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Oocyst production and immunogenicity of *Cryptosporidium muris* (strain MCR) in mice

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Abstract: Three-week-old ICR SPF mice were orally inoculated with one of 5 doses ranging from 2×10^2 to 2×10^6 oocysts of *Cryptosporidium muris* (strain MCR) per mouse. Oocyst inoculation was directly proportional to the amount of oocysts shed and was inversely proportional to the period required for peak oocyst production and to the prepatent period. Peak oocyst production occurred between fifteen and thirty-one days with a patent period from 61 to 64 days. Three days after all mice stopped shedding oocysts, they were orally challenged with a single dose of 2×10^6 oocysts of the same species. Marked seroconversion for IgG antibody accompanied recovery from mice inoculated with 5×10^5 oocysts. Mice administered with carrageenan excreted a small number of oocysts for 49.0 days on the average after challenge inoculation (ACI) and control mice for 14.2 days in a dose-independent fashion. Just before challenge infection, phagocytic activity of peritoneal macrophages (Mø) and the number of peripheral Mø were dramatically decreased. Mild challenge infection implies that the immunogenicity of *C. muris* (strain MCR) is very strong, despite Mø blocker carrageenan administration.

Key words: *Cryptosporidium muris* (strain MCR), immunogenicity, macrophage blocker carrageenan, mouse, oocyst production, immunoglobulin G

INTRODUCTION

Recently, it is suggested that two species of *Cryptosporidium* infected mammalian hosts. In 1907, the type species of *Cryptosporidium*, *C. muris*, was reported by Tyzzer, who described the life cycle of this small coccidian monoxenous parasite infecting the gastric glands of laboratory mice (Tyzzer, 1907 & 1910). In 1912, the second species, *C. parvum*, was isolated from the small intestine of laboratory mice (Tyzzer, 1912). Oocysts of the former species, measuring 7×5 μm, are distinctly larger than those of the latter species, which measure about 4.5 μm and parasites are localized to the stomach of mice in the former while the latter is confined to the small intestine of mice.

To investigate the biology of *Cryptosporidium* with special reference to oocyst production, immunogenicity and resistance to subsequent oral challenge infection in various hosts for confirming the kinetics of infection owing to fact that none of the literature concerned consistantly gives a satisfactory outcome, our preceding studies have experimentally clarified those respects of *C. baileyi* previously isolated

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from Korean chicken (*Gallus gallus*) in mallard and chicken (Rhee *et al.*, 1995). The present communication also concerns oocyst discharge pattern, resistance to subsequent challenge infection and serum antibody response following infection of *C. muris* in mice because comprehensive knowledge of those respects of this parasite in mice is lacking. In addition, the phagocytic activity and the number of Mø were monitored to evaluate the strength of immunogenicity utilizing Mø blocker carrageenan.

MATERIALS AND METHODS

***Cryptosporidium* isolate and preparation of inoculum:** *Cryptosporidium muris* (strain MCR) oocysts used in this study were the large type originally isolated from the mouse, *Mus musculus*, and passed in 3-week-old SPF mice (Rhee *et al.*, 1991a & b). Oocysts were collected from the feces of infected SPF mice by the Sheather's sugar flotation method. Preparation and storage of inoculum and enumeration of oocysts for inoculation have been described previously (Rhee *et al.*, 1995). Oocysts used for experimental inoculations were less than 3 months old.

Patterns of oocyst discharge: Three-week-old ICR SPF mice (*Mus musculus*) were orally infected with a single dose of 2×10^2, 2×10^3, 2×10^4, 2×10^5 or 2×10^6 oocysts per individual: twenty mice were used for each dosage group with ten uninfected age-matched ICR SPF mice served as a control for the experiments. Each dosage group of mice was separately housed and fecal examination for oocysts and calculation of the number of oocysts discharged per day (OPD) for each mouse were carried out daily (Rhee *et al.*, 1995).

Challenge infection: Three days after oocyst shedding ceased, mice were intraperitoneally injected with carrageenan type II at 50 mg and 12.5 mg/100 g body weight. The remainder of the trial was previously described (Rhee *et al.*, 1995).

Six hours following the 2nd carrageenan injection, splenic T-cells and Mø in the peripheral blood were quantified and the phagocytic activity of peritoneal Mø were checked from 5 mice per allocation group.

Count of peripheral macrophages (Mø): Blood was collected via the retro-orbital plexus, and the total number of leucocytes/mm^3 was counted after staining with Turk solution. A differential cell count and enumeration of the absolute number of peripheral Mø/mm^3 were subjected to the methods previously described by Rhee *et al.* (1995).

Phagocytic activity of peritoneal Mø and number of T-cells in splenocytes: The phagocytic activity of peritoneal Mø was carried out according to the method of Metcalf *et al.* (1986), and splenic T-lymphocytes were identified by an avidin-biotin-peroxidase complex method (ABC technic: Hsu *et al.*, 1981) using a rabbit anti-mouse lymphocyte serum as a primary antibody (Sigma) and biotinylated goat anti-rabbit IgG as a secondary antibody (Vector Laboratories), as described previously (Rhee *et al.*, 1995).

Detection of serum antibody: One hundred mice were orally infected with 5×10^5 oocysts of *C. muris* (strain MCR), and serum samples were obtained from five mice by cardiac puncture at each sampling time after an interval of two weeks until 20 weeks. Using purified *C. muris* (strain MCR) oocysts dried onto microscope slides as a antigen and goat anti-mouse IgG FITC conjugate (Sigma) as a secondary antibody, an indirect fluorescent antibody (IFA) assay for detection of IgG antibody to *C. muris* (strain MCR) was performed essentially as suggested by Maekawa (1988) and Rhee (1988).

RESULTS

Patterns of oocyst discharge: Each group of mice experimentally infected with one of 5 doses of oocysts initiated oocyst discharge within 10 and 12 days postinoculation (PI). Oocyst discharge increased daily with maximum numbers of oocysts observed in fecal samples from each group on days 17-37 to 47-51 following the first oral inoculation. Soon after, oocyst discharge declined rapidly, and oocysts were passed in the feces until days 71 to 75 PI (Table 1). While, the oocyst did not show up in fecal samples from control mice

Table 1. Mean daily oocyst production[a] in mice inoculated with *Cryptosporidium muris* (strain MCR)

Days after inoculation	Dose of *Cryptosporidium muris* oocysts				
	2×10^2	2×10^3	2×10^4	2×10^5	2×10^6
9	—	—	—	—	—
10	—	—	+	+	+
11	—	+	+	+	0.2
12	+	0.2	0.2	0.2	0.2
13	+	0.2	0.3	0.3	0.4
14	0.1	0.1	0.2	0.5	4
15	0.1	0.2	0.3	0.5	7
16	0.4	0.3	2	2	25
17	0.3	5	10	43	223
18	0.3	3	11	99	229
19	1	2	52	113	408
20	2	5	53	68	929
21	2	5	35	175	652
23	10	19	84	208	584
25	26	40	213	281	436
27	26	48	186	340	1692
29	32	18	134	750	636
31	11	68	124	360	964
33	31	188	155	225	830
35	61	232	177	240	596
37	129	285	189	737	1064
39	224	305	208	560	971
41	143	372	228	341	564
43	212	381	271	208	401
45	265	333	126	119	256
47	244	243	228	111	118
49	280	36	137	57	48
51	140	48	57	40	10
53	97	31	12	12	5
55	27	37	47	2	1
57	54	25	34	5	2
59	30	2	25	7	+
61	25	0.5	3	+	+
63	1	+	1	+	+
65	0.5	+	+	+	+
67	+	+	+	+	+
69	+	+	+	+	+
71	+	+	+	+	+
72	+	—	+	—	+
73	+	—	+	—	+
74	+	—	—	—	—
75	+	—	—	—	—
76	—	—	—	—	—
77	—	—	—	—	—

[a]The number (1×10^4) of oocyst detected by Sheather's sugar floatation method per mouse. —, negative; +, detectable oocysts by Kinyoun's modified acid-fast staining method.

during this period.

Resistance to reinfection: Primary infection in mice terminated 71 to 75 days PI with no fecal oocysts observed. Control mice injected with saline discharged a few oocysts on days 15.5-17.5 and 29.0-31.0 ACI. A small number

Table 2. Mean daily oocyst production in mice after primary and challenge inoculations with *Cryptosporidium muris*

Doses of primary inoculation	Initial appearance (days after challenge)		Clearance of infection (days after challenge)		Patent period (days)	
	Exp.	Con.	Exp.	Con.	Exp.	Con.
2×10^2	16.5	17.0	65.5	29.0	49.5	13.0
2×10^3	16.5	17.5	65.5	29.0	49.5	12.0
2×10^4	18.0	15.5	65.5	29.5	48.5	14.5
2×10^5	16.0	16.0	66.5	30.5	51.5	15.5
2×10^6	16.0	16.0	61.0	31.0	46.0	16.0
Mean	16.6	16.4	64.8	29.8	49.0	14.2

*All mice were challenged with a single dose of 2×10^6 oocysts 6 hr after the second carrageenan (Exp.) or saline (Con.) administration.

Table 3. Effect of carrageenan on the numbers of Mø in peripheral blood and T-cells in splenocytes and on the phagocytic activity of peritoneal Mø in mice

Doses of oocyst	Treatment	No. of peripheral Mø/mm^3	Phagocytic activity of peritoneal Mø (%)	T-cells in splenocytes (%)
2×10^3	Saline	55.20 ± 16.84	75.6 ± 5.4	65.0 ± 6.1
	Carrageenan	20.60 ± 7.30[a)]	60.3 ± 4.9[a)]	54.4 ± 4.5
2×10^5	Saline	66.98 ± 19.90	70.2 ± 6.4	62.8 ± 3.0
	Carrageenan	31.40 ± 5.17[a)]	57.2 ± 10.1[a)]	57.6 ± 4.2

[a)] $P < 0.05$ (SAS t-test) compared with the values for saline-injected group. Each value represents the mean of five determinations with the standard deviations per group.

of oocysts were observed in fecal samples obtained from carrageenan treated mice on days 16.0-18.0 and 61.0-66.5 ACI, which were dose-independent (Table 2). Simultaneously, intrinsic control mice (previously uninfected, inoculated with 2×10^6 oocysts) excreted a large number of oocysts.

Number and activity of Mø and T-cells: As shown in Table 3, the number of Mø in the peripheral blood and the phagocytic activity of peritoneal Mø were significantly decreased in the experimental group ($P < 0.05$), and the proportion of T-lymphocytes in splenocytes was slightly decreased in the experimental group.

Serum IgG antibody to *Cryptosporidium muris*: Serum IgG antibodies against *C. muris* were detected in mice inoculated with 5×10^5 oocysts by IFA assay. The mean titers of IgG antibody at 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 weeks PI were 1:5, 1:26, 1:384, 1:640, 1: 896, 1:768, 1:1,024, 1:768, 1: 896 and 1:896, respectively. The disappearance in faecal oocyst shedding was correlated with the time IgG levels peaked (Weeks 10-20).

All experiments in the current study were conducted at least three times with similar results.

DISCUSSION

Since Tyzzer (1907)'s original description, *C. muris* was not studied until the mid-1980s. However, it has gained considerable attention since Upton and Current (1985) found *C. muris* oocysts measuring 7.4 × 5.6 μm in fecal samples obtained from calves in the United States. Although several investigators have described fragmentary knowledge of this parasite (Maekawa, 1988; Iseki *et al.*, 1989;

Rhee *et al.*, 1991a & b; Matsui *et al.*, 1994), a complete, well documented infection kinetics has not been fully described at this time.

In the present study, mice inoculated orally with various oocyst doses of the coccidium had a prepatent period of 9-11 days, with an oocyst plateau production occurring between fifteen and thirty-one days with a patent period from 61 to 64 days. Generally, these effects were in an dose-dependant. Of coccidian species, the patency of the parasite was the longest so far as we know and was longer than that observed in chickens and mallards infected with *C. baileyi* (Rhee *et al.*, 1995) but was shorter than in cats infected with *C. muris* (strain RN 66) originally isolated from the house rat (Iseki *et al.*, 1989). The infectivity of *C. muris* (strain RN 66) in mice, rats, guinea pigs, rabbits and dogs has been described (Iseki *et al.*,1989). Based on previous studies and this experimental result, we propose that the mouse is the most favourable laboratory host for the parasite except for the cat. A host of studies on the parasite will be accomplished using the mouse/*C. muris* system as an experimental model. Although Matsui *et al.* (1994) reported that oocyst shedding temporarily decreased on days 12 and 13 PI in all mice inoculated with doses varying of *C. muris* (strain RN 66), we did not prove such a temporary depression of oocyst elimination. The prepatent and patent periods in our study were similar to those observed by Matsui *et al.* (1994).

Previously, it was demonstrated that chicken and mallard quickly acquired a strong immunity to *C. baileyi* by challenge transmission experiment regardless of carrageenan injection (Rhee *et al.*, 1995). In this study, the patent periods of challenge infection (previously infected, recovered, challenged) of *C. muris* were several times longer than those of *C. baileyi* (Rhee *et al.*, 1995), and the patent period of challenge infection with carrageenan injection was comparable to that of primary infection. The longer duration of mild challenge infection in the carrageenan treated group compared to the normal group may be explained by abrogation of protective immunity against *C. muris* owing to a significant decrease in the number of Mø in the peripheral blood and the phagocytic activity of peritoneal Mø, as described previously in regard to contribution of Mø to protective immunity (Rhee *et al.*, 1995). Therefore, mild challenge infection in mice implies that the intensity of immunogenicity of *C. muris* is strong regardless of Mø blocker carrageenan administration.

The present study reveals that mice inoculated with 5×10^5 oocysts were seropositive by the IFA assay 2 weeks following inoculation and increasingly, titers of IgG antibodies were visualized. High antibody levels (1:896-1:1,024) appeared on weeks 10 to 20 PI, when no oocysts were found in the feces by even Kinyoun's modified acid-fast staining method. Marked titer elevation was accompanied by the disappearance of fecal oocysts. Also, mice infected with *C. muris* by oral inoculation developed serum antibody responses that could be measured by the IFA assay. In the light of chronological pattern of the titer, it is suggested that IgG antibody may be present for an extended period. Also, Ungar *et al.* (1989) demonstrated that IgG antibodies to *Cryptosporidium* in man may be present for at least 1 or 2 years after infection, and an IgG response was detectable for at least a year in sera from five subjects with an acquired immunodeficiency syndrome (Campbell and Current, 1983).

In conclusion, regardless of the actual mechanism of acquired immunity, our study demonstrates that marked seroconversion usually results from a single oral exposure to oocysts, and clearance of heavy primary infection of *C. muris* and resistance to subsequent oral challenge by the parasite were accompanied by the appearance of serum antibodies detectable by the IFA assay using *C. muris* oocysts as the antigen.

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=초록=

마우스에 있어서 쥐와포자충(MCR주)의 오오시스트 배설상황과 면역원성

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Cryptosporidium muris(strain MCR)를 3주령의 각 마우스에 2×10^6개의 오오시스트와 그 10배 단계 희석계열을 2×10^2까지 5단계를 만들어 경구접종하여 오오시스트 배설상황을 조사하고 나서 초감염내과 마우스에 처음에 50 mg/100 g. 그 24시간 후에 12.5 mg/100 g의 carrageenan을 복강내 투여하고 그 6시간 후에 다시 2×10^6개의 오오시스트를 2차접종하여 면역원성을 조사하였다. 일반적으로, 오오시스트 투여량이 많을수록 오오시스트 배설량이 많았으나 prepatent period(9-11일간)와 오오시스트 생산이 피이크에 이르는 기간(16-36일간)은 짧았다. Patent period는 61-64일간이었으며, 15-31일간에 걸쳐 오오시스트가 많이 생산되었다. 한편, IgG 항체가의 현저한 상승과 분변내 오오시스트 소멸은 일치하였다. Carrageenan 투여군은 오오시스트 재차접종 후 16.0-18.0(16.6)일부터 61-66.5(64.8)일까지 46.0-51.5(49.0)일간, 대조군은 15.5-17.5(16.4)일부터 29.0-31.0(29.8)일까지 12.0-16.0(14.2)일간에 걸쳐 소수의 오오시스트가 검출되었다. 재차접종 직전의 말초혈액내 대식세포수 및 복강내 대식세포 활성은 대조군에 비하여 carrageenan 투여군이 현저하게 낮았다($P < 0.05$). 이는 Mø blocker인 carrageenan을 투여하였음에도 불구하고 이 원충의 2차감염이 미약하였다는 것은 이 원충의 면역원성이 매우 강력하다는 것을 뜻한다.

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