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Chemoprophylaxis of *Cryptosporidium parvum* Infection with Paromomycin in Kids and Immunological Study

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The anticryptosporidial activity of paromomycin, a natural antibiotic weakly absorbed when administered per os, was assessed in goat kids experimentally infected once via the oral route with 10^6 *Cryptosporidium parvum* oocysts. Paromomycin used prophylactically at a dose of 100 mg/kg of body weight per day from day −1 to day 10 (day 0 was the inoculation day) prevented infection during the period of drug administration. A delayed low infection was suggested by an antibody rise, but the infection developed below the microscopic detection limits. This low parasite development induced a partial immunity in kids, which reacted immunologically to a challenge on day 21 without symptoms or detectable oocyst shedding. So, paromomycin is a good candidate for field trials because it is prophylactically effective against experimental *C. parvum* infection and well tolerated by animals. This drug would be useful in an adapted form as an anticryptosporidial agent for neonatal ruminants.

In humans and cattle, primarily calves, lambs, and kids, cryptosporidiosis is a serious enteric disease caused by the coccidial protozoan parasite *Cryptosporidium parvum*, and it results in diarrhea, dehydration, weight loss, and sometimes death. *C. parvum* was detected in a goat kid in 1981 (11), but since that date, few papers have been concerned with cryptosporidiosis of kids. Nevertheless, the disease is frequently found on kid farms and causes watery diarrhea, weight loss, mortality (40%) (12, 20), and morbidity, which may rise to 100% by the end of the dropping period. As with other ruminants, such as calves and lambs, neonatal kids are susceptible at that time to cryptosporidiosis but become relatively resistant to *C. parvum* infection with age. Goat kids present the advantage, in spite of their seasonal production, of being less expensive to use as a model. To date, many drugs have been tested for prophylaxis or therapy (7). A few of them—lasalocid in calves (9, 17) and halofuginone in lambs (15) and calves (14, 16)—are effective, but none are commercially available, because their effective doses are too near to toxic levels for animals. Recently, several studies showed the efficiency of paromomycin in vitro (10) and in vivo for the treatment of cryptosporidiosis in human patients with AIDS (1–3, 8, 18, 19) and in animals, BALB/c mice (5), and calves (6).

The aminoglycoside paromomycin is weakly absorbed when administered per os (3.5 μg of the drug per ml of plasma after 10 g of paromomycin has been taken orally). A natural antibiotic, it is commercially available and has a broad-spectrum antimicrobial activity. It also shows an in vitro antiparasitic activity against such parasites of the gut as *Entamoeba histolytica* and *Giardia intestinalis* (6).

We report here on the efficacy of paromomycin used prophylactically against a single experimental *C. parvum* infection in kids and on the influence of the medication on the development of immunity, as monitored by enzyme-linked immunosorbent assay (ELISA) and immunoblot techniques.

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MATERIALS AND METHODS

Animals and groups. Nineteen 1-day-old male kids (French Alpin) separated from their dams at birth were used. Twice in the first 24 h, they were fed with heated goat colostrum (1 h at 56°C), and then they were randomly separated into two groups. Each group was reared in a pen (2.8 by 3.2 m) in an isolated room. Kids were fed twice daily with a milk replacement (Avrilait; Sodiavit, Metz, France) ad libitum.

Both groups were orally inoculated with a syringe at between 2 and 4 days of age with 10^6 *C. parvum* oocysts in 10 ml of water just before the kids drank milk (day 0 = inoculation day). The oocysts were isolated from the feces of calves experimentally infected at birth by sieving the oocysts (from 1,000 μm to 100 μm) and by ether sedimentation, and the oocysts were then treated aseptically in bleach (1.25% sodium hypochlorite) for 10 min. The purified oocysts were stored in 2.5% potassium dichromate at 4°C for 4 weeks until used for animal inoculations.

One group was not medicated and served as an infected control (group A). Twice a day, from day −1 to day 10, the second group (group B) was orally medicated with paromomycin (Humagel ND; Parke-Davis, Courbevoie, France) for a total daily dose of 100 mg/kg of body weight. Before the kids were fed milk, the Humagel powder was diluted in milk and orally administered with a syringe.

On day 21, half of each group was challenged with 10^7 oocysts of *C. parvum.*

The animals were weighed twice a week from day −1 to day 18. Blood was collected twice a week from day −1 to day 35. Serum samples were used for immunological assays (ELISA and immunoblot).

In order to monitor the development of the parasite, the feces were collected daily from day 0 to day 35, except for on days 8, 9, 15, 16, 22, 23, 29, and 30 postinoculation. Each day, for each animal, 0.25 g of feces was homogenized with 750 μl of water, and then 4 ml of a saturated sucrose solution was added. After they received a vigorous agitation, the oocysts were counted with a Thoma cell (volume, 0.01 mm^3). With the dilution taken into account, the number of oocysts shed per milliliter or gram of feces was the number counted on the Thoma cell multiplied by 200,000. When no oocyst was counted, a control was made by sucrose flotation. With regard to the sucrose solution, we replaced the phenol with sodium azide, which is less volatile than phenol (500 g of saccharose, 320 ml of water, 0.2 g of sodium azide per liter).

Immunological study. (i) ELISA procedure. An ELISA was performed to detect anti-*C. parvum* immunoglobulin G (IgG), IgA, and IgM antibodies in the serum of kids. A total of 96 flat-bottom wells in microdilution plates (Nunc, Roskilde, Denmark) were coated with 200,000 thawed oocysts suspended in 100 μl of distilled water per well, and the wells were dried overnight at 37°C. Then, 200 μl of 0.05% phosphate-buffered saline (PBS)–5% Tween 20–nonfat powdered milk (PBS-T-M) was added to each well to minimize nonspecific reactivity. The plates were incubated for 1 h at 37°C and then emptied, and 100 μl of kid serum diluted in PBS-T-M (optimal dilutions of 1:100, 1:25, and 1:25 for IgG, IgA, and IgM, respectively) was added to duplicate wells. After 1 h at 37°C, the wells were rinsed three times with PBS-T, and then 100 μl of a 1:9,000 dilution of rabbit anti-goat IgG (whole molecule)-alkaline phosphate conjugate (Sigma Chemical Co., St. Louis, Mo.) in PBS-T was added and the mixture was incubated for 1 h at 37°C. After three washes in PBS-T, 100 μl of a 1-mg ml^{-1}

p-nitrophenyl phosphate (disodium) (Sigma) solution in 1 M diethanolamine (pH 9.8) was added to each well and incubated for 1 h at 37°C. For IgA and IgM, the plates were incubated for 1 h at 37°C with a 1:2,000 dilution of swine anti-goat IgA (α chain specific; Nordic Pharma, Tilburg, The Netherlands) or swine anti-goat IgM (Fc) (Nordic) in PBS-T. After three washes, 100 μl of a 1:6,000 dilution of rabbit anti-swine Ig peroxidase conjugate (Dako) was added to each well, and then the wells were incubated for 1 h. The plates were emptied, the wells were rinsed three times with PBS-T, and then 100 μl of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in citrate buffer was added for 1 h.

The A_{405} was determined with an LP 400 spectrophotometer (Diagnostics Pasteur, Paris, France). This automatic ELISA reader was zeroed on eight wells containing only antigen, serum anti-species-enzyme conjugate, and substrate. Four kid serum samples taken at birth (before colostrum feeding) and four immunized kid serum samples were included on each plate as negative and positive controls, respectively.

(ii) Electrophoresis and immunoblotting procedures. Before electrophoresis, purified oocysts of *C. parvum* (4×10^7) were subjected to three cycles of freezing at −70°C, thawing in a buffer solution containing 50 mM Tris, 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 0.1% bromophenol blue, and then heating at 100°C for 4 min. After centrifugation at 8,000 × *g* for 10 min at room temperature, the proteins present in the supernatant were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide. Electrophoresis was run at 25 mA with a Minigel-Twin G42 (Biometra) apparatus under nonreducing conditions until the bromophenol blue marker dye reached the bottom of the gel. Then, the proteins were electrophoretically transferred onto nitrocellulose sheets (porosity, 0.1 μm) (Biometra) with the Milliblot SDE system (Millipore) apparatus at a constant current of 1 mA/cm^2. The transblotted nitrocellulose sheets were cut into strips and saturated for 1 h with PBS-T-M. Then, the strips were incubated for 1 h in serum samples diluted 1:50 in PBS-T-M. After three washes in PBS-T-M, the antigens recognized by IgG were revealed by incubation for 1 h in alkaline phosphatase-conjugated rabbit anti-goat IgG (whole molecule) (Sigma) diluted 1:2,000 in PBS-T. After three washes in PBS-T, the antigens recognized by IgA were revealed with nitroblue tetrazolium and bromo-chloro-indolyl phosphate (Gibco) as substrates. For antigens recognized by IgM, swine anti-goat IgM (Fc) (Nordic) diluted 1:2,000 in PBS-T followed by a rabbit anti-swine Ig peroxidase conjugate (Dako) diluted 1:2,000 in PBS-T were used for 1 h. 3,3′-Diaminobenzidine tetrahydrochloride was used as substrate for revelation. The reaction was stopped at the desired contrast with 0.1 M HCl and was followed by a water wash.

Prestained SDS-PAGE standards of a broad range (6.5 to 205 kDa) were used for calibration.

Statistical analysis. The statistical significance of the results was tested at the 0.01 level of confidence by an analysis of variance (the Fisher exact test) and comparisons of means (the Newman Keuls test).

RESULTS

Clinical signs and oocyst shedding. Kids from the unmedicated control group (group A) lost their appetites from day 3, one of them died on day 6, and four of eight lost weight during the period from day 4 to day 7 (Fig. 1). Oocyst shedding, which started on day 4 (Fig. 2), varied among the animals from 56×10^5 to 360×10^5 oocysts per g (mean, 183×10^5 oocysts per g). It lasted until day 12 but decreased from day 7. On days 11 and 12, respectively, four of eight and five of eight animals stopped shedding oocysts. The others shed fewer than 200,000 oocysts per g. Oocysts were observed only after sucrose concentration at a rate of fewer than one oocyst per microscopic field (250×). After day 12, no oocyst was detected in the feces.

In contrast, group B animals, which were medicated with paromomycin (100 mg/kg of body weight per day) from day −1 to day 10, had good appetites and gained weight (Fig. 1). Weight gains for both groups were significantly different for the periods from days 4 to 7 ($P < 0.01$) and 7 to 11 ($P < 0.01$). The group B kids had no watery diarrhea, but the feces were pasty throughout the period of medication. Only 1 kid (1 of 10) shed oocysts (4×10^5 oocysts per g) on one day (on day 6) (Fig. 2).

For both groups (A and B), no oocyst was detected in the feces up to day 35 in spite of the oocyst challenge on day 21 for half of each group.

Antibody responses. For the ELISA (Fig. 3), the optimal dilutions for the serum samples were defined as 1:25 for IgM and IgA and 1:100 for IgG.

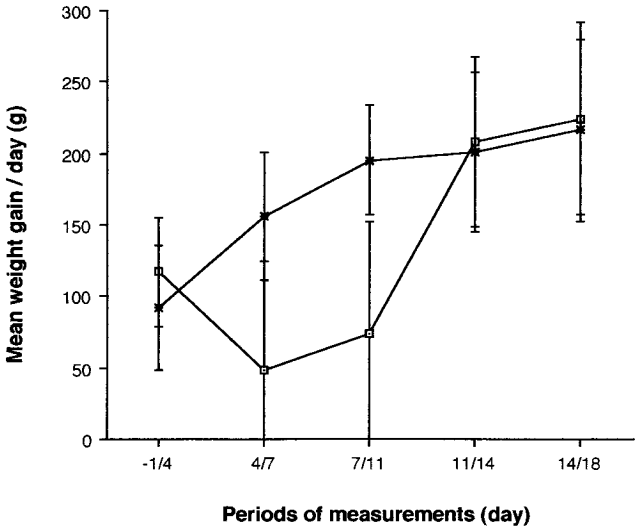

FIG. 1. Mean weight gains (in grams) per day in kids inoculated with 10^6 *C. parvum* oocysts on day 0. —⊡—, group A (unmedicated); —X—, group B (medicated with paromomycin). Error bars are standard deviations.

Anti-*C. parvum*-specific antibody (IgM, IgA, and IgG) levels decreased in two of the groups from day 0 to day 7.

For the infected unmedicated control group A, levels of IgM and IgA increased on day 11 and remained steady. IgG levels stopped decreasing from day 7 to day 14, increased from day 18 to day 27, and then remained steady. In this group A, no significant difference was seen up to day 35 between unchallenged and challenged animals.

In contrast, for the infected medicated group B, the decrease of the level of antibodies until day 7 was not followed by an increase in the level of antibodies on day 11. IgM levels remained steady up to day 21 and then increased from day 21 to day 27. The increase from day 27 to day 35 was more significant

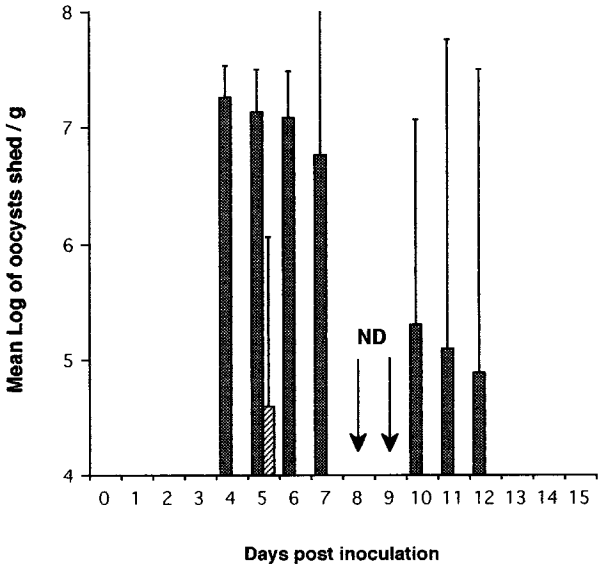

FIG. 2. Logs of mean oocyst shedding per gram of feces in kids inoculated with 10^6 *C. parvum* oocysts on day 0. ■, group A (unmedicated); ▨, group B (medicated with paromomycin). ND, not determined. Error bars are standard deviations.

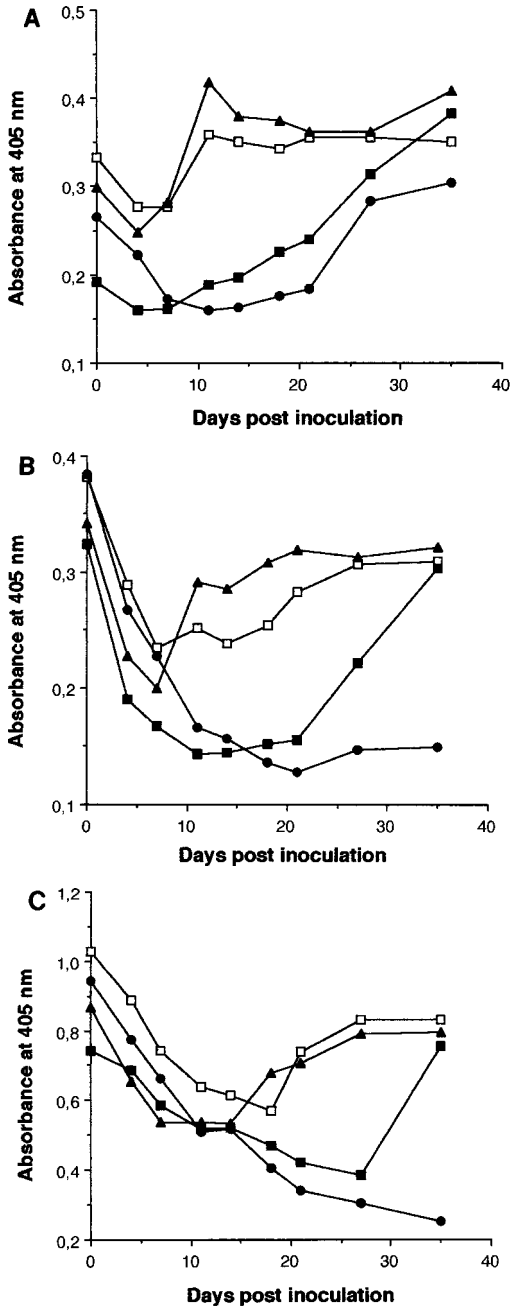

FIG. 3. Kinetics of serum IgM (A), IgA (B), and IgG (C) antibodies in kids by ELISA. □, group A (inoculated and unmedicated); ▲, group A (inoculated, unmedicated, and challenged on day 21); ●, group B (inoculated and medicated with paromomycin); ■, group B (inoculated, medicated, and challenged on day 21).

for the challenged animals. IgA levels decreased until day 11, remained steady until day 35 for the unchallenged animals, and increased from day 27 for the challenged animals. IgG levels for unchallenged animals decreased rapidly until day 21 and then decreased more slowly until day 35. This decrease was accompanied by a strong increase from day 27 to day 35 for challenged animals.

The antigens recognized in the immunoblot tests by serum antibodies of kids are shown in Fig. 4.

Serum anti-*C. parvum* IgG recognized a major 15- to 17-kDa band from days 11 to 35 in 8 kids that were infected and unmedicated and from days 18 (weakly) to 35 in 10 kids that were infected and were medicated with paromomycin.

DISCUSSION

Like calves or lambs, kids are susceptible to *C. parvum* infection in the days following birth. Unmedicated neonatal kids develop clinical cryptosporidiosis, with loss of appetite from day 3, severe weight loss during the periods from days 4 to 7 and 7 to 11 ($P < 0.01$), and mortality for one of nine. The prepatent period lasted 3 days, and the patent period lasted 9 days. A profuse oocyst shedding (mean, 1.83×10^7) was associated with diarrhea in 70% of the kids. The remaining 30% shed oocysts without diarrhea. This was frequently observed in other ruminants, such as calves and lambs, and Current indicated that 10^6 to 10^7 oocysts per g of feces were found in calves (4). The method of quantifying the oocysts with the Thoma cell has a low sensitivity (the total number is the number in the cell times 200,000), but it gave a better estimation of oocyst numbers than the semiquantitative methods currently used (from 0 to 5 in counting the oocysts per 250× microscopic field).

Paromomycin at a dose of 100 mg/kg/day from day −1 to day 10 prevented infection in kids inoculated orally with a single dose of 10^6 oocysts of *C. parvum* on day 0. Our results agree with those of Fayer et al. (6) for calves. In kids that were infected and medicated (group B), no mortality was observed and diarrhea was reduced. However, the feces remained pasty and were nearly liquid for some animals. Only 1 of 10 kids shed few oocysts (one sample from day 6). These clinical data were confirmed by the immunological results (ELISA and immunoblotting).

As early as day 0, anti-*C. parvum*-specific antibodies were detected in kid serum samples. These antibodies, levels of which decreased up to day 7, were probably of maternal origin since kids had drunk goat colostrum on the first day after their birth. The goat colostrum was heated to 56°C to destroy the caprine arthritis-encephalitis virus which is a pathogenic lentivirus inducing arthritis in goats and which is transmitted by colostrum and milk. Neonatal ruminants are agammaglobulinemic at birth. The maternal colostral antibodies are not destroyed by heat treatment, are absorbed in the first 24 h in the kid gut, and titered in the serum samples of kids as soon as day 0. As with other neonatal ruminants, the colostrum limits the mortality rate but does not prevent the development of the parasite and clinical disease. A cryptosporidiosis prophylaxis is obtained when animals are fed with hyperimmune colostrum for several days (13).

In kids (group A), an oral infection with *C. parvum* induced a rise in levels of IgM and IgA on day 11 and IgG on day 18, which reflected the protection of the animals against reinfection.

In medicated infected kids, antibody levels (IgM and IgA) rose only on days following day 21, suggesting that paromomycin prevents either the immune response or active parasite development during the medication period (days −1 to 10). This second hypothesis was supported by the absence of oocyst shedding during the medication period. The rise in levels of IgM and IgA antibodies 11 days later, on day 21, suggests that parasite development started when medication was withdrawn. The increase was more significant for IgM than for IgA levels, which remained steady. This rise was significantly higher in kids reinoculated on day 21. On day 35, the antibody levels (IgM and IgA) in challenged medicated kids were the same as those in unmedicated infected kids, challenged or not. The steady level of IgA antibodies and the slower decrease of IgG

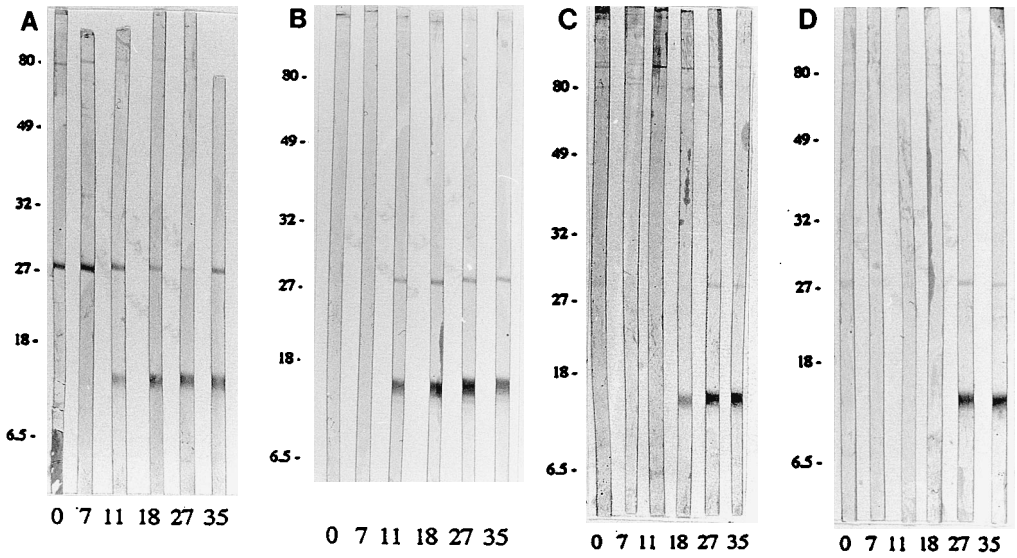

FIG. 4. Kinetics of serum IgG and analysis of antigens recognized by IgG by Western blot (immunoblot) techniques. The apparent molecular masses (in kilodaltons) of standards are indicated on the left. The postinoculation days are indicated at the bottom. (A) Group A (untreated and unchallenged). (B) Group A (untreated and challenged). (C) Group B (treated and unchallenged). (D) Group B (treated and challenged).

levels from day 21 also suggest that parasite development was low. In challenged kids, the IgG level increased to the same level as the group A IgG level.

Our results show that kids orally infected with *C. parvum* develop a protective immune response. The infection was prevented during the period of paromomycin medication (days −1 to 10), but a low development took place after the medication was withdrawn, as suggested by the rise in antibody levels. This development was very low since the shedding was below the microscopic detection limits and did not induce diarrhea. This low development induced only partial immunity in kids which immunologically reacted to a challenge without symptoms or detectable oocyst shedding.

The mechanisms of inhibition of *C. parvum* infection by the nonabsorbable aminoglycoside paromomycin are still unknown, but we think that this drug may act during the early stages of the life cycle since it delays antibody production.

Because paromomycin is prophylactically effective against a single experimental *C. parvum* infection and well tolerated by animals, it is a good candidate for field trials, especially in farms with a heavily contaminated environment. This drug, which is commercially available for use against other protozoan enteric parasites in humans (*Entamoeba histolytica* and *Giardia intestinalis*), would be useful in an adapted form as an anticryptosporidial agent for neonatal ruminants.

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