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Time gap between oocyst shedding and antibody responses in mice infected with *Cryptosporidium parvum*

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Abstract: We observed the time gap between oocyst shedding and antibody responses in mice (3-week-old C57BL/6J females) infected with *Cryptosporidium parvum*. Oocyst shedding was verified by modified acid-fast staining. The individually collected mouse sera were assessed for *C. parvum* IgM and IgG antibodies by enzyme-linked immunosorbent assay from 5 to 25 weeks after infection. The results showed that *C. parvum* oocysts were shed from day 5 to 51 post-infection (PI). The IgM antibody titers to *C. parvum* peaked at week 5 PI, whereas the IgG antibody titers achieved maximum levels at week 25 PI. The results revealed that IgM responses to *C. parvum* infection occurred during the early stage of infection and overlapped with the oocyst shedding period, whereas IgG responses occurred during the late stage and was not correlated with oocyst shedding. Hence, IgM antibody detection may prove helpful for the diagnosis of acute cryptosporidiosis, and IgG antibody detection may prove effective for the detection of past infection and endemicity.

Key words: *Cryptosporidium parvum*, ELISA, oocyst shedding, IgM, IgG

Cryptosporidium parvum is a parasitic protozoan responsible for cryptosporidiosis in humans as well as in certain animals, including domestic livestock (Tyzzer, 1907). In immunocompromised patients, this infection tends to be more serious, and can easily become chronic, or even fatal (Tzipori and Ward, 2002). This protozoan was initially identified in the stomach of autopsied mice (Tyzzer, 1907), and the first human cases were reported in an immunocompetent child and an immunosuppressed adult with diarrhea in 1976 (Nime et al., 1976; Meisel et al., 1997). The prevalence of C. *parvum* infection was previously found to be 10.5% in a study population of 67 human immunodeficiency virus (HIV)-infected patients in the Republic of Korea (Guk et al., 2005) and 1.0% (9/942 diarrhea patients) in a study of non-HIV Korean patients with diarrhea (Lee et al., 2005).

The enzyme-linked immunosorbent assay (ELISA) technique was previously evaluated as a rapid and reproducible method for the detection of *Cryptosporidium*-specific antibodies in captive snakes (Graczyk et al., 1997). However, several studies have demonstrated limitations of ELISA as a diagnostic tool for prediction of oocyst shedding. A seroprevalence study designed to detect IgG antibodies by ELISA in cattle, pigs, and cats reported a discrepancy in the prediction of oocyst shedding (Quilez et al., 1996; Lappin et al., 1997). Nydam et al. (2002) reported that there

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was little agreement between serum ELISA and fecal tests on samples collected from adult cows. According to them, ELISA evidenced a relatively high sensitivity (97.5%), but poor specificity (4.0%). Therefore, it is necessary to compare the durations of oocyst shedding and antibody response periods. The present study was conducted in order to determine the time gap between oocyst shedding and appearance of antibody responses in mice following infection with *C. parvum*.

For the infection of mice, *C. parvum* oocysts, a KKU isolate (Yu et al., 2002), were used. The oocysts were maintained in specific pathogen-free (SPF) female mice (C57BL/6J) following immunosuppression, as was previously described by Yu et al. (2002). Oocyst purification was conducted in accordance with the method described by Petry et al. (1995). Each mouse (3-week-old, 12-15 g) was infected orally with 2 x 10^6 oocysts. Five to 8 mice were allocated to each group. For the examination of oocyst shedding, from day 3 to 61 post-infection (PI), 1 g of fresh feces was collected from each group, and the numbers of oocysts per gram of feces (OPG) were determined in a fecal smear stained by a modified acid-fast technique after the feces were concentrated using formalin-ether sedimentation.

For antigen preparation and serum collection, the oocysts were excysted in 0.04% taurocholic acid in phosphate-buffered saline (PBS) at 37°C for 1 hr. After washing in PBS, the excysted oocysts in 1 ml of sample buffer were homogenized by sonication for 20 min at 4°C, as previously described by Bonnin et al. (1991). Sera were collected from infected C57BL/6J mice by heart puncture from week 5 to 25 PI, and centrifugation for 20 min at 1,500 g. Uninfected control sera were collected from 8 C57BL/6J female mice. The sera were aliquoted and stored at -20°C until testing.

For the ELISA procedure, antigen with approximately 2.5 x 10^5 oocysts was coated onto each well of flat-bottomed 96-well microtiter plates (Costar, Corning, Cambridge, Massachusetts, USA) diluted in 0.05 M carbonate buffer (pH 9.6) and incubated for 24 hr at 4°C. The plate was washed 3 times with PBS-T (PBS-Tween-20) for 5 min each, and then blocked with

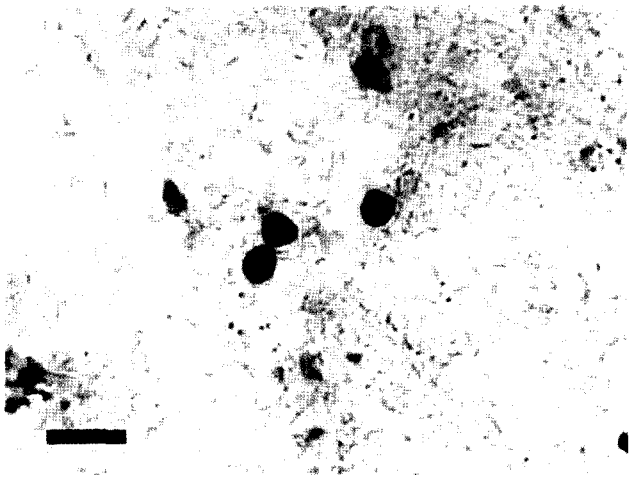

Fig. 1. *C. parvum* oocysts in mice feces detected by modified acid fast staining. Bar, 10 um.

1% bovine serum albumin in 0.05% Tween-20 for 1 hr at 37°C. The mouse serum (1: 100) diluted with PBS-T was added, followed by a 1 hr incubation at 37°C. Peroxidase-conjugated goat anti-mouse IgM or IgG (1: 100, Sigma, St. Louis, Missouri, USA) was allowed to react for 1 hr at 37°C as a secondary label. The color reaction utilized 40 mM ABTS, i.e., 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Sigma), for 10 min at 37°C, and was halted with 5% sodium dodecyl sulfate solution. Optical densities (OD) were determined at 405 nm with an ELISA reader (Bio-Rad M3550, Richmond, California, USA). Each serum sample was duplicated using 2 wells, and the mean OD was established as the final value.

The significance of antibody responses was compared between the non-infected and *C. parvum*-infected mice, and statistical analyses were conducted via *t*-test using dBSTAT^{TM} (http://www.dbstat.com, Korea). Differences between the groups were considered to be statistically significant when the *P*-values were lower than 0.05.

C. parvum oocyst excretion was detected in the feces at day 5 PI by a modified acid fast staining (Figs. 1 & 2). Oocyst shedding continued from day 5 to 51 PI, and OPG peaked at day 7 PI at 2.7 x 10^5; the number decreased sharply thereafter. From day 53 PI, no oocysts were detected in the feces. The IgM antibody titers to *Cryptosporidium* increased significantly and

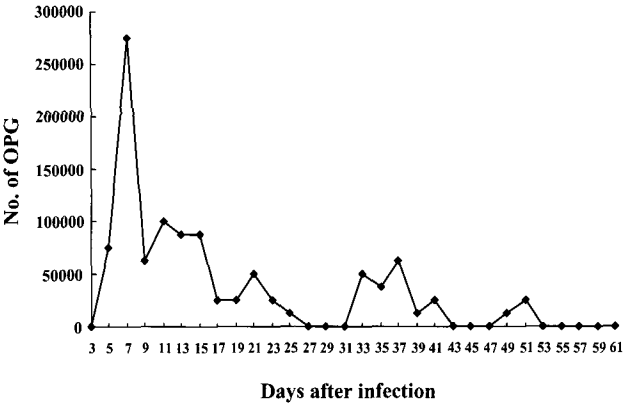

Fig. 2. Number of oocysts per gram of feces counted after modified acid fast staining from the C57BL mice after *C. parvum* infection.

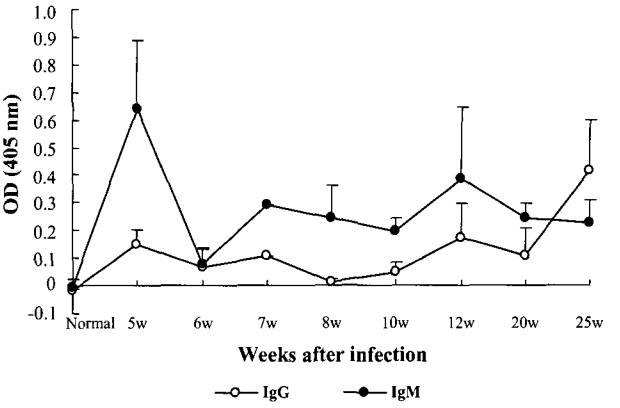

Fig. 3. Serum antibody titer of *C. parvum*-infected C57BL mice detected via ELISA.

peaked at week 5 PI, i.e., the earliest point of examination in the present study, and decreased thereafter ($P < 0.05$, Fig. 3). IgG antibody levels increased significantly, and remained steady from week 5 to 20 PI, then peaked at week 25 PI, the latest time point observed ($P < 0.05$) (Fig. 3). During the experiment, the IgM and IgG titers were maintained at levels significantly higher than in uninfected control mice.

In the present study, we noted that *C. parvum* oocysts were shed through feces from day 5 to 51 PI. In other words, oocyst shedding continued for approximately 8 weeks PI. Peak IgM antibody responses were observed during nearly the same period as oocyst shedding. The IgM responses peaked at week 5 PI and maintained significantly high OD values until week 25 PI. By contrast, the IgG responses were delayed until week 25 PI, and thus the periods of IgG responses did not coincide with the oocyst shedding period.

Previously, patients without acquired immunodeficiency syndrome (AIDS) evidenced an early rise and fall in IgM and a later elevation in IgG (Ungar et al., 1986). However, there was a great deal of variance in the timing of antibody responses in different hosts. Specific antibodies were generated from a calf at day 40 PI (Lazo et al., 1986). Specific IgG antibodies against *Cryptosporidium* were detected in serum from experimentally infected calves at days 6 to 7 PI and achieved peak levels at day 9 PI, whereas oocyst shedding occurred between days 5 and 10 PI (Williams and Burden, 1987; Whitmire and Harp, 1991). However, the IgM responses peaked at week 4 PI and declined rapidly in humans, and evidenced relatively low levels at week 22 PI, whereas the IgG responses peaked at week 14 PI (Moss et al., 1998). Based on the results of previous studies, the IgG responses occurred during a much earlier period in calves, within 1 week after infection, than was observed in humans. The results of our study demonstrated that the mouse antibody responses appear to follow a timing pattern more similar to humans than to calves.

In light of the overlapping period between oocyst shedding and the IgM responses, a serologic test for the detection of IgM might prove useful for the diagnosis of acute cryptosporidiosis, during which oocyst shedding occurs abundantly. The exact reason for the delayed IgG responses is difficult to explain. Although no oocysts were detected in the feces after day 51 PI, small quantities of oocysts below the detection limits of our method (fewer than 400 oocysts) or internal stages may have remained, and thereby stimulated antibody responses. Thus, the IgG antibody may be useful in evaluating a past infection status or a chronic infection. It remains difficult to ascertain whether the IgM responses might have started earlier than week 5 PI and the IgG responses might have been maintained for longer than 25 weeks after infection in mice, because we did not collect sera outside of this timeframe.

In conclusion, the IgM responses occurred during the early post-infection period (week 5 PI), and may prove useful for the diagnosis of acute cryptosporidiosis, whereas the IgG responses occurred during a later stage (week 25 PI), and may be useful for the diagnosis of a past infection and cryptosporidiosis endemicity.

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