JOURNAL OF CLINICAL MICROBIOLOGY, July 1983, p. 165–169
0095-1137/83/070165-05$02.00/0

Vol. 18, No. 1

Demonstration of Serum Antibodies to *Cryptosporidium* sp. in Normal and Immunodeficient Humans with Confirmed Infections

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Received 12 January 1983/Accepted 23 February 1983

Antibodies to *Cryptosporidium* sp. were detected in sera from 12 immunocompetent individuals recovered from cryptosporidiosis and from 5 subjects with an acquired immunodeficiency syndrome and persistent cryptosporidiosis by an indirect immunofluorescent (IIF) test. Marked seroconversion accompanied recovery from infection in immunocompetent individuals, and their IIF titers remained high (1:40 to 1:640) for at least 1 year. No antibodies to *Cryptosporidium* sp. were detected in sera from two subjects with hypogammaglobulinemia, normal T-cell function, and persistent cryptosporidiosis or in sera from individuals not previously exposed to *Cryptosporidium* sp. Very little or no cross-reactivity with the other coccidia—*Toxoplasma, Sarcocystis*, and *Isospora* spp.—occurred in the IIF test procedure. The application of this IIF procedure, along with recently developed techniques to detect oocysts in the feces, should provide the basis for a more accurate assessment of the number of individuals within any subject group with previous and active *Cryptosporidium* infections.

Organisms in the genus *Cryptosporidium* (phylum *Apicomplexa*, suborder *Eimeriorina*) are small intracellular parasites inhabiting only the microvillous region of epithelial cells, primarily enterocytes, of a variety of animals (11). The genus *Cryptosporidium* has gained considerable attention since it has been recognized as a diarrhea-associated enteropathogen of calves, lambs, and humans (15, 20). This zoonotic agent has been shown to produce a short-term, flu-like, gastrointestinal illness in immunocompetent individuals (2, 15) which contrasts sharply with the prolonged agonous course of severe diarrhea in most immunodeficient subjects (10, 18, 23, 24).

A previous study reported the presence of *Cryptosporidium* antibodies in the sera of 10 animal species, including humans (21). The present report describes an indirect immunofluorescence (IIF) test procedure for the detection of circulating antibodies specific to *Cryptosporidium* sp. The present paper also demonstrates the presence of antibodies to *Cryptosporidium* sp. in the sera of immunocompetent individuals who recovered from a short-term *Cryptosporidium* infection and in the sera of five subjects with the recently described acquired immune-deficiency syndrome (AIDS) (6) and persistent cryptosporidiosis. This IIF procedure, when used in conjunction with techniques to detect oocysts in the feces, should provide the basis for a more accurate assessment of the prevalence of cryptosporidiosis within any subject group.

MATERIALS AND METHODS

IIF test antigens. *Cryptosporidium* oocysts in feces from naturally infected calves or from an individual who had clinical and laboratory features of AIDS (6) and persistent cryptosporidiosis were stored in 2.5% $K_2Cr_2O_7$ at 4°C for up to 6 months before use. Before inoculation into mice, fecal samples containing oocysts were passed through a graded series of metal sieves to remove larger particulate material. The oocysts were then concentrated by centrifugation with Sheather sugar solution (15) and washed three times by centrifugation in phosphate-buffered saline. Six 3-day-old Swiss white mice were then inoculated with 200,000 to 500,000 *Cryptosporidium* oocysts of human or calf origin. Six age-matched mice were not inoculated and served as noninfected controls. All mice were necropsied 4 days post-inoculation. Portions of the ileum from each mouse were fixed in 95% ethanol at 4°C for use in the IIF test described below.

Serum samples. Serum samples for IIF were obtained from 31 individuals in five different subject groups (Table 1). Group I (infected and recovered) consisted of 12 individuals who had become infected with *Cryptosporidium* sp. while in contact with animals in three separate, unrelated outbreaks of calf cryptosporidiosis (2). Group I subjects experienced a short-term flu-like gastrointestinal illness (15). All of these individuals were healthy before and after infection with *Cryptosporidium* sp., had normal serum protein electrophoresis patterns, and had normal lym-

TABLE 1. IIF titers to *Cryptosporidium* sp. of various subject groups

Subject group	No. tested	No. of subjects with IIF titer[a] of:								
		10	20	40	80	160	320	640	1,280	2,560
Infected and recovered	12[b]	0	0	1	3	3	3	1	0	1
Exposed, infection not detected	4[b]	3	0	1	0	0	0	0	0	0
No known exposure	10	2	0	0	0	0	0	0	0	0
AIDS with prolonged infection	5	0	0	1	2	0	1	1	0	0
Hypogammaglobulinemia with prolonged infection	2	0	0	0	0	0	0	0	0	0

[a] Titers are expressed as the reciprocal of the highest dilution.
[b] Sera were obtained 60 to 90 days postexposure to infected calves.

phocyte responses to phytohemagglutinin (17). Sera from these individuals were collected 2 to 6 days after the onset of clinical symptoms and 60 to 90 or 360 to 400 days or both after clinical symptoms ceased. The four subjects in group II (exposed, infection not detected) had direct contact with *Cryptosporidium* sp.-infected calves, but had no detectable infections during, and for 1 to 2 weeks after, exposure. The 10 subjects in group III (no known exposure) had no recent diarrheal illness and no recent contact with calves. The five subjects designated as group IV (AIDS with persistent cryptosporidiosis) had clinical and laboratory features of the AIDS and were experiencing persistent infections with *Cryptosporidium* sp. The two individuals in group V (hypogammaglobulinemia with persistent cryptosporidiosis) had congenital hypogammaglobulinemia, normal T-cell function, and persistent infections with *Cryptosporidium* sp.

IIF test procedure. Ethanol-fixed tissues from control and *Cryptosporidium* sp.-infected mice were dehydrated and embedded in paraffin blocks (16). The tissues were sectioned at 5 to 7 μm, affixed to microscope slides without the aid of an adhesive, and subjected to the IIF test procedure described previously (16). Phosphate-buffered saline (pH 7.2) was used to dilute the test sera and fluorescein isothiocyanate (FITC)-conjugated antiserum and to wash the tissues between incubations. Twofold serial dilutions from 1:10 to 1:2,560 of sera from subjects in groups I through V were used for the first incubation, and rabbit FITC-conjugated antihuman polyvalent immunoglobulin (Miles Laboratories), diluted 1:320, was used for the second incubation. After the three 10-min washes to remove the unbound FITC-conjugated antiserum, the tissues were counterstained for 1 min in Evan blue, washed once, covered with 20% glycerin in phosphate-buffered saline, observed, and photographed with a Zeiss epifluorescence microscope. IIF titers were determined in a blind protocol. Serum dilutions were considered positive when the parasites in the microvillous border exhibited bright fluorescence as compared with mouse tissues.

Antigenic specificity. Serum samples from six individuals with IIF titers of 1:256 to 1:4,096 to *Toxoplasma gondii* (Rh strain) (14) were tested for cross-reactivity to *Cryptosporidium* sp. by the IIF procedure described above.

Serum from an animal caretaker with an IIF test titer of 1:2,560 to *Cryptosporidium* sp. (Table 2) was adsorbed separately with whole-parasite antigen of four different coccidian species: oocysts of *Cryptosporidium* sp. (human origin), sporozoites of *Isospora suis* (swine origin), tachyzoites of *T. gondii* (Rh strain), and bradyzoites of *Sarcocystis bovicanis* (calf origin). The sporozoites of *I. suis* were excysted by methods described previously (11a) and purified by a modified column filtering technique (19). The tachyzoites of *T. gondii* were harvested from a continuous cell culture (14). Bradyzoites of *S. bovicanis* were obtained by digestion of heart muscle tissue of an experimentally infected calf (13). Approximately 6×10^6 parasites of each species were pelleted in 2-ml conical centrifuge tubes, and the supernatant was replaced with 0.2 ml of test serum. After suspension in the test serum and incubation at 37°C for 60 min, the parasites were pelleted by centrifugation, and the serum samples were removed and then tested. To determine whether antibodies were bound to the surface of each coccidian species after the incubation period, the parasites were washed three times, smeared on glass slides, air dried, incubated in the FITC-conjugated antiserum, washed three times, and examined microscopically.

TABLE 2. IIF titers to *Cryptosporidium* sp. during and after infection of 7 of the 12 subjects in group I of Table 1

Occupation of subject[a]	IIF titer[b] at:		
	2–6 days (AOI)[c]	60–90 days (AI)[d]	360–400 days (AI)[d]
R	40	640	320
AC	NEG	2,560	320
VS	NEG	320	NA
R	NEG	80	160
V	40	1,280	NA
R	NEG	80	40
AC	NA	160	640

[a] R, Researcher; AC, animal caretaker; VS, veterinary student; V, veterinarian.
[b] Titers are expressed as the reciprocal of the highest dilution.
[c] AOI, After the onset of illness; NEG, negative; NA, not available.
[d] AI, After illness.

RESULTS

IIF titers. Fluorescent reactions of the positive sera were confined to parasites within the microvillous border of enterocytes from the lower small intestine of mice heavily infected with *Cryptosporidium* sp. (Fig. 1). Fluorescence was associated only with endogenous stages of the parasite and, in some cases, with other components of the parasitophorous vacuole (Fig. 2). Some of the parasites could be identified as schizonts, trophozoites, macrogametes, or oocysts by the shape and intensity of fluorescence. Oocysts and merozoites or sporozoites free in the lumen also fluoresced brightly. No detectable differences in the specificity, intensity of fluorescent reactions, or titers were observed between the parasites of calf origin and those of human origin within mouse tissues. Neither normal uninfected tissue nor infected tissues treated with negative control sera fluoresced (Fig. 3).

Distributions of the IIF titers in the five subject groups are shown in Table 1. The titers from immunocompetent subjects obtained 60 to 90 days after recovery from confirmed cryptosporidiosis (group I) ranged from 1:40 to 1:2,560 and, in all instances, were greater than the titers from subjects with no known exposure (group III). Of the four subjects who were in direct contact with *Cryptosporidium* sp.-infected calves and experienced no detectable infections during, and for 1 to 2 weeks after, potential exposure (group II), one had a titer of 1:40, whereas the other three had titers of 1:10. All five AIDS subjects with prolonged cryptosporidiosis (group IV) had titers of 1:40 to 1:640. The two subjects with hypogammaglobulinemia with persistent cryptosporidiosis (group V) had no detectable titers.

The IIF titers in serum samples obtained from six immunocompetent subjects during the acute phase of the *Cryptosporidium* infection (2 to 6 days after the onset of clinical symptoms) and 60 to 90 days after clinical symptoms had ceased and from five of the subjects 360 to 400 days after infection are shown in Table 2. A marked increase in the IIF titer occurred in each of the subjects tested 60 to 90 days postinfection, and the titers remained high (1:40 to 1:640) 360 to 400 days after recovery.

Specificity. Of the six human sera with high IIF titers (1:256 to 1:4,096) to *T. gondii* zoites, only two had fluorescent reactivity (1:20 titers) with *Cryptosporidium* sp.-infected mouse intestine.

When serum with a titer of 1:2,560 against *Cryptosporidium* sp. was adsorbed with sporozoites of *I. suis*, we observed no decrease in titer. When subjected to IIF testing after adsorption, these sporozoites showed little or no fluorescence. Although a decrease in titer from 1:2,560 to 1:1,280 was observed when the test serum was adsorbed with zoites of *T. gondii* or

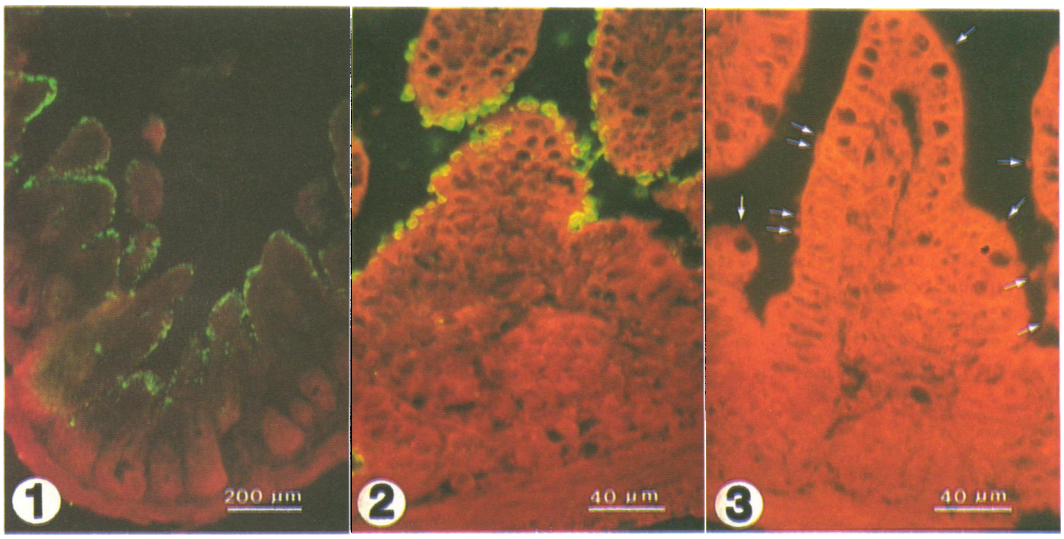

FIG. 1–3. Fluorescent micrographs showing specificity of IIF test for antibodies to *Cryptosporidium* sp. 1. Low magnification of a section of *Cryptosporidium* sp.-infected mouse ileum incubated in *Cryptosporidium* sp.-positive serum. Note that the fluorescent reaction is confined to parasites within the microvillous border. 2. Higher magnification of a portion of Fig. 1. Note that the fluorescence is confined to individual parasites in the microvillous border. 3. High magnification of *Cryptosporidium* sp.-infected mouse ileum (arrows point to some of the parasites) incubated in control serum. Note the absence of fluorescence.

S. bovicanis, zoites of these two species did not fluoresce when tested by IIF. The adsorption of this serum with oocysts of *Cryptosporidium* sp. reduced the IIF titer from 1:2,560 to 1:160. After adsorption, *Cryptosporidium* oocysts fluoresced brightly when tested by IIF.

DISCUSSION

This study demonstrates the presence of circulating antibodies to *Cryptosporidium* sp. in the sera of immunologically normal individuals recovered from cryptosporidiosis (2, 15) and in the sera of subjects with the AIDS and persistent cryptosporidiosis (3). The marked reduction in the IIF titer by the adsorption of antibodies onto the surface of *Cryptosporidium* oocysts, but not with equal numbers of zoites of other coccidian species tested; the absence of overlap in IIF titers between subjects recovered from cryptosporidiosis and subjects with no known exposure (Table 1); and the marked seroconversion of six immunocompetent subjects after recovery from *Cryptosporidium* infections (Table 2) are evidence of the specificity of the antibodies.

Although bile and pancreatic duct involvement in monkeys (9) and respiratory infection in poultry (4, 8) have been reported, *Cryptosporidium* sp. is thought to reside mainly in the microvillous border of the small and large intestines of humans. Because of its superficial position, a systemic antibody response may seem unlikely. However other pathogens, including *Giardia lamblia* (22), *Vibrio cholerae* (5), and *Escherichia coli* (12), which occupy a similar site in humans, stimulate systemic, as well as local, antibody responses. A systemic antibody response to *G. lamblia* has been attributed to organisms which invade the intestinal mucosa (1, 22). No such invasion has been reported for *Cryptosporidium* sp., which may produce a cholera-like illness in humans (23).

The actual role of systemic antibodies to *Cryptosporidium* sp. is uncertain, and the presence of local antibodies has not been determined. Immunocompetent individuals infected with *Cryptosporidium* sp. may develop a short-term flu-like gastrointestinal illness (15) that contrasts sharply with the potentially grave illness in immunocompromised subjects who contract cryptosporidiosis (10, 18, 24). High IIF titers were present in immunocompetent subjects recovered from cryptosporidiosis (group I) and in sera from the five individuals with the AIDS and persistent cryptosporidiosis (group III). The two subjects with normal T-cell function, hypogammaglobulinemia, and persistent cryptosporidiosis (group IV) had no detectable titers to *Cryptosporidium* sp. These data suggest that functional cellular and humoral immunities are necessary to clear an infection. In addition, infection with *Cryptosporidium* sp. may not result in protective immunity since one of the immunocompetent subjects in group I experienced three episodes of cryptosporidiosis during a 1-year period (15). However, the second and third infections were much shorter and less severe than the first.

Based on the results of IIF in the present study (Table 1) and the presence of antibodies to *Cryptosporidium* sp. long after recovery from infection (Table 2), we propose that IIF titers of 1:40 or greater can be considered as a positive test for cryptosporidiosis. In light of these findings, an earlier study that reported *Cryptosporidium* antibodies in the sera of 80 to 100% of 10 species of animals based on an IIF test using 1:10 serum dilutions (21) should be questioned. The 1:40 IIF titer in one of the subjects in group II was probably due to a previous *Cryptosporidium* infection since this individual had had contact with diarrhetic calves for 6 years. The positive IIF titers in sera collected from two subjects 2 to 6 days after infection (Table 2) could also be due to prior *Cryptosporidium* infections since these individuals had previous exposure to diarrhetic calves. The increase in the titers of two subjects 360 to 400 days after infection (Table 2) may be due to subsequent subclinical infections since both subjects were in contact with diarrhetic calves during this period.

Two of the six *T. gondii*-positive reference sera had 1:20 titers to *Cryptosporidium* sp. It is unlikely that this was due to cross-reactivity between these two protozoan parasites since the four reference sera with the highest IIF titers to *T. gondii* were *Cryptosporidium* sp. negative. It is possible, however, that these 1:20 titers were due to cross-reactivity not detected in the present study or to previous exposure to *Cryptosporidium* sp.

Since much of the medical community still relies on intestinal biopsy for the diagnosis of *Cryptosporidium* sp. and since only a small percentage of patients with diarrhea are subjected to this invasive procedure, it is probable that this protozoan is an unrecognized agent of gastroenteritis in humans. The application of our IIF test procedure, in conjunction with recently developed techniques to detect oocysts in the feces (7, 15), should allow a more accurate assessment of the number of individuals in any given subject group with previous and active infections of *Cryptosporidium* sp.

ACKNOWLEDGMENTS

We thank the following physicians and medical researchers for supplying sera from subjects with persistent cryptosporidiosis and the clinical and laboratory features of AIDS or hypogammaglobulinemia: Melvin Heyman, University of California, San Francisco; Wilfred Weinstein, University of California Los Angeles Center for Health Sciences, Los Angeles;

Pearl Ma, St. Vincent Hospital, New York, N.Y.; Edward Bottone, Mt. Sinai Hospital, New York, N.Y.; Joel Klompus, Pacific Coast Medical Center, San Francisco, Calif.; and Herbert Tanowitz, Albert Einstein Medical Center, Bronx, N.Y. The Rh strain of *T. gondii* and the positive reference sera was supplied by Marianna Wilson, Centers for Disease Control, Atlanta, Ga. The *S. bovicanis* antigen was kindly supplied by Ronald Fayer, Animal Parasite Institute, U.S. Department of Agriculture, Beltsville, Md. Also, we acknowledge the technical assistance of Norman C. Reese.

This study was supported in part by cooperative agreement no. ARS-58-7B30-2422 between the U.S. Department of Agriculture-Agricultural Research Service and Auburn University.

ADDENDUM IN PROOF

While this paper was in press, 10 additional serum samples from patients with AIDS and persistent cryptosporidiosis were tested for antibodies to *Cryptosporidium* by our IIF procedure. Three of the sera were positive, with titers ranging from 1:40 to 1:160, whereas the remaining seven had no detectible antibodies to *Cryptosporidium*. These data, along with those presented in Table 1, indicate that a significant proportion of patients with AIDS and persistent cryptosporidiosis do not have detectible antibodies to *Cryptosporidium*. A recent report has also shown that standard immunofluorescent assay for antibodies to *Toxoplasma* in AIDS patients with toxoplasmosis is not always helpful (A. E. Pitchenik et al., Ann. Intern. Med. **98**:277–284, 1983).

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