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Cryptosporidium parvum in Calves: Kinetics and Immunoblot Analysis of Specific Serum and Local Antibody Responses (Immunoglobulin A [IgA], IgG, and IgM) after Natural and Experimental Infections

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Fecal and serum anti-*Cryptosporidium parvum* immunoglobulin A (IgA), IgM, and IgG were monitored by an enzyme-linked immunosorbent assay after experimental and natural infection of calves with *C. parvum*. Although all experimentally infected calves showed high levels of colostral antibodies in the feces, they acquired *C. parvum* infection. Three of five animals died. Calves which acquired natural infection showed only diarrhea. Levels of colostral coproantibodies dropped quickly. Experimental infection was followed by a rise in local anti-*C. parvum* IgM levels from day 5 postinfection (p.i.). IgM peaked at day 14 p.i. and then disappeared quickly. Anti-*C. parvum* IgA levels rose between days 7 and 14 p.i. and decreased slowly. Rising levels of coproantibodies coincided with falling oocyst output. Fecal anti-*C. parvum* IgG levels rose slightly during oocyst output, and IgG disappeared 3 weeks p.i. Similar kinetics were established in naturally infected calves. Although fecal anti-*C. parvum* IgA levels declined slowly, reinfections were established 5, 7, and 14 weeks after the primary contact. Serum anti-*C. parvum* IgG levels rose during maximal oocyst excretion, whereas serum anti-*C. parvum* IgA levels peaked later than did local IgA levels. Challenge reinfection of naturally infected calves at day 112 was not followed by clinical signs or oocyst output or by a secondary antibody response. Sequential Western immunoblotting with fecal extracts revealed up to 32 different parasite antigens. Convalescent-phase sera recognized up to 23 antigens. Fecal IgA reacted intensely with antigens with relative molecular weights (M_r) of approximately 11,000 and 15,000. These antigens were not recognized by convalescent-phase serum IgG. Both local IgA and serum IgG also showed strong reactions with 23,000- and 44,000-M_r antigens and with several antigens of between 66,200 and 200,000 M_r. Most bands remained detectable for at least 16 weeks p.i.

Cryptosporidium parvum is a parasite distributed worldwide. It causes diarrhea and sometimes mortality in a broad range of mammals. In calves, the economic impact of cryptosporidiosis is considerable and comparable to that of rotavirus infection (23). *C. parvum* has been identified as the second most common infectious agent in outbreaks of diarrhea (1, 16). *C. parvum* is non-host specific and can easily be transmitted from one species to another. Infected calves and lambs pass up to 10^{10} oocysts by the feces between days 4 and 14 postinfection (p.i.) (7). Unlike other coccidial oocysts, *C. parvum* oocysts are fully sporulated and ready to initiate infection upon excretion (10). These oocysts were reported to contaminate drinking water resources (30). Contaminated drinking water may be a source of human cryptosporidiosis (12, 20).

The prevention of the disease is difficult. Whereas most other species of enteric coccidia have a genetically programmed series of developmental stages in the life cycle and are incapable of recycling within the host, *C. parvum* has two stages that initiate autoinfectivity: type I merozoites and sporozoites derived from thin-walled oocysts. Both characteristics are believed to be the life cycle features of *C. parvum* responsible for the development of severe infections in hosts exposed to only a small number of thick-walled oocysts (10). Only the development of sufficient immunity is able to stop the cycle.

Currently, little is known about the immunobiology or molecular biology of *Cryptosporidium* spp. Enteric cryptosporidiosis in immunocompetent hosts is self-limiting, and the immune status of the host appears to determine the severity and duration of infection (10). Calves that recover from infection are resistant to a second challenge with the same strain (19). Therefore, recovery depends on a specific, acquired immune response, although the exact mechanisms responsible for resistance have not been defined yet.

Ungar et al. (45) reported that chronically infected adult nude (T-cell-deficient) mice cease oocyst output after a transfer of lymphoid cells from immune mice, indicating that T-cell-dependent immune responses contribute to both resistance to and recovery from cryptosporidiosis. Nevertheless, Harp and Whitmire (17) were not able to protect infant mice against experimental infection after a similar transfer of lymphoid cells. On the other hand, several authors showed that infant mice can be protected against experimental infection by oral passive immunotherapy with hyperimmune serum or colostrum or with specific monoclonal antibodies (MAbs) (4–6, 15, 34, 35, 38). Also, neonatal calves can be partially protected with hyperimmune colostrum (14).

These studies stress the importance of a local humoral immune response. In this respect, a recent study showed

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that increasing levels of anti-*C. parvum* immunoglobulin A (IgA) antibodies in the gut were associated with decreasing oocyst output in lambs (22). This result might indicate that IgA may inhibit the penetration of host cells by sporozoites, as has already been demonstrated for *Eimeria tenella* infection in chickens (13). Whitmire and Harp (48) studied bovine cellular and serum antibody responses after experimental infection with *C. parvum*. Specific lymphocyte blastogenic responses to the parasite were detected as early as 2 days p.i. Parasite-specific serum antibodies were detected 7 days p.i. and achieved peak levels 9 days p.i. Both lymphocyte and antibody responses remained elevated until the termination of the experiment 23 days later. Concerning the kinetics of coproantibody responses in calves, no data are available. Since the gut lumen is the site of infection by *C. parvum* in calves, a more detailed examination of secreted antibody is required. The aims of this study were to describe the kinetics of specific serum and fecal anti-*C. parvum* antibody responses in naturally and experimentally infected calves, to quantitate antibody type, and to discover target antigens.

MATERIALS AND METHODS

Animals and husbandry. In the first experiment, five male 1-day-old calves of mixed Holstein-Friesian breed were purchased from traditional calf breeders in autumn 1990. Animals received colostrum before transfer to the Institut National de la Recherche Agronomique. They were allocated to five individual pens measuring 0.55 × 1.75 m and situated in the same room. Calves were kept separated from their feces by a wooden grid. Calves within adjacent pens were tethered to a side of the pen which was not shared to prevent contact between the animals. The room was aerated with filtered air, and hygienic measures were taken to avoid cross-contamination of the animals. Animals were fed a semisynthetic milk substitute ad libitum.

In the second experiment, a total of 17 male 3- to 6-day-old calves of mixed Red-White Belgian breed purchased from the local market and colostrum fed were tested from winter to early spring 1991. Animals were not vaccinated and did not receive any vitamins, other nutritional supplements, or any type of medication during the experiment. Before admission of the calves, the research unit was thoroughly cleaned and disinfected. Animals were allocated at random to individual wooden pens measuring 0.55 × 1.75 m. Solid walls between the boxes completely prevented contact between the animals. Calves were kept separated from their feces by a wooden grid. Animals were fed a semisynthetic milk substitute four times a day. During the first 2 weeks after arrival, the amount of milk substitute was kept restricted in comparison with that given to animals fed ad libitum, and all animals were fed the same amount of milk substitute. The same animals were used for the third experiment.

Strain. In the first experiment, a human strain of *C. parvum* isolated from a 3-year-old boy (3) and serially propagated for 8 years in lambs or calves without exposure to any anticoccidial drug was used. In the third experiment, a bovine strain recently isolated from a fattening unit (strain Slooten) was used. Oocysts were purified from fresh fecal material within 36 h of collection as described before (33). In brief, feces were washed through 150- and 45-μm-pore-size sieves (Endocotts Ltd., London, England) and centrifuged at 500 × *g* for 5 min. Subsequently, the sediment was shaken in a 1:1 (vol/vol) mixture of water and diethyl ether. After a further centrifugation, the supernatant was decanted. After being washed, the sediment was resuspended in distilled water, passed through a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) consisting of four 2.5-ml layers with densities of 1.13, 1.09, 1.05, and 1.01 g/ml, and centrifuged at 650 × *g* for 15 min. The band containing purified oocysts was removed, washed twice at 500 × *g* for 5 min each time, and suspended in distilled water. The number of oocysts present was counted in a modified Neubauer hemocytometer after 0.2 ml of the suspension was mixed with 0.8 ml of malachite green (malachite green, 0.16 g; sodium dodecyl sulfate [SDS], 0.1 g; 100 ml of aqua destillata). The suspension was then diluted to the desired concentrations. Animals were challenged with 10^6 (experiment 1) or 10^7 (experiment 3) oocysts suspended in 20 ml of phosphate-buffered saline (pH 7.2) (PBS).

Experimental design. (i) **Experiment 1.** The five 1-day-old calves were inoculated orally with 10^6 *C. parvum* oocysts at the Institut National de la Recherche Agronomique station. Over 1 month, fresh fecal material was collected daily and stored immediately at 4°C. The presence of *C. parvum* oocysts in the samples was evaluated after sucrose flotation and scored semiquantitatively at a magnification of ×250: 0, no oocysts; 1, fewer than one oocyst per microscopic field; 2, between 1 and 5 oocysts per field; 3, between 6 and 10 oocysts per field; and 4, more than 10 oocysts per field. Coproantibodies were extracted from the feces at 4°C within 24 h of collection. In brief, 2 g of fecal material was homogenized by being shaken with glass beads in 8 ml of PBS and centrifuged at 650 × *g* for 15 min at 4°C. The supernatant was stored at −30°C and checked later for IgA, IgG, and IgM by an enzyme-linked immunosorbent assay (ELISA). Fecal samples were forwarded to the Laboratoire de la Direction des Services Vétérinaires d'Indre et Loire to be examined for rotaviruses, coronaviruses, enterotoxinogenic *Escherichia coli* (K99), and *Salmonella* spp.

(ii) **Experiment 2.** At arrival, the 17 calves were allocated to individual boxes in a research unit (mean body weight, 47.8 kg; coefficient of variation, 9.3%). Only calves appearing healthy and in good condition were used. Animals were observed daily. Diarrhea was assessed as follows: 0, pasty feces; 1, semiliquid feces; 2, liquid stools; and 3, stools with mucus. Individual blood samples were taken from the jugular vein at arrival and 35, 63, and 98 days later. Serum was stored at −30°C until tested for circulating anti-*C. parvum* IgA, IgG, and IgM by the ELISA. Over a period of 4 weeks, fresh fecal material was collected twice a week by rectal sampling. Over the next 4 weeks, rectal samples were collected weekly. Over the next 8 weeks, rectal samples were collected every 2 weeks. As previous experiments (47) showed a close relationship between actual numbers of oocysts present and semiquantitative scoring of oocysts, oocyst excretion was evaluated semiquantitatively with carbolfuchsin stain (21) at a magnification of ×500 with a Leitz Laborlux 12 microscope. For each sample, the complete surface of the slide was examined and the average quantitative score for *x* number of microscopic fields was reported: 0, no oocysts; 1, fewer than 5 oocysts per microscopic field; 2, between 5 and 25 oocysts per field; 3, more than 25 oocysts per field. After this step, fecal material was homogenized in PBS and centrifuged as described above. The sediment was checked for the presence of *Salmonella* spp. by standard biological procedures, and the supernatant was checked for the presence of rotaviruses by immunodiffusion. An aliquot of the supernatant was stored at −30°C until tested for IgA, IgG, and IgM by the ELISA.

(iii) **Experiment 3.** At 112 days of age, five calves from

experiment 2 were challenged with 10^7 *C. parvum* oocysts (strain Slooten). Five other calves served as unchallenged controls. Diarrhea, shedding of *C. parvum* oocysts, rotaviruses, and *Salmonella* spp., and kinetics of local and serum antibody responses were evaluated over 4 weeks.

ELISA. Maxisorp microtitration plates (Nunc, Roskilde, Denmark) were coated with 100 μl of crude sonicated oocyst antigens in carbonate buffer (pH 9.6). After incubation for 1 h at 37°C and then overnight at 4°C, the plates were washed with PBS containing 0.05% Tween 20 (PBS-T). The wells were blocked with 1% horse serum (Life Technologies, Gaithersburg, Md.) at 37°C for 1 h to limit nonspecific reactivity. The plates were then processed immediately or stored for up to 14 days at 4°C. Fecal extracts were diluted 1:5 (original supernatant), 1:25, 1:125, and 1:625. After the wells were washed with PBS-T, 100 μl of fecal extract or serum dilution was added to the wells. The wells were then incubated with affinity-purified IgG raised in rabbits to bovine IgA (Nordic Pharma, Tilburg, The Netherlands) or to bovine IgM (ICN Biomedicals, Costa Mesa, Calif.). After the wells were washed again, they were incubated with peroxidase-conjugated affinity-purified goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Mo.). To test for the presence of anti-*C. parvum* IgG in fecal extracts or serum dilutions, we treated samples immediately after incubation with peroxidase-conjugated affinity-purified rabbit anti-bovine IgG. All steps were done at 37°C for 1 h, and the wells were washed three times between each step with PBS-T. Finally, tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, Md.) was added and allowed to react at room temperature (±23°C) for 15 min. The reaction was stopped by the addition of 1 M *o*-phosphoric acid, and the optical densities were read at 450 nm with a Titertek Multiscan spectrophotometer (Flow Laboratories, McLean, Va.). The absence of nonspecific binding of the various anti-immunoglobulins was tested by direct incubation on plates containing antigens. Moreover, each test was run with negative and positive fecal or serum samples. The last dilution of each sample with an optical density higher than twice the mean optical density of 10 negative control samples was considered the endpoint titer. Fecal extracts with titers higher than 625 were tested again at dilutions of 1:500, 1:1,000, 1:2,000, and 1:4,000. About 10% of ELISA tests were repeated, and all assays yielded consistently reproducible results.

Electrophoretic analysis. Oocysts (strain Slooten) were extracted from feces of infected male calves as described above, stored at −30°C in small aliquots, and used as the antigen for SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Before use, oocysts were thawed, diluted 4:1 in SDS solubilizing solution (4% SDS, 1.25 M Tris [pH 6.8], 20% glycerol, 10% 2-mercaptoethanol, 0.0004% bromophenol blue), and solubilized at 100°C for 5 min. The solubilized oocysts were then cooled in an ice bath and centrifuged for 2 min at 17,300 × *g*. Solubilized samples as well as prestained molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were electrophoresed on discontinuous SDS-polyacrylamide gels (15% resolving and 5% stacking) by the method of Laemmli (24) with a Mini Protean II gel apparatus (Bio-Rad). Antigen from approximately 1.5×10^6 oocysts was applied to each lane. Electrophoresis was done at 30 mA for 100 min.

Western immunoblot analysis. *C. parvum* antigens were electrophoretically transferred to nitrocellulose sheets (0.45-μm-pore size; Schleicher & Schuell, Kassel, Germany) with a Mini Trans-Blot Cell (Bio-Rad) for 1 h at 100 V (39). Following transfer, individual lanes were cut out and excess binding sites were blocked by incubation for 15 min with gentle rocking in wash buffer (50 mM Tris HCl, 140 mM NaCl, 5 mM EDTA [pH 7.4] containing 0.25% gelatin and 0.05% Nonidet P-40). The lanes were then probed with coproantibodies extracted from sequential fecal samples for 18 h at 4°C. One lane was not probed and was used to test for the absence of nonspecific binding of the anti-immunoglobulins used. All lanes were incubated with affinity-purified IgG raised in rabbits to bovine IgA (Nordic Pharma) for 1 h at room temperature with gentle rocking. Specific binding of rabbit anti-bovine IgA was subsequently detected by agitating the lanes with biotin-conjugated affinity-purified goat anti-rabbit IgG (Amersham Life Science, Amersham, England). The lanes were then incubated with alkaline phosphatase-conjugated streptavidin (Boehringer, Mannheim, Germany). All steps were done at an ambient temperature for 1 h with gentle rocking, and between each step the lanes were washed three times with wash buffer. After the last step, the lanes were washed with a buffer containing 100 mM Tris HCl, 100 mM NaCl, 50 mM $MgCl_2$ and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Immunoselect; Life Technologies). The reaction was stopped with a buffer containing 10 mM Tris HCl and 1 mM EDTA (pH 8.0). Nitrocellulose strips containing *C. parvum* preparation proteins transferred from SDS-polyacrylamide gels as described above were also probed with convalescent-phase sera from naturally infected calves and with two types of rabbit sera (diluted 1:10 in wash buffer): preimmune rabbit serum and antiserum produced by several parenteral inoculations of purified sonicated oocysts. Specific binding of calf IgG or rabbit IgG to nitrocellulose-bound parasite antigens was subsequently detected with, respectively, biotin-conjugated affinity-purified rabbit anti-bovine IgG (Sigma) or goat anti-rabbit IgG (Amersham Life Science). Relative molecular weights (M_r) of parasite antigens were estimated by comparing their relative mobilities with the relative mobilities of biotinylated molecular weight standards (Bio-Rad) which had been transferred to the same nitrocellulose strips.

RESULTS

Clinical findings and microbiology. (i) Experiment 1. The experimental infection caused anorexia, depression of weight gain during the whole observation period, and high mortality: three of five animals died. No *Salmonella* spp. *E. coli* K99, or coronaviruses were detected in any of the calves. Two calves shed rotaviruses in the feces. The kinetics of oocyst shedding are shown in Fig. 1.

(ii) Experiment 2. On the day of arrival 3 of 17 calves excreted oocysts (1 with a score of 3), and all calves became positive within 7 days. No depression of weight gain or anorexia was observed. At day 21 oocyst output dropped to the detection limit of 10^4 to 10^5 oocysts per g of feces. Most animals shed oocysts again 5, 7, and 14 weeks after arrival (Fig. 2), whereas calf 43 continued to shed oocysts continuously for at least 7 weeks. No *Salmonella* spp. were detected in any of the calves. Rotaviruses were identified in 14 of 17 calves, mainly between days 21 and 35. One calf shed low numbers of an *Eimeria* sp. 35 days p.i. *C. parvum* infection was associated with liquid to mucous feces in 11 of 17 calves between days 4 and 14. Another peak of diarrhea was observed between days 21 and 35 and was associated with a mixed *C. parvum*-rotavirus infection (Fig. 3).

Kinetics of serum antibody and coproantibody responses. (i) Experiment 1. Twenty-four h after intake of colostrum at birth, fecal extracts were positive for specific anti-*C. parvum*

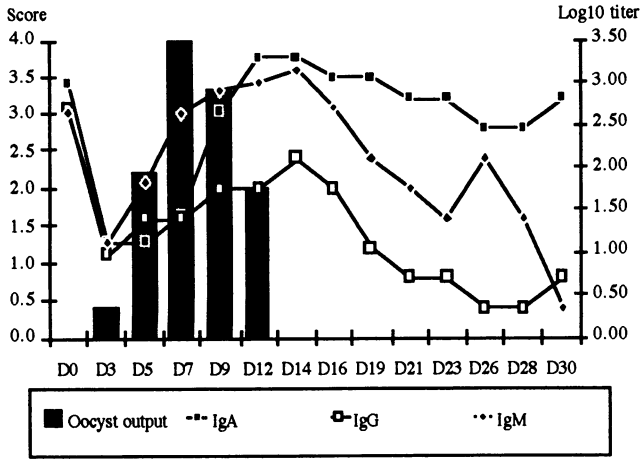

FIG. 1. Kinetics of mean oocyst output and mean titers of specific anti-*C. parvum* coproantibodies after experimental infection of five 1-day-old calves with *C. parvum*. D, day.

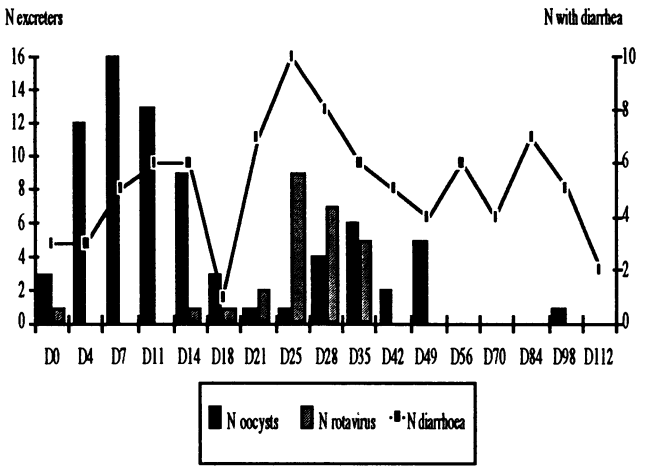

FIG. 3. Number (N) of animals with diarrhea and number of animals excreting *C. parvum* oocysts and/or rotaviruses after natural infection of 17 3- to 6-day-old fattening calves with *C. parvum*. D, day.

IgA, IgG, and IgM isotypes at dilutions of 1:1,000, 1:500 and 1:650, respectively. In 3 days these titers dropped to ≥1:25. Rising specific immunoglobulin levels were closely associated with increasing oocyst output. Once immunoglobulins had reached their maximal levels, oocysts were no longer detectable in the feces. IgM levels decreased very quickly, whereas fecal extracts were still positive for specific IgA 30 days after infection (Fig. 1).

(ii) Experiment 2. On the day of arrival of the calves, low levels of specific anti-*C. parvum* coproantibodies were detected (titers, ≤1:5). Anti-*C. parvum* IgA and IgM levels rose simultaneously and reached maximal titers of 1:125 to 1:2,000 and 1:25 to 1:625, respectively, 14 days after the mean first day of contact (Fig. 2). IgM levels declined very quickly. Once the oocyst output dropped, IgA levels declined slowly to low titers (≤1:25) 112 days after arrival. Levels of anti-*C. parvum* IgG remained low and rose only during the days preceding the mixed fecal *C. parvum*-rotavirus excretion. Figure 4 shows the kinetics of specific anti-*C. parvum* IgA, IgG, and IgM isotype responses in the serum. Levels of both IgG and IgM rose significantly ($P < 0.05$) between days 0 and 98. With regard to calf 43, which shed oocysts for 7 weeks, no difference in the kinetics of fecal and serum antibody responses was seen in comparison with those in the other animals.

Challenge experiment (experiment 3). At day 112 after arrival, five calves were challenged with 10^7 oocysts of *C. parvum*, 16 weeks after the primary contact with the parasite. The animals remained completely refractory to the infection: during the 4 weeks of observation no oocyst output or clinical signs were detected. Coproantibody levels did not increase in comparison with those in unchallenged but previously naturally infected controls, and neither did serum antibody levels.

Electrophoretic and immunoblot analyses. SDS-PAGE analysis of solubilized extracts of intact *C. parvum* oocysts revealed more than 50 different bands with M_r ranging from approximately 6,500 to 200,000 (Fig. 5). Sequential immunoblotting of the reduced preparations probed with fecal IgA from naturally infected calves detected up to 32 different

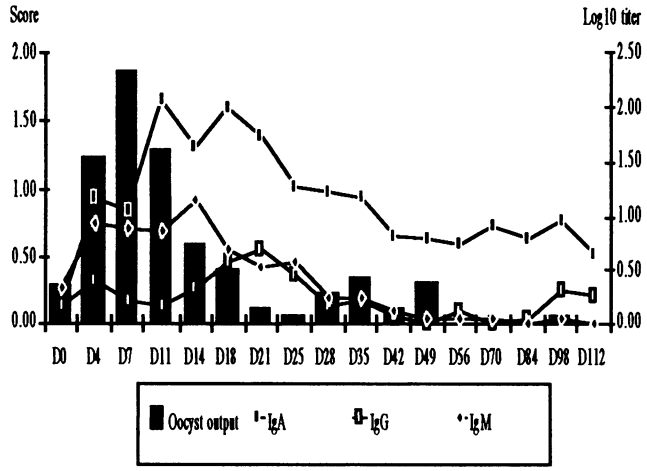

FIG. 2. Kinetics of mean oocyst output and mean titers of specific anti-*C. parvum* coproantibodies after natural infection of 17 3- to 6-day-old fattening calves with *C. parvum*. D, day.

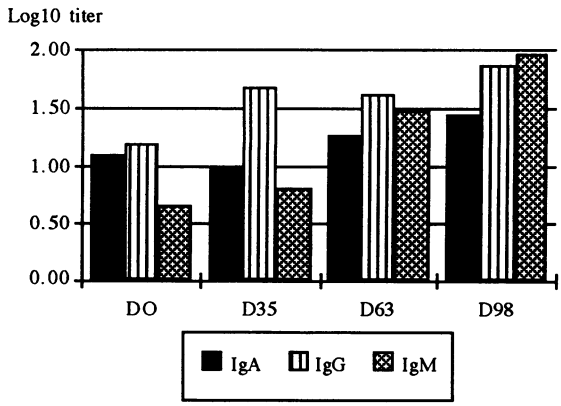

FIG. 4. Kinetics of mean titers of specific anti-*C. parvum* serum antibodies after natural infection of 17 3- to 6-day-old fattening calves with *C. parvum*. D, day.

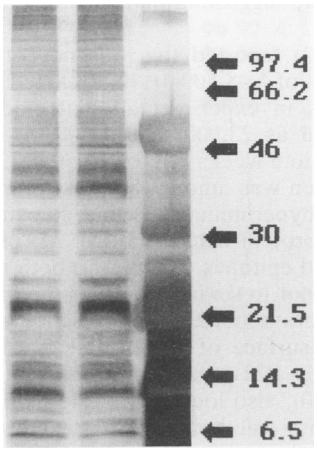

FIG. 5. Silver-stained SDS-polyacrylamide gel of *C. parvum* antigens (lanes 1 and 2). Lane 3 shows the positions of the molecular weight markers (in thousands).

parasite antigens (Fig. 6). All fecal extracts reacted intensely with a small antigen of approximately 11,000 M_r from 7 days p.i. until 16 weeks p.i. Samples from a total of 5 of 17 calves already reacted with this antigen on the day of arrival in the research unit. During the 16 weeks of observation, samples from most calves also reacted strongly with antigens of approximately 15,000, 23,000, and 44,000 M_r and with several high-molecular-weight antigens (66,200 to 200,000 M_r), including a double band of approximately 180,000 M_r. Convalescent-phase calf serum IgG recognized identical bands, with the exception of the 11,000- and 15,000-M_r antigens. Numerous parasite antigens, including those recognized by convalescent-phase fecal calf IgA, reacted with anti-oocyst antisera raised in rabbits. This was not the case with normal preimmunization rabbit serum.

DISCUSSION

The experiments demonstrate that *C. parvum* causes serious enteric problems in neonatal calves: experimentally infected calves showed high mortality, and 65% of naturally infected calves contracted diarrhea. It remains unclear whether the difference in clinical signs may be attributed to differences in strains of *C. parvum* or to differences in cattle breeds. Both possibilities remain open: Fayer and Ungar (16) reported differences in virulence between different *C. parvum* strains. On the other hand, we observed differences in the susceptibility of different cattle breeds to *C. parvum*: the Blue-White Belgian meat breed shows up to 20% mortality after a noncomplicated infection with *C. parvum* (33a). Similar data were reported for the French meat breeds Limousin and Charolais (49). Milk breeds or mixed milk-meat breeds, on the contrary, only show temporary diarrhea and recover spontaneously.

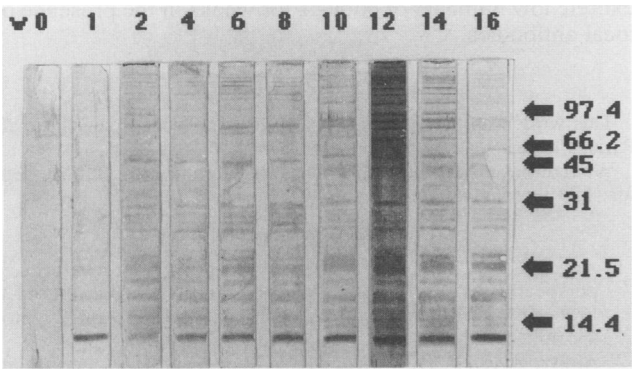

FIG. 6. Immunoblotting of *C. parvum* antigens (dye front not shown) performed with fecal IgA obtained from calf 16 between 0 and 16 weeks (w) after natural infection. The positions of the biotinylated molecular weight markers are marked in thousands.

All calves excreted large numbers of oocysts within 7 days after arrival on the farm (experiment 2). The prepatent period of cryptosporidiosis amounts to 3 to 5 days, so we may assume that virtually all animals became infected on the day of transit to the rearing unit. Oocysts disappeared almost completely from the feces 3 weeks after infection, indicating the development of resistance. However, protection was not complete, as small numbers of oocysts reappeared in the feces afterward. Moreover, it is likely that more animals shed oocysts than were reported here, as we cannot exclude the presence of oocysts in negatively screened samples. Indeed, the detection limit of the routinely used screening methods is about 10^4 to 10^5 oocysts per g of feces.

During this study, the kinetics of fecal and serum antibody responses were monitored by an ELISA. Evidence from the literature and from our data confirms that the test is reliable and reproducible. In a study of humans with an ELISA, no cross-reactivity with other intestinal parasites was found (46). With indirect immunofluorescence, very little or no cross-reactivity was found with the related coccidia *Toxoplasma*, *Sarcocystis*, and *Isospora* spp. (9, 32). Nevertheless, a recent study suggested cross-reactivity between *C. parvum* and *Eimeria* spp. in sheep (32). Anusz et al. (2) did not detect any evidence of cross-reactions between *C. parvum* and four bovine *Eimeria* spp., and neither did Current and Snyder (11) or Anusz et al. (2) with *C. baileyi* and *E. tenella* in chickens.

All animals showed high levels of specific maternal coproantibodies on the first postnatal day. These antibody levels dropped very quickly and did not inhibit the experimental infection. This result is not surprising, as different authors reported that normal colostrum does not protect calves from infection (18, 42). Fayer et al. (14) showed that even hyperimmune colostrum positive at 1:200,000 dilutions for specific IgG1, IgM, and IgA isotypes in the ELISA only provides partial protection. This result means that high antibody levels for prolonged periods are necessary to influence cryptosporidial infection distinctly. Only an acquired local immune response is able to guarantee such levels.

The analysis of fecal extracts established that IgA and IgM are the major immunoglobulins available for activity against endogenous stages of *C. parvum* in the gut. These isotypes were detected early during infection, probably reflecting the preferential stimulation of the mucosal IgA response via the Peyer's patches. The secretion of maximal IgA levels specific for *C. parvum* occurred during the same period of time in which oocyst levels in the feces had begun to drop noticeably. Thus, a good temporal association was indicated between *C. parvum* expulsion and the amount of specific IgA in secretions. Similar evidence was obtained with mice after *Giardia muris* infection (36) and in lambs after experimental infection with *C. parvum* (22). This evidence may indicate antibody-mediated inhibition of sporozoite and/or merozoite

penetration of host cells, as demonstrated for *E. tenella* infection in chickens (13).

Specific fecal IgA levels were maintained for at least 12 weeks, and as the half-life of bovine IgA in intestinal secretions is short, this result reflects a continuous antigenic stimulation of calves in fattening units by reingestion of oocysts or recolonization of the intestinal tract by hidden foci of organisms in the biliary or pancreatic duct. Despite the presence of specific IgA in intestinal secretions, recrudescence of *C. parvum* infection could not be avoided. Therefore, secretory antibodies alone are not sufficient to control reinfection completely. Moreover, a challenge infection with 10^7 oocysts was not followed by a rise in anti-*C. parvum* IgA levels, although animals remained completely refractory to infection. In the case of reinfection, age resistance may be ruled out, as calves raised in isolation from *C. parvum* for 3 months were shown to be as sensitive as 1-week-old calves (19). The latter study and a study with lambs (40) reported the absence of a secondary serum antibody response to *C. parvum*. Therefore, it is likely that secretory antibodies coupled with cell-mediated immune mechanisms are responsible for the clearance of parasites from the infected mucosa and for rendering the immunocompetent host resistant to reinfection.

Specific IgA, IgG, and IgM antibodies to *C. parvum* were also detected in the serum. On the day of arrival at the farm, most animals already had serum antibodies, probably of maternal origin. Like local IgA levels, serum IgG levels rose during the period of oocyst excretion and coincided with the expulsion of the parasite. In the gut, only low IgG levels were detected during and shortly after oocyst excretion, probably because of leakage of serum antibodies. Therefore, the role of local IgG in the expulsion of *C. parvum* seems questionable. Serum IgA levels rose later to a peak than did local IgA levels and remained the same until the end of the experiment at day 98. Similar observations were reported for mice after *G. muris* infection (36). On the other hand, sheep experimentally infected with *C. parvum* showed an early rise and fall in serum IgA levels coinciding with oocyst excretion, while IgG levels were still increasing by day 18 p.i. (22). The reason for the discrepancy between that study and ours is not clear, but the prolonged antibody response in our study and specifically the IgM response reflect continuous exposure to the parasite in a contaminated environment, with a subsequent boosting effect. A similar persistence of specific IgM after exposure to *Cryptosporidium* spp. has been reported in humans (25, 44).

All fecal extracts consistently recognized several soluble *C. parvum* antigens. Similar evidence was reported with sera obtained from humans and animals during and after active infection with *C. parvum* (22, 26–28, 31, 37, 48). Some of these antigens are known to be highly immunogenic, and MAbs to some of them have been effective in reducing the severity of *C. parvum* infection in experimentally infected mice (4–6, 34, 35, 38).

In a recent study, Tilley et al. (37) detected a 9,000- to 10,000-M_r antigen in immunoblots with hyperimmune colostrum. This band was only observed when anti-IgA was used to detect bound antigen and not when other anti-immunoglobulins were used. In our study, a highly reactive antigen of approximately 11,000 M_r was recognized only by fecal IgA and not by convalescent-phase serum IgG, suggesting that we have identified the same antigen. Whitmire and Harp (48) also detected an 11,000-M_r antigen which reacted on immunoblots with hyperimmune anti-oocyst and anti-sporozoite rabbit sera, suggesting that the antigen was of sporozoite origin.

Hill et al. (22) reported the presence of at least 25 *C. parvum* antigens of >48,500-M_r that reacted with serum and fecal antibodies in experimentally infected sheep. These antigens as well as 23,000- and 15,000-M_r antigens were detected for up to 6 weeks p.i. Tilley et al. (38) found that a 15,000-M_r antigen was among the most prominent antigens recognized by hyperimmune bovine colostrum and established its glycoprotein nature (GP15). MAbs raised against GP15 recognized epitopes common to both sporozoites and merozoites but not to sexual stages of the parasite.

Several authors reported the presence of a 23,000-M_r antigen on the surface of sporozoites (29, 31, 43). MAbs raised against this P23 protein and against a glycoprotein of 20,000 M_r (GP20), also localized in the pellicle of sporozoites, showed cross-reactions with merozoite surface proteins (4, 5). These MAbs were shown to share common epitopes with high-molecular-weight antigens (29). Arrowood et al. (4) described an antigen located at the anterior pole of sporozoites that was also identified on merozoites and had a complex Western immunoblot pattern, with several bands ranging from 25,000 to 200,000 M_r. This antigen may be related to glycoproteins characterized by MAbs recognizing molecules located in the apical *C. parvum* micronemes of both sporozoites and merozoites (8). Micronemes are believed to be involved in host cell invasion.

The evidence that different antigens located on both sporozoites and merozoites are recognized by immune colostrum and serum and fecal extracts helps to explain why passive immunotherapy is so effective in reducing developmental stages and oocyst output in experimentally infected mice but also in children and AIDS patients with cryptosporidiosis (10). Furthermore, Tilley et al. (38) established that at least the GP15 antigen was not recognized in the sexual stages of the parasite. This result made these authors believe that *C. parvum* is able to survive only by completing its life cycle rapidly and allowing the immune system the opportunity to eliminate specific recycling stages (sporozoites and type I merozoites). They hypothesized that, once differentiation into gametes has commenced, as for GP15, it would no longer be advantageous for surface antigens of sexual stages to be shared with sporozoites and merozoites. This hypothesis may explain the high prevalence of anti-*C. parvum* antibodies in many adult animal species (41) but also why local antibodies are not able to protect calves completely from reinfections with *C. parvum* and why calves naturally infected with *C. parvum* may excrete low numbers of oocysts for weeks in the presence of local antibodies.

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