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Neutralization-Sensitive Epitopes Are Conserved among Geographically Diverse Isolates of *Cryptosporidium parvum*

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Isolates of *Cryptosporidium parvum* from New York, Florida, Brazil, Mexico, and Peru were examined for the presence of two sporozoite surface epitopes originally identified in an Iowa isolate by neutralizing monoclonal antibodies (MAbs) 18.44 and 17.41. Immunofluorescence microscopy and immunoblotting demonstrated the presence of both epitopes on all isolates. Incubation of DEAE-cellulose-purified sporozoites of the New York, Florida, Brazil, and Mexico isolates with MAb 18.44 or 17.41 significantly neutralized their infectivity for 4- to 6-day-old BALB/c mice. The results indicate that two neutralization-sensitive epitopes are conserved on geographically diverse *C. parvum* isolates.

Cryptosporidium parvum is a protozoan parasite which has been recently recognized as an intestinal pathogen of calves, international travelers, children in day-care centers and developing countries, livestock handlers, and immunocompromised individuals such as patients with AIDS (1, 6, 8, 9, 18, 21, 22, 24). Cryptosporidiosis is an economically significant disease of calves and is the only major cause of neonatal calf diarrhea for which specific prophylaxis or therapy is unavailable. Current data suggest that only one species of *Cryptosporidium*, *C. parvum*, infects the intestines of calves and humans (1, 8, 23). However, reported differences in the severity of clinical disease associated with infection, variation in protein and genomic electrophoretic patterns, and differential immunofluorescence reactivities have suggested the possibility of isolate variation with *C. parvum* (1, 11, 14, 15, 22).

The development of immunotherapy and immunoprophylaxis against cryptosporidiosis is dependent on the selection of protective epitopes that are common to different geographic isolates. Recently, two distinct neutralization-sensitive surface epitopes defined by monoclonal antibodies (MAbs) 18.44 and 17.41 were found on both sporozoite and merozoite stages of a *C. parvum* isolate obtained from Iowa (H. Moon and D. Woodmansee, National Animal Disease Center, Ames, Iowa) (2, 3, 16). Incubation of sporozoites or merozoites with either MAb 18.44 (immunoglobulin G3 [IgG3] isotype) or MAb 17.41 (IgM isotype) neutralized their infectivity for mice, making the epitopes defined by these MAbs potential candidates for subunit immunogen testing as well as targets for passive immunotherapy. To this end, the present study investigated the conservation and neutralization sensitivity of the epitopes defined by MAbs 18.44 and 17.41 among North and South American isolates of *C. parvum* infecting humans and calves. Although polymorphisms in the antigens bearing these neutralization-sensitive epitopes may exist, the results demonstrate that two relevant epitopes on the isolates evaluated are conserved.

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***C. parvum* isolates.** Fecal samples containing oocysts from Holstein calves naturally infected with *C. parvum* were obtained from large dairy farms in New York (C. Guard and M. Frongillo, Cornell University, Ithaca, N.Y.) and Florida. Human isolates of *C. parvum* were obtained from diarrheic individuals in Brazil, Mexico, and Peru (C. R. Sterling, University of Arizona, Tucson). To obtain the amount of parasite material required for study, isolates were passaged in Holstein calves obtained at or immediately after birth and housed in isolation facilities (17). Prior to inoculation at 2 to 3 days of age, calves were determined to be free of *C. parvum* infection by fecal examination with a modified Kinyoun acid-fast stain.

Oocyst and sporozoite isolation. To separate oocysts from calf feces, ether extraction and sieving were used as previously described (17). Isolated oocysts were stored at 4°C for up to 4 months prior to use in Hanks' balanced salt solution containing 10,000 U of penicillin per ml, 0.01 g of streptomycin per ml, 1 μg of amphotericin B per ml, and 500 U of nystatin per ml and were treated with 1.75% sodium hypochlorite before excystation (17).

Hypochlorite-treated oocysts were excysted in Hanks' balanced salt solution (1.5 h, 37°C). Sporozoites were then isolated at 4°C by DEAE-cellulose anion-exchange chromatography (17). The viability of isolated sporozoites was assessed by using fluorescein diacetate and fluorescence microscopy (17) and exceeded 90% in all samples.

Immunofluorescence assay. Viable excysted preparations of each isolate were incubated with either MAb 18.44 (70 μg/ml) or MAb 17.41 (15 μg/ml) at 4°C for 30 min, washed, incubated with fluorescein-conjugated affinity-purified rabbit anti-mouse Ig (IgG, IgM, and IgA) (Kirkegaard & Perry, Gaithersburg, Md.), and washed again as previously described (17). Nonspecific antibody binding was evaluated by incubating excysted preparations with IgG3 or IgM isotype control MAb with irrelevant specificity before adding the fluorescein-labeled second antibody. Sporozoites and excysted oocysts were then examined by epifluorescence microscopy. The Iowa isolate, in which the surface epitopes under study were originally defined, was used as a control in all experiments. Specific surface immunofluorescence was observed on the majority of sporozoites of all isolates

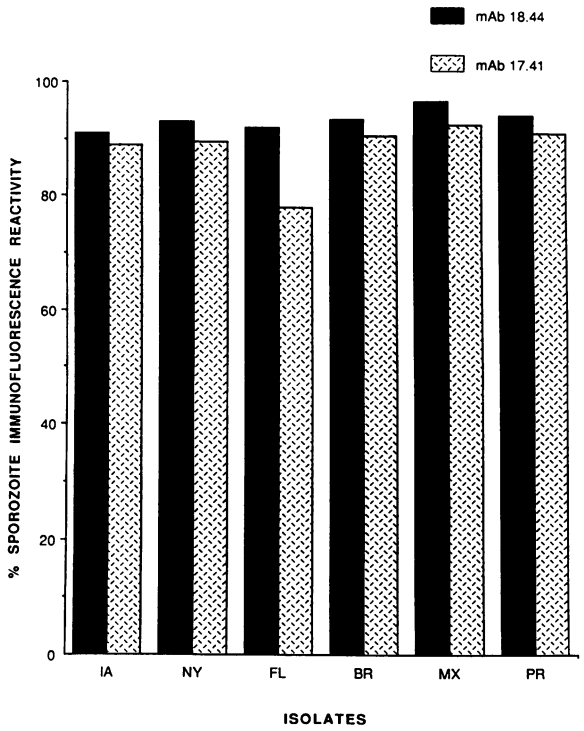

FIG. 1. Immunofluorescence detection of sporozoite epitopes recognized by MAbs 18.44 and 17.41 on different geographic isolates of *C. parvum*. IA, Iowa; NY, New York; FL, Florida; BR, Brazil; MX, Mexico; PR, Peru. Results are the means of two experiments for each isolate and MAb.

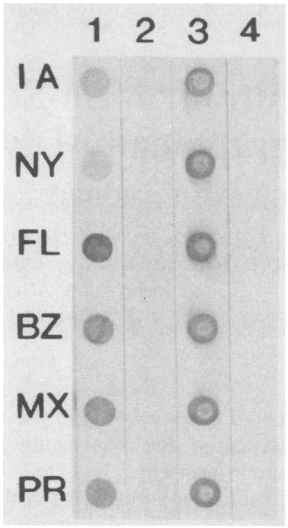

FIG. 2. Dot immunoblot detection of epitopes recognized by MAbs 18.44 (lane 1) and 17.41 (lane 3) on different geographic isolates of *C. parvum*. Lanes 2 and 4 are control immunoblots of *C. parvum* antigen with IgG3 (lane 2) and IgM (lane 4) isotype control MAbs. IA, Iowa; NY, New York; FL, Florida; BZ, Brazil; MX, Mexico; PR, Peru.

incubated with either MAb 18.44 or MAb 17.41 (Fig. 1). The immunofluorescence pattern was diffuse for MAb 18.44 and multifocal or focal polar for MAb 17.41. Specific binding of MAbs 18.44 and 17.41 to oocyst residual bodies and of MAb 17.41 to the inner wall of excysted oocysts was also observed for all isolates.

Immunoblot assay. As a second means of demonstrating epitope conservation, an immunoblot assay was performed. Dot immunoblots were performed instead of Western immunoblots because the objective was to determine epitope conservation rather than molecular weight conservation of the corresponding antigens. Additionally, the antigen recognized by MAb 18.44 is nonprotein and does not migrate according to molecular weight in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16). The molecular weights of the antigens recognized by MAb 17.41 in Western blot assay (Iowa isolate) have been previously reported (2). Hypochlorite-treated oocysts of each isolate were excysted, sonicated, and solubilized at 10^9 oocysts per ml in 0.05 M Tris buffer containing protease inhibitors (5 mM iodoacetamide, 5 mM EDTA, 0.1 mM TLCK (*N*-α-*p*-tosyl-L-lysine chloromethyl ketone), 1 mM phenylmethylsulfonyl fluoride) and 1% (wt/vol) octyl glucoside. The soluble fractions were dotted onto nitrocellulose membranes which were then processed as described previously (16), except that nonfat dry milk (5% [wt/vol]) was used as the blocking agent. After the blocking, the membranes were incubated for 40 min with MAb 18.44 (4 μg/ml), MAb 17.41 (0.25 μg/ml), or an isotype control MAb (IgG3 [4 μg/ml] or IgM [0.25 μg/ml]). Specifically bound antibody was detected with alkaline phosphatase-conjugated, affinity-purified rabbit anti-mouse IgG3 or IgM (Zymed, San Francisco, Calif.) and nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate substrates. Dot immunoblots with MAbs 18.44 and 17.41 for each isolate were positive (Fig. 2), further demonstrating conservation of the epitopes recognized by these MAbs.

Sporozoite neutralization assay. To determine whether conserved epitopes were neutralization sensitive, mouse sporozoite neutralization assays (17) were performed on the Florida, New York, Brazil, and Mexico isolates. Viable sporozoites of each isolate (2×10^5) were incubated with 12.5 μg of MAb 18.44 or 2.5 μg of MAb 17.41 (37°C, 30 min) before intraintestinal inoculation of 4- to 6-day-old BALB/c mice. Nonspecific neutralization was evaluated by inoculating control mice with sporozoites treated with irrelevant MAb IgG3 (12.5 μg) or IgM (2.5 μg). Positive-control mice were inoculated with sporozoites of the Iowa isolate treated with MAb 18.44 or 17.41. All mice were euthanatized 92 to 96 h postinoculation. Infection scores reflecting the density of organisms in the intestinal mucosa were determined histologically as previously described (0 = absence of infection; 9 = maximal infection) (17). Intestinal tracts of untreated mice from the same colony as the inoculated mice were free of endogenous *C. parvum* infection. Significant sporozoite neutralization was observed for all isolates treated with MAb 18.44 or 17.41 (Table 1). The significance of variation in infection scores among the isolates is unclear, as the isolates varied in age at the time of evaluation and 50% mouse infective doses for each isolate were not determined.

Intraspecific antigenic variation in different geographic isolates of several parasites has been demonstrated (5, 7, 19) and has been a significant problem in the development of immunoprophylactic measures. Differences in *C. parvum* isolates have mostly been implied by variation in clinical presentations (1, 22), and only recently have biochemical, genomic, and antigenic variations been investigated (4, 10–15, 20).

Field inversion gel electrophoresis of cryptosporidial chromosomal DNA and MAb reactivity with oocysts re-

TABLE 1. Neutralization of *C. parvum* isolates by MAbs 18.44 and 17.41

MAb and isolate[a]	Mean infection score ± SD[b] (no. infected/no. examined)	
	Test MAb	Isotype control MAb
18.44		
IA	1.3 ± 2.1[c] (2/6)	8.3 ± 0.5 (6/6)
NY	0[d] (0/8)	3.1 ± 2.6 (5/8)
FL	0.2 ± 0.4[d] (1/5)	6.8 ± 3.8 (4/5)
BZ	0.1 ± 1.2[c] (1/9)	6.4 ± 1.1 (10/10)
MX	0[c] (0/10)	8.7 ± 0.5 (10/10)
17.41		
IA	2.2 ± 0.9[c] (5/5)	8.8 ± 0.5 (6/6)
NY	0[d] (0/8)	2.8 ± 2.4 (5/6)
FL	0[c] (0/7)	5.0 ± 2.2 (6/6)
BZ	0[c] (0/5)	4.8 ± 1.3 (5/5)
MX	0[c] (0/9)	8.7 ± 0.5 (10/10)

[a] Isolates identified by geographic source as follows: IA, Iowa; NY, New York; FL, Florida; BZ, Brazil; MX, Mexico.
[b] Results of one replicate experiment for each isolate and MAb were consistent with those reported here.
[c] Score is significantly lower than that of the isotype control group when compared by Student's one-tailed *t* test ($P < 0.0005$).
[d] Score is significantly lower than that of the isotype control group when compared by Student's one-tailed *t* test ($P < 0.005$).

vealed differences between *Cryptosporidium baileyi* and *C. parvum* but not among bovine, equine, and human *C. parvum* isolates (12). Restriction endonuclease analysis of repetitive DNA from the isolates examined in the present study revealed similar banding patterns, although some variation between the bovine and human isolates and among the human isolates was observed (15). Common and, to a lesser extent, variable bands were also observed when one caprine and four human *C. parvum* isolates from different geographic regions of Australia were examined by SDS-PAGE (10). By two-dimensional gel electrophoresis, isolate-specific sporozoite proteins of *C. parvum* have been identified (14). Both conservation and restriction of sporozoite epitopes among human, cervine, and ovine isolates of *C. parvum* have been demonstrated by immunofluorescence assay with a panel of MAbs prepared against a human isolate (11). Recently, a 15-kDa surface glycoprotein recognized by a neutralizing MAb was identified in the sporozoite and merozoite stages of a bovine *C. parvum* isolate (20). The epitope defined by the neutralizing MAb was also present on a guinea pig *Cryptosporidium* isolate but not on *C. baileyi*, *Cryptosporidium muris*, or *Cryptosporidium serpentis* isolates (20). In addition, conservation of microneme epitopes defined by two MAbs on *C. parvum* isolates from humans, calves, and lambs has been demonstrated (4).

The results presented here demonstrate that two immunologically relevant sporozoite surface epitopes defined by neutralizing MAbs are conserved in six geographically diverse human and bovine *C. parvum* isolates. Conservation of these epitopes among the isolates suggests that they have an essential role in the infection process. Studies to determine the mechanisms of antibody-mediated parasite neutralization and to further evaluate the antigens defined by MAbs 18.44 and 17.41 as targets for vaccine development and passive immunotherapy against bovine and human cryptosporidiosis are in progress.

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