Cryptosporidium parvum: Intensity of Infection and Oocyst Excretion Patterns in Healthy Volunteers

Cynthia L. Chappell, Pablo C. Okhuysen, Charles R. Sterling, and Herbert L. DuPont

Data about human Cryptosporidium parvum infection have originated from travelers, community and day care center outbreaks, and persons infected with the human immunodeficiency virus. In addition, experimental infection in 29 antibody-negative, healthy, adult volunteers generated information on the dose-infection response of C. parvum (Iowa strain). In that report, low inocula were sufficient to cause infection in 18 and illness in 7 persons. To further define the duration and intensity of infection in this population, oocyst shedding patterns were investigated in the 18 subjects infected with C. parvum. Oocyst quantitation revealed that volunteers with diarrheal illness (n = 7) excreted more oocysts over the course of the infection than did volunteers without diarrhea (n = 11; P < .05). Symptomatic subjects were more likely to shed oocysts on consecutive days. Further, a statistical nonsignificant inverse trend (r² = .330, P = .136) was seen between challenge dose and total excreted oocysts. This paradox may relate to receptor saturation or a toxic effect on cells, parasites, or both afforded by a high inoculum.

Cryptosporidium parvum is a common enteric protozoan parasite of humans and other species that infects young farm animals (calves, lambs, goats) and pets (kittens, puppies). It is found worldwide in virtually every human population. The prevalence of infection varies widely but appears to be highest in young children and immunocompromised persons. Studies of childhood infections in developing countries suggest an average prevalence rate of ~8.3% (range, 2.3%-16.7%) [1]. In contrast, a review of studies in four industrialized nations indicated that ~3.6% of children with diarrhea were infected with C. parvum [2, 3]. The infection is common in day care centers in the United States. Of randomly selected children (12–42 months old) in day care in Denver, 0.8% were infected as were 2.7% of diaper-age children in Atlanta day care centers [4, 5]. In addition, outbreaks of cryptosporidiosis in this setting can result in infection rates of 27%–59% [6]. A high percentage of children infected with C. parvum are asymptomatic and may contribute to continued transmission (reviewed in [6]).

Adults may also become infected with C. parvum. The largest community outbreak of diarrhea due to any defined organism occurred in Milwaukee in 1993 when 403,000 people are thought to have contracted Cryptosporidium infection [7]. In non-outbreak situations, the rate of infection in adults with diarrhea is 0.4%–4.3% [8]. In AIDS patients with diarrhea, cross-sectional studies indicate that ~16% are positive for C. parvum [9, 10]; however, up to 50% of AIDS patients may acquire the parasite at some point during their illness.

The importance of C. parvum infection in humans was not appreciated before the human immunodeficiency virus epidemic. It was soon recognized that AIDS patients, especially those with CD4 cell counts of 200/mm³ or fewer, were at special risk for cryptosporidiosis [11]. The infection in this population is chronic, progressive, and sometimes fatal. Symptoms are initially similar to those in immunocompetent hosts but develop into a more severe disease and may spread to other sites.

In contrast, the infection in immunocompetent persons is self-limited. The clinical spectrum of cryptosporidiosis is well described in travelers to developing nations, in symptomatic individuals during C. parvum outbreaks, and in people with AIDS [12, 13]. However, little is known about incidence, length and intensity of infection, or excretion patterns in healthy subjects. Data from returning travelers and outbreaks are limited, since the timing and intensity of the infecting inocula as well as prior Cryptosporidium-specific antibody status are difficult to ascertain.
Recently, a human model of Cryptosporidium infection was established [14]. Healthy volunteers were challenged with 30 to 10⁶ oocysts to determine infectivity of C. parvum (Iowa strain). The percentage of those infected increased with challenge dose; however, symptoms occurred with both high and low doses. A dose-response curve indicated an ID₅₀ of 132 oocysts; however, as few as 30 oocysts were infectious for some persons. Further, 7 (39%) of 18 infected subjects had no symptoms.

Symptoms were similar to those seen in travelers with cryptosporidiosis, but the course of illness tended to be shorter than previously reported. In addition, the onset of infection showed a slight (not significant) delay with lower challenge doses. In comparison, the duration of oocyst excretion was somewhat longer with higher challenge doses (P = .06). The purpose of the present study was to extend these observations by quantitating oocyst excretion in healthy volunteers. Here we report the pattern of oocyst excretion in 18 infected volunteers, the relationship of intensity of infection to clinical outcome, and the correlation of oocyst production with challenge dose.

Materials and Methods

Study population. Volunteers and the C. parvum isolate used in this study have been described [14]. In brief, 29 specific antibody-negative, healthy adults were challenged with 30 to 10⁶ oocysts to determine the infectivity of C. parvum. All volunteers were screened for immunodeficiency before enrollment in the study and randomly assigned to challenge dose. Eighteen volunteers (61%) became infected (i.e., positive for fecal oocysts); 11 remained uninfected. Of the 18 infected, 7 had 1 or more enteric symptoms with diarrhea; 4 had 1 or more enteric symptoms without diarrhea; and 7 were completely asymptomatic.

Stool collection and preparation. All stools were collected daily for 14 days, and 24-h stools were collected twice weekly for 60 days. Each volunteer recorded the frequency of bowel movements and any symptoms in a diary that was audited daily by University of Texas-Houston Clinical Research Center nursing staff (Herman Hospital, Houston). Symptomatic subjects were defined as volunteers who experienced at least 1 enteric symptom and produced 3 unformed stools in 8 h or 4 unformed stools in 24 h. Volunteers were considered infected if oocysts were detected any time 36 h after challenge. Oocysts detected within 36 h of challenge were considered to be part of the inoculum and were not counted as infection.

Stools were kept at 4°C after collection and were delivered to the laboratory within 24 h of passage. Upon arrival in the laboratory, each stool was weighed, and an aliquot was preserved in 10% buffered formalin (1:4). A quantitative direct fluorescence assay was done as described [15] using a commercial kit (Merifluor Cryptosporidium/Giardia kit; Meridian Diagnostics, Cincinnati). Stool aliquots in a defined volume were assayed in triplicate, and mean oocysts were calculated to obtain the number of oocysts per milliliter. This number was multiplied by the stool weight to obtain the number of oocysts per sample. Finally, all samples from each day were summed to obtain the number of oocysts excreted per day (total daily oocysts). Intensity of infection was defined as total number of oocysts excreted over the entire study period. Oocyst yield was defined as the intensity of infection divided by the challenge dose [16].

Statistical procedures. Statistical analyses were done using INSTAT2 software (GraphPad Software, San Diego) and SPSS for Windows (SPSS, Chicago). Dunn's multiple comparison test was used to compare the intensity of infection in all groups of infected volunteers (i.e., those with diarrhea, those with enteric symptoms without diarrhea, and asymptomatic subjects). The Mann-Whitney test was used to compare onset or duration of infection for the various clinical groups (data not shown). To compare the relative increase in the number of oocysts recovered from each volunteer versus the challenge dose, we summed the total daily oocysts counted for all specimens collected throughout the trial for each subject, plotted the mean value for each challenge dose, and calculated a weighted linear regression. In this analysis, regression was weighted for the number of volunteers in each challenge group.

Results

Figure 1 shows the pattern of oocyst excretion for each of the 18 infected subjects. Initial evidence of replicating organisms began as early as day 4 and as late as day 24. Sixteen (89%) of 18 subjects began shedding within the first 14 days, and 2 excreted oocysts on a single day (days 19 and 24).

The mean period between challenge (ingestion of oocysts) and patenty (excretion of oocysts in stool) was 5 and 6.43 days in the 2 symptomatic groups, while asymptomatic persons had a longer prepatent period (10.7 days; figure 1). However, this
Table 1. Stool-to-stool variation in oocyst excretion by healthy volunteers.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Days after challenge</th>
<th>No. positive/total specimens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>1/3 (33.3)</td>
</tr>
<tr>
<td>2</td>
<td>5, 6</td>
<td>5/8 (62.5)</td>
</tr>
<tr>
<td>3</td>
<td>5–8</td>
<td>28/30 (93.3)</td>
</tr>
<tr>
<td>4</td>
<td>11, 13, 14</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td>5</td>
<td>9, 10</td>
<td>12/14 (85.7)</td>
</tr>
<tr>
<td>6</td>
<td>5, 6</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td>7</td>
<td>7, 8</td>
<td>6/12 (50)</td>
</tr>
</tbody>
</table>

NOTE. Samples were from persons with diarrhea on days during which ≥3 stools were produced.

Trend was not statistically significant. Oocyst excretion was limited to the first 14 days in 6 (85.7%) of 7 subjects with diarrhea but only in 2 (50%) of 4 with enteric symptoms and 3 (42.9%) of 7 asymptomatic volunteers. No statistically significant differences in the duration of infection were noted among these groups. Oocyst excretion was prolonged in 3 persons who shed for 18, 38, and 20 days (figure 1A–C, respectively).

All subjects with a diarrheal illness (n = 7) excreted oocysts on consecutive days; however, not all stools produced during illness were positive for oocysts (table 1). On shedding days, intermittent negative stools were seen in 6 of 7 subjects with diarrhea. The percentage of positive stools was 20%–100% (median, 62.5%).

Intensity of infection (log of total oocyst output) for all infected subjects is shown in figure 2. The overall intensity of infection (mean ± SD) was significantly higher (P < .05) in subjects with diarrhea than in those with enteric symptoms but no diarrhea (figure 2). Asymptomatic persons (figure 2C) had values similar to those in group B (figure 2B) but did not significantly differ from group A (figure 2A). Considerable variation in oocyst excretion was noted, with fluctuations of 3.9 and 2.6 logs in groups A and C, respectively. The percentage of positive stools in subjects with diarrhea was not directly related to the intensity of the infection. Indeed, persons who had 20% positive stools excreted 13-fold more oocysts than did the subject with 62.5% positive samples.

In 16 (89%) of 18 infected volunteers, the total oocysts excreted throughout the study exceeded the challenge dose (figure 3). The 2 subjects who did not show an increase in oocysts also received the highest challenge doses. The mean number of oocysts excreted was plotted for each challenge dose (figure 3). A trend towards an inverse relationship was seen between the number of ingested and excreted oocysts.
Linear regression analysis yielded a correlation coefficient of $-0.574; r^2 = .330 (P = .136)$. Volunteers receiving 500 oocysts showed the greatest variation in oocyst excretion ($5.52 \times 10^4 - 3.85 \times 10^5$), resulting in a mean value well above the expected trend. When the highest value from this group was excluded, $P = .014$. The oocyst yield (figure 3, bottom) also revealed a trend of increasing numbers of oocysts in subjects receiving the lower challenge doses.

**Discussion**

*Cryptosporidium* infection studies in volunteers provide a controlled context in which to analyze the host-parasite interaction in immunocompetent humans. Using the experimental design described earlier [14], we have extended our observations to demonstrate that the number of excreted oocysts and the pattern and the duration of excretion vary widely among healthy persons.

In volunteers, the intensity of infection was associated with clinical outcome; that is, statistical significance was seen between persons with diarrhea and those who had enteric symptoms without diarrhea (figure 2). However, no significant difference was found when asymptomatic subjects were compared. Thus, the significance seen between groups A and B may have resulted from the low number of study subjects in group B; additional volunteers might well increase the variability of results so that statistical significance would no longer be reached. Even though the small number of volunteers does not allow firm conclusions, it is noteworthy that the mean number of oocysts excreted by subjects with a diarrheal illness was ~50-fold higher than excreted by asymptomatic persons or those with milder enteric symptoms. In contrast, in AIDS patients, diarrhea was seen in those with high ($10^9$) or low ($10^4$) daily oocyst excretion [15], suggesting that in AIDS-associated diarrhea, factors other than the number of oocysts contribute to symptoms.

The prepatent period was similar in all symptomatic subjects regardless of severity of symptoms and was only slightly longer in asymptomatic subjects (not statistically significant). Further, neither the prepatent period nor the duration of oocyst excretion correlated with intensity of infection. Also, most volunteers quickly cleared infection, regardless of total oocysts excreted, and none were positive beyond day 38 after challenge.

Variability in oocyst shedding was seen. Only 1 of 7 volunteers with diarrhea had oocysts detected in every stool throughout the shedding period. In the other 6 subjects, oocysts were detected each day, but not in each stool. Stool-to-stool variability in the majority of diarrheal patients showed 2- to 8-fold differences in the number of oocysts excreted; 1 subject had a 30-fold difference. Thus, it appears that oocyst production is not a constant process and may be influenced by unknown factors. In addition, persons excreting fewer oocysts showed an intermittent day-to-day pattern of shedding.

This observation in lighter infections could in part be explained by the asynchronous parasite development, limitations of the detection method, or both. Taken together, these data suggest that a single random sample from a person, even someone with diarrheal illness, may not be adequate to detect infection. The intermittent shedding pattern in *C. parvum* infection helps to explain the epidemiologic observation that fewer than 50% of persons who acquired illness during an outbreak of waterborne cryptosporidiosis have stool samples positive for the parasite when only 1 stool sample is analyzed [16].

In the 2 subjects who received the highest challenge doses, the number of excreted oocysts was equal to or less than the number of ingested oocysts. Nevertheless, these volunteers were considered to be infected for the following reasons. In the subject given $10^9$ oocysts, the organisms seen in stool at days 0 and 1 were likely to be washout of the challenge dose. However, it is unlikely that oocysts would remain for the 4–15 days of excretion documented in this person. Likewise, in the volunteer who received $10^7$ oocysts, organisms were not detected until day 9, which makes it likely that they originated from replicating parasites.

In both cases, we interpret oocyst excretion 2 days after challenge to be indicative of *C. parvum* replication. All other infected volunteers excreted more oocysts than were ingested. Further, oocysts were not detected in any uninfected volunteers during the initial 36 h of infection or thereafter. Of interest, the number of oocysts excreted by volunteers tended to decrease with increased challenge dose (figure 3), which may be related to the lack of association found between rates of illness and increasing doses of *C. parvum* [14]. The phenomenon of decreasing intensity of infection with higher inocula has been described in *Eimeria* infections of rats and chickens (reviewed in [17]). Several explanations for the decreased yield have been offered: A molecule may be produced that is toxic to host cells, merozoites, or both [18]; available target cells may be saturated; or there may be an increased host immune response to larger challenges [19]. All of these possibilities lack confirmation.

The studies described here are important steps towards understanding the host-parasite interaction between *C. parvum* and healthy humans. The volunteers studied were antibody-negative (IgM and IgG) by ELISA before challenge [14]; however, 7 (39%) of 18 infected subjects remained asymptomatic and 11 (37.9%) of 29 were uninfected. Further studies are underway to define the basis of the observed differences in susceptibility, infection intensity, and oocyst excretion in this population. The model of infection described can monitor a subject’s immune response to *C. parvum* before challenge and at intervals after exposure. The role of serum and secretory antibody in the establishment, clearance, and prevention of subsequent infections is being actively investigated.

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References