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Analysis of Oocyst Wall and Sporozoite Antigens from Three *Cryptosporidium* Species

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A comparison was made of the antigenic composition of oocyst walls and sporozoites from *Cryptosporidium baileyi* from turkeys, *C. muris* from rodents, and *C. parvum* from ruminants, employing immunoblotting and immunofluorescence. In immunoblotting, oocyst antigens were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) and detected with rabbit polyclonal anti-*C. muris* or -*C. parvum* antibodies or murine monoclonal antibodies developed against *C. parvum*. Immunofluorescence was used to investigate the reactivity of these monoclonal antibodies with air-dried excystation mixtures of sporozoites and oocysts of the different species. The results from both types of experiment indicated that the three *Cryptosporidium* species could be differentiated immunologically. In comparison, few antigenic differences were found between a number of isolates of *C. parvum* in immunoblotting. There was also evidence to suggest that *C. parvum* and *C. baileyi* were more closely related antigenically to one another than to *C. muris*.

The coccidian genus *Cryptosporidium* is the causative agent of cryptosporidiosis, a disease found worldwide in a variety of vertebrates. Since the first report of the genus by Tyzzer (20), a large number of species have been recorded in the literature, each usually being distinguished by descriptive features including parasite morphology, site of infection, and host species of origin (6). Because little information is available on the biology of the parasite, including strain variation, it is not clear whether information on these features alone is sufficient to determine species of *Cryptosporidium*. Certainly, the inclusion of host species of origin as a factor in identifying "new" species has caused some confusion. It has often been assumed that *Cryptosporidium*, like many other coccidia, has strict host specificity, but recent evidence suggests that the genus has a relatively wide host range. *C. parvum* and *C. muris*, for example, are each able to infect diverse mammalian species, and the development of *C. baileyi* may occur in separate avian host species (6, 7, 21). The recent finding of disseminated *C. baileyi* infection in an AIDS patient (5) also suggests that immunosuppression of the host weakens any barrier to infection with cryptosporidia of other vertebrate classes.

The employment of techniques in biochemistry, immunology, and molecular genetics is likely to be helpful in the identification of *Cryptosporidium* species and strains and should be valuable in epidemiology and taxonomy.

In studies by earlier workers, *Cryptosporidium* species were differentiated by polypeptide composition of the oocyst wall (12, 19) and electrophoretic mobility of chromosomes (15). Antigen profiles of oocysts in Western blots (immunoblots) have been reported (9, 15), and significant differences were observed between the profiles of *C. parvum* and *C. baileyi* by using a polyclonal anti-*C. parvum* serum (2). Some antigenic diversity has also been observed between separate isolates of *C. parvum* (17, 18), and a sporozoite antigen of this species was found not to be conserved among different isolates (3).

In the present report, results are presented of a new comparative investigation of the antigenic composition of separate *Cryptosporidium* species. Oocyst antigens from three species, *C. baileyi*, *C. muris*, and *C. parvum*, were examined by Western blotting and immunofluorescence. Polyclonal antisera against two of the species or monoclonal antibodies (MAbs) developed against *C. parvum* were used for antigen detection. The results demonstrated that there was greater antigenic diversity between the three species of parasite than there was between isolates of a single species (*C. parvum*). There was also evidence that *C. parvum* and *C. baileyi* are more closely related antigenically to one another than to *C. muris*.

MATERIALS AND METHODS

Parasites. The MD isolate of *C. parvum*, originally isolated from a deer, was passaged in calves. This isolate was used to represent *C. parvum* in experiments to compare antigens of different *Cryptosporidium* species. Other isolates of this species were provided by clinicians or field veterinary workers. The RN 66 strain of *C. muris* (7) was passaged in C57BL/6 mice or BALB/c mice with severe combined immunodeficiency, and an isolate of *C. baileyi* was obtained from turkeys. Purified oocysts of each species were obtained by sucrose or salt flotation (7, 14). Excystation of sporozoites was obtained by incubation of oocysts at 37°C in RPMI 1640 medium with 0.4% bovine bile salts for 60 to 120 min. Bile salts were found not to be required for *C. muris*.

Preparation of antibodies against oocyst antigens. Polyclonal antibodies against *C. parvum* or *C. muris* antigens were produced in New Zealand White rabbits by three intramuscular injections, at intervals of 14 days, of 5×10^6 homogenized oocysts (13) of a human isolate of *C. parvum*

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or the RN 66 strain of *C. muris* incorporated in Freund's complete adjuvant. The serum was collected 10 days after the final injection of antigen.

Murine MAbs against the same human isolate of *C. parvum* were prepared from fusions between BALB/c spleen cells, following immunization of animals with disrupted oocysts, and SP2/0 myeloma cells, as described by McDonald et al. (13). The specificity of the MAbs for parasites was determined by the indirect fluorescent-antibody test (IFAT), using an air-dried and acetone-fixed excystation mixture of oocysts and sporozoites as the antigen (13).

Electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (13), based on the method of Laemmli (8), using 5% stacking gels and 7.5% separating gels. Oocyst antigens were prepared by freeze-thawing, using liquid nitrogen, of fresh oocysts in a standard electrophoresis sample buffer containing 1.5 mM dithiothreitol. Particulate matter was removed from the freeze-thawed preparation by centrifugation at 13,000 rpm for 5 min, and the soluble material, which formed the electrophoretic samples, was heated at 100°C for 90 s. Each sample contained a similar amount of protein, estimated by the method of Lowry et al. (11); thus, for *C. baileyi*, *C. muris*, and *C. parvum*, respectively, samples contained the equivalent of 1.0×10^6, 4×10^5, and 2.5×10^6 oocysts. Electrophoresis was done in a Biotech Easy apparatus (15 by 15 cm) with a constant current of 20 mA.

Antigens were transferred electrophoretically from gels to Immobilon-P membranes (Millipore) by using a semi-dry Western blotting system (Biotech). Transfer was obtained with a constant current of 100 mA for 90 min.

After incubation of the blots with anti-*Cryptosporidium* antibodies, blocking was performed with 4% bovine serum albumin in Tris-buffered saline (pH 10.3) (13). The blots were then treated with a second antibody, goat anti-rabbit immunoglobulin G or anti-mouse immunoglobulin, conjugated with alkaline phosphatase (Sigma), and antigens were visualized following the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma).

RESULTS

Antigenic differences between species were observed in IFAT. The anti-*C. parvum* MAbs used in this study were known to recognize the oocyst wall (181B5) or part(s) of the sporozoite (all remaining antibodies). The reactivities of the MAbs with sporozoites and oocysts from *C. baileyi*, *C. muris*, and *C. parvum* are presented in Table 1. As in previous experiments, all the antibodies reacted strongly with *C. parvum*. The oocyst wall-reactive antibody, 181B5, also bound to the same component of *C. baileyi* and *C. muris*, although with less intensity. Only two of the sporozoite-reactive MAbs, 182C1 and 182C2b, were observed to bind to sporozoites of *C. muris*, and the fluorescence was weak. Sporozoites of *C. baileyi* reacted strongly with MAbs 181C3 and 182C1, moderately with 182C2b, and weakly with 182A6. The remaining five sporozoite-reactive antibodies failed to bind to *C. baileyi*.

Immunoblots with polyclonal antisera demonstrated antigenic differences between *Cryptosporidium* spp. Oocysts isolated from a number of unrelated human cases of cryptosporidiosis in southeast England had the typical shape and size (almost spherical and approximately 5 μm in diameter) of those associated with *C. parvum*. Freeze-thawed oocyst material was subjected to SDS-PAGE and immunoblotted with a polyclonal anti-*C. parvum* antiserum. The antigenic profiles obtained of these isolates (Fig. 1) were found to be similar.

TABLE 1. Interaction in IFAT of murine MAbs developed against *C. parvum* with sporozoites or oocysts of *C. parvum*, *C. baileyi*, and *C. muris*

MAb	Staining pattern[a]	*C. parvum*[b]	*C. baileyi*[b]	*C. muris*[b]
181B5	Oocyst wall	++	+	+
172A4	Whole	++	−	−
181A4	Whole	++	−	−
181C3	Interior	++	++	−
182A6	Surface	++	±	−
182B2	Surface	++	−	−
182B4	Surface	++	−	−
182B7	Whole	++	−	−
182C1	Interior and surface	++	++	±
182C2b	Surface	++	+	±

[a] The part(s) of the sporozoite of the homologous isolate of *C. parvum* recognized by each MAb is given; the exceptional antibody is 181B5, which reacted only with the oocyst wall.

[b] A semiquantitative assessment was made of fluorescence: ++, strong; +, moderate; ±, weak; −, none.

In contrast, differences were observed between the antigenic profiles of oocyst antigens from the separate species, *C. baileyi*, *C. muris*, and *C. parvum* (Fig. 2). Variations between species were obtained with either anti-*C. parvum* or anti-*C. muris* serum. In immunoblots obtained with anti-*C. parvum* serum, 30, 7, and 36 antigens were detected from *C. baileyi*, *C. muris*, and *C. parvum*, respectively; with anti-*C. muris* serum, 25, 18, and 22 antigen bands, respectively, from the three species were found. *C. muris*, therefore, had the lowest number of antigen bands with both homologous and heterologous antisera. With this species, however, a large antigenic mass extending from around 45 to 0 kDa was consistently detected with either antiserum.

In blots with each antiserum, a number of major bands were found to be unique to individual species. Examples of this in experiments with the anti-*C. muris* serum were *C. baileyi* antigens of 100 (double band) and 65 to 56 kDa, *C.*

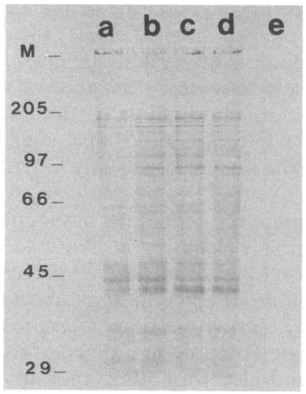

FIG. 1. Antigenic profiles in Western blots of freeze-thawed oocysts from human isolates of *C. parvum* (lanes a to d) from unrelated cases of cryptosporidiosis in southeast England, following incubation with 1:100 rabbit anti-*C. parvum* serum. Antigens were visualized immunoenzymatically with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. A control sample was treated with normal rabbit serum (lane e). M, molecular weight marker proteins (10^3).

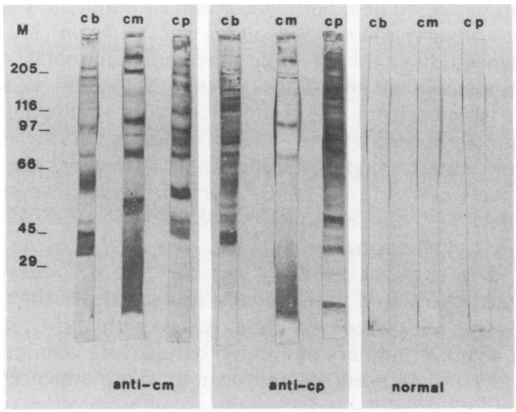

FIG. 2. Antigenic profiles in Western blots of freeze-thawed oocysts of *C. baileyi* (cb), *C. muris* (cm), and *C. parvum* (cp) after incubation with 1:100 serum from rabbits which either were immunized with oocyst antigens of *C. muris* (anti-cm) or *C. parvum* (anti-cp) or remained unimmunized. Antigens were visualized enzymatically as described in the legend to Fig. 1. M, molecular weight marker proteins (10^3).

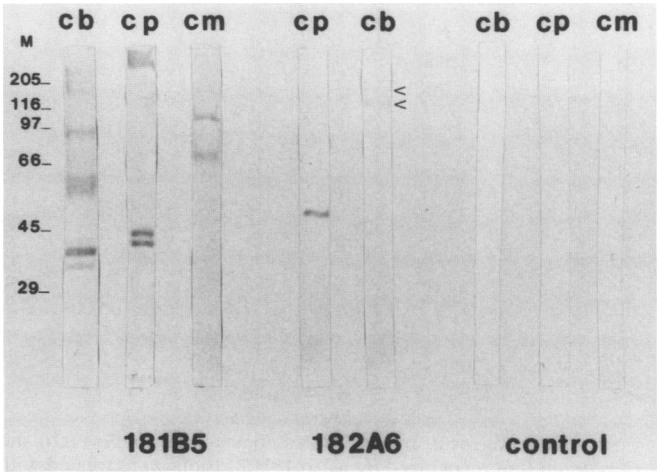

FIG. 4. Comparison of antigens of freeze-thawed oocysts of different *Cryptosporidium* species detected by MAbs 181B5 and 182A6 in ascites diluted 1:50. The parasite species were *C. baileyi* (cb), *C. muris* (cm), and *C. parvum* (cp). Controls were treated with 1:50 normal mouse ascites. Antigens were visualized as described in the legend to Fig. 3. The positions of the faint bands of *C. baileyi* recognized by 182A6 are marked with arrowheads. M, molecular weight marker proteins (10^3).

muris antigens of 110 and 51 to 54 kDa (more than one band), and *C. parvum* antigen bands of 58 and 48 kDa. With the anti-*C. parvum* serum, bands of 56 and 51 kDa and of 48, 42, and 38 kDa were seen only with *C. baileyi* and *C. parvum*, respectively. Some antigen bands of each species were detected with only one antiserum or were more prominent in blots with one antiserum than with the other. At least two bands of *C. parvum* (58 and 48 kDa) were observed more prominently with the heterologous antiserum than with the homologous one.

Immunoblots with MAbs showed antigenic differences between species. The MAbs used in IFAT were also used in immunoblots of oocyst antigens from the three species of parasite. Similar antigenic profiles were usually obtained with different isolates of *C. parvum*, and examples of this are presented in Fig. 3. The oocyst wall-specific MAb, 181B5, reacted strongly with a 44-kDa band from each of four animal isolates and, except in one case, similarly reacted with a 41-kDa band (Fig. 3, lanes a to d). Many isolates of *C. parvum* have now been examined in immunoblots with 181B5, and the 44- and 41-kDa bands were detected in most instances. Treatment of blots from three human isolates of *C. parvum* with the sporozoite surface-reactive MAb, 182A6, resulted in the detection of a 47-kDa antigen (Fig. 3, lanes f to h).

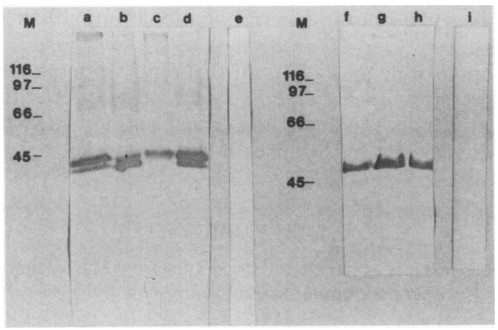

FIG. 3. Detection of parasite antigens in Western blots from different freeze-thawed oocyst isolates of *C. parvum* from animals and humans, following treatment with MAb 181B5 (lanes a to d) or 182A6 (lanes f to h) in ascites diluted 1:50. Antigens were visualized immunoenzymatically with goat anti-mouse immunoglobulin conjugated to alkaline phosphatase. Samples treated with 181B5: lane a, cervine isolate, passaged in calves; lane b, calf isolate; lane c, sheep isolate 1; lane d, sheep isolate 2; lane e, same isolate as in lane a but treated with 1:50 normal mouse ascites. Samples treated with 182A6: lane f, immunocompetent child; lane g, AIDS patient 1; lane h, AIDS patient 2; lane i, control (same as lane e). M, molecular weight marker proteins (10^3).

The oocyst wall-reactive MAb 181B5 also recognized antigens of *C. baileyi* and *C. muris* in blots, but the antigenic profiles from the three species were different (Fig. 4). The 44- and 41-kDa bands of *C. parvum* previously recognized by this antibody were again evident in the cervine isolate of this species. In contrast, the most prominent antigenic bands of *C. muris* observed were of 110 and 72 kDa (which probably corresponded with two of the seven bands recognized by the polyclonal anti-*C. parvum* serum). Furthermore, with *C. baileyi*, a number of bands were observed between >200 and 35 kDa; this included a 41-kDa band which was fainter than that of the same size found in *C. parvum*. In the same experiment, a comparison was made between the antigens of *C. baileyi* and *C. parvum* recognized by MAb 182A6 (Fig. 4). A single prominent 48-kDa band of *C. parvum* was observed, whereas with *C. baileyi*, two weak bands (positions marked with arrowheads in Fig. 4) were just detectable at positions corresponding to 178 and 140 kDa. In a similar experiment to examine the reactivity of MAb 182C2b (data not presented), a 48-kDa band of *C. parvum* was again recognized, as were a number of less intense bands of larger molecular masses (>150 kDa), but with *C. baileyi*, a single band of 42 kDa was identified.

The sporozoite-reactive MAb 181C3 recognized a double antigen band from *C. parvum* at 168 kDa (Fig. 5). With *C. baileyi*, this MAb detected a number of bands between 207 and 40 kDa, the most prominent band being the smallest. A similar result was obtained with 182C1 (data not shown).

MAbs 182C1 and 182C2b, which reacted weakly with *C.*

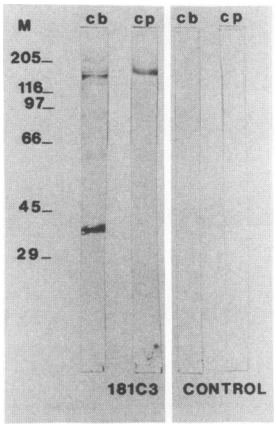

FIG. 5. Antigens of freeze-thawed oocysts of *C. baileyi* (cb) and *C. parvum* (cp) recognized by MAb 181C3. Blots were treated with 1:50 ascites containing the MAb or normal ascites. Antigens were visualized as described in the legend to Fig. 3. M, molecular weight marker proteins (10^3).

muris in IFAT, did not detect any antigens of this species in blots. In addition, MAbs which did not provide a positive result in IFAT with *C. baileyi* or *C. muris* also failed to identify antigens of these species in blots.

DISCUSSION

The results of this study clearly demonstrated that oocysts from three *Cryptosporidium* species, *C. baileyi* (from turkeys), *C. muris* (from mice), and *C. parvum* (from deer and cattle), could be readily distinguished immunologically.

In IFAT, each MAb from a panel of 10 anti-*C. parvum* MAbs reacted with *C. parvum*, but only 50 and 33% of these antibodies, respectively, recognized *C. baileyi* and *C. muris*, and the binding to these parasites was usually weaker than to *C. parvum*.

Antigenic differences were also found between the three species by immunoblotting with either polyclonal antisera or MAbs. Using immunoblotting with an anti-*C. parvum* serum, Current (2) had previously shown variation between the antigenic profiles of *C. parvum* and *C. baileyi* oocysts. In the present study, unique antigenic profiles were obtained with each of the three species in blots treated with either rabbit anti-*C. parvum* or anti-*C. muris* polyclonal serum. In addition, the two polyclonal antisera produced different profiles for each species. The MAbs used in the study each detected one or more antigens of *C. parvum* in blots (unpublished data), but usually only the MAbs which reacted strongly or moderately with *C. baileyi* or *C. muris* in IFAT (i.e., a minority of the antibodies) were able to detect antigens of these two species in blots (182A6 was an exception). Furthermore, when MAbs reacted with more than one species in blots, the antigen(s) recognized by each MAb varied in size from species to species. For example, 181B5, an oocyst wall-reactive antibody and the only MAb to recognize all three species in blots, bound strongly in blots with 44- and 41-kDa antigens of *C. parvum*, but antigens of these sizes were not found in *C. muris* and only a weak antigen of 41 kDa was found in *C. baileyi* (Fig. 4). A similar MAb, described by other workers (1), reacted with the oocyst wall of both *C. parvum* and *C. muris*; it recognized a 40-kDa antigen of *C. parvum* which may correspond with the 41-kDa band detected by 181B5 in the present study.

In previous studies, some variation of antigenic composition was observed between isolates of *C. parvum* (17, 18). In our own studies, for example, the molecular mass of the antigen recognized by MAb 182A6 was usually 47 kDa in human isolates from the United Kingdom or Portugal, but the equivalent antigen of bovine, ovine, or cervine isolates was consistently slightly larger (18a). However, the differences between strains of *C. parvum* were less marked compared with those found in the present study between separate *Cryptosporidium* species.

Our data suggested that *C. parvum* and *C. baileyi* were more closely related antigenically to each other than either species was to *C. muris*. In blots treated with anti-*C. parvum* serum, similar numbers of antigen bands were obtained with *C. baileyi* and *C. parvum*, and visually there appeared to be a degree of homology between the antigenic profiles of these species. In contrast, the profile of *C. muris* obtained with this antiserum contained only about 25% of the numbers of bands found with the other species, and in addition, a large diffuse antigenic mass found in the lower-molecular-weight range of immunoblots of *C. muris* was not detected with the other species. Similar conclusions about the antigenic relationships between the three species of parasite could be made from results of experiments involving sporozoite-reactive MAbs developed against *C. parvum*, since a larger number of antibodies recognized *C. baileyi* than *C. muris* in IFAT and immunoblotting.

The antigenic relationships between the three species may reflect biological functions of the parasites associated with the normal sites of development in the host. *C. parvum* and *C. baileyi* may develop in a number of mucosal sites. In the immunocompetent host, *C. parvum* is normally found in the intestine (21) and *C. baileyi* affects the respiratory system, intestine, and bursa of Fabricius (4, 10); in the immunocompromised host, *C. parvum* has been observed throughout the alimentary tract, including the stomach and respiratory system (21), and *C. baileyi* infection of an AIDS patient affected the intestine, the gall bladder, and urinary bladder (5). In contrast, *C. muris* has been reported to develop only in the stomachs of immunocompetent animals (7, 20), and in our experience, even in immunodeficient (severe combined immunodeficiency) mice, no developing stages of *C. muris* were observed in extragastric locations (unpublished observations).

In conclusion, the results of this study demonstrated that *Cryptosporidium* species could be readily differentiated antigenically with MAbs or polyclonal antibodies, while antigenic differences between isolates of one species, *C. parvum*, were less marked.

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