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Concurrent response to challenge infection with *Cryptosporidium parvum* in immunosuppressed C57BL/6N mice

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We investigated the response to challenge infection with *Cryptosporidium parvum* oocysts in immunosuppressed C57BL/6N mice. In the primary infection, fecal oocyst shedding and parasite colonization were greater in immunosuppressed mice than in nonimmunosuppressed mice. Compared with primary infection, challenge infection with *C. parvum* didn't show any oocyst shedding and parasite colonization. Especially, oocyst shedding and parasite colonization from the mice infected with heat-killed oocysts were not detected. After challenge infection with *C. parvum* oocysts, however, these mice were shedding small numbers of oocysts and parasite colonization. Except normal control and uninfected groups, the antibody titers of other groups appear similar. Based on the fecal oocyst shedding, parasite colonization of ilea, and antibody titers in the mice, these results suggest that the resistance to challenge infection with *C. parvum* in immunosuppressed C57BL/6N mice has increased.

Key words: antibody response, *Cryptosporidium parvum*, immunosuppression, infection

Introduction

Since the first report of cryptosporidiosis in cattle in 1971 [15], *Cryptosporidium parvum* has proven to be significantly important as a cause of neonatal diarrhea in most domesticated animals [21]. *C. parvum* (Apicomplexa: Cryptosporididae) is an intracellular protozoan parasite that colonizes in epithelial cells of the respiratory and digestive tracts in humans and other animals [5,11,14]. The most severe consequence of human cryptosporidiosis occurs in the immunodeficient host, and *C. parvum* is recognized as a significant opportunistic pathogen in the acquired immunodeficiency syndrome patient population [14,17]. The infection is usually mild and self-limiting in hosts with a normal immune system, but can be chronic and life-threatening in immunocompromised individuals [3,12]. The prevalence of *C. parvum* infections in the general population has reportedly been 2.2~8.5% [4]. Despite decades of research on hundreds of chemo- and immunotherapeutic agents either in vitro or in vivo in animal models and clinical trials, there is still no specific therapeutic or preventive modality approved for cryptosporidiosis [22].

In the immune response to *C. parvum* infection, cell-mediated and human immune responses are believed to be involved in the resolution of infections and the development of protection [19], but the specific immune mechanisms to *C. parvum* are not well understood. Cell-mediated immunity has been suggested to play an important role in clearing cryptosporidial infections [10]. Especially, $CD4^+$ T cells and Interferon (IFN)-γ activity play a major role in immune system. For example, adult athymic nude mice infected with *C. parvum* were reported to develop chronic infections [7] and IFN-γ seemed to inhibit reproduction of *C. parvum* in epithelial cell lines [18]. These results suggest that cell-mediated immune responses are necessary for both resistance to and recovery from cryptosporidiosis by *C. parvum* oocysts.

Meanwhile, antibody responses to *C. parvum* antigens, particularly secretory IgA response to mucosal antigens, suggest that examination on the local immune response may be of interest in seroepidemiological studies. Benhamou *et al.* [2] reported that *Cryptosporidium*-infected patients develop both serum and secretory antibodies to *C. parvum*. However, despite the presence of *C. parvum*-specific serum and antibodies, infection can persist with protracted diarrhea in AIDS patients. Thus, cell-mediated immunity has shown only a limited degree of efficacy in cryptosporidiosis.

The objective of the present study was to investigate the effect of challenge infection to *C. parvum* oocysts in immunosuppressed C57BL/6N mice.

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Materials and Methods

Animals and parasites

Female C57BL/6N mice (Simonson Laboratories, USA) aged 6 to 8 weeks weighing 15 to 20 g each were used. The mice were immunosuppressed with dexamathasone phosphate (DEXp; Sigma, USA) administered ad libitum in drinking water (10 μg/ml) [23]. They were maintained in isolation during the course of the study and were housed in wire-floored cages. The cages were placed on trays containing 1.8% potassium dichromate solution to prevent the feces from drying out.

The mice were inoculated with the Iowa isolate of *C. parvum*. Oocysts were maintained by passage in experimentally infected mice and purified from feces using discontinuous sucrose gradients [1]. Purified oocysts were stored in 2.5% potassium dichromate solution at 4°C for less than 4 months prior to use. Oocyst inocula were prepared by washing purified oocysts with distilled water 3 times to remove the potassium dichromate. Washed oocysts were enumerated on a hemocytometer using microscopy and then administered to mice by orogastric intubation (10^6 *C. parvum* oocysts/mouse) as reported previously [24].

Experimental design

Mice were randomly distributed into six groups of 20 mice/group and housed in different isolation cages. The mice in groups 1 and 2 were inoculated orogastrically with 10^6 *C. parvum* oocyst per each on the first day of immunosuppression. These mice have received DEXp continuously until the experiment was terminated. Indeed, on the 25th day, the mice of group 2 were inoculated with *C. parvum* oocysts (secondary infection). Group 3 was inoculated with heat-killed *C. parvum* oocysts on the first day of immunosuppression. These mice were challenged with *C. parvum* oocysts on the 25th day (secondary infection). Groups 4 (positive control) and 5 (negative control) were inoculated with *C. parvum* oocysts on the 1st day of the experiment, but did not receive DEXp. By the way, group 5 was inoculated with *C. parvum* oocysts on the 25th day (secondary infection). Group 6 (normal control) was neither immuno-suppressed with DEXp nor inoculated with *C. parvum*.

Determination of oocyst shedding in infected mice

Fecal pellets were collected from mice in order to monitor oocyst shedding throughout the experiment at an interval of 5 days. Following collection, the fecal pellets were mixed with distilled water and one bacterial loop of suspended material per fecal sample was smeared onto a glass slide. Monoclonal antibody 9D10, prepared in our laboratory and specific for oocyst stage of *C. parvum*, was used in an indirect immunofluorescent assay (IFA) technique [9]. Smears were examined microscopically in a blind fashion and scored 0 to 4+ based on oocyst numbers. Scoring was as follows; 0, no oocyst detected; 1+, less than 5 oocysts per smear; 2+, 5 to 50 oocysts per smear; 3+, 50 to 100 oocysts per smear; and 4+, more than 100 oocysts per smear.

Histologic examinations

Ten mice from each group were killed on the 10th day and the remaining 10 mice/group were killed on the 40th day. Tissue samples were collected postmortem from the terminal ileum. The tissue samples were fixed in 10% formal saline and embedded in paraffin wax. Sections 4-5 μm in thickness were cut and stained with hematoxylin and eosin. The tissue sections were then mounted and examined microscopically with a 400× objective on a compound microscope. The tissue samples of randomly selected 10 villi/mouse were examined and then the number of parasites/epithelial cells on each villus was counted. The infection was then quantitated by scoring on a scale of 0 to 3 as follows: 0 = no parasite observed; 1 = small numbers of parasites focally distributed in the tissue (less than 10% of the tissue colonized); 2 = moderate numbers of parasites widely distributed throughout the tissue (10 to 50% of the tissue colonized); 3 = large numbers of parasites widely distributed throughout the tissue (more than 50% of the tissue colonized).

Preparation of parasite homogenate

For antigen preparation, freshly collected oocysts were purified by sucrose-gradient centrifugation. The purified oocysts were further purified using a cesium chloride gradient [8]. These oocysts were sonicated 25 × 50 sec (50mW) on ice to produce the *C. parvum* homogenate (CPH). After homogenization, CPH was subjected to 3 times snap frozen in liquid nitrogen and thawed in a 37°C waterbath. CPH prepared in this fashion was assayed for total protein concentration (Bicinchoninic Acid Protein Assay; Pierce Scintific, USA) and the final protein concentration was adjusted to 40-60 μg/ml. CPH was stored at −20°C prior to use.

Serum antibody titers

The blood samples were collected from each mouse by cardiac puncture on the 10th and 40th days. The titer of anti-*C. parvum* antibody in the serum was monitored by using a modified enzyme-linked immunosorbent assay (ELISA) [6]. Briefly, ELISA plates were coated with the oocyst homogenate of *C. parvum* in 0.025 M phosphate buffered saline (pH 7.4) at a concentration of 1 μg protein in 100 μl per well overnight at 4°C. The blocked plates were washed with PBS and a 100 μl of a diluted mouse serum was added. We used the anti-mouse IgG developed in a rabbit, and conjugated with horseradish peroxidase for secondary antibody. Optical density (OD) was read at 450 nm using an ELISA plate reader (Microplate Autoreader; Bio-Tek, USA). Serum antibody titers between 10th and 40th day

were analyzed by Student's t-test in each group. The $p < 0.05$ was considered as statistically significant.

Results

One mouse in group 1, 2, and 3 died prior to the end of the experiment, undoubtedly due to the toxic effects of DEXp. Other mice in group 1, 2, and 3 exhibited poor health and hair loss. However, the mice in group 4, 5, and 6 all appeared healthy and active.

Fecal oocyst detection

The observed oocyst shedding patterns of all groups appear in Table 1. Oocyst shedding began initially after 5 days' postinoculation(PI) in group 1, 2, 4, and 5. The mice in group 3 were inoculated with heat-killed oocysts. Group 6 was served as normal control. All mice in group 1 and 2 were shedding oocysts throughout the duration the 40-day experiment. Oocyst shedding intensities between group 1 and 2 were very similar on the 5th to 25th days. Group 3 did not shed oocysts until the 25th day. The mice in group 2 and 3 were infected with *C. parvum* oocysts on the 25th day. The intensities of oocyst shedding were significantly greater in group 1 than in group 2, and 3 from the 30th through 40th day. Group 4 and 5 inconsistently shed lower numbers of oocysts until the 10th day.

Histologic examination of terminal ileum

Ten mice from all groups were euthanized by carbon dioxide inhalation on the 10th and 40th day after the infection. Parasite colonization in the terminal ilea of mice from all groups is provided in Table 2. *C. parvum* colonization of the terminal ilea of all groups showed similar pattern to that of the oocyst shedding. The largest number of cryptosporidia was found in group 1. Group 4 and 5 inconsistently shed lower numbers of oocysts until the 10th day, and from these groups developmental stages of *C. parvum* were observed at the 40th day. No parasites were detected in group 6.

Table 1. Patterns of *Cryptosporidium parvum* oocysts shedding intensity in the mice of all groups

Days	5	10	15	20	25	30	35	40
G1	4+	4+	3+	4+	3+	4+	4+	3+
G2	4+	3+	4+	4+	3+	2+	2+	1+
G3	0	0	0	0	0	1+	2+	1+
G4	1+	2+	0	0	0	0	0	0
G5	1+	1+	0	0	0	0	0	0
G6	0	0	0	0	0	0	0	0

*Scoring was as follows; 0, no oocyst detected; 1+, less than 5 oocysts per smear; 2+, 5 to 50 oocysts per smear; 3+, 50 to 100 oocysts per smear; and 4+, more than 100 oocysts per smear.

Table 2. Parasite colonization of the terminal ileum in the mice of all groups

Group	G1	G2	G3	G4	G5	G6
10th day	3	3	0	1	1	0
40th day	3	2	1	0	0	0

*The index of infection was determined as follows: 0: no parasite observed; 1: small numbers of parasites focally distributed in the tissue (less than 10% of the tissue colonized); 2: moderate numbers of parasites widely distributed throughout the tissue (10 to 50% of the tissue colonized); 3: large numbers of parasites widely distributed throughout the tissue (more than 50% of the tissue colonized).

Serum antibody titers

ELISA titers of all groups are graphically illustrated in Fig. 1. The blood of normal control (group 6) showed a very low titer and was considered to be negative (range: 0.16~0.22). The groups inoculated with *C. parvum* oocysts were revealed positive titers (range: 0.27~0.42). The mice inoculated with heat-killed oocysts (group 3) also had positive antibody titers. In addition, these antibody titers were similar to each other. The antibody titers of all groups were higher on the 40th day than on the 10th day except normal control.

Discussion

The apicomplexan parasite *Cryptopsporidium parvum* infects the intestinal tract in humans, calves and other agriculturally important animals and is a leading cause of diarrhea throughout the world [20]. However, despite decades of researches on hundreds of chemo- and immunotherapeutic agents either in vitro or in vivo in animal models and clinical trials, there is no specific therapeutic or preventive modality approved for cryptosporidiosis [22]. Passive immunization with bovine hyperimmune colostrums or monoclonal antibodies has been used for the treatment of cryptosporidiosis in both animals and humans [16].

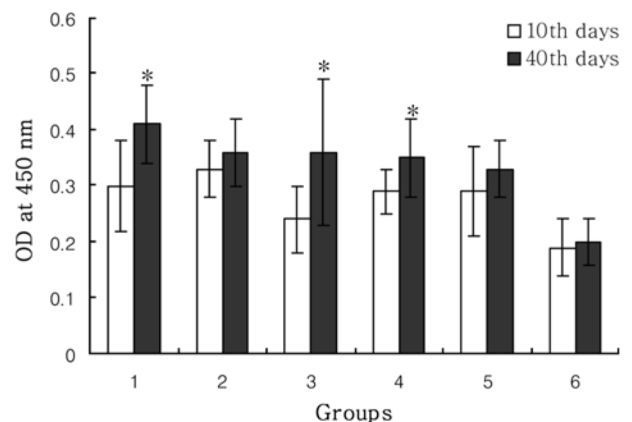

Fig. 1. Patterns of antibody response for sera from the mice of all groups as detected by ELISA. Data are presented the mean ± SD. *$p < 0.05$ compared with each 10th days, respectively.

The objective of the present study was to investigate the effect of challenge infection with *C. parvum* oocysts in immunosuppressed C57BL/6N mice. The oocysts from the groups of primary infection were detected on the 5th day, although the intensities of oocyst shedding were significantly greater in the immunosuppressed mice than in the nonimmunosuppressed one. The nonimmunosuppressed mice stopped shedding after 10 days' PI, whereas the group of immunosuppressed mice continued to shed oocysts through the experimental period. Especially, despite the reinfection of *C. parvum* oocysts in the negative control group, oocysts were not detected in the mice. The heat-killed oocyst inoculation group did not shed oocysts until the 25th day. These mice were shedding oocysts from the 30th to 40th day. In addition the amount of shedding oocysts in the immunosuppressed mice of primary and secondary infection group, and heat-killed oocyst inoculation group was very similar to each other after secondary infection. The mice of normal control did not shed oocysts. These results indicate that dead and live oocysts of *C. parvum* have protective effect against *C. parvum* infection. Although challenge infection has resulted in reduction of oocyst shedding, it did not truly eliminate the infection.

Through microscopic examination of tissue sections, it was revealed that *C. parvum* was localized predominantly in the small intestines. Specifically, the greatest levels of parasites were located in terminal ileum [19]. In the present study, parasite colonization of all groups except normal control was observed to have the same pattern of fecal oocyst shedding.

The diagnosis of cryptosporidiosis is commonly done by microscopic detection of oocysts in feces, but this method is relatively slow, subject to the expertise of the microscopist, and thus is often not cost effective. The serological study by ELISA had a high sensitivity and low specificity [13]. In the present study, the infection of *C. parvum* was investigated by using an ELISA technique. Antibody titers of the primary infection group were very similar each other. Indeed, the mice inoculated with heat-killed oocysts revealed positive titers. Especially, the titers of the heat-killed oocyst inoculation group were not different from other groups. Furthermore, the ELISA titers of these groups were higher on the 40th day than on the 10th day. These results indicate that the *C. parvum* and the whole *C. parvum* extracts have a moderate protection against the reinfection. Thus, these results demonstrate that the adult C57BL/6N mice infected with *C. parvum* is more resistant to a challenge infection following immunosuppression with DEXp as determined by decreased fecal oocyst shedding, reduced parasite colonization of the ilea and increased serum antibody titers.

Meanwhile, nonimmunosuppressed mice with primary infection have acquired identical levels of immunity to *C. parvum* throughout experimental period regardless of challenge infection, and it seems that mice with primary infection have acquired high immunity.

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