Korean Journal of Parasitology
Vol. 45, No. 3: 175-180, September 2007

DOI: 10.3347/kjp.2007.45.3.175

Role of murine Peyer's patch lymphocytes against primary and challenge infections with *Cryptosporidium parvum*

Sang-Mee GUK and Jong-Yil CHAI*

Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine, and Institute of Endemic Diseases, Seoul National University Medical Research Center, Seoul 110-799, Korea

Abstract: In order to determine the role of Peyer's patch lymphocytes (PPL) in self-clearing of *Cryptosporidium parvum* infection in murine models, changes in PPL subsets, their cytokine expression, and in vitro IgG1 and IgA secretions by PPL were observed in primary- and challenge-infected C57BL/6 mice. In primary-infected mice, the percentages of CD4+ T cells, CD8+ T cells, sIgA+ B cells, IL-2+ T cells, and IFN-γ+ T cells among the PPL, increased significantly ($P < 0.05$) on day 10 post-infection (PI). Secretion of IgG1 and IgA in vitro by PPL also increased on day 10 PI. However, all these responses, with the exception of IgG1 and IgA secretions, decreased in challenge-infected mice on day 7 post-challenge (= day 13 PI); their IgG1 and IgA levels were higher ($P > 0.05$) than those in primary-infected mice. The results suggest that murine PPL play an important role in self-clearing of primary *C. parvum* infections through proliferation of CD4+, CD8+, IL-2+, and IFN-γ+ T cells, and IgG1 and IgA-secreting B cells. In challenge infections, the role of T cells is reduced whereas that of B cells secreting IgA appeared to be continuously important.

Key words: *Cryptosporidium parvum*, Peyer's patch lymphocytes, mucosal immunity, cytokines

INTRODUCTION

Cryptosporidium species are apicomplexan parasites that can cause mild to severe diarrheal illnesses in animals and humans (Griffiths, 1998; Leav et al., 2003). In particular, immunosuppressed hosts infected with this protozoan, including patients with acquired immunodeficiency syndrome (AIDS), may undergo chronic intractable diarrhea and life-threatening illnesses (O'Donoghue, 1995; Chai et al., 1999; Guk et al., 2003, 2005). In immunocompetent hosts, the infection is generally subclinical and resolved within 2-4 weeks, and they become resistant thereafter (O'Donoghue, 1995). From studies of human and animal cryptosporidiosis, it is apparent that adoptive immunity, in the forms of CD4+ T cells and IFN-γ, is important for resistance to and clearance of the infection (Abrahamsen, 1998; Theodos, 1998; Leav et al., 2005).

The major effectors responsible for the self-resolution of the host from *C. parvum* infections have been reported to be lymphocytes in the small intestine, i.e., intraepithelial lymphocytes (IEL), lamina propria lymphocytes (LPL), and mesenteric lymph node lymphocytes (MLNL) (O'Donoghue, 1995; Wyatt et al., 1996; Abrahamsen et al., 1997; Pasquali et al., 1997). Our previous studies have reported that CD8+ IEL were involved in protection against a challenge infection with *C. parvum*, through enhanced cytotoxicity,

• Received 28 June 2007, accepted after revision 7 August 2007.

*Corresponding author (e-mail: cjy@snu.ac.kr)

whereas LPL played a more important role in protection against a primary infection with *C. parvum*, through production of IFN-γ and IgA (Chai et al., 1999; Guk et al., 2003). In mucosal immunity, detection of antigens from the gut is essential, and Peyer's patches (PP) are the major site of antigen sampling and presentation by microfold (M) cells, and transportation to T- and B- cell areas (Boher et al., 1994; Kelsall and Strober, 1996; Aleksandersen et al., 2002; Macdonald, 2003; Mariotte et al., 2004). Nevertheless, there have not been many reports on the role of PP lymphocytes (PPL) against *Cryptosporidium* infection in mice. Thus, the present study aimed to investigate the role of PPL in self-resolution of mice from primary and challenge infections with *C. parvum*.

MATERIALS AND METHODS

Experimental animals

Specific pathogen-free C57BL/6 mice, 4-week-old females, with a weight of 13-15 g, were obtained from the Daehan Laboratory Animal Center, Ltd. (Jinju, Korea). Oocysts of *C. parvum* were obtained from the stool of a Korean leukemic child, who suffered from this protozoan infection, after obtaining an informed consent. This isolate was maintained by one passage in a calf (Holstein), a 1-week-old male weighing approximately 65 kg.

Parasite infection

In the primary infection group, each mouse was infected orally with 10^6 oocysts and killed on day 10 post-infection (PI). In the challenge infection group, mice were challenged with the same dose of oocysts on day 6 PI after the primary infection and killed 7 days later (on day 13 PI). In the control group, the mice were inoculated orally with only the phosphate-buffered saline (PBS) and killed on the same day.

Lymphocyte isolation

PP were removed aseptically from the small intestine of mice and kept in cold RPMI 1640 medium (GIBCO BRL, Grand Island, New York, USA) containing 10% fetal bovine serum (FBS) (GIBCO). PPL were isolated by gentle tease of the tissues through a stainless-steel wire mesh screen (Sigma, St. Louis, Missouri, USA) and by filtering the suspension through a nylon membrane to exclude dead cells and debris.

Phenotype analysis

To analyze lymphocyte subsets, a total of 10^6 cells were washed in the staining buffer (PBS containing 1% FBS and 0.1% sodium azide) and resuspended in the same buffer. Nonspecific Fc-binding was blocked using an antibody specific for FcγII/III receptors. Cells were washed with PBS twice, and monoclonal antibodies (mAbs) against murine leukocyte differentiation molecules were used for single and dual fluorescence flow cytometry; mAbs against murine CD4 (IgG2b) (PharMingen, San Diego, California, USA), CD8 (IgG2b) (PharMingen), surface IgG1 (Serotec Ltd., Kidlington, UK), and surface IgA (IgG1) (Serotec) were used. Labeled cells were assayed with a fluorescence-activated cell sorter (FACScan) (Becton Dickinson, Sparks, Maryland, USA). The proportion (%) of cells expressing a given molecule was determined as the average of 3 sample replicates.

Analysis of intracellular cytokines

To analyze the proportion of intracellular cytokines, membranes of cells were permeated with the Cytofix or Cytoperm solution (PharMingen), a combination of fixatives with paraformaldehyde was added and incubated at 4°C for 20 min. Cells were stained with fluorochrome-conjugated anti-cytokine antibodies and then analyzed immediately using the FACScan.

Analysis of immunoglobulin production

To measure the production of IgG1 and IgA in vitro from PPL cultures, isolated PPL were re-suspended in RPMI 1640 containing 10% FBS at a density of 10^6 cells per well in 96-well plates (Costar, Cambridge, Massachusetts, USA). Lymphocytes were stimulated by culture with the mitogen, concanavalin A (Con A, 5 μg/ml; Sigma) for 3 days at 37°C in a 95% air-5% CO_2 atmosphere. After incubation for 3 days, the culture supernatant of each well was collected and the

Table 1. Phenotypes of Peyer's patch lymphocytes (PPL) isolated from *C. parvum*-infected mice

Lymphocyte subset	% PPL (mean ± SD; n = 3)		
	Controls	Primary-infected mice[a)]	Challenge-infected mice[b)]
CD4+	14.6 ± 2.9	36.2 ± 1.2[c,d)]	15.9 ± 2.2
CD8+	4.9 ± 1.5	21.8 ± 1.5[c,d)]	1.6 ± 0.2[e)]
sIgG1+	75.8 ± 10.5	82.4 ± 10.3	82.7 ± 0.2
sIgA+	2.9 ± 0.2	7.6 ± 2.9[c,d)]	3.4 ± 1.1

[a)]Mice were infected orally with 10^6 oocysts and killed at day 10 PI.
[b)]Mice were challenged with the same oocyst dose at day 6 PI and killed at day 7 post-challenge, i.e., day 13 PI.
[c)]Significantly higher ($P < 0.05$) than controls.
[d)]Significantly higher ($P < 0.05$) than challenge-infected mice.
[e)]Significantly lower ($P < 0.05$) than controls.

Table 2. Kinetics of intracellular cytokine expressions by PPL isolated from mice infected with *C. parvum*

Intracellular cytokine	% PPL (mean ± SD; n = 3)		
	Controls	Primary-infected mice	Challenge-infected mice
IL-2	8.0 ± 0.1	11.9 ± 8.3[a)]	0.7 ± 0.2[b)]
IFN-γ	23.8 ± 5.7	63.8 ± 7.1[a,c)]	40.7 ± 9.2[c)]

[a)]Significantly higher ($P < 0.05$) than challenge-infected mice.
[b)]Significantly lower ($P < 0.05$) than controls.
[c)]Significantly higher ($P < 0.05$) than controls.

Table 3. IgG1 and IgA productions in vitro from PPL isolated from mice infected with *C. parvum*

Antibody	Absorbance at 410 nm (mean ± SD; n = 3)[a)]		
	Controls	Primary-infected mice	Challenge-infected mice
IgG1	0.052 ± 0.001	0.133 ± 0.017[b)]	0.149 ± 0.050[b)]
IgA	0.174 ± 0.008	0.429 ± 0.060[b)]	0.441 ± 0.018[b)]

[a)]All groups were stimulated in vitro with lipopolysaccharides (5 μg/ml).
[b)]Significantly higher ($P < 0.05$) than controls.

absorbance was determined by the mAb-based mouse immunoglobulin isotyping kit (PharMingen).

Statistical analysis

Statistical significance of differences was determined with the two-tailed Student's *t*-test. $P < 0.05$ was considered significant for all comparisons.

RESULTS

Among the PPL, the proportions of CD4+, CD8+, and sIgA+ T cells increased significantly ($P < 0.05$) in primary-infected mice compared to the controls (Table 1). In challenge-infected mice, the proportions of these cells were only slightly higher than the controls (CD4+ PPL and sIgA+ PPL), or significantly ($P < 0.05$) lower than the controls (CD8+ PPL). The proportion of sIgG1+ PPL did not change significantly in any of the infected groups.

The proportions of the PPL expressing IFN-γ+ or IL-2+ in primary-infected mice increased greatly ($P < 0.05$) compared to the controls (Table 2). However, in challenge-infected mice, the proportion of the PPL expressing IFN-γ+ became lower than that in primary-infected mice, though it was maintained at a significantly higher ($P < 0.05$) level than the controls.

Meanwhile, the proportion of the PPL secreting IL-2 decreased significantly ($P < 0.05$) compared to both primary-infected mice and the controls (Table 2).

The in vitro productions of IgG1 and IgA from the isolated PPL in both primary-infected and challenge-infected mice were significantly greater ($P < 0.05$) than those of the controls (Table 3). The levels of both antibody isotypes were higher in challenge-infected mice than in primary-infected mice, although the differences were statistically not significant.

DISCUSSION

Our previous studies showed that mice began to shed *C. parvum* oocysts on day 4 PI, peaking on day 10 PI, and oocysts were not observed after day 13 PI in primary-infected mice (Guk et al., 2003). In challenge-infected mice, oocyst excretion peaked on day 4 after challenge, i.e., day 10 PI, the number of oocysts being smaller than that in primary-infected mice, and oocysts were not observed after day 10 after challenge, i.e., day 16 PI (Guk et al., 2003). These results suggested that secondary immune responses of the hosts are more rapid than primary immune responses.

In the present study, experimental *C. parvum* infections in immunocompetent C57BL/6 mice resulted in alterations of lymphocyte subsets, cytokine expressing cell proportions, and antibody secreting patterns among the PPL. In primary-infected mice, increases in CD4+, CD8+, sIgA+ and IFN-γ+ PPL were observed at the beginning of oocyst clearing on day 10 PI, but the proportions of these cells, with the exception of IFN-γ+ PPL, were normalized or decreased to lower levels than those of the controls in challenge-infected mice on day 13 PI, i.e., day 7 post-challenge. This result suggests that the mechanisms implicated in the control of a challenge infection may be different from those of a primary infection with *C. parvum*.

According to our previous study which targeted on murine LPL and IEL, CD4+ LPL played an important role in protection against a primary infection with *C. parvum*, through induction of IFN-γ+ and IgA (Guk et al., 2003). Whereas, against a challenge infection, CD8+ IEL and CD8+/TCR $\gamma\delta$+ IEL involved more, increasing cytotoxicity, and aided by the sustained activity of the specific IgA (Guk et al., 2003). In the present study, which focused on PPL, CD4+ T helper cells were also important in protective mechanisms of primary-infected mice. Similar reports on the role of PPL in murine cryptosporidiosis have been available. For example, in neonatal BALB/c mice, *Cryptosporidium* infection induced proliferations of both CD4+ and CD8+ lymphocytes on days 4, 10, and 16 PI in ileal PP, and CD4+ cells increased on day 4 PI in jejunal PP (Boher et al., 1994). In suckling Naval Medical Research Institute (NMRI) mice infected with *C. parvum*, all CD4+, CD8+, TCR α/β+, and CD45R/B220+ PPL were shown to be more than 10-fold increases than the uninfected controls on day 17 PI (Mariotte et al., 2004).

In challenge infections, 2 major effecter mechanisms seem to be involved. One is CD8+ IEL and CD8+/TCR $\gamma\delta$+ IEL, as suggested by our previous study (Guk et al., 2003). However, this is contradictory to the results of the present study. The proportions of CD8+ T cells among the PPL in this study decreased remarkably in challenge-infected mice, down to lower levels than those of the uninfected controls. IEL are known to be derived from dividing precursor cells, originating from PP, circulating in the mesenteric lymph nodes and the thoracic duct lymph, and then selectively returning to the gut wall (Guy-Grand et al., 1978). This discrepancy is difficult to explain. A possible speculation is that CD8+ PPL, which is destined finally to migrate into the gut epithelium, might be depleted after a primary infection, and time is needed for them to restore before a challenge infection or late phages of infection. In this respect, it is worth referring that, in suckling NMRI mice infected with *C. parvum*, more than 10-fold increased numbers of T and B cells among the PPL decreased rapidly and remained at lower levels than the controls on days 19-23 PI, i.e., the termination of the infection (Mariotte et al., 2004).

The other effecter mechanism in challenge infections is the role of B cells, among the LPL and PPL, which produce IgA. In the present study, the number of IgA expressing cells among the PPL and the level of

IgA secretion by the PPL in vitro elevated both in primary- and challenge-infected mice. It is well known that, in the mucosal immune system, most of secretory IgA responses are generated in PP, and the B cell progeny migrates to the lamina propria (Macdonald, 2003). In previous studies on *C. parvum*, IgA+ and IgG1+ B cells increased among the LPL on day 6 and day 10 PI, and antigen-specific IgA secretion from intestinal mucosa increased markedly in a challenge infection (Huang et al., 1996; Guk et al., 2003).

The IgA secretion is known to be generated by 2 ways. One is T helper cell-dependent and the other is T cell-independent way. With regard to the T-dependent way, it has been suggested that a challenge infection with *C. parvum* may drive the immune response towards a Th2 type, enhancing IL-4 production by LPL, and may stimulate sustained production of the specific IgA (Guk et al., 2003). Most B cells in PP are known to express IgA; however, other immunoglobulin classes are also present. To enhance IgA production, regulatory T cells in PP promote the immunoglobulin class switch to IgA, and support differentiation of IgA-committed B cells (Reynolds, 1987). As to the T-independent way, it is of note that about 25% of the mouse secretory IgA response is T-independent and largely of the B1 lineage (Macpherson et al., 2000).

In the present study, PPL expressing IFN-γ+ were shown to increase after primary and challenge infections with *C. parvum*. The extent of IFN-γ+ PPL increase was greater in the primary infection compared with the challenge infection. Reports have indicated importance of IFN-γ in systemic and mucosal immune responses against cryptosporidiosis (Leav et al., 2005), the mechanisms of innate immune responses as well as the source of IFN-γ remain poorly defined. The lack of innate mucosal IFN-γ expression was noted in humans and animals, and this may be an explanation for differences between species in susceptibility to *Cryptosporidium* (Leav et al., 2005). Further studies of IFN-γ mediated innate immune responses in cryptosporidiosis will be useful to develop strategies for improving resistance to the infection.

In conclusion, we suggest that PPL in the small intestines of mice play an important role in self-clearing of a primary *C. parvum* infection through proliferation of CD4+, CD8+, IL-2+, and IFN-γ+ T cells, and IgG1 and IgA-secreting B cells. In a challenge infection, the role of T cells seems to be reduced, whereas the role of B cells secreting IgA may be continuously important.

REFERENCES

Abrahamsen MS (1998) Bovine T cell responses to *Cryptosporidium parvum* infection. *Int J Parasitol* **28:** 1083-1088.

Abrahamsen MS, Lancto CA, Walcheck B, Layton W, Jutila MA (1997) Localization of α/β and γ/δ T lymphocytes in *Cryptosporidium parvum*-infected tissues in naive and immune calves. *Infect Immun* **65:** 2428-2433.

Aleksandersen M, Lie KI, Gjerde B, Landsverk T (2002) Lymphocytes depletion in ileal Peyer's patch follicles in lambs infected with *Eimeria ovinoidalis*. *Clin Diagn Lab Immun* **9:** 83-91.

Boher Y, Perez-Schael I, Caceres-Dittmar G, Urbina G, Gonzalez R, Kraal G, Tapia FJ (1994) Enumeration of selected leukocytes in the small intestine of BALB/c mice infected with *Cryptosporidium parvum*. *Am J Trop Med Hyg* **50:** 145-151.

Chai JY, Guk SM, Han HK, Yun CK (1999) Role of intraepithelial lymphocytes in mucosal immune responses of mice experimentally infected with *Cryptosporidium parvum*. *J Parasitol* **84:** 125-130.

Griffiths JK (1998) Human cryptosporidiosis: epidemiology, transmission, clinical disease, treatment, and diagnosis. *Adv Parasitol* **40:** 37-85.

Guk SM, Yong TS, Chai JY (2003) Role of murine intestinal intraepithelial lymphocytes and lamina propria lymphocytes against primary and challenge infections with *Cryptosporidium parvum*. *J Parasitol* **89:** 270-275.

Guk SM, Seo M, Park YK, Oh MD, Choe KW, Kim JL, Choi MH, Hong ST, Chai JY (2005) Parasitic infections in HIV-infected patients who visited Seoul National University Hospital during the period 1995-2003. *Korean J Parasitol* **43:** 1-5.

Guy-Grand D, Griscelli C, Vassalli P (1978) The mouse gut T lymphocyte, a novel type of T cell: nature, origin and traffic in mice in normal and graft-versus-host conditions. *J Exp Med* **148:** 1661-1677.

Huang DS, Lopez MC, Wang JY, Martinez F, Watson RR (1996) Alteration of the mucosal immune system due to *Cryptosporidium parvum* infection in normal mice. *Cell*

Immunol **173:** 176-182.

Kelsall BL, Strober W (1996) Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. *J Exp Med* **183:** 237-247.

Leav BA, Mackay M, Ward HD (2003) *Cryptosporidium* species: new insights and old challenges. *Clin Infect Dis* **36:** 903-908.

Leav BA, Yoshida M, Rogers K, Cohen S, Godiwala N, Blumberg RS, Ward H (2005) An early intestinal mucosal source of gamma interferon is associated with resistance to and control of *Cryptosporidium parvum* infection in mice. *Infect Immun* **73:** 8425-8428.

Macdonald TT (2003) The mucosal immune system. *Parasite Immunol* **25:** 235-246.

Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM (2000) A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* **288:** 2222-2226.

Mariotte D, Comby E, Brasseur P, Ballet JJ (2004) Kinetics of spleen and Peyer's patch lymphocyte populations during gut parasite clearing in *Cryptosporidium parvum* infected suckling mice. *Parasite Immunol* **26:** 1-6.

O'Donoghue PT (1995) *Cryptosporidium* and cryptosporidiosis in man and animals. *Int J Parasitol* **25:** 139-195.

Pasquali P, Fayer R, Almeria S, Trout J, Polidori GA, Gasbarre LC (1997) Lymphocyte dynamic patterns in cattle during a primary infection with *Cryptosporidium parvum. J Parasitol* **83:** 247-250.

Reynolds JD (1987) Peyer's patches and the early development of B lymphocytes. *Curr Top Microbiol Immunol* **135:** 43-56.

Theodos CM (1998) Innate and cell-mediated immune responses to *Cryptosporidium parvum. Adv Parasitol* **40:** 87-119.

Wyatt CR, Brackett EJ, Perryman LE (1996) Characterization of small intestine mucosal lymphocytes during cryptosporidiosis. *J Eukaryot Microbiol* **43:** 66S.