reactive with I. bellii tissue cysts at a 1:100 dilution. Rabbit anti-S. neumora antiserum reacted variably with zoites and positively with the tissue cyst wall at a 1:100 dilution but was negative at a 1:250 dilution. Reactivities of the other antisera

<table>
<thead>
<tr>
<th>Parasite antiserum</th>
<th>Serum dilution</th>
<th>Zoite reactivity</th>
<th>Tissue cyst wall reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isospora suis</td>
<td>1:100</td>
<td>Variable</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:250</td>
<td>Negative</td>
<td>Variable</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>Negative</td>
<td>Variable</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td>No. 1</td>
<td>1:100</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:250</td>
<td>Negative</td>
<td>Variable</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:2,000</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Neospora caninum</td>
<td>No. 1</td>
<td>1:100</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:250</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>Variable</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>Variable</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:2,000</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>No. 2</td>
<td>1:100</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>No. 3</td>
<td>1:100</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hammondia hammondi</td>
<td>No. 1</td>
<td>1:100</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>Variable</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>Variable</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:2,000</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>No. 2</td>
<td>1:100</td>
<td>Variable</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>
examined are presented in Table 1. In general, reactivity to the tissue cyst wall was observed more often than reactivity to the zoite (Fig. 3). Reactivity to zoites was often variable, with some being positive and others being negative.

**Histochroscopic examinations**

No reactivity was observed with Gridley’s fungus stain, Grocott’s methenamine silver stain, Bodin’s silver stain, Wilder’s silver stain, Brown and Hopp’s Gram stain, or Ziehl–Neelsen’s acid-fast stain. The tissue cyst wall of *T. gondii* stained positive by Bodin’s silver stain but was negative by Wilder’s and Grocott’s methenamine silver stains, indicating that the stains performed as expected. Zoites of *I. belli* contained several PAS-positive granules, but the tissue cyst wall was negative. Tissue cysts of *T. gondii* were PAS positive as expected. *Isospora belli* zoites stained red and the tissue cyst wall was light green with Gomori’s 1-step trichrome stain.

**Ultrastructural findings**

Forty distinct tissue cysts were examined from photomicrographs (Figs. 4–8). No multinucleate stage or tissue cyst with more than 1 zoite was observed. Two of the 40 tissue cysts photographed appeared to be immature (Fig. 4) because the granular material of the tissue cyst wall was not as thick and dense as that observed in the other tissue cysts. On 1 occasion, 2 tissue cysts were observed in single host cell (Fig. 5). The tissue cyst wall was immediately beneath the parasitophorous vacuole (PV) membrane and was composed of granular material that contained randomly arranged electron-dense bodies and tubular structures. The tubular structures were often clearly evident at the internal surface of the tissue cyst wall and were also present in the lumen of the tissue cyst admixed with fibrillar material that surrounded the centrally located zoite (Figs. 5, 8). The fibrillar material appeared to originate from the surface of the zoite (Fig. 7). Zoites were in the central portion of the tissue cyst. Several zoites appeared to have grooves or projections in their pellicles (Figs. 6, 8). Rhoptries were electron dense and extended to the level of the nucleolus. The numbers of rhoptries present could not be determined. Dense bodies were present anteriorly to the nucleus and rarely posteriorly. Micronemes were numerous and were located both anteriorly and posteriorly to the nucleus. Zoite mitochondria were usually swollen and vacuolated (fixation artifact). Numerous polysaccharidelike and lipidlike granules were present in the zoite cytoplasm; they were concentrated slightly more in the anterior region of the zoite. Micropores were not observed in zoites.

**DISCUSSION**

In the early 1970s, extraintestinal stages of feline and canine *Isospora* species were found in cats and dogs (Dubey and Frenkel, 1972; Dubey, 1975, 1978, 1979). These stages probably were similar to those of *I. belli* observed in the present study; however, these extraintestinal stages in dogs and cats have not been examined extensively because only rarely are they present in tissue sections, and most information has come from tissue-feeding studies.

The large numbers of tissue cysts observed in the present study provided a fortuitous sample for the examination of these stages in the definitive host. After examination of 1,000 tissue cysts, we determined that only 1 zoite was present in tissue cysts of *I. belli*, and this is in agreement with reports for the canine and feline *Isospora* species. The apparent grooves or projections of the zoite surface that we observed may be mistaken for additional zoites if close observation under oil immersion is not used. This may explain the reports of 2 to 3 zoites in tissue cysts of *I. belli* by Restrepo et al. (1987); however, we examined the material obtained from the same case as Michiels et al. (1994), and the difference may result from *I. belli* strain differences or other parasite/host factors. Michiels et al. (1994) reported that *I. belli* zoites contained a posteriorly located refractile body. No typical highly eosinophilic coccidial refractile body was observed in *I. belli* zoites in the present study. However, we did observe a pale eosinophilic area by H&E stain and lightly blue staining area in hematoxylin or toluidine blue stained sections of *I. belli* zoites (Fig. 2). This is the area referred to by Michiels et al. (1994), and the authors misidentified the pale area as a refractile body.

The large numbers of *I. belli* tissue cysts present in our study make it difficult to conceive that the zoites in the tissue cysts are sporozoites that originated from ingested oocysts. It is more likely that they represent merozoites that are produced by a meront in the intestine or an unidentified extraintestinal site. The presence of a crystallloid body in the zoites does not conclusively demonstrate that they are sporozoites (Lindsay, Blagburn, and Toivio-Kinnucan, 1991; Pinckney et al., 1993) because crystallloid bodies are also present in *I. suis* merozoites (Lindsay, Blagburn, and Toivio-Kinnucan, 1991) and therefore, possibly, in other *Isospora* species merozoites.

We did not observe sexual stages or oocysts in our samples. Asexual and sexual stages and oocysts of *I. belli* have been observed in the bile duct epithelium of an AIDS patient with acalculous cholecystitis (Benator et al., 1994). No lymph nodes were examined in this patient, and the relationship between bile duct infections and disseminated infections with tissue cysts is presently not known.

Results of our immunohistochemical studies were unexpected because several genera cross-reacted with *I. belli* tissue cysts.
FIGURES 7, 8. Transmission electron micrographs of tissue cysts of *Isospora belli* in the mesenteric lymph node of an AIDS patient. 7. Zoite that demonstrates a rhoptry (arrow), crystallloid body (CB), dense bodies (D), and numerous micronemes (arrowheads) anterior and posterior to the crystallloid body. Fibrillar material is present around the zoite and appears to be associated with its surface (open arrow). Bar = 1 μm. 8. Longitudinal section of a tissue cyst and zoite. Note the thick tissue cyst wall (CW) that is beneath the parasitophorous vacuole membrane (open arrows). The zoite is surrounded by fibrillar material, and tubules (arrowheads) are present in the central portion and at the inner surface of the tissue cyst wall. An anvil-like projection (arrow) is on the surface of the zoite, which also contains a nucleus (N) and crystallloid body (CB). Bar = 1 μm.
Preimmune sera studies and procedural controls indicate that the reactivity is not nonspecific binding of antibodies. Because of the within-genera variability, no clear-cut aid in diagnosis or stage localization was found with the various antisera examined. The greater frequency of reactivity to the tissue cyst walls by the various antisera over reactivity to the enclosed zoites suggests that a preserved, possibly apical complex-associated or surface-associated antigen is being recognized. Apical complex-associated and surface-associated antigens of other coccidia have been shown to be conserved across different genera (Lindsay et al., 1989; Lindsay, Dubey, and Fayer, 1991; Herzenberg et al., 1995). The formation of the I. belli tissue cyst wall is most likely a complex process involving many parasite induced and host cell-stimulated responses. Our findings suggest a role for excreted/secreted or shed zoite products in I. belli tissue cyst wall formation.

The results of most of the histochemical stains (Gridley’s fungus stain, Grocott’s methamine silver stain, Bodin’s silver stain, Wilder’s silver stain, Brown and Hopp’s Gram stain, or Ziehl-Neelsen’s acid-fast stain) were unremarkable and provide no diagnostic or localization value. Restrepo et al. (1987) indicated that the tissue cyst wall of I. belli was PAS positive. In the present study, and the study by Michiels et al. (1994), PAS-positive granules were observed in zoites, but the tissue cyst wall was PAS negative. Differences in the procedures used to conduct the PAS staining procedure may be the reason for these differences in PAS reactivity. The red staining of I. belli zoites and light green staining of the tissue cyst wall with Gomori’s 1-step trichrome stain may aid in locating tissue cysts in future studies.

Results of our TEM studies expand the description of I. belli zoites and tissue cysts reported by Restrepo et al. (1987) and Michiels et al. (1994). Conin and Santucci (1994: fig. 6) illustrated a TEM photomicrograph that is an I. belli tissue cyst in a mesenteric lymphatic channel but erroneously labeled it a merozoite; it is similar to I. belli tissue cysts observed in extraintestinal tissues in the present and previous studies (Restrepo et al., 1987; Michiels et al., 1994) of I. belli tissue cysts. New findings in the present study consisted of identifying what are probably early tissue cysts that lack a developed tissue cyst wall, demonstrating that more than 1 tissue cyst can occupy a host cell, describing the distribution of micronemes and the shedding of zoite membranes, and identifying tubular structures in the inner tissue cyst wall and inner compartment. Restrepo et al. (1987) indicated that the crystallloid body of zoites was anterior (rostral) to the nucleus. However, their micrograph (Restrepo et al., 1987; fig. 6) depicts a zoite with a posteriorly located crystallloid body. Numerous micronemes were present in the posterior portion of the zoite and probably were mistaken for rhoptries.

The cause of relapse in I. belli infection in immunocompetent and AIDS patients is probably the extraintestinal tissue cysts. The zoites within extraintestinal tissue cysts are most likely dormant because they are not susceptible to anticoccidial treatment. This situation is similar to that observed in toxoplastic encephalitis in AIDS patients, where relapse is common and is caused by the dormant tissue cyst stage with Bradyzoites (Luft and Remington, 1988). Presently, no model for relapsing isosporosis exists in any mammalian species.

**LITERATURE CITED**


