ABSTRACT: The purpose of this study was to determine whether gamma-irradiated Cryptosporidium parvum oocysts could elicit protective immunity against cryptosporidiosis in dairy calves. Cryptosporidium parvum Iowa strain oocysts (1 × 10⁶ per inoculation) were exposed to various levels of gamma irradiation (350–500 Gy) and inoculated into 1-day-old dairy calves. The calves were examined daily for clinical signs of cryptosporidiosis, and fecal samples were processed for the presence of C. parvum oocysts. At 21 days of age, the calves were challenged by oral inoculation with 1 × 10⁵ C. parvum oocysts and examined daily for oocyst shedding and clinical cryptosporidiosis. Calves that were inoculated with C. parvum oocysts exposed to 350–375 Gy shed C. parvum oocysts in feces. Higher irradiation doses (450 or 500 Gy) prevented oocyst development, but the calves remained susceptible to C. parvum challenge infection. Cryptosporidium parvum oocysts exposed to 400 Gy were incapable of any measurable development but retained the capacity to elicit a protective response against C. parvum challenge. These findings indicate that it may be possible to protect calves against cryptosporidiosis by inoculation with C. parvum oocysts exposed to 400-Gy gamma irradiation.

Cryptosporidiosis remains a significant health threat to humans and animals because of the resiliency of the oocyst stage and the lack of approved drugs to prevent or treat infection. It is highly prevalent in preweaned cattle, constituting a health threat for these young animals and a significant source of environmental contamination. Although passive immunotherapy with monoclonal antibodies, immune serum, or hyperimmune bovine colostrum specific for Cryptosporidium parvum antigens has shown some efficacy against cryptosporidiosis, most of these studies have been conducted in rodent models (for review, see Rigs, 1997, 2002; Crabb, 1998). Passive immunotherapy with hyperimmune bovine colostrum has reduced clinical signs in humans or dairy calves, but C. parvum oocysts continue to be shed in high numbers (Fayer et al., 1989; Ungar et al., 1990; Okhuyzen et al., 1998; Perryman et al., 1999). Preliminary research has shown that individuals experiencing a patent C. parvum infection, i.e., diarrhea, oocyst shedding, are more resistant to oocyst challenge (Chappel et al., 1999). An ideal vaccine against cryptosporidiosis would protect susceptible individuals against C. parvum infection without causing clinical symptoms during the primary “immunization.” Exposing C. parvum oocysts to gamma irradiation may prevent schizont development and thus overt cryptosporidiosis. Gamma irradiation of various protozoa has been used to prevent associated diseases such as malaria (Clyde et al., 1975; Schell and Azad, 1995; Chatterje et al., 1999), avian coccidiosis (Jenkins, Augustine et al., 1991; Jenkins, Danforth et al., 1991; Jenkins et al., 1993), babesiosis (Phillips, 1971; Purnell et al., 1979), and toxoplasmosis (Dubey et al., 1996). The present study was designed to identify a dose of gamma irradiation that inhibits oocyst development and determine whether gamma-irradiated C. parvum oocysts could be used to vaccinate calves against cryptosporidiosis.

Cryptosporidium parvum Iowa strain oocysts were propagated by infecting a 1-day-old calf with 1 × 10⁶ oocysts as described (Jenkins et al., 1999). The oocysts were purified by continuous flow centrifugation (Vetterling, 1969), followed by CsCl gradient centrifugation (Kilani and Sekla, 1987), suspended in distilled H₂O stored at 4°C before inoculation. Cryptosporidium parvum oocysts used in primary inoculation were 2.1 ± 0.9 mo old (range 0.9–3.6 mo). Oocysts used in challenge studies were 3.1 ± 0.9 mo old (range 1.6–4.3 mo). The C. parvum oocysts were suspended at a concentration of 1 × 10⁶ oocysts/ml and exposed to various doses of gamma irradiation using a ¹³⁷Cs Gammarot M radiation source at 10 Gy/min. The excitation rate of both nonirradiated and irradiated oocysts was determined using a standard in vitro protocol (Gut and Nelson, 1999) and subjected to a 1-way analysis of variance using a Tukey–Kramer multiple comparisons test (GraphPad InStat Version 4.1, GraphPad Software, San Diego, California). Within 1 h after irradiation, the oocysts were inoculated per os into newborn (1- to 2-day-old) male Holstein calves obtained from the Beltsville Agricultural Research Center Dairy Unit. Calves were examined daily for clinical signs of cryptosporidiosis; fecal samples (~40 g) were collected daily from days 1–21 and processed for detecting C. parvum oocysts as described (Fayer et al., 2000). In brief, fecal samples were homogenized by vortexing, a 10-g subsample was mixed with water and sieved, centrifuged, and the pellet was suspended in 1.4 g/ml aqueous cesium chloride, followed by centrifugation at 16,000 g. After centrifugation, the supernatant was aspirated, and the pellet was washed with deionized water, stained with Merifluor reagent (Meridian Diagnostics, Cincinnati, Ohio), and examined by fluorescence microscopy for the presence of oocysts. For RNA extraction, purified oocysts were subjected to 3 freeze–thaw cycles of ~80 and 55°C, after which the oocyst lysate was processed using the Qiagen Viral RNA kit (Valencia, California). Purified RNA (10 µl) was used as template for reverse transcriptase–polymerase chain reaction (RT-PCR) using the CP0716F/CP0992R primer pair, which amplifies a 315-bp fragment of the gene coding for an uncharacterized protein of the C. parvum double-stranded RNA viral symbiont ("KSU-1" strain, GenBank
I). Increasing the irradiation dose to 400 Gy eliminated detectable oo-
cysts. High numbers of oocysts were found in feces from these calves (Table I). PCR products were cycle-sequenced using the Big Dye automated fluorescence sequencing instrument (Applied Biosystems).

- Clinical signs: -, indicates no diarrhea; +, loose stools; ++, watery diarrhea for at least 3 consecutive days.
- Oocysts detected by immunofluorescence staining, identified as Iowa strain oocysts used in the inoculation rather than from contaminated sources, e.g., Beltsville-1. For purposes of
  titration studies revealed no apparent diminution in oocyst production.
- Oocyst shedding as well as clinical cryptosporidiosis as did higher doses (450 or 500 Gy) (Table I). Although the prepatent period for 350- or 375-Gy-exposed C. parvum oocysts was similar to that of nonirradiated oocysts, the duration of oocyst shedding arising from irradiated oocysts was shorter (M. Jenkins, unpubl. obs.). Also, calves inoculated with 450- or 500-Gy-treated C. parvum oocysts showed prepenty and patency periods after challenge infection similar to those of naive animals (M. Jenkins, unpubl. obs.).

Irradiation appeared to have an effect on oocyst excystation. A significant decrease (P < 0.01) in excystation rate was observed between nonirradiated oocysts and oocysts that were exposed to greater than 350-Gy gamma irradiation. For instance, in C. parvum oocysts exposed to 400-Gy irradiation, excystation was 20% less than in nonirradiated control oocysts (Table II).

In a subsequent challenge, calves that received a primary inoculation of C. parvum oocysts irradiated at 400 Gy were resistant to oocyst challenge infection at 21 days of age. Clinical symptoms were absent after challenge in all 3 calves (calves 4–6) that had been inoculated at 1–2 days of age with 400-Gy-irradiated oocysts, and only calf 6 shed 6 oocysts after challenge (Table I). Oocyst production (5 x 10^4 oocysts/g) in this calf was 2.5% of that observed (2 x 10^4 oocysts/g) in naive control calves that had been challenged at 3 wk of age with C. parvum Iowa oocysts. Although it is possible that oocysts may have been shed by calves 4 and 5 in this group, the number of oocysts would have been extremely low based on the sensitivity of this assay (Fayer et al., 2000).

Irradiation doses (450 and 500 Gy) not only inhibited parasite development in the primary “infection” but also ablated any protective effects. These results indicate that there is a narrow range of optimum irradiation dose that can inhibit parasite development but still maintain the capacity of the radiation-attenuated parasites to elicit a protective immune response. These findings also indicate that exposure of C. parvum oocysts to 400-Gy gamma irradiation does not affect the capacity of oocysts to induce a protective response in calves but does prevent the parasite from developing to the oocyst stage. Determining at what point development is blocked in the C. parvum life cycle would be difficult in calves and probably not relevant to test in mice. Studies in related protozoans indicate that development is blocked after sporozoite penetration of host cells (Jenkins, Augustine et al., 1991; Jenkins, Danforth et al., 1991; Silvie et al., 2002). Presumably, irradiation doses above the optimum level prevent sporozoite invasion or intracellular metabolism (or both), which are necessary for expression of parasite antigens on host cells and the induction of immunity.

Comparing the present study with findings in mouse models of cryptosporidiosis indicates that results in mice may not be predictive of findings in calves (Jenkins et al., 1998; Yu and Park, 2003). In neonatal BALB/c mice, C. parvum development was blocked by exposure of oocysts to 250-Gy irradiation (Jenkins et al., 1998). Preliminary dose titration studies revealed no apparent diminution in oocyst production in calves inoculated with C. parvum oocysts exposed to less than 300-Gy irradiation (M. Jenkins, unpubl. obs.). Somewhat conflicting with

### Table I. Efficacy of Cryptosporidium parvum oocysts exposed to various doses of gamma irradiation to confer protection in each of 8 dairy calves against clinical cryptosporidiosis and C. parvum oocyst shedding.

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>Irradiation dose (Gy)</th>
<th>Primary infection*</th>
<th>Secondary infection†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical signs*</td>
<td>Oocyst shedding§</td>
<td>Clinical signs</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>350</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>375</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>400</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>400</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>400</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>450</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>500</td>
<td>–</td>
<td>++</td>
</tr>
</tbody>
</table>

* Calves were infected at 1-2 days of age with 1 x 10^5 C. parvum Iowa strain oocysts at irradiation level shown.
† Cows were challenged at 21 days of age with 1 x 10^5 C. parvum Iowa strain oocysts.
‡ Clinical signs: –, indicates no diarrhea; +, loose stools; ++, watery diarrhea for at least 3 consecutive days.
§ Oocysts detected by immunofluorescence staining, identified as Iowa strain C. parvum oocysts by RT-PCR, purified by CsCl density gradient centrifugation, and enumerated by hemacytometer counting. –, indicates no oocysts detected; +, <10^4 oocysts/g; ++, 10^4–10^5 oocysts/g; ++++, >10^5 oocysts/g.
¶ ND, not done.

### Table II. Percent excystation of Cryptosporidium parvum oocysts exposed to various doses of gamma irradiation.

<table>
<thead>
<tr>
<th>Irradiation dose (Gy)</th>
<th>Percent oocyst excystation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>350</td>
<td>84 ± 9</td>
</tr>
<tr>
<td>375</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>400</td>
<td>70 ± 8</td>
</tr>
<tr>
<td>450</td>
<td>66 ± 4</td>
</tr>
<tr>
<td>500</td>
<td>60 ± 8</td>
</tr>
</tbody>
</table>

CPI959996; Khamtsov et al., 1997; Kozwich et al., 2000. RT-PCR was carried out using the Superscript®-1 step reagent (Invitrogen, Carlsbad, California), with the reverse transcription performed at 55°C for 30 min, followed by 4 min at 95°C, and 40 cycles of 15 sec at 95°C, 45 sec at 55°C, and 1 min at 72°C. The sequence of the forward primer is 5'-CCGAGACGCTGAACTTGGTCCTACCTCCTTACTCAT-3', and the sequence of the reverse primer is 5'-GAATCTATCCTATTGATATCCGGAGCGAAGTCATCGGTA-3'. RT-PCR products were cycle-sequenced using the Big Dye® 3.1 reagent (Applied Biosystems, Foster City, California) and an ABI Model 3100 automated fluorescence sequencing instrument (Applied Biosystems). Sequence data were analyzed using MacVector® 6.5 software (Oxford Molecular; Madison, Wisconsin). The purpose of this assay was to ensure that oocysts shed by calves had originated from the C. parvum Iowa strain oocysts used in the inoculation rather than from contamination with local C. parvum strains, e.g., Beltsville-1. For purposes of testing vaccine efficacy of irradiated oocysts, the calves were then challenged at 21 days of age by oral inoculation with 1 x 10^5 C. parvum Iowa strain oocysts. This challenge dose was based on preliminary studies wherein reproducible clinical signs and shedding of high numbers of C. parvum oocysts were achieved. Calves were challenged at 3 wk of age to allow sufficient time for development of immunity. Inoculation of naive 21-day-old calves with 1 x 10^5 C. parvum Iowa strain oocysts produced an average of 1.9 x 10^9 ± 0.6 x 10^9 oocysts/g of feces (M. Jenkins, unpubl. obs.). Fecal samples were collected twice daily for 3–4 wk after challenge, and fecal smears were examined by immunofluorescence staining for the presence of C. parvum oocysts following the manufacturer’s instructions (Meridian Diagnostics). All oocyst-positive fecal samples were pooled, weighed, and processed for total oocyst counts by sieving, sucrose flotation, and CsCl gradient centrifugation. Similar to primary infection, the calves challenged at 21 days of age were examined daily for clinical signs, and samples of feces were collected for oocyst enumeration. Calves that shed oocysts after a primary infection with nonirradiated or irradiated C. parvum Iowa strain oocysts were not challenged at 21 days of age because the goal of this study was to develop an immunization regimen that did not entail clinical signs or oocyst shedding.

Although calves that were infected at 1–2 days of age with 350- or 375-Gy-irradiated C. parvum oocysts exhibited reduced clinical signs, high numbers of oocysts were found in feces from these calves (Table I). Increasing the irradiation dose to 400 Gy eliminated detectable oo-cysts and reduced clinical signs and oocyst shedding.
the current study in calves and our previous studies in mice is a recent report that 25,000 Gy was necessary to prevent *C. parvum* oocyst shedding in weaned C57Bl/6 mice (Yu and Park, 2003). The observed discrepancy may be due to differences in age and strain of mice used. These authors also found that oocysts were shed for an extended period of time, and that peak oocyst shedding was delayed in mice receiving irradiated oocysts. In the present study, calves were examined for at least 3 wk after the primary and challenge infections to ensure that absence of oocyst secretion was due to the effect of gamma irradiation or immunity rather than delayed development. This precaution was taken because *C. parvum* is unique among protozoans in that an autoinfective oocyst stage is produced during the life cycle, and thus small numbers of viable oocysts may eventually lead to excretion of high numbers of oocysts, albeit with some time delay.

The present study suggests that irradiated *C. parvum* oocysts can be used to prevent clinical signs and oocyst shedding in dairy calves. Also, irradiation doses greater than 450 Gy inhibit *C. parvum* from producing patent infection, which is considerably less than the irradiation level required to destroy bacteria on the surface of foods. The stability of 400-Gy-irradiated *C. parvum* oocysts is unknown and will require additional studies before this approach is considered useful in preventing cryptosporidiosis in dairy calves.

The authors acknowledge the excellent technical assistance of Christina Hohn, Kristi Ludwig, Robert Palmer, and Marisol Ponte. The authors thank James Harp for providing the original *C. parvum* Iowa strain oocysts used in this study.

**LITERATURE CITED**


