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Evidence of Thymus-Independent Local and Systemic Antibody Responses to *Cryptosporidium parvum* Infection in Nude Mice

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Differences in susceptibility to persistent cryptosporidial infection between two strains of adult athymic nude mice prompted us to investigate the immune mechanism(s) that may control resistance to infection in these T-cell-deficient mice. We studied fecal oocyst shedding, serum and fecal parasite-specific antibody responses, and fecal immunoglobulin levels in athymic C57BL/6J nude and athymic BALB/cJ nude mice following oral inoculation with *Cryptosporidium parvum* oocysts at 8 to 9 weeks of age. C57BL/6J nude mice had significantly higher fecal parasite-specific immunoglobulin A (IgA) (days 27, 31, 35, and 42 postinoculation) and IgM (days 10, 17, 24, 28, 31, 38, 42, and 48 postinoculation) levels than BALB/cJ nude mice ($P < 0.05$) and significantly higher serum parasite-specific IgA levels at 63 days postinoculation ($P < 0.03$). Moreover, C57BL/6J nude mice shed significantly fewer *C. parvum* oocysts than BALB/cJ nude mice from days 52 to 63 postinoculation ($P < 0.05$). In contrast, BALB/cJ nude mice had higher levels of non-parasite-specific IgA (days 38 to 63 postinoculation) and IgM (days 24, 35, 38, and 52 postinoculation) than C57BL/6J nude mice in feces ($P < 0.05$). These data suggest that parasite-specific fecal antibodies may be associated with resistance to *C. parvum* in C57BL/6J nude mice.

During the past two decades, *Cryptosporidium parvum* has gained recognition as an important enteropathogen of mammals, including humans (17, 18, 48). In immunocompetent hosts, *C. parvum* generally causes a self-limited diarrheal illness. However, in immunocompromised hosts, *C. parvum* may cause a life-threatening, prolonged cholera-like illness (12, 31, 37). Numerous attempts to treat cryptosporidiosis in both humans and animals have met with limited success (8, 41). The absence of consistently effective anticryptosporidial chemotherapeutic agents exacerbates the consequences of this disease. Moreover, the lack of a suitable immunocompetent adult laboratory model of infection has hindered our understanding of functional immune responses to this parasite.

The discovery that mice homozygous for the nude mutation (*nu*/*nu*, T-cell deficient) and SCID mice (which lack both T and B cells) develop persistent *C. parvum* infection led to their use as models for the study of disease pathogenesis and evaluation of potential anticryptosporidial agents (24, 32, 49). Evidence from the above studies and others (2, 10, 36, 50, 51) indicates that the control of cryptosporidial infection in mice involves $CD4^+$ cells and T-cell receptor (TCR) $\alpha\beta$ $CD4^+$ cells. Gamma interferon (IFN-γ) is also important in the control of parasite development, as treatment of mice with anti-IFN-γ antibodies increases susceptibility to infection (11, 47, 50). Serum and local parasite-specific antibodies have been detected following infection, but their contribution to resistance to cryptosporidiosis remains unclear (7, 44). Several investigators have examined the kinetics of *C. parvum*-specific serum and fecal antibody responses associated with oocyst shedding in infected mice, lambs, and calves. While some investigators have found an association between declining oocyst shedding and rising titers of specific immunoglobulin A (IgA) and IgM (25, 38), others have provided no evidence for such an association (44, 46).

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Published studies on cryptosporidiosis in athymic nude mice have used primarily the BALB/cJ strain; to our knowledge, little work has been done with athymic nude mice of other inbred strains (24, 32). In our laboratory, inoculation of 8-week-old athymic C57BL/6J nude mice with *C. parvum* oocysts resulted in fecal oocyst shedding significantly lower than that observed in athymic BALB/cJ nude mice, suggesting non-thymus-dependent strain differences in susceptibility to infection. Recently, other investigators have noted differences in susceptibility to cryptosporidial infection in immunodeficient mice (13, 33, 47). These studies suggest that genetic differences, the presence or absence of certain cytokines (such as IFN-γ and interleukin-4 [IL-4]), and other host factors yet unidentified may influence the outcome of cryptosporidial infection. While cell-mediated immunity is required for control of cryptosporidiosis, mucosally delivered neutralizing antibodies may be involved in resistance to and recovery from infection (38, 40). We herein examine the relationships between local antibody (IgA and IgM) responses and oocyst shedding in adult C57BL/6J nude and BALB/cJ nude mice. Our results indicate that parasite-specific serum (IgA) and fecal (IgA and IgM) antibodies may be associated with resistance to *C. parvum* infection in C57BL/6J nude mice. In addition to providing insight into the role of anti-*C. parvum* serum and fecal antibody responses, these two mouse strains may serve as models to allow evaluation of thymus-independent responses to *C. parvum*.

MATERIALS AND METHODS

Animals. Female C57BL/6J nude and BALB/cJ nude mice (23 to 26 g), ages 8 to 9 weeks, were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice were housed in microisolator cages in high-efficiency particulate air (HEPA)-filtered laminar flow racks and allowed to acclimate for 1-week prior to oocyst challenge. All manipulations were done in a HEPA-filtered hood. Mice received sterilized normal mouse diet (Tekland, Harlan, Md.) containing 18% (wt/wt) protein and 6% (wt/wt) fat as well as sterilized water throughout the experimental period (63 days).

***C. parvum* oocyst inoculation.** Purified *C. parvum* oocysts (IOWA isolate) were obtained from Pleasant Hill Farms (Mount Pleasant, Idaho). Oocysts were stored at 4°C in 2.5% (wt/vol) potassium dichromate for up to 2 weeks prior to use. Before mice were inoculated, oocyst preparations were treated with 1.75%

(wt/vol) sodium hypochlorite (1 min, 22 to 23°C) and then washed with 0.025 M phosphate-buffered saline (PBS; pH 7.2). Each mouse was inoculated intragastrically with 10^6 oocysts in 100 μl of PBS, using an 18-gauge gavage needle (Thomas Scientific, Swedesboro, N.J.).

Quantification of oocysts in feces. Fecal pellets from each mouse in each group were collected twice weekly from days 3 to 63 postinoculation (p.i.) to monitor oocyst shedding by immunofluorescence assay. Briefly, four fecal pellets were collected from each mouse into individual microcentrifuge tubes and suspended in 1.5 ml of PBS overnight at 4°C. The fecal pellets were then vortex homogenized and centrifuged (1,500 × *g*, 4°C, 10 min), after which the supernatant fractions were collected and kept at −80°C prior to specific IgA or IgM antibody measurements. Ethyl acetate (300 μl) was then added to the fecal pellets in each tube; the feces were vortexed (60 s) and then centrifuged (1,500 × *g*, 10 min, 4°C) to concentrate oocysts. The supernatants were then aspirated, and the oocyst-containing sediments were resuspended in 300 μl of PBS/sample. Ten microliters of each suspension was then placed in individual wells of multiwell glass slides previously treated with poly-L-lysine (0.01% [vol/vol]; Sigma Chemical Co., St. Louis, Mo.). Slides were heat fixed and processed for immunofluorescence assay using oocyst-specific monoclonal antibody (MAb) OW50 conjugated with fluorescein isothiocyanate as instructed by the manufacturer (Meridian Diagnostics, Cincinnati, Ohio). Oocysts in each sample were counted in an Olympus (La Palma, Calif.) epifluorescence microscope (400× objective). Thirty arbitrarily selected fields in each well were examined.

Preparation of CpA. *C. parvum* oocyst antigen (CpA) was prepared by using a modification of a previously described protocol (35). Briefly, oocysts (7×10^8) were washed with PBS, centrifuged (1,500 × *g*, 5 min, 4°C), resuspended in 1.75% (wt/vol) sodium hypochlorite for 1 min, centrifuged (1,500 × *g*, 5 min, 4°C), and washed three times (4°C) with PBS. They were then resuspended in 2 ml of sterile deionized water containing 1 mM phenylmethylsulfonyl fluoride (Sigma) and disrupted by ultrasonication (10 min, 4°C) followed by freezing and thawing five times. The lysate was then centrifuged (1,500 × *g*, 10 min, 4°C) to remove insoluble material; the supernatant was dialyzed (molecular weight exclusion limit, 6,000 to 8,000) against PBS (12 h, 4°C) and then stored at −80°C. Protein content was determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.) according to the manufacturer's instructions.

Fecal and serum antibody determination. To determine the local antibody response, four fecal pellets per mouse (collected from days 3 to 63 p.i. from both strains of mice) were suspended in 1.5 ml of PBS, vortex homogenized, and centrifuged (1,500 × *g*, 4°C, 10 min) to remove insoluble material. The antibody-containing supernatants were then collected and kept at −80°C prior to IgA and IgM determination by an indirect enzyme-linked immunosorbent assay (ELISA). Each mouse was also bled from the retro-orbital plexus on days 0, 3, 21, and 63 p.i. Serum was separated and stored at −80°C prior to IgA, IgG, and IgM determination by ELISA.

To measure *C. parvum*-specific antibody responses by ELISA, Falcon Pro-Bind (Becton Dickinson, Mountain View, Calif.) ELISA plates were coated overnight (12 h, 4°C) with 50 μl of CpA (2 μg/ml in 0.1 M carbonate buffer [pH 9.6]). The plates were then washed thrice (5 min per wash) with PBS containing 0.05% Tween 20 (PBS-T) and twice with PBS and then blocked (37°C, 1 h) with 100 μl of 1% (wt/vol) nonfat milk in PBS; 50 μl of fecal supernatant (described above) or serum (for IgA, 1:20 dilution; for IgG and IgM, 1:100 dilution in 1% nonfat milk–PBS solution) from individual mice was then added in triplicate to wells and incubated (12 h, 4°C). Controls included various concentrations of anti-*C. parvum* MAbs (IgA, 4D4 G9H4 [16]; IgM, OW50 [43]; IgG1, 3F7-F11 [42]). After washing, 50 μl of horseradish peroxidase-labeled goat anti-mouse IgA, IgG, and IgM (1:1,000 dilution; Sigma) was added to each well and incubated (37°C, 2 h). After additional washing, ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)]-H_2O_2 (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to each well and incubated (22 to 23°C, 30 min), after which optical densities (ODs) were measured at 450 nm with an ELISA Reader (Dynatech Laboratories Inc., Chantilly, Va.). Concentrations of parasite-specific serum IgA, IgG, and IgM in each sample were calculated from positive controls determined on each plate. Total fecal IgA or IgM levels were measured by sandwich ELISA using various concentrations of class-specific antibodies as controls. Capturing MAbs (Kirkegaard & Perry) included rat anti-mouse IgA (catalog no. 01-18-01) and rat anti-mouse IgM (catalog no. 01-18-09). Falcon Pro-Bind (Becton Dickinson) ELISA plates were coated with each capture antibody (1 μg/ml in carbonate buffer [pH 9.6]; 12 h at 4°C), washed thrice with PBS-T and twice with PBS as described above, and blocked (1% [wt/vol] nonfat milk–PBS solution; 37°C, 1 h). Fecal supernatants and standards were then added in triplicate to the wells. Positive controls used as standards consisted of murine polyclonal antibodies derived from mouse myeloma cells (IgA, MOPC-315; IgM, MOPC-104E; each at 0.0156 to 1.0 μg/ml; Sigma) and were included independently in each ELISA plate. Plates were incubated (12 h, 4°C), washed as described above, and incubated (37°C, 2 h) with 50 μl of horseradish peroxidase-labeled goat anti-mouse IgA or IgM (1:1,000 dilution; Kirkegaard & Perry). After additional washing, ABTS-H_2O_2 substrate (Kirkegaard & Perry) was added to each well and incubated (22 to 23°C, 30 min). Following incubation, ODs were measured at 450 nm. The immunoglobulin concentrations in each sample were calculated from immunoglobulin standard curves determined on each plate.

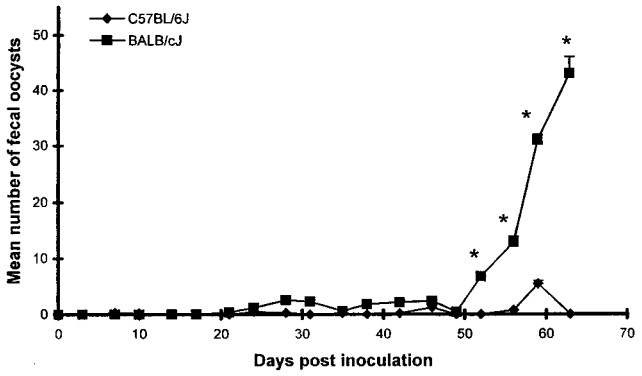

FIG. 1. Fecal oocyst shedding by *C. parvum*-inoculated athymic C57BL/6J ($n = 6$) and BALB/cJ ($n = 5$) nude mice. *, values are significantly different ($P < 0.05$).

Statistical analysis. Parasite-specific fecal antibody and total fecal immunoglobulin values for individual mice of each strain were averaged, and differences between the means were analyzed for significant differences by a random-effects repeated-measures model using Stata Software (Computer Resource Center, Santa Monica, Calif.). Differences in the mean serum anti-*C. parvum* IgA and IgM antibody levels for each strain were analyzed for significant differences by using the Wilcoxon rank sum test. Mean fecal oocyst shedding results (days 3 to 63 p.i. from each strain) were analyzed for significant differences by one-tail Student's *t* test.

RESULTS

Oocyst shedding. Oocyst shedding was undetectable in both strains of mice from days 3 to 24 p.i. (Fig. 1). Variable, low-level oocyst shedding was noted in BALB/cJ nude mice from days 28 to 48 p.i. The intensity of oocyst shedding in BALB/cJ nude mice increased at days 52, 56, and 59 and again at day 63 p.i. and was significantly higher than C57BL/6J nude mice oocyst shedding ($P < 0.05$). Oocyst shedding in C57BL/6J nude mice was generally below detectable levels before day 60 p.i., at which time transient oocyst shedding was observed. The differential responses between C57BL/6J and BALB/cJ nude mice described herein were observed in replicate experiments.

Fecal *Cryptosporidium*-specific antibody. Although there was considerable variability both between days and between individuals within each group, on days 27, 31, 35, and 42 p.i., C57BL/6J nude mice had higher levels of anti-*C. parvum* IgA than BALB/cJ nude mice ($P < 0.05$) (Fig. 2A). The titer of anti-*C. parvum* fecal IgM was higher in C57BL/6J nude mice than in BALB/cJ nude mice on days 10, 17, 24, 28, 31, 38, 42, and 48 p.i. ($P < 0.05$) (Fig. 2B).

Fecal immunoglobulin levels. The kinetics of total fecal IgA and IgM levels (Fig. 3) differed from that of fecal *C. parvum*-specific antibody responses (Fig. 2). Total fecal IgA in BALB/cJ nude mice remained at a low level from day 0 until day 31 p.i. On day 35 p.i., fecal IgA in BALB/cJ nude mice increased sharply and remained significantly higher (days 38 to 63 p.i.) than in C57BL/6J nude mice ($P < 0.05$) (Fig. 3A). Conversely, total fecal IgA in C57BL/6J nude mice remained at a moderate level from days 0 to 35 p.i. (Fig. 3A). On days 17, 21, 24, 28, and 31 p.i., fecal IgA levels in C57BL/6J nude mice were significantly higher than in BALB/cJ nude mice ($P < 0.05$) (Fig. 3A). Levels of fecal IgA in C57BL/6J nude mice dropped and remained significantly lower than those in BALB/c mice from days 42 to 63 p.i. ($P < 0.001$). The total fecal IgM in C57BL/6J nude mice changed little over the course of the experiment (Fig. 3B). However, in BALB/cJ nude

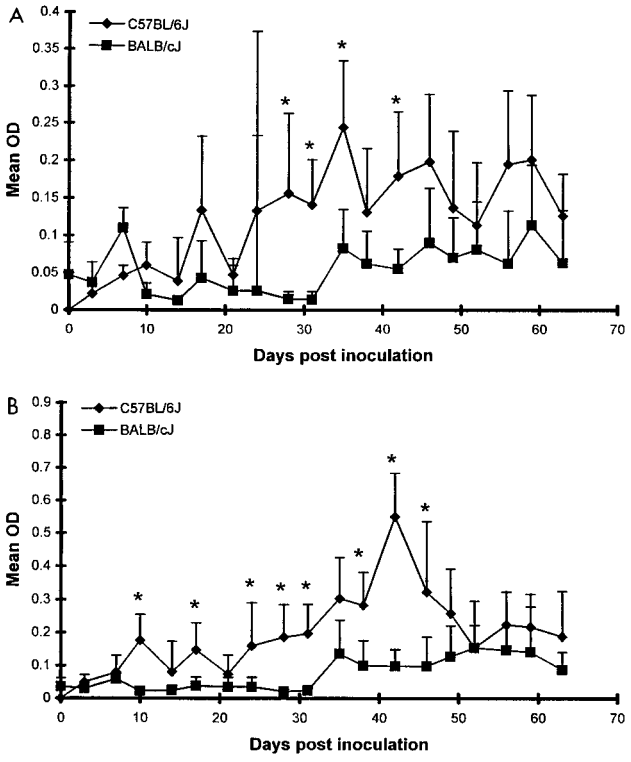

FIG. 2. Kinetics of fecal *C. parvum*-specific IgA (A) and IgM (B) antibody responses in *C. parvum*-inoculated athymic C57BL/6J ($n = 6$) and BALB/cJ ($n = 5$) nude mice measured by ELISA. *, values are significantly different ($P < 0.05$).

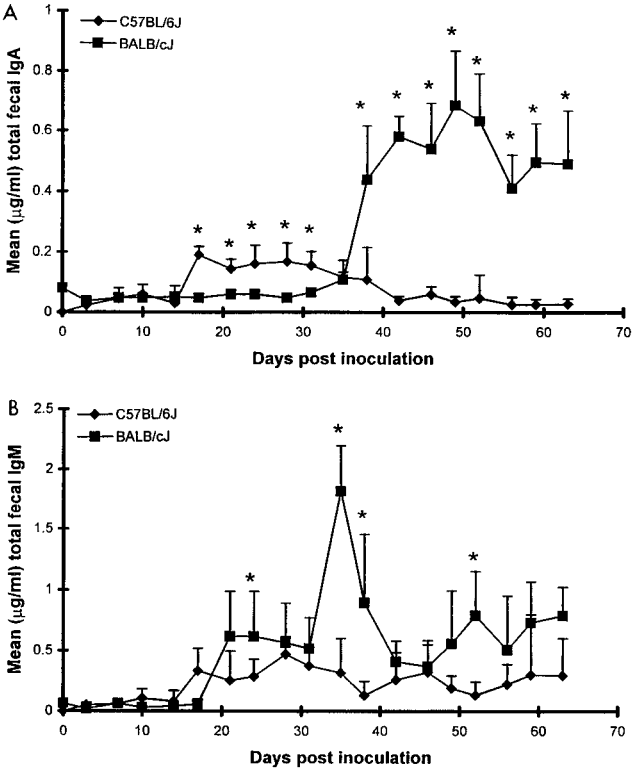

FIG. 3. Concentration of total fecal IgA (A) and IgM (B) antibody responses in *C. parvum*-inoculated athymic C57BL/6J ($n = 6$) and BALB/cJ ($n = 5$) nude mice measured by ELISA. *, values are significantly different ($P < 0.05$).

mice, fecal IgM increased and was significantly higher than in C57BL/6J nude mice on days 24, 35, 38, and 52 p.i. ($P < 0.05$).

Serum *Cryptosporidium*-specific IgA, IgG, and IgM levels. On days 0, 3, and 21 p.i., anti-*C. parvum* serum IgA was not detected in either C57BL/6J nude mice or BALB/cJ nude mice (data not shown). On day 63 p.i., four of the five BALB/cJ nude mice had no detectable anti-*C. parvum* serum IgA (Fig. 4). Anti-*C. parvum* serum IgA in the one BALB/cJ nude mouse that responded was 44.1 μg/ml (Fig. 4). In contrast, five of the six C57BL/6J nude mice showed a high level of anti-*C. parvum* serum IgA ranging from 208 to 470 μg/ml (Fig. 4). One C57BL/6J nude mouse had no detectable anti-*C. parvum* serum IgA. The mean serum anti-*C. parvum* IgA (BALB/cJ, 8.8 μg/ml; C57BL/6J, 310 μg/ml) on day 63 p.i. was significantly different ($P < 0.05$) between the two strains (Fig. 4).

Anti-*C. parvum* serum IgG levels at 3, 21, and 63 days p.i. were highly variable between individual animals in both mouse strains. There were no significant differences in serum anti-*C. parvum* IgG levels between BALB/cJ nude mice (day 3 p.i., 16.71 ± 10.43; day 21 p.i., 37.01 ± 21.58; day 63 p.i., 9.53 ± 5.19) and C57BL/6J nude mice (day 3 p.i., 8.841 ± 5.68; day 21 p.i., 30.22 ± 12.15; day 63 p.i., 40.02 ± 33.54) at any time point studied ($P > 0.05$). Similarly, there were no significant differences in serum anti-*C. parvum* IgM levels between BALB/cJ nude mice (day 3 p.i., 5.00 ± 1.58; day 21 p.i., 6.02 ± 0.72; day 63 p.i., 2.24 ± 0.52) and C57BL/6J nude mice (day 3 p.i., 3.841 ± 2.88; day 21 p.i., 9.65 ± 4.82; day 63 p.i., 7.40 ± 3.37) at any time point studied ($P > 0.05$).

DISCUSSION

Athymic BALB/cJ nude mice have been used extensively as models of *C. parvum* infection to investigate the role of humoral and cell-mediated immune responses (9, 24, 32, 41). However, to our knowledge, no work on the immune response to *C. parvum* has been done with mice carrying the *nu/nu* mutation on other strain backgrounds. Resistance to *C. parvum* requires both cellular (primarily) and humoral responses (27, 41, 54). These responses have been studied systemically in mice, humans, and domestic animals. However, the role of intestinal mucosal immune responses has not been thoroughly explored in athymic nude mice. During our studies of mucosal immune responses, we observed that C57BL/6J nude mice, despite the absence of functional thymus-dependent responses, develop only low-grade *C. parvum* infection, while BALB/cJ nude mice exhibited greater infection. Specifically, BALB/cJ nude mice shed significantly more oocysts in feces than C57BL/6J nude mice. A comparison of antibody responses between BALB/cJ nude mice and C57BL/6J nude mice revealed marked differences. *C. parvum*-inoculated BALB/cJ nude mice had significantly lower production of fecal *C. parvum*-specific IgA (days 27, 31, 35, and 42 p.i.) and IgM (days 10, 17, 24, 28, 31, 38, 42, and 48 p.i.) than C57BL/6J nude mice, and only one of five BALB/cJ nude mice produced anti-*C. parvum* serum IgA. In contrast, BALB/cJ nude mice had significantly more total IgA (days 38 to 63 p.i.) and IgM (days 24, 35, 38, 48, and 52 p.i.) in feces than C57BL/6J nude mice, suggesting that in BALB/cJ nude mice either secretory responses were not completely impaired, there was increased leakage via increased intestinal permeability, or parasite-specific responses were suppressed or not appropriately expressed. Our results suggest that intestinal mucosal immune responses temporally associated with fecal parasite-specific antibodies may accompany resistance to *C. parvum* infection in C57BL/6J nude mice. Moreover, the results further suggest that differ-

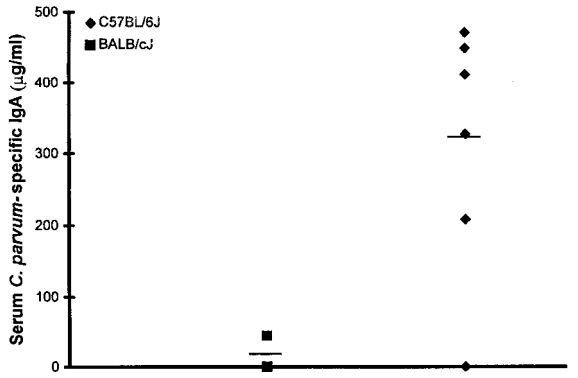

FIG. 4. Concentration of serum anti-*C. parvum* IgA antibody on day 63 p.i. in athymic C57BL/6J ($n = 6$) and BALB/cJ ($n = 5$) nude mice measured by ELISA. Medians (——; BALB/cJ, 8.82 μg/ml; C57BL/6J, 310 μg/ml) are shown. Anti-*C. parvum* IgA antibodies in C57BL/6J nude mice were significantly higher than in BALB/cJ nude mice ($P < 0.03$).

ences in the two athymic nude mouse strains examined can have a significant effect on susceptibility to *C. parvum* infection even though both strains are T-cell deficient. Recent reports have demonstrated a relationship between oocyst shedding and local IgA immune responses in *C. parvum*-infected lambs, calves, and mice (25, 38, 40, 46). In the experiments reported here, association of parasite-specific fecal IgA and oocyst shedding is also observed in C57BL/6J nude mice.

Athymic nude mice lack functional systemic T lymphocytes (34), have poorly developed or absent Peyer's patch germinal centers, where progenitors of IgA plasma cells reside (21, 29). These defects are corrected by adoptive reconstitution with T cells (22). In addition, production of IgA antibodies in mice requires the cooperation of cytokines, including IL-4, IL-5, IL-10, IFN-γ, and transforming growth factor β, which have been found at low levels in athymic mice (14, 29, 30).

Recently, functional T lymphocytes at the mucosal level have been detected in nude mice and identified as small intestinal intraepithelial lymphocytes (IEL), which are predominantly TCR γδ IEL (23, 26, 52, 53). These γδ T cells have been shown to promote mucosal IgA immune responses and have also been shown to produce an array of Th1-type (e.g., IFN-γ), and Th2-type (e.g., IL-4, IL-5, and IL-10) cytokines, as well as transforming growth factor β and tumor necrosis factor alpha (3–6, 19, 30, 45). We have found significantly higher numbers of TCR αβ and γδ IEL T cells in the small intestines of *C. parvum*-inoculated C57BL/6J nude mice infected with *C. parvum* than in those of BALB/cJ nude mice (1). The reasons for these differences between the two strains of mice are difficult to explain. However, the putative lack of mature or functional γδ IEL in athymic BALB/cJ mice may have contributed to the low fecal antibody responses observed. Although the present study does not address whether γδ IEL in C57BL/6J nude mice are responsible for the lower oocyst shedding and increased serum and fecal parasite-specific antibody levels observed, it is possible that in the absence of systemic T cells, γδ IEL had an effect on either IgA^+ B-cell differentiation, induction of IgA switch, or other functional mucosal responses. Alternatively, it is possible that γδ IEL in C57BL/6J nude mice had influenced αβ T cells to up-regulate parasite-specific IgA and IgM mucosal responses (15, 20, 39). Studies are now in progress in our laboratory to explore these possibilities, and to define the factors influencing development of mucosal and systemic antibodies in athymic C57BL/6J nude mice and their potential involvement in the control of *C. parvum* infection.

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