Quantification of Specific Antibody Response to *Cryptosporidium* Antigens by Laser Densitometry

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Cryptosporidium spp. is a protozoan parasite with worldwide distribution associated with diarrhea in immunocompromised patients (particularly those with acquired immunodeficiency syndrome [AIDS]) and in immunocompetent humans. Immunoglobulin M (IgM) and IgG antibody responses are readily detected by an enzyme-linked immunosorbent assay. To determine which *Cryptosporidium* antigens invoke antibody responses in humans, we performed polyacrylamide gel electrophoresis using purified oocysts, followed by Western blots with human sera from various populations. Of 40 sera from persons with cryptosporidiosis (24 AIDS and 16 non-AIDS patients), in 37 (93%) a 23,000-dalton antigen measured quantitatively by laser densitometry was recognized. Of 63 sera from IgM- or IgG-positive individuals, as determined by enzyme-linked immunosorbent assay, in 58 (92%) this same antigen was recognized. Up to three additional bands between 125,000 and 175,000 daltons were identified by some of these sera. These results suggest that most persons infected with *Cryptosporidium* spp. produce antibodies which recognize at least one common low-molecular-weight antigen. Isolation of this antigen will be useful in development of diagnostic tests and may be important in the study of immunity.

Cryptosporidium spp. is a protozoan parasite with worldwide distribution that is found in the intestinal and respiratory epithelia of birds, reptiles, fish, and mammals (16, 25). It has been associated with an unremitting and frequently life-threatening diarrhea in immunocompromised patients, particularly those with acquired immunodeficiency syndrome (AIDS) (8, 13, 18, 20, 21, 23, 29–31), and a self-limited diarrhea in immunocompetent persons, including children in day-care centers (1, 7), animal handlers (2, 4, 9; A. S. M. H. Rahaman, S. C. Sanyal, K. A. Al Mahmud, A. Sobhan, K. S. Hossain, and B. C. Anderson, Letter, Lancet ii:221, 1984), travelers (11, 14, 22) and close contacts of infected individuals (3, 12, 19). On a global basis, prevalence rates as high as 10.8% have been reported based on examination of stools from patients with diarrhea (17). Enzyme-linked immunosorbent assays (ELISAs) were developed which readily detect immunoglobulin M (IgM) and IgG responses in serum to antigens of *Cryptosporidium* oocysts (28). In randomly selected populations, 20 to 50% of sera contain anti-*Cryptosporidium* IgG, suggesting that at sometime in life, infection is common. To further define the ability of human antibodies to recognize individual *Cryptosporidium* oocyst antigens, antigens were separated by polyacrylamide gel electrophoresis; band recognition by various sera was analyzed by an enzyme-linked immunoelectrotransfer blot technique (EITB). One low-molecular-weight antigen band was frequently identified, and antibody to this antigen was measured quantitatively by laser densitometry. Results by the standard ELISA and the technique described above were compared.

MATERIALS AND METHODS

Cryptosporidium oocysts were obtained, purified, and sonicated as described previously (28). They were then stored frozen at −20°C until used to coat the solid phase in the ELISA or as antigen in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Serum specimens were obtained from 40 patients with diagnosed cryptosporidiosis, including 24 AIDS patients and 16 immunocompetent patients evaluated in the metropolitan Washington, D.C., area or at the New York Hospital-Cornell Medical Center in New York City (courtesy of Rosemary Soave). Of 17 additional sera from persons with stool examinations negative for *Cryptosporidium* spp., five were from AIDS patients and 12 were from presumed immunologically healthy hospital or laboratory personnel or patients at the National Institutes of Health. Another 30 sera were from persons with other parasites or potential exposure to intestinal parasites but without stool examinations for *Cryptosporidium* spp. Of these 87 sera, 63 had IgM or IgG antibody to *Cryptosporidium* spp. detectable by ELISA. All sera were stored at −20 to −70°C for less than 5 years.

EITB was performed by the procedure of Tsang et al. (24). Thawed oocyst sonicate was dissolved in sample buffer containing 2.5% sodium dodecyl sulfate and 5% mercaptoethanol. Phenylmethylsulfonyl fluoride (34.8 mg/cm³ of methanol), iodoacetamide (37 mg/cm³ of H_2O, made fresh), sodium EDTA (336 mg/cm³ of H_2O), and pepstatin (20 mg/cm³ of dimethyl sulfoxide) were added as inhibitors of proteases. This preparation was boiled for 2 min and centrifuged for 10 s at 12,000 rpm (Eppendorf centrifuge 5414; Brinkman Instruments, Inc., Westbury, N.Y.) to remove particulate matter and then electrophoresed for 4 h at 200 V with a 5 to 15% gradient of polyacrylamide gel. Transblotting to nitrocellulose was performed overnight at 4°C at 60 V. Examination of the electrophoresed gel with Coomassie blue dye, before and after transblotting, showed that all major antigen bands transferred. For the enzyme-linked assay performed on transblotted nitrocellulose strips, 1:200 dilutions of test sera in phosphate-buffered saline (pH 7.4) containing 0.3% Tween 20 and 1% bovine serum albumin were used. Simultaneous testing of several dilutions of sera
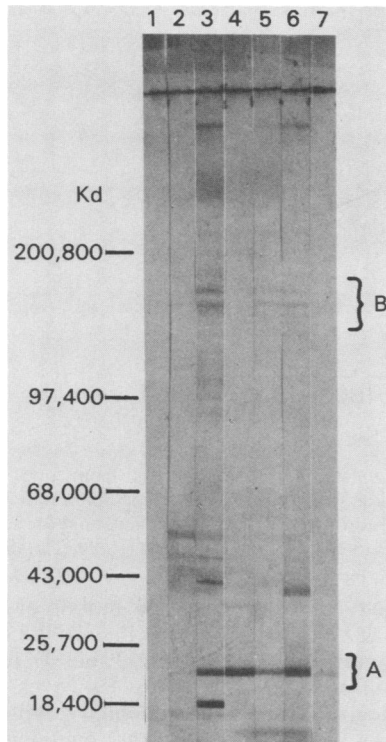
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FIG. 1. Immunoblots of *Cryptosporidium* antigens performed with serum specimens from AIDS and non-AIDS patients with or without cryptosporidiosis. A, 23,000-Da antigen; B, 125,000- to 175,000-Da antigens; Lane 1, conjugate control (density ratio [DR] = 0); lane 2, non-AIDS uninfected serum (DR = 0); lane 3, non-AIDS infected control serum (DR = 1.000); lane 4, AIDS infected serum (DR = 1.692); lane 5, AIDS infected serum (DR = 0.605); lane 6, AIDS infected serum (DR = 2.018); lane 7, AIDS infected serum (DR = 0.104).

(1:10, 1:50, 1:75, 1:100, 1:200) from persons with or without cryptosporidiosis and with or without ELISA-detectable antibody to *Cryptosporidium* spp., showed that the 1:200 dilution yielded results comparable to other dilutions and allowed conservation of specimens. Peroxidase-labeled goat anti-human IgM (Cappel Laboratories, Cochranville, Pa.) and IgG (Cappel) were pooled and used at a 1:250 dilution in the same diluent. Both serum and conjugate exposures were for 1 h with agitation at room temperature, and both were followed by three 10-min washes with phosphate-buffered saline containing 3% Tween 20. Bands appeared after exposure to diaminobenzidine substrate (Sigma Chemical Co., St. Louis, Mo.) for 5 to 10 min, and the reaction was then stopped with water. Negligible nonspecific binding was noted.

Density measurement of the 23,000-dalton (Da) band was obtained on reverse photographic negatives of the tested nitrocellulose strips with a laser densitometer (2202 Ultroscan; LKB Instruments, Inc., Bromma, Sweden). To allow comparisons between EITB runs, results were expressed as the ratio of the density of a test specimen to a 1:200 dilution of a single positive control standard which was examined with each set of nitrocellulose strips (density ratio). No qualitative variations were identified for the positive control standard on any of more than a dozen EITB runs; quantitative variations correlated with time exposure to substrate. A single negative control sample was also

examined with each set of nitrocellulose strips, and results remained consistent.

ELISA testing for specific IgM or IgG in serum to *Cryptosporidium* spp. was performed by a previously described protocol (28). A clinical specimen was positive if the mean optical density from duplicate wells minus the mean plus two standard deviations of 3 to 7 simultaneously examined, presumed negative, sera was greater than zero.

RESULTS

Sera from 37 of 40 patients with *Cryptosporidium* infection identified by fecal examination and from 7 of 17 presumed uninfected persons reacted with a 23,000-Da oocyst antigen band, as determined by EITB. The degree of reactivity judged by direct visualization correlated well with quantitative measurement, as shown in selected examples in Fig. 1. Immunoblots and individual laser densitometer area tracings were completed for multiple dilutions of one positive serum sample designated as the positive control standard. The standard curve of density ratios of these dilutions was linear and reproducible (Fig. 2).

The range of density ratios for the 23,000-Da antigen band elucidated with sera from various populations is shown in Fig. 3. Of 40 patients with cryptosporidiosis, all of whom were positive by ELISA for IgM or IgG, 37 had a density ratio to the standard positive control greater than zero. Of the three who did not, one was a presumed immunologically healthy host who had only a transient and minimal antibody response detected by ELISA, and two were AIDS patients with strongly positive ELISA-detectable anti-*Cryptosporidium* IgG.

Of sera from 17 persons with one or more stool examinations negative for *Cryptosporidium* spp., 10 did not identify this band by EITB and had no detectable antibody by ELISA; of the remaining 7, 5 had ELISA-detectable antibody and EITB recognition of this 23,000-Da antigen, including one AIDS patient with *Isospora belli*. In the two others no antibody was detectable by ELISA but nevertheless the 23,000-Da antigen was identified: one was another AIDS patient with *I. belli* infection and the other was a Peace Corps volunteer who had recently returned from Africa with other parasitic infections (*Entamoeba coli* and *Endolimax nana*).

By using sera from 30 persons without stool examinations for *Cryptosporidium* spp. but with other parasites or potential exposure to other parasites, 23 reacted with the 23,000-Da antigen, and 16 of these were also positive for antibody, as determined by ELISA. Seven had no antibody by ELISA but did recognize the 23,000-Da antigen. Two others had ELISA-detectable antibody, but the 23,000-Da band was not identified. There were no apparent distinguishing features to explain these discordant results, and there was no correlation between the magnitude of the optical density readings for the standard ELISA and laser densitometry readings for the EITB. There were also no apparent distinctions between AIDS and non-AIDS patients in any group of sera examined.

One to three additional antigen bands between 125,000 and 175,000 Da were detected by serum from 20 of 38 persons with stool examinations positive for *Cryptosporidium* spp. (Fig. 1). Seven of nine sera from persons with stool examinations negative for *Cryptosporidium* spp. did not detect bands in this region; the two sera that did detect bands were from AIDS patients with *I. belli* infections. (Two sera from persons with stool examinations positive for *Crypto-*
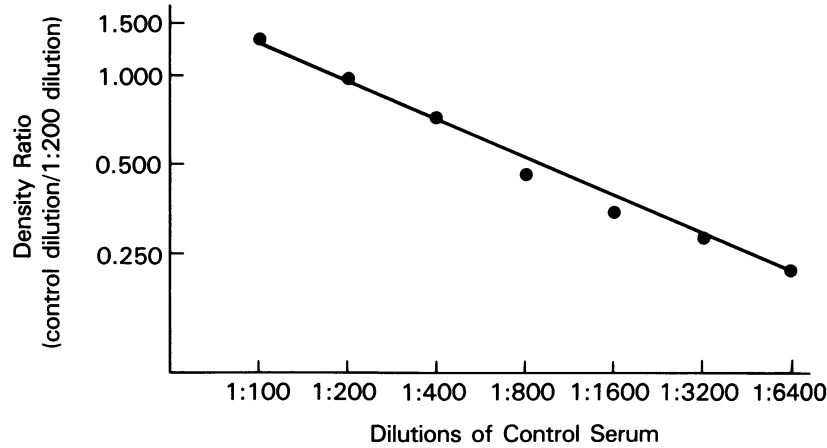
FIG. 2. Immunoblot density ratio standard curve of 23,000-Da *Cryptosporidium* antigen using serum from one patient with cryptosporidiosis (designated the positive control). Quantitative results were expressed as the ratio of the test specimen to a 1:200 dilution of the positive control standard examined with each set of nitrocellulose strips (density ratio). Areas (in millivolt seconds) for serial dilutions were as follows: 1:100, 2,076,600; 1:200, 1,518,000; 1:400, 1,116,200; 1:800, 749,480; 1:1,600, 540,570; 1:3,200, 447,590; 1:6,400, 341,420.

sporidium spp. and eight sera from persons with stool examinations negative for *Cryptosporidium* spp. were not included in this analysis because of technical problems and the need to conserve specimens.)

In three infected patients, EITB detection of IgM or IgG to the *Cryptosporidium* 23,000-Da antigen was examined at various time intervals after infection. Density ratios were

greatest during or within the first month of illness and declined 2 to 12 months postillness (Fig. 4); the corresponding ELISA optical density values (Table 1) followed the same pattern in these three instances. However, serum from one parasitologist (without documented cryptosporidiosis but who works with stool specimens) contained IgG antibody to *Cryptosporidium* spp., as detected by ELISA, as well as EITB-detectable antibody to the 23,000-Da antigen in samples collected 9 years apart. Another laboratory worker had no ELISA-detectable antibody prior to the onset of diarrhea, possibly due to *Cryptosporidium* spp., but detectable IgG (and no IgM) approximately 2 months afterward; both serum specimens were positive by EITB for the 23,000-Da antigen, which is perhaps suggestive of a second infection.
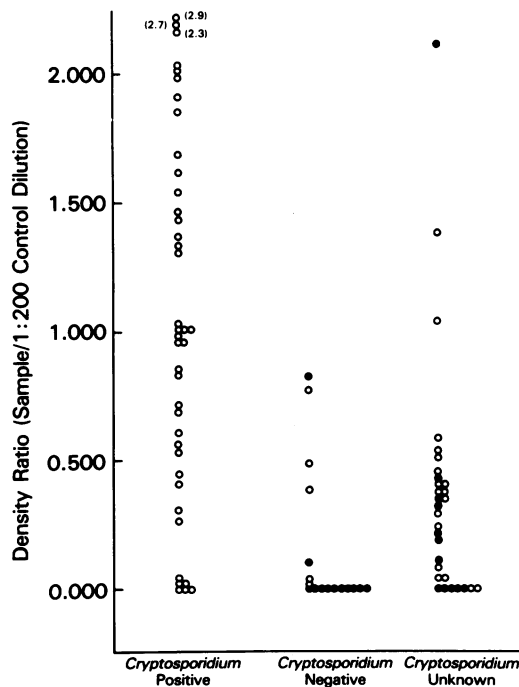

FIG. 3. Immunoblot detection of IgM or IgG to *Cryptosporidium* 23,000-Da antigen performed on serum specimens from patients with *Cryptosporidium* spp. found on stool examination, without *Cryptosporidium* spp. found on stool examination, and without stool examination. Quantitative results were expressed as the ratio of the test specimen to a 1:200 dilution of the positive control standard examined with each set of nitrocellulose strips (density ratio). Symbols: O, positive by ELISA for IgM or IgG to *Cryptosporidium* spp.; ●, negative by ELISA for IgM or IgG to *Cryptosporidium* spp.
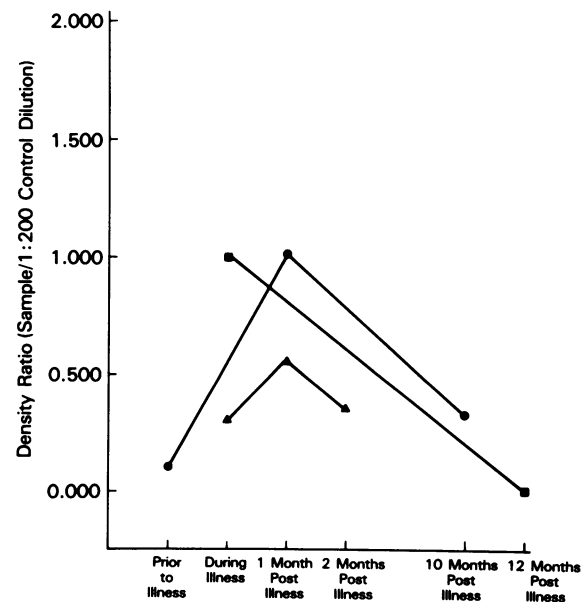

FIG. 4. Immunoblot detection of IgM or IgG to *Cryptosporidium* 23,000-Da antigen in three infected patients over time. Quantitative results were expressed as the ratio of the test specimen to a 1:200 dilution of the positive control standard examined with each set of nitrocellulose strips (density ratio).

TABLE 1. ELISA optical densities for anti-*Cryptosporidium* IgM or IgG in three infected patients over time

Patient	Time	Optical density, as determined by ELISA, for[a]:	
		IgM	IgG
A	Prior to Illness	−0.013	−0.189
	1 mo postillness	−0.154	0.104
	10 mo postillness	−0.275	−0.277
B	During illness	0.288	−0.179
	1 mo postillness	0.167	0.218
	2 mo postillness	0.000	0.128
C	During illness	0.521	0.299
	12 mo postillness	−0.153	−0.127

[a] The mean and standard deviation of three to seven simultaneously examined presumed negative sera were calculated. For clinical specimens the mean optical density from duplicate wells was calculated, and the mean optical density plus + 2 standard deviations of the negative controls was subtracted. A specimen was positive if the optical density was greater than 0.000. A corresponds to the closed circle in Fig. 4, B to the triangle, and C to the square.

DISCUSSION

Cryptosporidium spp., recognized for at least a decade as a parasite of veterinary importance, recently has been acknowledged as a potentially important agent of diarrhea in humans (16, 25) and is emerging as a noteworthy pathogen in children (5, 15, 17; D. P. Casemore and B. Jackson, Letter, Lancet ii:679, 1983; N. Hojlyng, K. Molbak, S. Jepsen, and A. P. Hansson, Letter, Lancet i:734, 1984). The indirect immunofluorescence assay (6, 12, 22, 26) and, more recently, the ELISA (28) have demonstrated anti-*Cryptosporidium* IgG response in immunocompromised and immunocompetent patients. In the latter group, the IgG response persists for at least 2 months (6, 28); it is found in at least 20 to 50% of randomly collected serum samples (26, 28) or samples from persons without documented cryptosporidiosis or exposure to *Cryptosporidium* spp. (12, 28), suggesting that undiagnosed infection is common. Specific IgM response has also been detected by ELISA (28). No apparent cross-reactivity with other intestinal or protozoan parasites has been noted by these methods (6, 28).

Results of the current investigation show that most infected individuals (37 of 40; 93%) and most IgM- or IgG-positive individuals, as determined by ELISA (58 of 63; 92%), recognized a single 23,000-Da *Cryptosporidium* antigen by EITB. The latter group includes sera from 21 of 47 persons (45%) not known to harbor or to have been exposed to *Cryptosporidium* spp. This supports the notion that *Cryptosporidium* infections have frequently been undetected and complements the high prevalence rates emerging from epidemiologic surveys (5, 10, 11, 15, 17, 19, 27, 32; Casemore and Jackson, Lancet ii:679, 1983; Holjyng et al., Lancet i:734, 1984). Other higher-molecular-weight antigens were detected less frequently by EITB using sera from patients with cryptosporidiosis (20 of 38; 53%). They were also detected using sera from two patients infected with the related coccidian parasite *I. belli*, suggesting that these antigens may be shared, although the possibility that these patients had once been exposed to *Cryptosporidium* spp. cannot be excluded.

The ELISA probably does not detect antibodies only to the 23,000-Da *Cryptosporidium* antigen or to any other complex of antigens identified by EITB. This is suggested (i) by the inability of sera from three ELISA-positive patients

with cryptosporidiosis and from two other ELISA-positive persons to recognize the 23,000-Da antigen by EITB, (ii) by the ability of sera from nine persons without demonstrated *Cryptosporidium* spp. and with negative ELISA serology to identify the 23,000-Da band, and (iii) by the ELISA seroconversion with persistent EITB recognition of the 23,000-Da antigen in one laboratory worker. However, the possibility that preparation of *Cryptosporidium* sonicate for electrophoresis destroyed some ELISA-detectable antigens or vice versa, or that some other aspect of either procedure destroyed a relevant antigen or antibody, cannot be discounted. Alternatively, it is possible that the 23,000-Da antigen is shared with a yet unidentified coincidental additional organism; further work in this area, particularly that involving *I. belli*, would be useful.

Several patterns of antibody response have been identified by both standard ELISA and EITB recognition of the 23,000-Da band. Sera from one parasitologist spanning a 9-year interval contained antibody that was detected by both methods. Another laboratory worker showed seroconversion by ELISA with EITB recognition of the 23,000-Da antigen both times. Two of three patients developed increased antibody response with infection, and in all three the response diminished after infection, as detected by both methods. These different patterns suggest that duration of antibody response after infection varies; the possibility of persistent antigenic stimulation of antibody production through an undetected reservoir of infection in some cases needs further investigation.

The sera of AIDS patients with cryptosporidiosis, all of which were positive for IgG by ELISA, recognized the 23,000-Da antigen by EITB in all but two instances. There were no clinically distinguishing features in these latter cases, again implying that ELISA and EITB do not necessarily present the same antigens for antibody recognition.

Finally, although EITB does not appear to offer any advantage over ELISA in routine detection or measurement of specific antibody response to *Cryptosporidium* spp., and in fact is more cumbersome, laser densitometry does offer the advantage of quantification of antibody responses to specific antigens without purification. This technology allowed identification of a low-molecular-weight *Cryptosporidium* antigen of potential significance in elucidating mechanisms of immunity and in developing diagnostic tests.

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