The Antibody Response to 27-, 17-, and 15-kDa Cryptosporidium Antigens following Experimental Infection in Humans

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Previous studies have suggested that persons infected with Cryptosporidium parvum develop antibody responses to 27-, 17-, and 15-kDa C. parvum antigens. Studies of volunteers infected with Cryptosporidium species provided an opportunity to evaluate the relationship between antibody reactivity to these antigens and infection outcome. As monitored by immunoblot, increases in specific antibody reactivity were more prevalent among volunteers who developed signs and symptoms of cryptosporidiosis (n = 11) than among asymptomatic infected (n = 7; P = .05) or oocyst-negative volunteers (n = 11; P = .02). Volunteers with preexisting IgG antibody to the 27-kDa antigen excreted fewer oocysts than volunteers without this antibody (P = .003). IgG reactivity to the 17-kDa antigens and IgM reactivity to the 27-kDa antigens were higher at day 0 for asymptomatic infected persons than for those who developed symptoms (P = .03 and P = .04, respectively). These results suggest that characteristic antibody responses develop following C. parvum infection and that persons with preexisting antibodies may be less likely to develop illness.

Infection with Cryptosporidium parvum causes an acute enteritis characterized by a profuse watery diarrhea that is self-limited in immunocompetent persons but may be chronic and life threatening in immunocompromised persons [1]. Recent waterborne outbreaks of cryptosporidiosis [2–4] have focused attention on the lack of understanding of the epidemiology of this infection. Efforts to quantify risk factors for acquisition of C. parvum infection would be facilitated by the development and validation of sensitive and specific serologic tools for population-based studies.

When an immunoblotting technique was used to evaluate antibody responses of persons exposed to Cryptosporidium infection in outbreak settings, serum samples from persons with confirmed infection showed changes in IgG reactivity to the 27-, 17-, and 15-kDa oocyst antigen groups, in IgM reactivity to the 27-kDa group, and in IgA reactivity to the 17-kDa group [5]. On the basis of these observations, antibody responses to these antigens may be of value for seroepidemiologic studies. Monoclonal antibodies that recognize the 15-, 17-, and 27-kDa antigens also react with the surface of C. parvum sporozoites, suggesting that these antigens are major targets of the antibody response elicited by infective stages of the parasite [5, 6]. Volunteer studies conducted to determine the infective dose of Cryptosporidium oocysts provided us with an opportunity to analyze the relationship between antibody responses to these antigens and to well-characterized infections [7, 8].

Materials and Methods

Study design and patient population. Volunteers were recruited for participation in studies designed to define the infectious dose of C. parvum oocysts. To rule out the participation of volunteers with evidence of prior exposure to Cryptosporidium species, serum samples from volunteers were screened prior to enrollment by ELISA, as previously described [7]. Subjects considered negative by ELISA were subsequently exposed to measured numbers (30 to 10⁶) of Cryptosporidium oocysts. After the inoculum was administered, stools were examined for oocysts by direct immunofluorescence assay (DFA) [8, 9] to demonstrate and quantitate oocyst excretion, and volunteers were monitored for the development of clinical signs and symptoms for a total of 45 days. Serum samples were collected immediately before oocyst ingestion (day 0) and then serially at ~5, 12, 32, and 60 days after inoculation for all volunteers except 6 from whom only day 0 and day 60 specimens were available. Serum specimens were collected at ~1 year after inoculation for 19 of the 29 volunteers.

Antigen preparation. Extracts of C. parvum oocysts were prepared as previously described [10]. In brief, oocysts that had been purified by discontinuous sucrose gradients from the feces of calves experimentally infected with the Iowa isolate of C. parvum...
Sediment was solubilized in 8 M urea, dialyzed against PBS, and centrifuged as previously described [10]. This supernatant was labeled insoluble (IN) fraction. Protein content of these two fractions was determined by the Bradford method [12].

Immunoblotting. The SO fraction was separated by analytical polyacrylamide gradient gel electrophoresis (PGGE), using 3%–25% gradients, and electrophoretically transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon; Millipore, Bedford, MA) as described [5, 13]. Strips from the PVDF membranes (2 or 3 mm) were incubated with serum samples from the volunteers and diluted 1:200 for monitoring of IgG responses and 1:50 for IgM and IgA. Bound antibodies were detected with biotinylated monoclonal antibodies directed against IgG (clone HP6017), IgM (clone HP6083), and IgA (clone GA112) (Zymed, South San Francisco) followed by streptavidin–alkaline phosphatase (Life Technologies Gibco BRL, Gaithersburg, MD). Bands were developed using 5-bromo-4-chloro-3-indolyl phosphate as substrate and nitroblue tetrazolium as chromogen. Blots were evaluated visually for the presence of or change in IgG responses to the 27-, 17-, and 15-kDa antigen groups, IgM responses to the 27-kDa group, and IgA responses to the 17-kDa group.

In addition, the presence or absence of bands at days 0 and 32 and at ~1 year after inoculation was assessed for the five combinations of isotype and antigen group. For each combination, an antibody score of 1 was assigned for each visible band, for a possible cumulative score ranging from 0 to 5 for each volunteer.

For the initial serum specimens (day 0), blots also were evaluated quantitatively by densitometry. A tagged image format file was created for the strips, using a scanner (Scanjet IIc; Hewlett-Packard, San Diego), and the areas of the bands of interest were analyzed by use of the Wilcoxon test.

Statistics. Data were analyzed with Epi Info (version 6.0; CDC) and SAS (Cary, NC) software. The relationship of blot results to infection outcome and blot results to clinical outcome were analyzed by $\chi^2$ or Fisher’s exact tests. The Wilcoxon signed rank test and the Mann-Whitney rank sum test were used to determine significant changes in antibody scores within each category of volunteers and between categories of volunteers, respectively. The relationship of antibody reactivity, as assessed visually and by median band area (immunoblot) or optical density (ELISA), to infection outcome for different infection groups was analyzed by use of the Wilcoxon rank sum test. An advantage of using the Wilcoxon rank sum test is that we do not have to make any assumptions about the underlying distribution of the data; however, this test is not amenable to any multiple comparison procedure other than the Bonferroni adjustment, which is overly conservative. The relationship of log oocyst output to day-0 antibody status also was analyzed by use of the Wilcoxon test.

Results

Study population. In studies done to determine the infectious dose, 18 of 29 volunteers who were exposed to C. parvum doses ranging from 30 to 10^6 oocysts were infected, as determined by the detection of oocysts in stools by DFA [7]. Linear regression of the proportion of volunteers infected as a function of dose indicated that the ID_{50} was 132 oocysts. Eleven of the 18 infected persons had enteric symptoms; 7 of these persons had diarrhea and met the clinical definition for having cryptosporidiosis [7].

Blot reactivity following exposure. The development of antibody responses of the volunteers to the 27-, 17-, and 15-kDa antigens was determined with SigmaGel software (Jandel Scientific Software, San Rafael, CA). Band intensity values for a given antigen group were expressed as a ratio of the area of the band obtained with the test serum to the area of the band obtained with a positive control serum. The latter was collected from a cryptosporidiosis patient in the 1987 outbreak in Carrollton, Georgia [2]. The positive control was performed on each blot.

Purification of the 17-kDa antigens. The IN fraction of the oocyst antigen was treated with SDS without reducing agents and separated on preparative SDS-PGGE, using 3%–25% gradients. The portion of the gel containing the 17-kDa antigen group was excised, and the proteins were electroeluted in an Elutrap (Schleicher & Schuell, Keene, NH) using methods previously described [13]. Recovered proteins were analyzed by analytical SDS-PAGE, silver stain, and immunoblot.

ELISA. Microtiter plates were sensitized with the crude SO fraction (100 ng/well) or purified 17-kDa antigens (25 ng/well) overnight at 4°C in 0.1 M sodium carbonate–bicarbonate buffer, pH 9.7. After removing unbound antigen and washing with PBS containing 0.05% Tween 20, we blocked the plates with PBS containing 0.3% Tween 20 for 1 h at room temperature. All subsequent incubation and washing steps were done in PBS containing 0.05% Tween 20. Day 0 serum samples were added to plates in triplicate at a 1:200 dilution. After reacting bound primary antibodies with biotinylated mouse anti-human IgG (clone HP6017, Zymed), we exposed bound secondary antibodies to streptavidin–alkaline phosphatase. About 15 min after the addition of p-nitrophenyl phosphate to the bound enzyme, optical densities were read at 405 nm.

<table>
<thead>
<tr>
<th>Isotype/antigen</th>
<th>Oocyst positive</th>
<th>Oocyst negative, asymptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symptomatic $\times$ 11</td>
<td>Asymptomatic $\times$ 7</td>
</tr>
<tr>
<td>IgG/27 kDa</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>IgG/17 kDa</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>IgG/15 kDa</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>IgM/27 kDa</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>IgA/17 kDa</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Blot positive</td>
<td>10</td>
<td>3</td>
</tr>
</tbody>
</table>

NOTE. Volunteers were considered blot positive if changes in reactivity were observed for ≥2 listed responses. Overall reactivity of symptomatic volunteers was significantly greater than that of oocyst-positive or oocyst-negative asymptomatic volunteers (Fisher’s exact test, P = .05 and .02, respectively).
antigen groups was monitored by immunoblot. For the three categories of volunteers described above, the number showing an increase in immunoblot reactivity to these antigen groups over time, as assessed by visual inspection of the blots, is summarized in table 1. Those who showed an increase in antibody reactivity to two or more of the five combinations of isotype and antigen group following exposure were defined as blot positive (representative blot-positive responses are shown in figure 1). Ten of the 11 symptomatic volunteers were blot positive. Of the 7 asymptomatic oocyst-positive volunteers, only 3 demonstrated an increase in antibody reactivity and were considered blot positive. Four of 11 oocyst-negative volunteers showed an increase in antibody reactivity and were considered blot positive (representative blot-negative responses are shown in figure 2). Overall, the prevalence of increased reactivities among symptomatic volunteers was significantly greater than that of the other volunteers ($P = .008$). No relationship was observed between the development of antibody reactivity and oocyst dose; however, the number of persons infected with a given dose may have been too small to observe an effect.

**Kinetics of antibody responses.** For most volunteers who developed antibody responses, IgM and IgG reactivity to the three antigen groups appeared to peak by day 32 after inoculation (representative data for volunteers 21 and 5 are shown in figure 1, IgM and IgG columns). IgA reactivity to the 17-kDa group typically was most intense at day 32 and decreased rapidly to baseline level by day 60 after inoculation. Increased IgG reactivity to the three antigen groups and IgM reactivity to the 27-kDa group did not diminish as rapidly as IgA reactivity.

Blot reactivities for the volunteers are summarized in table 2 as an antibody score. The antibody score reflects the presence or absence of bands but not their magnitude; thus, scores for some persons remained the same, although the intensity of the responses increased. The median antibody score of symptomatic volunteers increased significantly from day 0 after inoculation (median, 2) to day 32 after inoculation (median, 5; $P = .008$). For the 7 symptomatic volunteers who were followed up to 1 year after inoculation, the antibody scores at 1 year after inoculation (median, 3) were higher, but not significantly, than those at day 0 after inoculation (median, 2; $P = .06$).

**Figure 1.** Immunoblot of Cryptosporidium oocyst antigens exposed to serum specimens from volunteer (VOL) 21 and VOL 5. Both were oocyst positive, symptomatic, and blot positive. Panels show IgA reactivity to 17-kDa group, IgM reactivity to 27-kDa group, and IgG reactivity to 27-, 17-, and 15-kDa antigen groups (indicated by brackets at extreme right). Nos. at bottom of strips indicate no. of days after inoculation that serum specimens were obtained. Strip A, protein profile by Aurodye. Molecular mass protein standards in kDa are at extreme left.
Each of these 7 subjects showed increased band intensities to at least one of the five combinations of isotype and antigen group at 1 year after inoculation over that observed at day 0 after inoculation (e.g., figure 1, volunteer 5, IgG column, 27-kDa antigen group, strips at day 0 and day 364 after inoculation). Median antibody scores for the oocyst-positive asymptomatic volunteers did not change significantly; however, all oocyst-positive volunteers who showed no change in blot reactivity had relatively high antibody scores at day 0 after inoculation (median, 3). Antibody scores at day 0 after inoculation for these 7 oocyst-positive asymptomatic volunteers were higher than those for the 11 oocyst-positive symptomatic volunteers ($P = .046$). Similarly, although 7 of 11 oocyst-negative volunteers were considered to have negative blot results, on the basis of the absence of change in antibody reactivity (figure 2), most recognized multiple bands at day 0.

Initial blot reactivity (day 0). By immunoblot, day-0 serum samples from 21 (72%) of 29 volunteers showed two or more IgG, IgM, or IgA reactivities to the 27-, 17-, and 15-kDa antigen groups (e.g., volunteer 17 and 14 in figure 2). To examine the relationship between levels of preexisting antibody to these antigens and the subsequent clinical response to oocyst exposure, day-0 band areas were quantified by intensitometry. Median IgG and IgA reactivity did not differ significantly between oocyst-positive and oocyst-negative volunteers. Median IgM reactivity to the 27-kDa antigen group was higher among oocyst-positive volunteers ($P = .05$); however, this result reflected a chance association between oocyst dose and initial IgM response ($P < .05$).

When the analysis was restricted to oocyst-positive persons, median IgG reactivities to the 17-kDa antigen group and median IgM reactivity to the 27-kDa antigen group were significantly greater ($P = .03$ and .04, respectively; Wilcoxon rank sum test) for asymptomatic volunteers ($n = 7$) than for those who developed enteric symptoms ($n = 11$; table 3). The geometric mean oocyst inoculum did not differ between infected volunteers with or without symptoms (1552 vs. 501 oocysts, $P = .36$; Wilcoxon rank sum test).
Table 2. Summary of antibody scores for Cryptosporidium-exposed volunteers.

<table>
<thead>
<tr>
<th></th>
<th>Median oocyst dose (log10)</th>
<th>Median antibody score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Day 0</td>
</tr>
<tr>
<td>Oocyst-positive/symptomatic</td>
<td>11</td>
<td>2.7</td>
</tr>
<tr>
<td>Oocyst-positive/asymptomatic</td>
<td>7</td>
<td>2.7</td>
</tr>
<tr>
<td>Oocyst-negative/asymptomatic</td>
<td>11</td>
<td>2.0</td>
</tr>
</tbody>
</table>

NOTE. Antibody score is defined, on basis of bands listed in table 1, as total no. of bands observed for given volunteer at given point. PI, postinoculation.

* For volunteers with no sample at day 32, antibody score at day 60 was used.

† 1-year specimens were collected from 7, 6, and 6 volunteers in each group, respectively.

To confirm the observation that symptomatic and asymptomatic volunteers had different initial levels of IgG reactivity to the 17-kDa antigens, reactivity to the purified antigens was analyzed by ELISA. The purity and reactivity of the electrophoretic fractions of the 17-kDa antigen group as assessed by SDS-PAGE and immunoblot are shown in figure 3A, B. By ELISA, the reactivity of the day-0 serum specimens of asymptomatic oocyst-positive volunteers was significantly greater (P = .04) than that of asymptomatic oocyst-positive volunteers (figure 3C). IgG ELISA reactivity and immunoblot intensity values for the 17-kDa group were significantly correlated at day 0 (n = 29; P = .001, Spearman’s correlation).

To evaluate the relationship of day-0 antibody status to quantitative measurements of infection intensity, oocyst excretion was compared for groups defined according to the presence or absence of blot reactive antibodies at day 0. Infected persons with IgG reactivity to the 27-kDa antigen at day 0 (n = 14) had significantly lower geometric mean levels of oocyst excretion (6.3 × 10⁵) than persons without IgG reactivity to this antigen (n = 4; 1.4 × 10⁶; P = .003). The presence or absence of antibodies to these antigens had no detectable influence on the duration of oocyst excretion (data not shown).

Table 3. Relationship between day 0 antibody reactivity and subsequent development of symptoms in oocyst-positive persons.

<table>
<thead>
<tr>
<th>Band</th>
<th>Asymptomatic (n = 7)</th>
<th>Symptomatic (n = 11)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-27</td>
<td>0.482 (0.136–2.443)</td>
<td>0.126 (0.000–4.018)</td>
<td>.39</td>
</tr>
<tr>
<td>IgG-17</td>
<td>0.321 (0.000–0.675)</td>
<td>0.000 (0.000–0.271)</td>
<td>.03</td>
</tr>
<tr>
<td>IgG-15</td>
<td>0.054 (0.000–0.675)</td>
<td>0.090 (0.000–0.163)</td>
<td>.18</td>
</tr>
<tr>
<td>IgM-27</td>
<td>0.940 (0.587–5.019)</td>
<td>0.262 (0.000–7.117)</td>
<td>.04</td>
</tr>
<tr>
<td>IgA-17</td>
<td>0.000 (0.000–0.247)</td>
<td>0.000 (0.000–1.504)</td>
<td>.71</td>
</tr>
</tbody>
</table>

* Arbitrary units.
† Wilcoxon rank sum test.
Figure 3. Purification of 17-kDa antigens. A, Silver stain profile of oocyst antigens separated by SDS–polyacrylamide gradient gel electrophoresis. B, Immunoblot corresponding to A. Lanes in A and B: 1, crude soluble fraction; 2, insoluble fraction; 3, electroeluate containing 17-kDa antigen group; and 4, molecular mass protein standards in kDa. Positive control serum mentioned in Materials and Methods was used in immunoblot. C, IgG reactivity to purified 17-kDa antigen group was assessed by ELISA. Optical density (OD) values for asymptomatic (Sx–) and symptomatic (Sx+) oocyst-positive volunteers are plotted. Median reactivity for each group is shown as horizontal line across column of data points. Median reactivity of asymptomatic persons at day 0 was significantly higher than that of persons who subsequently developed symptoms (P = .04).

Symptomatic infection but not with protection from infection. In addition, IgG antibody to the 27-kDa antigen was associated with lower levels of oocyst excretion. Given that the 27- and 17-kDa antigen groups contain proteins recognized by monoclonal antibodies that bind to sporozoite antigens [5, 6], these results imply that immune responses to sporozoite antigens can ameliorate subsequent infection; however, we do not know if antibodies were directly responsible for mediating these effects or if they served as markers of other humoral or cellular immune responses.

In contrast to the immunoblot data presented here, ELISAs performed with crude oocyst antigens and serum specimens collected from 19 volunteers after primary challenge demonstrated no increases in IgG reactivity, whereas increases in IgM reactivity were observed in 58% of the 19 individuals. No correlation was seen between the development of IgM reactivity after primary challenge and protection from infection or disease [18]. In the current study, we also observed that day-0 antibody responses to crude oocyst antigens (as detected by ELISA) and to specific antigen groups (as measured by immunoblot) were not well correlated (data not shown). This may reflect differences between the two assay systems in the results imply that immune responses to sporozoite antigens can ameliorate subsequent infection; however, we do not know if antibodies were directly responsible for mediating these effects or if they served as markers of other humoral or cellular immune responses.

There are two possible explanations for the existence of antibodies at day 0 that react with Cryptosporidium antigens by immunoblot. Either some of the volunteers were exposed to Cryptosporidium prior to their entry into the study or they were exposed to heterologous antigens that elicited cross-reactive antibodies. If the former possibility is correct, ELISAs using crude oocyst extracts, such as that used for initial screening of the volunteers, may not be as sensitive as the immunoblot, a conclusion that is consistent with our previous results [14].
Alternatively, it is possible that day-0 antibody reactivity represents responses directed against non-
Cryptosporidium antigens. Reactivity to one or more C. parvum proteins, including high-molecular-mass bands, was seen on virtually all blots. Thus, some immunoblot reactivity may represent cross-reactive antibody, perhaps to carbohydrate epitopes stimulated by unidentified species. However, in parallel studies to address the specificity of the immunoblot using serum samples from persons infected with Toxoplasma or Giardia species or other protozoan parasites, we were unable to identify a source of cross-reactive IgG and IgA responses to the 27-, 17-, and 15-kDa antigens (Priest et al., unpublished observation).

As determined by immunoblot, Cryptosporidium seroprevalence in the volunteer population is relatively high (72%) compared with previous ELISA-based seroprevalence surveys of healthy US residents [19]. To our knowledge, there are no published studies of seroprevalence in non-outbreak settings that compare ELISA and immunoblot reactivity or that correlate these responses with known risk factors for cryptosporidiosis.

In summary, changes in antibody reactivity to small molecular mass antigens were observed in 13 of 18 volunteers who were oocyst positive and in 4 of 11 volunteers who were oocyst negative. Increases in antibody responses were significantly more common in symptomatic than asymptomatic volunteers. At the time of inoculation, volunteers with existing antibodies to the 27-, 17-, and 15-kDa antigen groups appeared to be less susceptible to disease than volunteers without existing antibodies. Further study of the antibody reactivity that existed at the time of exposure may provide additional insight into the relationship between antibody reactivity and clinical outcome.

References