Cryptosporidium parvum infection in bovine neonates: dynamic clinical, parasitic and immunologic patterns

R. Fayer, a* L. Gasbarre, a P. Pasquali, b A. Canals, a S. Almeria a and D. Zarlenega a

a United States Department of Agriculture, Agricultural Research Service, Immunology and Disease Resistance Laboratory, Beltsville, MD 20705, U.S.A.
b Istituto di Parassitologia, Facolta' di Medicina Veterinaria, Universita' degli Studi di Perugia, Perugia, Italy

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Abstract

Twenty-six experimentally infected calves were monitored daily for oocyst excretion. All began excreting oocysts 3–6 days p.i. Most calves (n = 23) excreted oocysts for 6–9 days, with a daily range from 4 × 10^7 to 4.15 × 10^7 oocysts g^-1 of faeces. Over half the calves excreted peak numbers of oocysts 6–8 days p.i. Diarrhoea, observed intermittently beginning as early as day 3 p.i., lasted 4–16 days and varied greatly in severity from calf to calf. In a second study, nine of 18 calves were orally inoculated with 5 × 10^6 oocysts between birth and 2 days of age and nine remained uninfected. Monoclonal antibodies for cell surface markers indicated substantial increases in CD4+ and CD8+ T cells in the intraepithelial lymphocyte population of the ilea of infected calves at 7–9 days of age. RT-PCR demonstrated increases in mRNA for interleukin-12 and interferon-γ that correlated with increases in both CD4+ and CD8+ T cells. Increased mRNA for interleukin-12 and interferon-γ from lamina propria lymphocytes correlated with increased numbers of CD8+ cells. No changes were found in interleukin-2, interleukin-4 or interleukin-10 mRNA levels. However, interleukin-15 mRNA, possibly from epithelial cells contaminating intraepithelial lymphocytes, was decreased in infected calves and had a negative correlation with increases in CD4+ and CD8+ cells. No differences were detected in mRNA levels for cytokines from lymph node lymphocytes.

1. Introduction

Cryptosporidiosis due to Cryptosporidium parvum has been documented in people and animals in 95 countries [1]. Although it has been reported in people from 3 days of age to 95 years of age, the highest prevalence of infection has been recognised in both children and young animals [2]. It is also evident from recent large-scale waterborne outbreaks [3] that many adults with apparently normal healthy immune systems, but presumably not previously exposed to the organism, also are susceptible to infection. The most severe clinical infections have been associated with immunologically incompetent individuals. These include people with the acquired immune deficiency syndrome (AIDS), malnourished individuals, those taking exogenous immunosuppressive medications, hypogammaglobulinaemic patients, individuals with concurrent viral infections such as measles, and possibly pregnant women [4]. Animals
such as immunodeficient Arabian and thoroughbred foals [5–8] and T-cell-deficient mice [9] are so severely affected that they die despite therapy and special care.

Although C. parvum-like infections have been reported for 79 species of mammals [10], the most frequently reported and most widely recognised host species other than humans are the ruminants [11]. Of all the wild and domesticated species of ruminants examined, Bos taurus, which includes all the domesticated European breeds of cattle, has been studied most extensively with nearly 400 references to date. The prevalence of C. parvum infection in preweaned calves is high. When faeces were examined twice weekly for a month, beginning with 3- to 6-day-old calves purchased from a local market, 93% of 42 non-medicated calves were found to be infected [12]. When only one or two faecal specimens were examined per calf during the entire preweaning period, 22% of 7369 calves on 1103 farms in 28 states in the U.S.A. had oocysts in their faeces [13]. Likewise, C. parvum oocysts were observed in faeces from 51% of 445 calves in Washington state, U.S.A. [14], 39% of 325 calves in Holland (PW DeLeeuw, A Moerman, JMA Pol, FP Talmon, FG van Zijderveld, Epidemiological observations on cryptosporidiosis in dairy herds in the Netherlands. Proceedings of the World Congress on Diseases of Cattle 1984; 104–109. Durban, Republic of South Africa), 44% of 284 calves in Germany [15] and 82.7% of 29 calves in Spain [16, 17]. All of the aforementioned reports, and others worldwide [11] in which only one or two faecal specimens were examined per calf, likely underestimate the actual prevalence of bovine cryptosporidiosis. Cryptosporidium parvum has been detected in the faeces of adult cattle and calves over 20 weeks of age in France [18], Canada [19], the U.S.A. [20–22], Spain [12, 23], the U.K. [24–26] and Brazil [27].

To separate the effects of immunity acquired after natural exposure from the effects of age-related resistance, Harp et al. [28] raised calves in isolation from C. parvum and found that they were susceptible to infection at 3 months of age. These results suggest that older cattle might not become infected because of immunity they acquired from exposure to the parasite as calves.

Whereas no strong correlation could be demonstrated between humoral immunity and resistance to infection with C. parvum in humans, mice and calves [9, 28], reports of cryptosporidiosis in certain immunocompromised humans and animals suggested a strong cellular immune component [9].

Little information is available on differences in infectivity, excretion patterns, virulence, or immunogenicity among different isolates of C. parvum from cattle or other sources. There are no reports of single oocyst cloned isolates, all are heterogenetic mixtures. Few isolates are maintained because they do not survive freezing and therefore require repeated passage through animal hosts to maintain viability. We have three isolates stored frozen for molecular and biochemical studies, two (Utah-1 and Beltsville-1) from calves and one (Georgia-1) from white-tail deer passed through calves. Reports of natural infections and most experimental infections do not describe or record isolates by name or number. An isolate obtained from a calf in Alabama in 1983 caused mortality in calves when 3.2 x 10⁶ oocysts were administered, but no calves even became seriously ill when oocysts were cleaned and treated with antibiotics before being administered at 30 x 10⁶ oocysts/calf [29]. Unfortunately, this isolate was not maintained. The IOWA isolate has been widely distributed and utilised in numerous experimental infections of animals and humans since it was first isolated by Moon and Bemrick [30] and is maintained in several laboratories. The KSU-1 isolate, originally from a calf in Kansas [31] and the AUCP-1 isolate from a calf at Auburn University [32] both have been widely distributed and maintained. An attempt to subculture a precocious strain from the latter isolate was unsuccessful after passage through 10 calves [33]. The AUCP-1 isolate was used in the following studies described herein.

Based on the foregoing, we designed a series of experiments to determine why preweaned calves are so highly susceptible to infection with C. parvum. First, patterns of oocyst excretion and resultant diarrhoea (the most noteworthy sign of cryptosporidiosis) were recorded for experimentally infected preweaned calves. Then, both C. parvum infected and uninfected neonatal calves were examined to determine the status of T cells and cytokines at the site of infection in the lower ileum and in
adjacent lymph nodes. In this paper we present a compilation of data from our laboratory along with reports from colleagues that summarise the state of knowledge on neonatal bovine cryptosporidiosis.

2. Materials and methods

Animals and infection

Forty-four Holstein-Friesian male calves were obtained at birth from the dairy herd at the Beltsville Agricultural Research Center and housed in separate 4 x 6-m concrete floored pens with cinder block walls in a sanitised masonry building. Calves were fed thawed pooled colostrum within 24 h of birth and then commercial milk replacer twice daily with water available ad libitum. In the first study, data on oocyst output and diarrhoea were recorded for 26 calves over 3 years. Each calf was fed an aqueous suspension of 1.5 x 10^6 oocysts of C. parvum (AUCP-1 isolate) at 1-3 days of age and was monitored daily for oocyst excretion and diarrhoea for the following 25-28 consecutive days. Some calves were held in wire-bottom metabolic crates to collect total faecal output, but most had faeces collected one to six times daily directly from the rectum into plastic cups. Because total faeces were not collected daily from all calves, neither total output nor total daily output of oocysts could be determined. Instead, the number of oocysts per gram of faeces per day was determined, providing data on the onset, peak, and termination of oocyst excretion. For each specimen, debris was removed from 5 g of faeces by centrifugation over cesium chloride solution [34], leaving clean oocysts which were counted with the aid of a haemocytometer. To classify the severity of diarrhoea a score of 1 was recorded for a soft stool that was formless upon collection, 2 indicated a liquid stool that contained much organic material, 3 indicated a watery stool with little organic material but opaque, and 4 indicated a watery stool with little or virtually no organic material and appearing translucent or nearly clear. As an overall estimate of the severity of infection and to facilitate comparisons among calves, daily scores were totalled for each calf to determine a “diarrhoea severity index”. Specimens were not examined for bacterial or viral enteropathogens. However, oocyst inocula contained no observable contaminants as determined by bright field, Nomarski and phase-contrast microscopy, and most were incubated at room temperature in 0.5% sodium hypochlorite before infecting calves. Many neonatal calves have soft formless stools in the first few days of life and therefore scores of 1 recorded before and up to 2 days after oral inoculation with oocysts were not considered related to infection and are not included in the data (Table 1).

Cell-mediated immunity had been shown to play a prominent role in the control of C. parvum infection in mouse models, but little information has been available on the immune responses of calves with cryptosporidiosis. Therefore, the second study was designed to identify lymphoid cell populations at the site of infection in calves where, as effector cells, they respond directly to the parasite [35]. Lymphocytes were isolated from 5-g segments of ileum 60-cm anterior to the ileocaecal junction, just anterior to the continuous ileal Peyer’s patch, and from lymph nodes at the ileocaecal junction. Collection and isolation of lymph node cells (LNLs), intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were as described elsewhere [35]. The 10 mAbs used in the study for phenotypic analysis by indirect immunofluorescence, their isotype, specificity and methods of use are also described elsewhere [35].

In the second study, to investigate the changes in lymphoid cell populations and cytokine responses in the ileum and adjacent lymph nodes of uninfected versus infected calves, 18 additional calves were obtained [35, 36]. Nine of the calves were infected with 5 x 10^6 oocysts at 1–2 days of age and tissues were collected for cell and cytokine analysis from both infected and non-infected groups at 7–9 days of age.

To determine what cytokine responses occurred in cattle and specifically in the intestinal mucosa, we conducted a third study. IELs, LPLs and LNLs from our second study [36] and from four additional calves (two infected and two uninfected) were lysed in guanidinium isothiocyanate buffer and RNA was extracted as described elsewhere [36]. Competitive reverse transcriptase polymerase chain reaction (RT–PCR) was performed by co-amplification of the target cDNA and the internal control cytokine
gene competitor molecules with the same primers [36].

3. Results and discussion

3.1. Oocyst excretion patterns

The prepatent period for excretion of oocysts in male calves, infected at 1–3 days of age with $1.5 \times 10^6$ oocysts of *C. parvum* ranged from 3 to 6 days after experimental infection. Fourteen of the 26 calves began excreting oocysts at 4 days (Table 1). Twenty calves excreted oocysts for 6–8 days but two calves excreted oocysts for 13 days. The day that peak numbers of oocysts were excreted varied greatly, but occurred on days 6–8 p.i. for over half the calves. Although the number of oocysts excreted varied greatly over the patent period ($4 \times 10^6$ to $4.15 \times 10^4$ oocysts g$^{-1}$ of faeces), 16 of 19 calves excreted over 1 million oocysts g$^{-1}$ for 1 day or more during the infection. The actual number of oocysts per gram of faeces was undoubtedly greater than our counts revealed, because many remained with faecal debris at various steps during the cleaning process. Estimates of oocyst numbers or even the presence or absence of oocysts in faeces can vary greatly, based on the methods of detection. For example, we weighed 50 glass microscope slides before and after applying direct faecal smears and found that each received an average 0.001 g of wet faeces. One oocyst found on each slide would rep-
resent 1000 oocysts g⁻¹. However, if none were detected, theoretically, as many as 999 oocysts g⁻¹ might be present and the faecal specimen would be regarded as negative. An apparently negative adult bovine excreting this level of oocysts and 20 kg of faeces daily would produce nearly 20 million oocysts a day. The lack of sensitivity in detection, which might account for the infrequent reporting of positive adult cattle, has led some investigators to determine infectivity based on examination of 10 g of faeces per animal [24], 10,000 times more faeces than found in a faecal smear. Of 14 healthy 6–10-year-old cows, Scott et al. [24] detected 900 oocysts g⁻¹ of faeces (range 25–1.8 x 10⁴ g⁻¹), all of which would have been undetected by other conventional techniques.

3.2. Diarrhoea

Despite attempts to reduce variability by using only male calves from the same herd, at the same age, housed and fed similarly, and given the same number of oocysts from the same aged inoculum from the same isolate of C. parvum, great variability was observed in the severity and duration of diarrhoea. Of 26 calves from which data were recorded, diarrhoea lasted from 4 to 17 days (Table 1). Eighteen of 19 calves had diarrhoea begin 3–5 days p.i. Diarrhoea was often intermittent with no clear patterns of recurrence of diarrhoea between normal stools. The diarrhoea severity index varied greatly, with scores ranging from 5 to 31 with 4 and 16 days of diarrhoea, respectively. These findings indicate a great range in responses between calves exposed under similar conditions.

3.3. Lymphocyte patterns in the ileum and adjacent lymph nodes

A significant increase in CD2+, CD3+, CD4+ and CD8+ T cell subsets was observed in the IELs of infected versus uninfected calves (Table 2). Marked or significant increases in CD2+, CD3+ and CD8+ T cell subsets were also observed in the LPLs of infected versus uninfected age-matched calves. Our findings were supported by Wyatt et al. [37] who infected 3-day-old naive calves with C. parvum oocysts and examined ileal mucosal lymphocytes from them and 9–12-day-old age matched uninfected controls. Using numerous mAbs to bovine leukocyte differentiation markers they found significantly more IEL CD8+ T cells in infected

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Ig isotype</th>
<th>Predominant specificity</th>
<th>Percentage of positive-stained IEL cells from calves</th>
<th>Percentage of positive-stained LPL cells from calves</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAQ95A</td>
<td>IgG₁</td>
<td>CD2</td>
<td>59.8</td>
<td>18.2</td>
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<tr>
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<td>IgG₁</td>
<td>CD3</td>
<td>51.6</td>
<td>14.5</td>
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<tr>
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<td>IgG₁</td>
<td>CD4</td>
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<td>3.7</td>
</tr>
<tr>
<td>CACT80C</td>
<td>IgG₁</td>
<td>CD8a</td>
<td>3.7</td>
<td>4.4</td>
</tr>
<tr>
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<td>IgM</td>
<td>WC1</td>
<td>12.0</td>
<td>8.6</td>
</tr>
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<tr>
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<tr>
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<td>13.0</td>
<td>8.6</td>
</tr>
<tr>
<td>BIG312D3</td>
<td>IgG</td>
<td>Ig</td>
<td>8.0</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Derived from Pasquali et al. [35].

*Values express the mean percentage.
versus control calves and found only in infected calves that some IEL CD4+ T cells coexpressed CD25. Our findings were supported in part by Abrahamsen et al. [38], who infected 3-day-old naive calves with *C. parvum* oocysts and 3 days later stained tissue sections from them and uninfected age-matched control calves with mAb. They found a dramatic increase in the number of CD4+ and CD8+ T cells in lamina propria and perifollicular areas of Peyer’s patch. These findings also support those of a previous study in mice in which there was an increase in CD4+ and CD8+ T cells in ileal Peyer’s patches following infection with *C. parvum* [39]. Furthermore, Abrahamsen et al. [38] found increases in γ/δ T cells in lamina propria of ileum from infected versus control calves, which we did not observe, although we used the same mAb. This difference may be due to the fact that they obtained tissue 3 days p.i. whereas we obtained cells 7 days p.i.

We found no significant changes in the percentages of T cell subsets in LNLs from infected versus uninfected calves; however, there was an increase in CD2+ T cells and a decrease in CD8+ T cells, possibly due to recruitment into the ileum. Such findings suggest that the immune response to acute initial infection resides primarily in the gut with little involvement of adjacent lymph nodes. In a previous study in calves [40], phenotypes of lymphocytes from peripheral blood, spleen, mesenteric and prescapular lymph nodes of infected and control calves were compared. They reported more null (γ/δ) lymphocytes in all tissues of infected versus uninfected calves; however, there was an increase in CD2+ T cells and a decrease in CD8+ T cells, possibly due to recruitment into the ileum. Such findings suggest that the immune response to acute initial infection resides primarily in the gut with little involvement of adjacent lymph nodes.

3.4. Cytokine patterns in the ileum and mesenteric lymph nodes

Identifying changes in cytokine production in response to infectious agents is essential for determining the immune effector mechanisms. The effects of different cytokines on cryptosporidiosis have been studied primarily in immunocompromised human patients [46, 47] and in mice either depleted *in vivo* of cytokines with specific mAbs or injected with exogenous cytokines [43, 44, 48, 49]. A specific lymphocyte blastogenic response against *C. parvum* was detected 2 days p.i. of calves [50], but no cytokine data were obtained. DeGraaf and Peeters [51] found that beginning 6 days p.i. of calves, an infection-induced interferon-γ (IFN-γ) response could be obtained in cultures of peripheral blood-derived monocytes stimulated with *C. parvum* oocyst.

Results from the competitive RT-PCR demonstrated elevated levels of IFN-γ and interleukin (IL)-12 mRNA in IELs and LPLs but not in LNLs. This was the first demonstration of a significant increase in mRNA levels for IFN-γ in freshly isolated mucosal cells from the ileum of bovine calves in response to cryptosporidiosis. IEL-derived IFN-γ correlated with increases in CD4+ and CD8+ subsets of IELs [36] and LPL-derived IFN-γ correlated with increases in the CD8+ subset of LPLs [36] found by Pasquali et al. [35]. The findings also supported studies that demonstrated elevated mRNA levels in ileal homogenates from mice experimentally infected with *C. parvum* [49, 52]. Increases in IL-12 mRNA from IELs and LPLs were not significant due to great variation among calves; however, elevated levels for specific calves correlated with increased percentages of CD8+ cells within the same calves. The variation within this group may have resulted from inappropriate sampling time relative to peak IL-12 production in response to infection, as demonstrated in mice by Urban et al. [49] wherein IL-12-induced increases in IFN-γ occurred as early as 3 days after injection of IL-12. Wyatt et al. [37] reported that IFN-γ was expressed by IELs from the ilea of infected and uninfected calves, but that tumour necrosis factor-α was expressed by uninfected calves and was downregulated by infected calves. IFN-γ and tumour
necrosis factor-α, but not IL-2, IL-4 or IL-10, were expressed by LPLs from ilea from infected as well as uninfected calves. These findings suggested to them an altered cytokine expression in IELs but not in LPLs from infected ilea. As in our study, sampling time versus response time can strongly influence interpretation of the data.

We found that calves with elevated CD4+ and CD8+ cells had correspondingly lower mRNA levels of IL-15. Presumably, the IL-15 originates from epithelial cells contaminating the IELs, since IL-15 is not produced by T cells [53]. Consequently, IL-15 may have a regulatory role in the function of the intestinal epithelial cells [53]. Damage to epithelial cells from C. parvum infection may be associated with the reduction in mRNA levels for IL-15.

No significant or consistent differences in mRNA levels were obtained in any of the cell populations analysed for IL-2, IL-4, or IL-10.

Changes in cytokine transcription appeared restricted to the mucosa with greatest changes in IELs, changes of less magnitude in LPLs, and no evidence of change in LNls. Furthermore, alterations in cytokine mRNA levels correlated with our observations of cell changes in these sites [35].

Although these initial studies are significant in their demonstration of cytokine changes and phenotypic cell changes during acute primary infection in the neonatal bovine, they are all limited in scope of time. They point the way and demonstrate the need to clearly define the kinetics of these changes so that rational approaches can be made in the development of methods to enhance a protective immune response to infection with C. parvum.

References


