Passive transfer of immunity against *Cryptosporidium* infection in neonatal mice using monoclonal antibodies

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Abstract: Monoclonal antibodies (mAb) against merozoites and sporozoites of the protozoan parasite *Cryptosporidium parvum* were examined for potential modulation of cryptosporidial infections *in vivo* by daily oral mAb administration to oocyst-inoculated neonatal mice. Monoclonal-treated neonatal mice were sacrificed four and eight days post infection (pi). Differences in infection rates were observed among the treatment groups at the $p < 0.05$ level. Suckling mice treated daily with orally administered mixtures of mAbs (ascitic fluids) showed significantly reduced parasite loads compared to control mice at four and eight days pi, while suckling mice receiving mAb Cmg-3 alone showed significant differences only at 4 days pi., suggesting that passive transfer of mAb may be of value in controlling cryptosporidial infections.
Key words: *Cryptosporidium*, monoclonal antibody, passive transfer, BALB/c neonatal mice.

INTRODUCTION

Cryptosporidium parvum is a protozoan parasite that infects the intestinal epithelium of a variety of mammals. Infection is often accompanied by symptoms of gastroenteritis and diarrhea (Current, 1985). In immunocompetent hosts the disease is self-limiting, and resolution is accompanied by antibody production to various life-cycle stages (Casemore, 1987; Petersen, 1993). Immunodeficient humans (those with acquired immune deficiency symdrome and hypogammaglobulinemia) and mice (athymic) may exhibit persistent infection (Petersen C, 1992). Humoral and cell-mediated immune responses are apparently necessary for recovery from cryptosporidiosis.

Treatment of cryptosporidiosis, especially in immunodeficient persons, has been unsuc-cessful in most cases. Despite the testing of more than ninety chemotherapeutic agents, no regimen provides sustained symptomatic relief or parasitologic cure against cryptosporidiosis. The development of an effective treatment has been limited by the lack of a reliable small-animal model of clinical disease for screening the efficacy of drug compounds (Fayer and ungar, 1986; Sterling and Arrowood, 1993). The present study was initiated to determine whether orally administered anti-*C. parvum* monoclonal antibodies could modulate infections in neonatal mice.

MATERIALS AND METHODS

1. Monoclonal antibodies used for treatment

Experimental neonatal mice groups were treated with two different ascitic fluids containing anti-*Cryptosporidium* monoclonal antibodies, which were developed from Dr. Charles R. Sterlings laboratory at the University of Arizona, U.S.A., for mouse

passive protection tests; i) ascites containing mAb (Cmg-3), an IgG3, which was directed against a 3.5-kilodalton (kDa) merozoite antigen, and ii) a mixture of ascites of Cmg-3 and IgG1 (C6B6) and IgM (C4A1) monoclonal antibodies. C6B6 and C4A1 were derived from mice immunized with purified sporozoites and directed against 20-kDa and different molecular weight above 25-kDa sporozoite antigen, respectively, while Cmg-3 was generated from mice immunized with partially purified merozoites as antigens. Ascitic titers were determined using an indirect immuno-fluorescent assay on merozoite and sporozoite-coated microscopic slides.

2. Experimental BALB/c neonatal mouse groups

Untimed pregnant BALB/c mice were purchased from Harlar Sprague Dawley (Indianapolis, Ind., USA). Within two days of birth, mouse pups were randomized and placed back with the original mothers (eight pups/litter) to minimize maternal effects on experimental outcome. All neonatal mice were infected at 4 days of age and sacrified 4 or 8 days post infection. Infected neonatal mice were kept with parents until sacrificed. Sacrifice was performed by cervical dislocation. Six experimental groups were established; control (CL), treatment 1 (Kor), and treatment 2 (Ea) for 4 or 8 day treatment. Each group was consisted of a minimum of 7 litters and each litter had four to ten pups. Animals were maintained in isolator cages throughout experiment.

3. Oocyst production and purification

Cryptosporidium parvum oocysts originally isolated from Holstein calves were used to infect 2-5-day-old Holstein calves (10^8/animal). Following the onset of oocyst shedding, feces were collected daily, mixed with an equal volume of 5% potassium dichromate ($K_2Cr_2O_7$), and stored at 4°C. Feces were sieved sequentially through stainless steel screens with a final mesh size of 230 (63 μm porosity).

Oocysts were purified from feces using discontinuous sucrose and isopycnic Percoll (Pharmacia, Piscataway, New Jersey) gradient centrifugation techniques. In brief, sieved feces were centrifuged over 2 sequential discontinuous sucrose gradients prepared using 0. 025 M phosphate-buffered saline (PBS, pH 7.2) and 1% Tween 80. Each gradient tube was composed of 2 10-ml lower sucrose layers (1. 064 g/ml and 1.103 g/ml) and a 5-ml upper layer of sieved feces. The tubes were centrifuged at 1,500 g for 30 min and oocysts were recovered from the interface of the sucrose layers and washed with PBS at 1,500 g (10 min × 3). A 1-ml aliquot of sucrose gradient-recovered oocysts in PBS was layered over 9 ml of Percoll in a high-speed centrifuge tube and centrifuged at 22,000 g for 30 min at room temperature. Oocysts were recovered from the centrally located band in the Percoll gradient.

4. Experimental protocol

Experimental groups received three different treatments: i) an ascitic fluid containing IgG3 mAb Cmg-3 (Kor), ii) mixtures of ascitic fluids containing mAbs of C6B6, C4A1, and Cmg-3 (Ea), iii) and saline (CL). Mice treated with Cmg-3 received 10 μl ascitic fluid and mice treated with the ascitic fluid mixture received a total volume of 30 μl per dose (10 μl of each mAb) based on the dosage reported for neonatal mice (Letchworth and Appleton, 1983) and for calves (Sherman *et al.*, 1983). Controls received 10 μl saline.

Neonatal BALB/c mice were infected orally with an inoculum of approximately 3-5 μl containing 10^4 Percoll-cleaned *C. parvum* oocysts at 4 days of age. Infected BALB/c mice were maintained in an isolation room separate from control mice. They received the primary antibody dose or saline one hour prior to oocyst infection and subsequent doses were administered for 4 or 8 days until sacrificed. Pups were fed using an micropipette with pipette tip and were allowed to swallow the inoculum. Parasite infection was assayed using terminal ileum. Terminal ileum was fixed in 10% buffered formalin, embedded in paraffin, microtome sectioned longitudinally and stained with hematoxylin and eosin. These longitudinal sections were examined for parasite life cycle stages along villus surfaces.

The quantitation of parasite density along villus surface was performed by counting the

number of parasitic forms per high power (400 ×) field in the tissue sections for each mouse. Three randomly chosen mice from each litter were examined for the presence of parasites. Two randomly chosen high power fields were selected for each tissue segment and total numbers of parasites were counted for each.

5. Data analysis

A model II nested analysis of variance (Sokal and Rohlf, 1981) accommodating unequal sample size was applied to square root transformed numeric data collected on parasite numbers along villus surface in an effort to determine the significance of treatment of parasite infection. The null hypothesis of equal treatment means was tested by the F-value measuring whether or not there is a systematic difference between the treatment means for valence in the analysis of variance table.

Multiple comparisons among the treatment group means (Tukey's studentized range test) were performed to study the magnitudes of the differences shown in ANOVA table. The sample means were taken as estimates of the corresponding treatment means and the 95% confidence intervals were put around the sample means and the evidence in these intervals were examined to study these differences. Analysis of variance was computed using statistical analysis system program (SAS) accessed through RVAX.

RESULTS

1. Treatment conditions

The characteristics of experimental groups and anti-C. parvum monoclonal antiboies used in passive transfer treatments with BALB/c neonatal mice are presented in Table 1.

2. Infection rates among mAb treatment groups

Table 2 presents cryptosporidial infection rates for all experimental groups. Rates varied from a low of 86.7% in group CL to 100% in group Ea. The mean infection rate for all treatment groups was 94.6%. Infections were confirmed by the presence of parasites in histologic sections of the terminal ileum.

3. ANOVA results for treatment group data

The ANOVA results for experimental groups are presented in Table 3. The between- and within- subjects nature of the design dictates a repeated measure analysis of variance. The ANOVA model tested includes treatments (forty levels), days (two levels), mice (three levels), litters (forty seven levels), and counts (two levels).

The null hypothesis of equal treatment efficacy is tested by the F-value, measuring whether or not there is a systematic difference between the treatment means, for valence in the analysis of variance table in table 3, where $F = 32.73$. From a table of the F distribution for 2 and 92 degrees of freedom, a value of F larger than 3.07 with a 5% significance level and 4.79 with a 1% significance level is required in order to reject the null hypothesis. Because the observed F value is larger than either of these cutoffs, the null hypothesis of equal treatment means is rejected. If the treatment means are equal, the probability is 0.001 that F is larger than or equal to the observed value. This probability is small, which

Table 1. Experimental groups and anti-cryptosporidial monoclonal antibody used in passive transfer prophylaxis of BALB/c neonatal mice

Group	Ascites treatment	Subclass	IFA Titer	Treatment volume (ml)	Treatment period (days)
Kor	Cmg-3	IgG3	1024	10	4 and 8
Ea	C6B6	IgG1	1024	30	4 and 8
	C4A1	IgM	512	(10 ml each)	
	Cmg-3	IgG3	1024		
CL	saline			10	4 and 8

Kor: treatment 1, Ea: treatment 2, CL: control

Table 2. Cryptosporidial infection rates among mice in monoclonal antibody passive transfer prophylaxis groups

Group	Days	No. of litters	Total No. of mice	No of mice infected	Infection rates (%)
Kor	4	8	38	36	94.7
	8	7	31	30	96.8
Ea	4	10	39	37	94.9
	8	9	33	33	100.0
CL	4	7	35	33	94.3
	8	6	30	26	86.7
Totals	47		306	195	94.6

Kor: treatment 1, Ea: treatment 2, CL: control

Table 3. Repeated measures analysis of variance for between and within subjects effects in anti-cryptosporidial mAb passive transfer prophylaxis groups

Source	DF	ANOVA SS	Mean square	F	P
Between subjects					
Treatment	2	750.660	375.330	32.73	0.0001
Day	1	824.999	824.999	71.95	0.0001
Mice	2	36.339	18.169	1.58	0.2106
Error	92	1054.959	12.467		
Within subjects					
Count	1	3.620	3.620	1.68	0.1982
Count treatment	2	4.308	2.154	1.00	0.3721
Count day	1	0.152	0.162	0.07	0.7849
Count mice	2	2.180	1.090	0.551	0.6048
Error	92	198.281	2.155		

DF: Degree of Freedom, SS: Sum of Squares

is strong evidence to reject the null hypothesis.

Difference between days was significant (P < 0.05), indicating that significant differences exist in the number of parasites between treatment days. There was no difference between counts of two randomly chosen fields at the 0.05 level, i.e. the variance to which the counts made on each tissue/slide differ from one another and thus contribute to the overall variance was negligible. No significant difference existed between three randomly chosen mice from each litter at p < 0.05, suggesting that the selection of more than three mice from each litter would not be required to reduce the variance components contributed by the mice.

4. Multiple comparisons among means using the T-method

The sample means were taken as estimates of the corresponding prophylactic means to obtain an idea of the magnitudes of differences shown in the ANOVA table. The 95% confidence intervals were put around the sample means and the evidence in these intervals were examined to study these differences. The groups, group means and their coresponding 95% confidence intervals are presented in Table 4. Treatment groups Kor and Ea differed significantly from the control group CL at 4 days pi, that is, the 95% confidence intervals for groups Kor and Ea did not overlap those of group CL, but only group Ea showed significant difference from group CL

Table 4. Multiple comparisons—differences between monoclonal antibody passive transfer prophylaxis group means

Mean[a]	Day 4		
	6.745	2.870	3.150
Treatment	CL	Kor	Ea
Kor	3.875[b]		
Ea	3.595[b]	0.280	

Table values are the differences between treatment means.
Confidence = 0.95, DF = 48, MSE = 4.757
Critical value of Tukey's studentized range (HSD) = 3.420

Mean[a]	Day 8		
	9.845	7.731	5.627
Treatment	CL	Kor	Ea
Kor	2.114		
Ea	4.218[b]	2.104	

Confidence = 0.95, DF = 42, MSE = 8.499
Critical value of Tukey's studentized range (HSD) = 3.436
Kor: treatment 1, Ea: treatment 2, CL: control.
[a] = mean of square root transformed data
[b] = significant at $p < 0.05$

Fig. 1. Hematoxylin and eosin stained ileal sections from mice infected at 4 days of age and treated with saline (A) and monoclonal antibodies (B) until sacrificed 4 days later (8 days of age). Parasitic life cycle stages are present on the surface of the villous epithelium. A: × 100, B: × 400

at 8 days pi. Monoclonal antibodies used in the study have been shown to be effective in controlling *Cryptosporidium* infection in neonatal mice (Table 4 and Fig. 1).

DISCUSSION

Treatment of cryptosporidiosis, especially in immunodeficient persons, has been unsuccessful in most cases. Despite the testing of more than ninety chemotherapeutic agents, no regimen provides sustained symptomatic relief or parasitologic cure against cryptosporidiosis (Collier *et al.*, 1984; Petersen, 1992; sterling and Arrowood, 1993). Of the numerous antimicrobial and antiparasitic drugs administered to immunodeficient parsons with intestinal cryptosporidiosis, the macrolide antibiotic spiramycin is the only one reported to date to have some efficacy (Collier *et al.*, 1984). Most patients receiving this treatment, however,
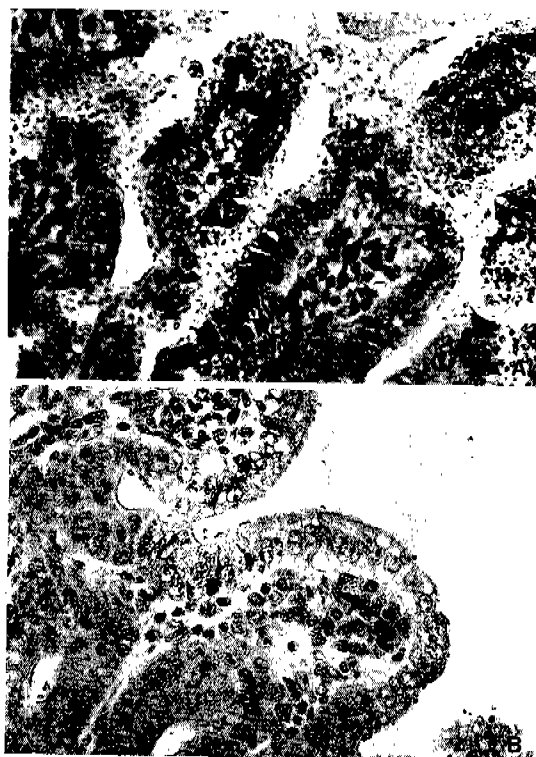
continued to shed *Cryptosporidium* oocysts in their feces (Collier *et al.*, 1984).

At present, supportive care with oral or intravenous hydration is the primary therapeutic intervention available for humans with cryptosporidiosis (Sterling and Arrowood, 1983). The disease is self-limiting in immunocompetent individuals. For the majority of immunocompromised patients, such as those with AIDS whose illness is life-threatening, no agent of antidiarrheal compound offers clear benefit (Petersen, 1992). There are currently no vaccines available to prevent cryptosporidiosis in humans.

The major factor that appears to determine the severity and duration of a *Cryptosporidium* infection in humans is the immune status of the host (Ungar *et al.*, 1985). Discontinuing immunosuppressive chemotherapy, which

results in a restoration of immune function, has eliminated cryptosporidiosis from the intestinal tract of several patients (Current, 1986). suckling mice treated daily with orally administered mixtures of anti-sporozoite monoclonal antibodies showed significantly reduced parasite loads compared with control mice at four days postinfection, even in infections were not completely interrupted (Arrowood et al., 1989). A study of crypto-sporidiosis in Costa Rican children demon-strated a lower prevalence in breast-fed infants less than 1 year of age compared to children of the same age who were fed artificial diets (Mata et al., 1984). These findings suggest that immunomodulation or passive transfer of antibodies may be of value in controlling cryptosporidial infections.

The partial reduction in the numbers of sporozoites of Eimeria found within the epithelium of immune animals was due to the inhibitory effects of antibodies on invasion, as demonstrated in vitro (Rose et al., 1975; Davis et al., 1978; Guy-Grand nd Vassalli, 1982; Lawn and Rose, 1982; Whitmire et al, 1988). Sherman et al. (1983) succeeded in protecting calves from serious enterotoxigenic Escherichia coli infections through the prophylactic oral administration of murine anti-E. coli mAbs in ascitic fluid.

Control of enteric cryptosporidiosis by the host immune system is indicated by the following observations. (i) The disease is self-limiting in immunocompetent hosts and stimulates the production of antibodies to C. parvum (Campbell and Current, 1983), but is persistent in immunodeficient hosts and in athymic (nude) mice (Fayer and Ungar, 1986; Petersen, 1992); (ii) recovered immuno-competent calves and humans are resistent to reinfection (Current, 1985). For these reasons, investigations on immunologic approaches to control this disease were initiated. Monoclonal antibodies to sporozoites and merozoites of C. parvum were applied to assess a role in controlling cryptosporidial infections in neonatal mice.

The passive transfer of immunity using monoclonal antibodies to sporozoites and merozoites of Cryptosporidium parvum monoclonal antibodies has shown to limit the number of parasites which complete the life cycle of Cryptosporidium. Monoclonal antibodies transferred passively into the gut lumen appear to be ideally suited for interaction with the extracellular invasive forms (sporozoites, merozoites, and micro-gametes) of Cryptosporidium for several reasons. First, antibodies may act directly on extracellular stages to damage them, or by interacting with components of the complement system. Secondly, antibodies may neutralize sporozoites and merozoites directly by blocking their attachments to new host cells. Finally, antibodies may enhance phagocytosis of extracellular forms mediated by Fc receptors on macrophages.

It has been also suggested that the hindering of penetration may prolong the exposure of the parasites to lytic factors normally present in the intestinal contents which can damage surface antigens and thereby inhibit the growth of those that do succeed in entering cells (Davis and Porter, 1979). This may explain why in immunocompetent hosts cryptosporidial infection is transient (Current et al., 1983; Petersen, 1992). Treatment or prophylaxis of other diarrheal diseases may be afforded by the oral administration of specific immunologic agents. Further studies are required, however, to answer the question of longer term treatment impact on crypto-sporidial infections, especially regarding parasite load and severity of clinical symptoms.

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=국문초록=

단클론항체를 이용한 생쥐에서의 크립토스포리디움 감염의 수동면역

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조명환

*Cryptosporidium parvum*의 sporozoite에 대한 단클론항체(C6B6, C4A1)와 merozoite에 대한 단클론항체(Cmg-3)를 크립토스포리디움 오오시스트로 감염시킨, 생후 4일된 생쥐에 4일 혹은 8일간 경구 투여하여 크립토스포리디움 감염에 미치는 효과를 조사하였다. 투여는 Cmg-3만으로, 혹은 C6B6, C4A1, Cmg-3를 함께 섞은 것으로, 그리고 식염수로 실행하였다. $p < 0.05$ 수준에서 항체투여를 받은 것과 받지 않은 그룹 사이에 현격한 차이가 관찰되었다. Cmg-3 한개만으로 투여하였을 때는 4일간 투여하였을 때만 효과가 있었던 반면, 3개의 단클론항체를 함께 투여하였을 때는 4일간, 8일간 모두 기생충의 증식을 현격히 감소시켰다. 이것은 경구투여된 항체가 장내에 기생하는 크립토스포리디움의 증식을 억제한 것을 보여주는 것이며, 크립토스포리디움증에 대한 치료약이 없는 상황에서 치료법 개발에 새로운 접근 방법을 제시한다고 판단된다.

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