Infectivity of a Cryptosporidium parvum Isolate of Cervine Origin for Healthy Adults and Interferon-γ Knockout Mice

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The infectivity of a Cryptosporidium parvum isolate of cervine origin (type 2, Moredun) propagated in calves was investigated simultaneously in healthy adult human volunteers and in interferon-γ knockout (GKO) mice. After exposure to 100–3000 oocysts, 16 volunteers recorded, for a duration of 6 weeks, the number and form of stools that they passed and any symptoms that they experienced. Oocyst excretion was assessed by enzyme-linked immunosorbent assay and direct immunofluorescence assay. Eleven subjects (69%) became ill, and 8 subjects (50%) shed oocysts in stool. The median duration of illness was 169 h, and the median number of unformed stools passed was 24. The duration and intensity of symptoms were more severe than were those associated with previously studied isolates. The median infectious dose was estimated to be 300 oocysts for humans and 1 oocyst for the GKO mouse model. The Moredun isolate was more pathogenic than the reference GCH-1 isolate. The GKO mouse model of cryptosporidiosis is useful for discerning isolate-specific differences in pathogenicity.

Exposure of healthy persons to Cryptosporidium parvum results in transient infection that may be asymptomatic or may result in self-limited diarrhea. Previous studies involving volunteers demonstrated that as few as 10 oocysts from experimentally infected calves can cause infection in otherwise healthy adults and that isolates differ in infectivity and, perhaps, virulence [1]. Similar differences were observed in studies involving patients with AIDS, in whom cryptosporidiosis has diverse manifestations, even after the analysis was adjusted for level of immunosuppression [2–4], and in studies involving calves, in which variability in infectivity has been observed [5]. Other researchers have noted differences in mouse infectivity [6], protein composition [7], immunoreactivity [8], and genotype [9, 10]. The biological basis for these differences is not understood.

Analysis of multiple genetic loci has identified 2 major groups of isolates (designated “type 1” and “type 2”) that result in 2 independent human transmission cycles [11–14]. Type 1 isolates are associated with human and nonhuman primate infection, whereas type 2 isolates follow a zoonotic cycle that causes infection in mammals, including humans. Analysis of microsatellite loci indicates an abundance of genetic polymorphisms within both type 1 and type 2 isolates. Infectivity models that can investigate phenotypic differences in infectivity that correlate with genotypes are needed to better define differences among the numerous C. parvum isolates.

Although human challenge studies with cryptosporidia provide valuable data for risk assessment and the study of Cryptosporidium pathogenesis and provide a suitable model for investigation of the relative infectivity of different isolates, they are limited by expense and the number of subjects available for study. Thus, surrogate models that reflect human infection are needed.

In the 1990s, several rodent models for cryptosporidiosis were developed, principally for testing of immunotherapeutic and chemotherapeutic agents [15]. Of the currently available models, the interferon-γ knockout (GKO) mouse is perhaps the most susceptible to infection with Cryptosporidium [16, 17]. The objective of the present study was to investigate and compare the infectivity of the Moredun isolate in humans and in a surrogate animal model in parallel by estimating the infectious dose needed to cause illness or infection in humans and GKO mice, investigating the clinical illness experienced, and measuring the number of parasites excrated after exposure.

Materials and Methods

C. parvum isolates. Oocysts of the Moredun isolate were originally isolated from a red deer calf (Cervus elaphus) in 1986 but have been passaged for many years in sheep. The isolate for this study (obtained from S. Wright, Moredun Research Institute, Edinburgh, Scotland) has been passaged in bovine calves since 1999 at the University of Arizona. The oocysts were purified according to
the method of Arrowood et al. [18, 19]. Before they were used in volunteers and in mice, the oocyst preparations were cultured for bacteria, mycobacteria, and fungi. Transmission electron microscopy and a variety of cell lines (WI-38, MRC-5, RKC, and A549 [American Type Culture Collection] and PRMK 1 and 2 [BioWhittaker]) were used for adventitious virus detection [20]. Studies were conducted in volunteers and in mice in parallel by use of the same batches of oocysts. The GCH-1 isolate (type 2), a reference laboratory isolate included in the mouse studies, was isolated in 1991 from a patient with human immunodeficiency virus/AIDS and chronic cryptosporidiosis and has been propagated continuously since then in calves [15].

Preparation of oocysts. Oocysts suspensions were washed twice in 10 mL of cold sterile PBS, pH 7.2, to remove potassium dichromate storage solution and then were resuspended in cold sterile PBS and serially diluted before use. Oocysts were counted by hemocytometer and adjusted to the desired dose. A minimum of 5 additional counts were done to get an accurate estimate of the inoculum. Volunteers ingested oocysts in gelatin capsules, as described elsewhere [21]. Oocysts were ingested within 14–51 days of calf passage and in all cases were found to have an excystation rate >85% at the time of challenge. Actual oocyst concentrations delivered to the volunteers were typically within a median coefficient of variance of ≤5% of the target dose. Volunteers received oocyst doses of 100, 300, 1000, or 3000 oocysts and were assigned to receive the different dose levels by a mathematical model [22]. The final distribution allocated 4 subjects to the 100-oocyst dose, 5 to the 300-oocyst dose, 3 to the 1000-oocyst dose, and 4 to the 3000-oocyst dose.

Parasite inoculation of GKO mice followed the same methods. Oocyst stock concentration was estimated from 5 independent counts with a hemocytometer. From this stock, final inocula were prepared by serial dilution in distilled water to obtain 100, 10, or 1 oocyst(s) per 30 μL of oral inoculum per mouse. The mouse experiment was repeated 4 times, and each experiment used the same batch of oocysts that was used for the volunteers.

Studies in human subjects. Volunteers were asked for a detailed medical history. Subjects were included for further study if the results of screening for the presence of anti-C. parvum antibodies by ELISA were negative. Before oocyst challenge (study entry), participants underwent a physical examination and extensive laboratory studies for general health assessment, as described elsewhere [20]. Volunteers collected all stools passed for the first 2 weeks of the study and in two 24-h collections every week thereafter, for a total of 6 weeks after challenge. Each volunteer kept a diary and described the time and characteristics of all stools passed and symptoms experienced. All stools collected were examined initially for the presence of cryptosporidial by ELISA (Meridian Bioscience). Samples found to be positive by ELISA were then studied by quantitative direct immunofluorescence assay (DFA) [20]. Stools from all episodes of diarrhea were cultured for enteric bacterial pathogens, including Shigella, Salmonella, Campylobacter, Aeromonas, and Plesiomonas species.

Diarrheal illness was defined as any 1 of the following: the passage of 3 unformed stools in an 8-h period; the passage of ≥4 unformed stools in a 24-h period; or the passage of unformed stools weighing >200 g total in a 24-h period, accompanied by at least 1 enteric symptom. Enteric symptoms included nausea, vomiting, abdominal pain, flatulence, tenesmus, fecal urgency, and incontinence. A confirmed infection was defined as the presence of fecal oocysts in the stool at ≥36 h after challenge. Presumed infection was defined as manifestation of a diarrheal illness or enteric symptoms without demonstrated oocysts within 30 days after challenge. We use the term “presumed” because the detection limit for the DFA was 10,000 oocysts/mL [23]. The time to onset of diarrhea was the number of days that elapsed between oocyst challenge and passage of the first unformed stool after which illness was declared. The duration of diarrhea in hours was defined as described elsewhere [24]. The attack rate was the percentage of persons who developed diarrhea after oocyst exposure.

Studies in GKO mice. Four-week-old GKO mice weighing 10–19.5 g were purchased from Jackson Laboratories. Mice were weighed

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### Table 1. Clinical and parasitologic outcomes for 16 healthy adult volunteers after ingestion of Cryptosporidium parvum (Moredun isolate).

<table>
<thead>
<tr>
<th>Inoculum size in oocysts</th>
<th>No. of subjects</th>
<th>No. of ill subjects</th>
<th>Median onset, days (range)</th>
<th>Median duration, h (range)</th>
<th>Median no. of unformed stools</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>4</td>
<td>3</td>
<td>5 (4–5)</td>
<td>122 (73–169)</td>
<td>19</td>
</tr>
<tr>
<td>300</td>
<td>5</td>
<td>3</td>
<td>4 (3–7)</td>
<td>169 (57–264)</td>
<td>14</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>2</td>
<td>5 (3–7)</td>
<td>145 (6–284)</td>
<td>27</td>
</tr>
<tr>
<td>3000</td>
<td>4</td>
<td>3</td>
<td>3 (3–5)</td>
<td>342 (110–360)</td>
<td>23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of subjects with ELISA-positive stools</th>
<th>Median onset, days (range)</th>
<th>Median duration, days (range)</th>
<th>Median excretion, oocysts × 10,000 (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7.5 (6–9)</td>
<td>3.5 (2–5)</td>
<td>187 (119–250)</td>
</tr>
<tr>
<td>2</td>
<td>5 (5)</td>
<td>3 (1–5)</td>
<td>21 (21–690)</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>11</td>
<td>31,000</td>
</tr>
<tr>
<td>3</td>
<td>5 (6–9)</td>
<td>6 (4–7)</td>
<td>2070 (1060–2700)</td>
</tr>
</tbody>
</table>

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**Figure 1.** Infectious dose curves for the Moredun isolate in healthy volunteers. The cumulative percentage of volunteers confirmed to be infected or presumed to be infected after exposure to Cryptosporidium parvum Moredun isolate was determined by the method of Reed and Muench [25].
and randomly assigned to groups of 7 in such a manner that the total weight of each group was within 0.1 g of the others. Each mouse was inoculated by per os gavage directly into the esophagus with a 30-μL aliquot of oocyst suspension. Mice were observed daily for symptoms. GKO mice do not develop diarrhea; symptoms of *C. parvum* infection typically include lethargy, anorexia, a ruffled coat, and reduced weight gain. Beginning 5 days after inoculation, fecal smears were stained with a modified acid-fast stain and monitored microscopically for oocyst excretion. Oocysts were quantified by averaging of the number of oocysts observed in 30 high-power fields. Each mouse was weighed at the start of the experiment and at 5-day intervals thereafter until death, at which point gut sections were obtained for histologic examination. At 16 or 17 days after challenge, surviving mice were euthanized, and gut sections were obtained.

Mucosal infection scores were determined at 6 gastrointestinal sites: the pyloric region of the stomach, 3 equally spaced small-intestinal sites, the cecum, and the colon. The 6 gut sections were combined to calculate the mucosal infection score for each mouse, which reflected the extent of *C. parvum* infection in the luminal portion of the intestine. Each site was scored as follows: 0, no infection; 1, parasite forms present but very difficult to find; 2, parasite forms sparse but easy to find; 3, abundant presence of parasite forms but focal distribution; 4, extensive presence of parasite forms covering most mucosal surfaces; 5, extensive presence of parasite forms covering the entire mucosal surfaces. The isolate-specific mucosal infection profiles, like the oocyst-shedding profiles, were determined by plotting mean score against dose.

**Statistical methods.** For human infectivity studies, we developed a dose-response curve by the method of Reed and Muench [25]. Data were analyzed by linear regression fit on the basis of least-square estimation. The clinical outcomes experienced by the volunteers who received the Moredun isolate were compared with the outcomes for volunteers in previously published studies [1]. For data on duration and onset of diarrhea, we used the Kruskal-Wallis method. We used analysis of variance to compare the number of stools passed. For all statistical analyses, we used Statview for Windows 5.0.1 software (SAS Institute).

**Results**

**Studies in human subjects.** In total, 16 volunteers were challenged with the Moredun isolate at the doses shown in table 1. Infectious dose curves (figure 1) were plotted using data from subjects classified as having a presumed infection and from subjects who only had documented oocyst shedding. The ID₅₀ value for the Moredun isolate was ~300 oocysts in subjects with presumed infection. A similar dose response was seen when data from volunteers shedding oocysts were used (ID₅₀, 375 oocysts).

During the study, 11 (69%) of 16 volunteers developed diarrhea (table 1) (median onset, 3 days after exposure; range, 3–7 days). The median duration of diarrhea was 169 h (range, 6–360 h), and the mean number of unformed stools was 19 (range, 2–52 stools).

Eight of the 16 volunteers had detectable oocysts in their stools after challenge (table 1). All participants in whom oocysts were detectable developed diarrheal illness. Three subjects with diarrhea did not shed oocysts. The mean onset of oocyst excretion was 5 days (range, 4–9 days), and the mean duration was 5 days (range, 1–11 days).

The clinical outcomes of onset, duration, and intensity of diarrhea were compared with those associated with 3 isolates (Iowa, TAMU, and UCP) previously studied [1]. Subjects exposed to the Moredun isolate experienced an earlier median onset of illness, compared with those exposed to the Iowa isolate (3 vs. 7 days; *P* < .05), but an onset similar to that seen in subjects exposed to the TAMU and UCP isolates (5 and 6 days, respectively). The

**Figure 2.** Infectious dose curves for *Cryptosporidium parvum* Moredun (MD; inocula of 1, 10, and 100 oocysts) and GCH-1 (inocula of 1 and 10 oocysts) isolates in interferon-γ knockout mice. Both isolates are genotype 2. The Y-axis shows the log of the mean number of oocysts seen for 30 fields of a modified acid fast–stained fecal smear. Bars, 95% confidence intervals.

**Figure 3.** Mortality in interferon-γ knockout mice 9–16 days after challenge with various doses of 2 distinct *Cryptosporidium parvum* genotype 2 isolates: Moredun (MD; inocula of 1, 10, and 100 oocysts) and GCH-1 (inoculum of 10 oocysts).
Moredun-exposed subjects also experienced a longer median duration of diarrhea, compared with the Iowa-exposed subjects (169 vs. 93 h; \( P < .05 \)), but duration was similar to that associated with the TAMU and UCP isolates (\( P \) was not significant [NS]). Similarly, volunteers in the Moredun group experienced diarrheal illness that was more intense, as determined by the median number of unformed stools passed per diarrheal episode, than that in the TAMU or UCP groups (19 vs. 8 and 9 stools, respectively; \( P < .05 \)) but similar to that in the group receiving the Iowa isolate (\( P \) was NS). No differences in the number of parasites excreted were noted between groups of subjects.

Oocysts excreted by one volunteer were purified and genotyped using 2 microsatellite markers (5B12 and 1F07) to determine whether passage through humans affected the genetic profile of the parasite population. For both loci, the alleles detected in the volunteer’s oocysts were identical to those of the original oocyst inoculum.

**Studies in GKO mice.** Oocyst shedding in GKO mice showed a similar pattern in each of the 4 replicated experiments (figure 2). Both the onset of oocyst excretion and the intensity of that excretion appeared to be dose dependent throughout. For example, mice receiving the Moredun isolate at a dose of 100 oocysts had an earlier and higher intensity of shedding than mice receiving 1 or 10 oocysts. Compared with the mice challenged with the reference isolate, GCH-1, the mice that received the Moredun isolate also had an earlier onset of oocyst shedding. None of the mice that received the GCH-1 isolate died. In contrast, 50% of the mice that received the Moredun isolate died before the conclusion of the experiment (figure 3).

We also observed a dose-dependent and isolate-specific response in GKO mouse experiments with respect to mucosal infection scores for the various intestinal sites (figures 4 and 5). The site of infection correlated with severity of the disease; the more proximal the intestinal site affected, the more severe was the clinical outcome. A trend was seen for the Moredun isolate toward a more proximal involvement of the gut than that for the reference GCH-1 isolate. Figure 6 shows the impact of the infectious dose on weight gain in the 4 experiments. After challenge, all mice gained weight throughout the course of the experiment; however, the infected mice gained weight more slowly than uninfected mice. At the end of the experiment, the groups of mice receiving the larger inocula were lighter than groups of uninfected controls. A trend toward an inverse correlation between weight gain and challenge dose was apparent.

**Discussion**

The *C. parvum* Moredun isolate was studied in healthy adults and GKO mice in parallel to determine whether there are correlations between the infectivity for humans and GKO mice. We chose this isolate because methods for its propagation are well established, and it is used in research laboratories worldwide in a multitude of projects, including genomic libraries [26, 27]. Defining the infectivity of this isolate in humans and in mice will be of assistance in correlating basic science observations to infectivity and pathogenicity.

When it was measured by the same approach used elsewhere to determine the infectivity of other isolates in humans [1], the \( \text{ID}_{50} \) in this study for the Moredun isolate was 300 oocysts. This value falls within the range of \( \text{ID}_{50} \) values obtained for 3 other type 2 *C. parvum* isolates (TAMU, Iowa, and UCP) studied in seronegative subjects (\( \text{ID}_{50} \), 10–1042 oocysts) [1]. In accordance with previous studies, diarrheal illness was frequently associated with oocyst excretion as determined by DFA, and little relationship was observed between the dose ingested and the severity of the clinical outcome, the onset of illness or infection, or the number of parasites excreted [28]. However, when clinical outcomes for subjects receiving the Moredun isolate were compared with our previously published observations [1], the subjects who received the Moredun isolate experienced a longer duration of diarrhea, a shorter incubation period, and more-intense diarrheal illness than subjects who received other isolates. Although we studied few volunteers and isolates and the challenges with different isolates were done at different times, the data suggest that the infectious dose (infec-
trusivity) of a given isolate may not accurately reflect its pathogenic potential for humans.

In contrast to the volunteers, the GKO mouse was highly susceptible to the Moredun isolate. All mice became infected and shed oocysts, and the GKO mouse model provided a clearer dose-response effect in terms of oocyst excretion and pathogenicity. Of interest, compared to the reference isolate, GCH-1, the Moredun isolate appeared to be more pathogenic for mice; this was reflected in the increased number of deaths and was in concert with the findings in humans. When outcomes associated with the Moredun isolate were compared with outcomes associated with the other 3 isolates previously studied in humans, the Moredun isolate also proved to be more infectious (data not shown).

Several limitations of our study deserve mention. Considerable variability in the mouse infectivity experiments was observed. It is possible that small variances in oocyst viability or excystation rates can have a significant impact on infection outcomes when a highly susceptible animal such as the GKO mouse is used. Nonetheless, a consistent pattern was evident. First, oocyst shedding in GKO mice showed a characteristic pattern, rising to peak levels and then subsiding. Second, oocyst shedding tended to be higher for the Moredun than the GCH-1 isolate. Third, the onset of shedding was dependent on challenge dose—higher the number of oocysts administered, the earlier oocyst shedding peaked. Oocyst shedding in lower-dose groups usually reached the same intensity but required a longer prepation period.

The genotype of the Moredun isolate was characterized elsewhere on the basis of multiple microsatellite polymorphisms [10] and was different from that of other type 2 isolates studied in human subjects. However, we cannot determine whether this is the same population that was originally isolated from a deer or whether it was overgrown by exogenous genotypes during its long history of propagation in the laboratory. Moreover, this isolate population, like any other C. parvum isolate, is not a clonal population and is therefore likely to include multiple subpopulations, the relative abundance of which may change in response to the host or environmental conditions, as was shown in a recent study of another type 2 laboratory isolate that revealed extensive intraisolate heterogeneity [29]. The infectivity of the Moredun isolate should be interpreted in light of these observations.

Taken together, these data support previously published observations of variations in isolate-specific infectivity and virulence [1, 5]. The susceptibility of GKO mice to small numbers of oocysts and the differences in mortality suggest that the GKO mouse model of cryptosporidiosis may be useful in comparing the infectivity and pathogenicity of distinct C. parvum isolates. This model will be useful in the study of the relative contribution of putative virulence factors to infectivity. Further parallel studies that correlate the infectivity of genotypically diverse isolates in humans and mice are warranted.

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References


