Novel In Vitro and In Vivo Models and Potential New Therapeutics to Break the Vicious Cycle of Cryptosporidium Infection and Malnutrition

Lourrany B. Costa,1,2 Francisco Jose Noronha,1,2 James K. Roche,1 Jesus Emmanuel Sevilleja,1 Cirle A. Warren,1 Reinaldo Oria´,2 Aldo Lima,2 and Richard L. Guerrant1

1Center for Global Health, Division of Infectious Diseases and International Health, School of Medicine, University of Virginia, Charlottesville; and 2Institute of Biomedicine, Federal University of Ceará, Fortaleza, Brazil

Background. Although several animal models of cryptosporidiosis have been reported, most involve genetically or pharmacologically immune-suppressed hosts.

Methods. We report challenge with excysted (in vitro and in vivo) and unexcysted (in vivo) Cryptosporidium parvum oocysts in human colonic adenocarcinoma (HCT-8) cells and weaned nourished and malnourished C57BL/6 mice, following outcomes of growth rate, stool shedding, and tissue burden. We tested treatment with an oligodeoxynucleotide containing unmethylated CpG motif (CpG-ODN) and alanyl-glutamine in vivo and in vitro.

Results. C. parvum–challenged mice showed prolonged weight loss (>10% over 4 days), robust stool shedding (>3 logs/d over 7 days), and epithelial infection in the ileum, cecum, and colon. Of 2 potential therapeutic compounds evaluated in the model, CpG-ODN reduced body weight loss (to <6% on days 3–7 after challenge), reduced shedding of organisms (by 25% on days 1 and 3 after challenge), and decreased the burden of parasites in the ileum. Alanyl-glutamine showed similar benefits. In vitro findings suggested that effects on the epithelial component of the mucosa probably likely responsible for beneficial effects seen in vivo.

Conclusions. Weaned mice provide a convenient and reproducible model of cryptosporidial disease, including its vicious cycle with body weight loss and heavier infection with malnutrition, and this model may be useful in exploring innovative therapeutic solutions for this challenging infectious disease.

Cryptosporidium parvum is an important cause of diarrhea among young children in developing countries in association with malnutrition [1–3], as well as in persons with immune deficiencies such as AIDS, in whom it can lead to chronic diarrhea and death [4]. Treatment options for cryptosporidiosis are limited, and a mouse model to test new therapeutics is needed, one that mirrors particularly vulnerable populations such as young children who are malnourished [5].

C. parvum oocysts adhere to epithelium of the small intestine in humans, which they then invade and reproduce under the plasma membrane in parasitophorous vacuoles. Succession of parasitic life forms ensue, including trophozoites and merozoites able to invade adjacent epithelial cells and/or be expelled as oocysts in stool, able to survive for a prolonged period before entering another host [6]. Knowledge of the pathogenesis may suggest options for therapy or prevention of cryptosporidiosis, when pharmacological options are not optimal. These include activation of the local immune response against C. parvum and enhancing the barrier function of the epithelial cells.

Oligodeoxynucleotides and alanyl-glutamine (AQ) are compounds with therapeutic potential against C. parvum. Oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) act as an immune adjuvant for vaccines boosting immune responses [7, 8]. CpG-ODN

Received 25 July 2011; accepted 16 November 2011; electronically published 26 March 2012.

Presented in part: This study has been presented in part at the 59th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Atlanta, Georgia, November 2010. Abstract 327.

Correspondence: Richard L. Guerrant, MD, PO Box 801379, Center for Global Health, Division of Infectious Diseases and International Health, University of Virginia, 345 Crispell Dr, Harrison-Carter Bldg, Rm 2520, Charlottesville, VA 22908 (rlg9a@virginia.edu).

The Journal of Infectious Diseases 2012;205:1464–71
© The Author 2012. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com
DOI: 10.1093/infdis/jis216
selectively binds to Toll-like receptor 9 (TLR9), that activates production of proinflammatory cytokines [9]. AQ is an amino acid necessary for polyamines production, cell division, and DNA replication [10–15]. This compound has been studied in in vitro and in vivo studies and clinical trials and has shown promise as therapy for patients with enteric infections [16, 17].

The pathogenesis of cryptosporidiosis also suggests that several experimental approaches are available to explore these therapeutic options. Major involvement of C. parvum with epithelial cells suggests that reductionist tissue culture models may be useful, because they are amenable to highly controlled studies while remaining relevant to disease. Second, animal model systems that permit exploration of all the host’s anti-C. parvum capabilities may be instructive as well. In the current study, we use both approaches to examine potential therapeutic candidates for C. parvum disease.

Our goals in the current work are 3-fold: (1) define a simple convenient model not requiring oocysts excystation yet causing severe disease; (2) determine whether CpG-ODN or AQ can be therapeutic in C. parvum infection; and (3) explore whether any of the beneficial effects found in vivo might be accounted for by their effects on intestinal epithelium as observed in vitro.

MATERIALS AND METHODS

In Vivo Studies

Animals and Malnutrition

The protocol described below is in accordance with the Institutional Animal Care and Use Committee (IACUC) policies of the University of Virginia. Weaned 21-day-old female C57BL/6 wild-type mice were purchased from Charles River Laboratories. On arrival, mice were acclimated, weighed, and distributed in groups. On day 24 of life, nourished groups received chow containing 20% protein, and malnourished groups received isocaloric chow containing 2% protein (Harlan Laboratories). The animals were kept on their respective diets for 7 days to establish malnutrition before infection and during the whole experiment after infection. Diet and water were given ad libitum. Each mouse was weighed daily throughout the duration of the experiment, and stools were collected daily during 10–15 days of the infection phase. Some animals were killed on day 4 after infection when intestinal segments were collected.

Administration of Therapeutics In Vivo

Mice received solutions of AQ (4.3 mg per mouse) (Ala-Gln A8185; Sigma-Aldrich) and CpG-ODN (50 μg per mouse) (1668 5’TCCATGAGCTTCCTGATGCT 3’; Sigma-Aldrich). Mice received 50–150 μL (in 1× phosphate-buffered saline [PBS], pH ~7) daily of the AQ solution by gavage for 10 days (starting 3 days before C. parvum challenge and continuing until day 6 after challenge). We have used up to 44 g of AQ daily to treat adults with AIDS in Brazil and thus in the current study used a similar dose adjusted on a weight basis. The groups with CpG-ODN received it intraperitoneally 3 times (3 days before, at same time, and 3 days after C. parvum challenge). The dose of CpG-ODN administered was based on a prior published study (see below) that used 100 μg given intraperitoneally daily in neonatal mice with success in ameliorating growth shortfalls.

Cryptosporidium Parvum Infection

Oocysts of C. parvum (Iowa isolate) were purchased from Waterborne. Concentration of the stock solution, as received from the vendor (1 × 107/50 mL PBS) was measured using a hemocytometer to estimate the number of oocysts needed. In some experiments, mice received excysted oocysts. Oocysts were excysted by adding 1 part of sodium hypochlorite to 4 parts of the oocyst stock solution, then briefly vortexed and incubated at room temperature for 10 minutes. After incubation, the number of oocysts was counted to compute the excystation rate (30%–35% in these experiments). Each infected mouse received an inoculum of 5 × 107 C. parvum excysted oocysts in 100 μL of freshly prepared oocysts solution via oral gavage directly into the stomach; controls received 100 μL of PBS alone. In other experiments, mice received 1 × 106–5 × 107 unexcysted oocysts. Excysted oocysts were used in tissue culture studies.

DNA Extraction for Parasite Detection

The DNA was extracted from the thawed stool samples using the QIAamp DNA Stool Kit (Qiagen) with minor modifications. DNA from tissue samples was extracted from frozen tissue samples using the QIAamp DNA Tissue Kit (Qiagen), following the protocol exactly.

Real-Time Polymerase Chain Reaction for Cryptosporidium parvum Quantification

Quantification of the infection was performed in a Bio-Rad iCycler iQ PCR Detection System by interpolating C, values of each run with a standard curve of known amounts of C. parvum DNA and transformed into number of organisms per milligram of stool or tissue sample [18]. The master mix solution and primers were used as described elsewhere [19]. Amplification consisted of 15 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 15 seconds at 52°C, and 20 seconds at 72°C and then 40 cycles of 10 seconds, starting at 75°C with 0.5°C increments for the melt curve.

In Vitro Studies

Cell Culture

Human colonic adenocarcinoma (HCT-8) cells were cultured in 75 cm² flasks using Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum (FBS) and 100 U/mL of penicillin-streptomycin in 5% carbon dioxide incubator at 37°C. Cells were grown to 80%–90% confluence in 12-well culture plates with regular media (n = 3–6 wells per group) and then submitted to different FBS concentrations (10% and 1%) in glutamine-free medium (21870; Gibco) (“nourished” and
“malnourished” cells). After 24 hours, cells were treated with AQ (10 mmol/L) or CpG-ODN (2, 20, and 100 μg/mL) (purchased from same sources used for the in vivo studies described above). After 24 hours, cells were incubated with serum-free and glutamine-free media with respective treatments and infected with 1–3 × 10^6 excysted C. parvum oocysts per well. At 6 hours after incubation, the media were aspirated and the cell monolayers were washed with PBS and then given fresh media with the respective treatments and FBS concentrations. Cells were harvested at 6, 12, and 30 hours using 0.25% trypsin-EDTA. Samples were stored at −20°C until DNA extraction.

Measure of Cryptosporidium parvum Infection
DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) by following the manufacturer’s protocol exactly. A ratio of number of parasites per 10^6 cells was calculated using standard curves and the C_i values of quantitative polymerase chain reaction (PCR) for parasite DNA sequence and HCT-8 18S ribosomal RNA reference gene (forward, 5’-CCGATAACGAGGAGACTCTGG-3’; reverse, 5’-TAGGTTAGGCACACGGCTGAGCC-3’) (accession No. NR_003286.1). The primers used to detect parasite DNA and the PCR conditions were the same as those used for the animal studies.

Statistical Analysis
Data analyses were performed with GraphPad Prism 5 software (GraphPad Software) and SPSS software, version 19 (SPSS Inc). All statistical analyses were done from raw data with the use of analysis of variance, Student t tests, and Bonferroni post hoc analysis where applicable. Differences were considered significant at P < .05. Data are represented as means ± standard errors of the mean.

RESULTS
Cryptosporidium parvum Infection Using Unexcysted Oocysts
Because weaned mice have a more mature digestive system, we reasoned that C. parvum oocysts that are unexcysted might be fully infective as excysted ones in this age group. To test this, mice malnourished on 2% protein diet were challenged with 10^7 unexcysted oocysts per mouse, either unexcysted or excysted ex vivo (treated with sodium hypochlorite and then extensively washed) before administration. Compared with malnourished mice given oocysts already excysted, those given unexcysted oocysts lost more weight (about 15% in 4 days) (Figure 1A) (P < .001; days 3–10). These results suggested that the intraluminal environment of the intestine within the weaned mouse is sufficient to enable excystation of oocysts administered by gavage.

To explore the number of unexcysted oocysts needed to have clinical impact in the model, weaned malnourished C57BL/6 mice were administered different doses of unexcysted oocysts and followed for 7 days after challenge (Figure 1B). Remarkably, a reduction to 20% of our original challenge dose caused the same prolonged decrement in body weight as the 2 larger challenge doses, when compared with uninfected (PBS-challenged) control mice (P < .001). Nourished mice challenged with C. parvum oocysts demonstrated marked weight loss as well (Figure 1C). Together, these studies show that a reproducible animal model of cryptosporidiosis can be induced in the malnourished weaned mouse, challenged with as few as 1 × 10^7 unexcysted C. parvum oocysts, leading to a prolonged and substantial loss of body weight of ≥10% or more.

Stool Shedding and Tissue Burden After Challenge With Unexcysted Oocysts
Because the intensity of infection, as quantified by the extent of stool shedding of challenge parasites, and the importance of the host’s nutritional status to disease intensity were not known, we gave an equal inoculum of 5 × 10^7 unexcysted oocysts to 2 groups of 58-day-old C57BL/6 mice, differing only in diet (2% or 20% protein) and followed their shedding pattern daily (Figure 1D). At day 5 after challenge, nourished mice shed few parasites per mg of stool, whereas malnourished mice showed robust but gradually declining shedding (up to 10^4 parasites/mg of stool) over the next 6 days. This shedding pattern was confirmed in a second independent study, which showed, in addition, that unexcysted oocyst challenge doses of 1, 2.5, and 5 × 10^7 per mouse did not differ significantly in the subsequent intensity of stool shedding (data not shown).

To test the effect of oocyst excystation on tissue burden, we challenged 37-day-old weaned malnourished C57BL/6 mice with an identical inoculum of C. parvum oocysts and then harvested 3 regions of bowel on day 4 after infection for analysis. Both colon and ileum from animals challenged with unexcysted oocysts showed significantly increased intestine-associated C. parvum organisms, compared with those given excysted oocysts (P < .05 and <.001, respectively). Thus, by the criteria of stool shedding and tissue attachment of organisms to colon and ileum, the malnourished C57BL/6 weaned mouse challenged with unexcysted oocysts is a convenient and reproducible model of cryptosporidiosis, with easily measured and quantifiable biological end points.

Testing Therapeutics in the Model
To determine whether CpG-ODN and/or AQ [20, 21] have an effect on C. parvum infection in vivo, we used the murine model described above to test the notion that 1 or more of them would be associated with less weight loss, more limited stool shedding, and/or a reduced tissue burden of parasites. Malnourished infected mice lost >12% of body weight by day 4 after challenge, clearly different from malnourished noninfected controls (Figure 2A). Among infected groups, mice treated with CpG-ODN maintained 95% of their weight, compared with the malnourished infected controls (P < .05, days 3–6). Those administered AQ also showed benefit compared with infected malnourished controls (Figure 2B).
To determine whether the positive results observed clinically with weight change would be reflected in reduced intensity of infection, we followed stool shedding by quantitative PCR. CpG-ODN treatment showed a significant reduction in the number of parasites per mg of stool on all study days ($P < .05$ on days 1 and 3) (Figure 2C). The AQ group had the lowest oocyst excretion rate of all treatment groups, and this difference was statistically significant on day 3 compared with the infected-only group (Figure 2D).

To determine whether CpG-DN treatment resulted in a difference in *C. parvum* infection of intestinal tissue, mice were killed on day 4 after challenge and *C. parvum* quantified in stool-free intestine by quantitative PCR (Figure 2E). CpG-ODN was associated with a lower burden of organisms in the ileum.
Figure 2.  

A, Ability of oligodeoxynucleotides containing unmethylated CpG motif (CpG-ODN) to reduce body weight loss in malnourished female Cryptosporidium parvum oocyst–challenged C57BL/6 mice. CpG-ODN was administered intraperitoneally daily, beginning 3 days before C. parvum challenge, at $5 \times 10^7$ unexcysted oocysts per mouse (n = 5–7 mice/group); *P < .05 for CpG-ODN on days 3–7, compared with infected-only mice. B, Effect of alanyl-glutamine (AQ) in decreasing body weight loss in malnourished female C. parvum oocyst–challenged C57BL/6 mice. AQ was administered by gavage daily, beginning 3 days before C. parvum challenge at $2 \times 10^7$ unexcysted oocysts per mouse (n = 5 mice/group); *P < .05 on days 4–7, compared with infected-only mice. C, Stool shedding over time in mice described in A; *P < .05 (mice treated with CpG-ODN vs infected-only mice). D, Stool shedding over time in mice described in B; *P < .05 (day 3) (mice treated with AQ vs infected-only mice). E, Tissue burden of C. parvum in stool-free intestine on day 4 after challenge of female C57BL/6 mice with $5 \times 10^7$ C. parvum unexcysted oocysts and treatment with CpG-ODN. Quantification was by quantitative polymerase chain reaction for C. parvum DNA, normalized by wet weight of colon or ileum (n = 2 mice per group).
Together with the findings above, these results suggest that CpG-ODN administered systemically beginning 3 days before challenge with unexcysted C. parvum oocysts can ameliorate cryptosporidiosis in the weaned malnourished mouse.

**In Vitro Correlates of Cryptosporidiosis**

Prior studies have shown that C. parvum becomes established in parasitophorous vacuoles of intestinal epithelium but rarely invades more deeply into the mucosa [22–24]. It seemed reasonable, then, to use epithelial monolayers to explore the effect of our study compounds (CpG-ODN, AQ) as potentially therapeutic against C. parvum attachment to intestinal epithelial cells. After a period (24 hours) of exposure to a study agent, monolayers were washed, exposed to 1–3 × 10⁶ excysted oocysts per monolayer for 6 hours in glutamine-free and FBS-free medium, washed again to diminish unattached oocysts, and exposed to the same concentration of study agent; parasitic DNA and the number of epithelial cells were quantified by PCR. CpG-ODN at 100 μg/mL was associated with a decrease in monolayer-associated parasites at 12 hours after challenge in monolayers incubated with 1% FBS (Figure 3B) (*P < .05). AQ showed little or no effect on the burden of parasites associated with intestinal epithelium (Figure 3C). These data suggest that a primary site for the anticryptosporidial effect of CpG-ODN seen in the in vivo studies above (Figure 2) is the epithelium, and that the AQ benefit may occur through repair of intact mucosa.

**DISCUSSION**

Animal models with which to explore therapeutics for C. parvum disease in vivo are limited, primarily to those that are genetically derived where an important cytokine is deleted [25, 26], or in which an antibody or drug is used to immunosuppress the host [27, 28]. We report a weaned mouse model of cryptosporidiosis in which malnutrition is easy to initiate (change diet), a larger amount of tissue is available to study, and costs are lower because 80% fewer oocysts when unexcysted are needed. Further, 3 quantifiable end points (weight loss, stool shedding, and the tissue burden of parasites in the ileum and colon) were significantly higher in mice receiving unexcysted oocysts, compared with those receiving excysted ones. Finally, because of the presence of malnutrition and reliance on an oral diet, the model closely parallels a particularly vulnerable human population, children shortly after being weaned.

---

Figure 3. A, Impact of undernutrition of human colonic adenocarcinoma (HCT-8) epithelial monolayers on parasite attachment. Monolayers were incubated for 24 hours in glutamine-free medium containing 1% or 10% fetal bovine serum (FBS), then challenged with Cryptosporidium parvum excysted oocysts (1 × 10⁶/well) for 6 hours in FBS- and glutamine-free media. After washes, monolayers were again incubated in their respective glutamine-free medium (1% or 10% FBS) and then harvested (at 6 and 30 hours after challenge) to quantify parasite and cell numbers. B, Effects of several concentrations of oligodeoxynucleotides containing unmethylated CpG motif (CpG-ODN) on epithelial attachment by excysted C. parvum oocysts at 6 and 12 hours after challenge in vitro. CpG-ODN was administered 24 hours before oocyst challenge (n = 3 HCT-8 monolayers per condition); *P < .05 at 12 hours after initiation of oocyst challenge, comparing monolayers exposed to 100 μg/mL CpG-ODN with those that were not. C, Trend for alanyl-glutamine (AQ) in reducing the attachment of C. parvum oocysts to human epithelial monolayers, at 6 and 30 hours after initiation of oocyst challenge (1 × 10⁶/well) (n = 6 monolayers per condition).
The notion that malnutrition increases vulnerability to infection of the host and vice versa has been characterized as a vicious cycle [29], described primarily in young children in developing countries, and associated with growth shortfalls and impaired cognitive development [30–32]. Only recently, animal models have been developed in neonatal mice that mirror the malnutrition-infection cycle in children [19, 33]. Stool shedding data in the current study confirmed this cycle in an older weaned animal model with unexcysted oocysts (Figure 1).

The literature on therapeutics for enteric infection suggested each of the agents studied in the current experiments might be beneficial [20, 21], although mechanisms responsible for their benefit are not clear. In our study, CpG-ODN, administered intraperitoneally starting 3 days before, during infection, and continuing through 3 days after, stabilized the weight of mice by day 2 after challenge, with statistically significant differences from malnourished, infected but untreated control mice (Figure 2A) (P < .05; days 3–6). Coincidentally, stool shedding data suggested that mice given CpG-ODN had less intense infection with C. parvum on days 1 and 3 (Figure 2C), as well as a lower tissue burden of parasites in the ileum on day 4 (Figure 2E).

Barrier and colleagues [34, 35] reported benefit of CpG-ODN in neonatal mice challenged with C. parvum. Specifically, they found that intraperitoneal administration of CpG-ODN 1668 led to an 80%–95% reduction in intestinal parasite load 6 days later, through interaction with TLR9 leading to production of cytokines and lymphocyte activation. Neither weaned nor adult mice were included in their report, and the issue of malnutrition was not addressed. Thus, ours is the first report to our knowledge to show benefit of oligodeoxynucleotides in weaned mice that are malnourished. Benefit was also seen with AQ, when administered by gavage several days before and through 6 days after oocyst challenge. Although oral administration in drinking water may be more acceptable clinically, we used intraperitoneal injection and gavage of the study therapeutics to be sure the agent entered the host in a known dose. The intraperitoneal route argues against an effect attributable to the agent interacting directly with the parasite, as does the temporal (6 hour) separation between AQ administration and C. parvum challenge.

The in vitro findings we report confirm our in vivo results and highlight the epithelium as a primary site of effect of several of the candidate therapeutic agents. That is, a 6-hour exposure to CpG-ODN at 100 μg/mL before C. parvum challenge, reduced parasite attachment to human epithelial monolayers at 6 and 12 hours after exposure (Figure 3A) (P < .05). AQ had a modest but not statistically significant effect on parasite invasion of epithelium at 6 and 30 hours (1% and 10% FCS). Our results suggest an early effect of CpG-ODN and a prolonged effect of AQ, lasting ≥30 hours after exposure to them was begun.

Our study has several limitations. Administration of CpG-ODN slowed weight loss but did not reverse it. Furthermore, at present, this agent is expensive, and the results we report may not carry over to effects in human populations. Prophylactic use, as reported here, may also not be practical in clinical medicine, and postchallenge benefit alone would be of interest. With regard to the mouse model we report, there was no overt diarrhea in C. parvum–challenged mice, and one of the read-outs (PCR on DNA extracted from stool) is time-consuming and expensive. On the other hand, body weight change was remarkably easy and convenient to monitor in the model.

In summary, the current work reports that challenge with unexcysted C. parvum oocysts in a malnourished weaned C57L/J mouse generates a convenient and reproducible model of overt C. parvum disease. It is associated with prolonged and substantial weight loss (>10% over 4 days), robust stool shedding (>3 logs/d over first 7 days), and attachment of organisms to the ileum, cecum, and colon. Of the putative therapeutic compounds evaluated in the model, CpG-ODN showed ability to reduce body weight loss (to <6%, days 3–7 after challenge), reduce shedding of organisms (by 25% days 1 and 3 after challenge), and decrease (by 30%) the burden of parasites in the ileum. AQ administered by gavage showed benefit as well. In vitro studies suggested that effects of study compounds on the epithelial component of the mucosa might be responsible for beneficial effects seen in vivo. We conclude that a usable model for C. parvum disease with quantifiable end points is available and may be useful to identifying mechanisms and therapeutics for C. parvum–induced disease.

Notes

Financial support. This work was supported in part by the Middle Atlantic Regional Center of Excellence for Biodense and Emerging Infectious Diseases Research (grant U54 AI57168); L. B. C. was supported by the National Institutes of Health (Fogarty GIDRT Training grant D43 TW006578).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References