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Thymic Dependence of Immunity to *Eimeria falciformis* var. *pragensis* in Mice

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Athymic (nude) mice, their normal littermates, and Swiss white mice were infected with 750 oocysts of *Eimeria falciformis* var. *pragensis* and reinfected twice with 20,000 oocysts, 20 and 40 days after the primary infection. The prepatent and patent periods of the primary infection were similar in each group of mice; however, the athymic mice discharged more oocysts. The normal littermates and Swiss white mice developed immunity to the parasite after the first or second infection, whereas the athymic mice never developed immunity. Infection with *E. falciformis* var. *pragensis* induced the production of antibodies in the normal littermates and Swiss white mice, whereas antibody production was impaired in athymic mice. Passive administration of immune serum did not protect athymic mice from reinfection by the parasite. Immunity to the parasite was thymus dependent, and "effector" T-lymphocytes seemed to be required for protection.

Acquired resistance to coccidial infection is well recognized (6). Whether functional immunity to eimerian parasites is primarily humoral or cell mediated remains unresolved. Passively administered immune serum has been reported to provide minimal (24, 25; W. Wittchow, Dr. Med. Vet. thesis, Freie Universität Berlin, Berlin, Federal Republic of Germany, 1972) or no (1, 4, 23) protection against coccidiosis. Although neonatal thymectomy had no influence on the acquisition of immunity (12, 22, 27), cell-mediated immunity to coccidia has been demonstrated with transferred immune lymphocytes (8, 9, 11; Wittchow, Dr. Med. Vet. thesis), transfer factor (13), the macrophage migration inhibition test (20), delayed hypersensitivity (8, 10, 26), and in vitro lymphocyte blastogenesis (10). The immune response to *Eimeria falciformis* var. *pragensis* does not interfere with penetration of host cells by the parasite, but it does act against parasitic stages as they develop within intestinal epithelial cells (18). In general, cell-mediated immune mechanisms are more important in the immune response to intracellular parasites.

In this study, congenitally athymic (nude) mice, which are known to be incapable of mounting a cell-mediated immune response (16, 30), were used to determine the influence of the thymus on the life cycle of *E. falciformis* var. *pragensis* and the thymic dependence of immunity to the coccidium.

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MATERIALS AND METHODS

Experimental animals. Congenitally athymic mice (of BALB/c background), hereafter designated as nude (*nu/nu*), their phenotypically normal (*nu/+*), thymus-bearing littermates (NLM), obtained from the Laboratory Animal Division of the Mogul Corp., Madison, Wis., and Swiss white (SW) mice, obtained from the Animal Resources Center, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, were used in this study. The NLM and SW mice were kept in the same room maintained at 26 to 27°C. Mice, in groups of three to five, were kept in metal cages that were changed every other day during the time of oocyst production and every 3 to 4 days when oocyst discharge had ceased. Individual mice used to study oocyst production were kept in similar cages fitted with wire net floors. The cages and the feed and water supplied to the nude mice were autoclaved for 20 min at 120°C. Sterile disposable masks and gloves were used when handling the nude mice.

Oocysts. *E. falciformis* var. *pragensis* (2) was maintained by frequent passage through coccidium-free SW mice, sporulated in 2.5% potassium dichromate, and stored at 4°C for not more than 8 weeks before it was used.

Infection. Nude, NLM, and SW mice were infected orally with 750 oocysts. Twenty days after the primary infection, recovered and noninfected nude mice were infected with 20,000 oocysts. The third challenge infection, of 20,000 oocysts, was given 20 days after the second infection.

Oocyst production. Daily oocyst production was determined by the method of Long and Rowell (15). The sugar concentration method was used when there were too few oocysts to count with a McMaster chamber. The cages and the wire mesh were washed with water to recover all of the oocysts.

Sera. Immune sera obtained from SW, NLM, and nude mice 16 to 20 days after the second immunizing dose of 20,000 oocysts of *E. falciformis* var. *pragensis* and nonimmune serum obtained from uninfected SW mice were kept at −20°C until used. At −12, 0, 24, and 48 h after the third immunizing infection, 0.5 ml of immune or nonimmune sera, obtained from SW mice, was given intraperitoneally to each of four nude mice. Six control nude mice received 0.5 ml of physiological saline over the same period of time.

Indirect fluorescent antibody reaction. The indirect fluorescent antibody reaction (29) was used to determine the humoral immune response to the coccidium. Sera were serially diluted with saline to 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. Third-generation merozoites of *E. falciformis* var. *pragensis* were used as antigens.

Histopathological methods. On days 2 to 7, two nude mice were infected with 20,000 oocysts, and on day 16 after the third immunizing infection, all surviving mice were killed by atlantooccipital dislocation. The intestines, mesenteric lymph nodes, spleen, liver, kidney, heart, lungs, anterior mediastinal tissue, and tissue at the thoracic inlet from all mice were fixed in Bouin's fluid, postfixed in 70% alcohol, processed routinely, and stained with hematoxylin–eosin.

Statistical methods. The *t* test was used to compare the total numbers of oocysts produced by the different groups of mice (28).

RESULTS

Life cycle, oocyst discharge, and pathological changes in nude mice infected with *E. falciformis* var. *pragensis*. The life cycle of *E. falciformis* var. *pragensis* in nude mice was identical to that in SW mice (17). First-, second-, third-, and fourth-generation schizonts and gamonts matured at 2 to 3, 3 to 4, 5, 6, and 7 days postinfection, respectively. Parasitic development was restricted to the cecum and colon except in one mouse where a few gamonts were seen in the crypt epithelium of the lower ileum.

The clinical signs of the primary infection, which included anorexia, depression, diarrhea, and dehydration, as well as colonic mucosal necrosis and inflammation, were less marked in nude mice than in SW mice (18a). There was a higher concentration of parasites in the colonic sections of nude mice killed on days 6 and 7 than in those of SW mice. Thymic tissue was not found in any of the nude mice. Oocyst discharge began on day 7, peaked on days 8 and 9, and declined rapidly thereafter in all of the infected mice (Table 1). A similar pattern of oocyst discharge occurred in each group of mice, but total oocyst production was higher in the nude mice than in the NLM and SW mice.

The NLM and SW mice exposed to the second infection showed no clinically detectable indication of the infection. In contrast, the nude mice infected for either the first or the second time had diarrhea and dysentery, and 4 of the 22 mice died during the second infection. The oocyst production of mice reinfected with 20,000 oocysts is shown in Table 2. The total number and pattern of oocyst discharge in the nude mice were the same as those in the primary infection, whereas in the NLM and SW mice oocyst production was markedly reduced. The pathological findings in all nude mice that died during the first and second infections were similar. The

TABLE 1. *Daily oocyst discharges by nude and normal mice infected with 750 oocysts of E. falciformis var. pragensis*

Days post-infection	Oocyst discharge (×10⁴)/day[a]		
	Nude mice[b] (*nu/nu*)	NLM (*nu/+*)	SW
1–6	0	0	0
7	55 ± 35	23 ± 20	73 ± 43
8	1,150 ± 380	660 ± 140	790 ± 170
9	1,030 ± 150	630 ± 200	510 ± 170
10	360 ± 80	120 ± 60	230 ± 90
11	110 ± 50	42 ± 24	83 ± 21
12	7 ± 4	6 ± 4	5 ± 3
13	2 ± 1	0.7 ± 0.6	0.9 ± 0.7
14	0.4 ± 0.2	0.1 ± 0.1	0.2 ± 0.2

[a] Values represent the mean ± standard deviation of six mice except at day 14, where values are of three to five mice.

[b] Total oocyst discharge by nude mice was significantly higher ($P < 0.05$) than that by either of the other two groups.

TABLE 2. *Total oocyst discharges by nude and normal mice repeatedly infected with E. falciformis var. pragensis*

Infection no.	Infective dose (oocysts)	Oocyst discharge (×10⁵)[a]		
		Nude (*nu/nu*)	NLM (*nu/+*)	SW
1	750	272 ± 46[b]	148 ± 35	170 ± 45
	20,000	196 ± 47[c]	ND[d]	ND
2	20,000	219 ± 29[c]	6 ± 6[e]	0.4[f]
3	20,000	232[f]	0.5	0.0

[a] Oocyst discharge was measured for 15 days postinfection. Values represent the mean ± standard deviation of six mice, except where noted otherwise.

[b] Significantly higher ($P < 0.05$) than in the corresponding groups.

[c] Four mice.

[d] ND, Not determined.

[e] Five mice.

[f] Two mice.

prominent histological findings were submucosal edema and intestinal epithelial necrosis. The colonic crypts and lumen were filled with large numbers of shrunken oocysts, but the inflammatory cellular response was not remarkable. Numerous giant cells were found in association with retained oocysts within the colonic mucosa of mice that died after the second infection. Pathogenic bacteria were not isolated from any of the dead mice. Three of the nude mice kept within one cage failed to gain weight despite their apparent recovery from coccidiosis. The mice became progressively dehydrated, and two of them eventually became dyspneic at 16 to 20 days after the second infection. Histological examination of the lungs revealed pneumonitis. Neither bacteria nor viruses could be isolated from the lungs.

The clinical signs and mortality of nude mice infected for the third time were more marked than those during the first or second infection. Some of the mice passed frank blood during the acute stage of the disease. None of the NLM and SW mice showed any indication of the infection.

The clinical signs of the disease as well as the mortality were as severe in the nude mice that received immune serum as in those that received nonimmune serum or saline. Eleven of the 14 infected nude mice died of coccidiosis on days 9 through 11, including all of those that received immune serum intraperitoneally. Only 1 of the nonimmune serum recipients and 2 of the control mice that received saline survived the infection after severe dysentery and dehydration. These mice passed as many oocysts in the third infection as they had passed in the second infection (Table 2). The oocyst production for the first 7 to 10 days postinfection in the mice that eventually died was similar to that discharged by the survivors and to the oocyst production of the corresponding periods of the first and second infections. None of the SW mice challenged for the third time passed oocysts. At 8 through 10 days postinfection, a few oocysts were seen in the feces of two of the NLM mice.

The pathological changes and bacteriological results during the third infection were similar to those found during the second infection, but more neutrophils were seen in the colonic lumen and in association with retained oocysts. Fewer microgranulomas and "microabscesses" were seen in the colonic sections of the NLM and SW mice.

Humoral immune response to the coccidium. Merozoites treated with immune sera obtained from NLM and SW mice fluoresced intensely, whereas merozoites treated with sera obtained from infected nude mice and uninfected SW mice fluoresced weakly and unevenly (Fig. 1).

DISCUSSION

The life cycle of *E. falciformis* var. *pragensis* in nude mice was similar to that observed in nonimmune SW mice (17), suggesting that the immune response of the host has little influence on either the number or the timing of the development of the different endogenous stages of the parasite during the primary infection. Nude mice are deficient in cell-mediated immunity (16, 30) and were not shown to produce antibodies against the coccidium. Enhanced oocyst production in animals treated with dexamethasone is accompanied by prolonged patency of the infection (14, 21), presumably due to the development of an additional asexual generation(s) (14). The present study does not support this idea. The increased oocyst production during the first infection in the nude mice seemed to be related to the less extensive inflammation and less nonspecific mucosal injury in these mice compared with those seen in SW mice (18a).

Of the nude mice, 33 and 78% died after the second and third infections, respectively. The apparent increase in the pathogenicity of the parasite during the third infection could not be explained. Although it may have been partly due to debilitation of the nude mice, none of the age-matched uninfected nude mice died during this period. Latent infection, frequently seen in nude mice kept under "conventional" husbandry (3), was not established through routine pathological and bacteriological investigations. The three nude mice that had respiratory problems

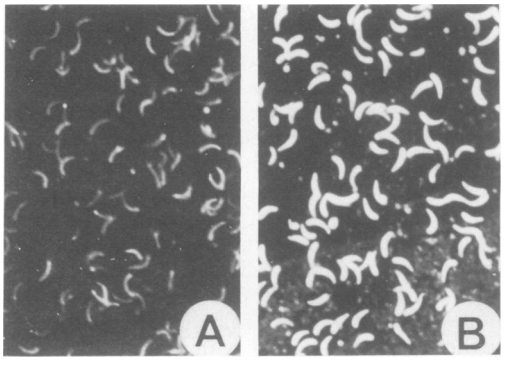

FIG. 1. *Merozoites of E. falciformis var. pragensis treated with serum obtained from athymic mice exposed to two infections were weakly and unevenly fluorescent (A), compared with the intensely and uniformly fluorescent merozoites treated with serum obtained from NLM (B). Merozoites were stained with fluorescein-conjugated antiglobulin.* ×480.

after the second coccidial infection had pathological changes suggestive of infection by Sendai virus, an agent reported to be one of the common causes of mortality in nude mice (3).

The protective immune response of the NLM mice appeared to be weaker than that of the SW mice. It is possible that the reduction in the number of θ-bearing lymphocytes reported in NLM (5) may have accounted for their weaker immunological response. However, since the NLM were of BALB/c background, the strain difference more likely accounts for the differences in immunological response.

Nude mice exposed to three infections of *E. falciformis* var. *pragensis* failed to develop immunity whereas NLM and SW mice were resistant to reinfection after the first or second infection. The inability of nude mice, animals known to have no functionally significant T-lymphocytes (7), to develop resistance to the coccidium provided direct evidence for the T-cell dependence of immunity to the parasite. The acquisition of immunity in birds infected with *E. tenella* and in rats infected with *E. nieschulzi* was not affected by neonatal thymectomy (12, 22, 27). It seems unlikely that the immune mechanisms acting against coccidia would differ greatly between rats and mice. The apparent conflict between the previous results and the present findings could be due to the existence of a residual "thymic influence" in the neonatally thymectomized hosts. Thymectomized animals are not necessarily comparable to athymic animals, since T-cells can enter the circulation before birth in many mammals (19).

Passive immunization with immune serum failed to protect the nude mice from a challenge infection; this suggests that T-lymphocytes are necessary as "effectors" of immunity to the parasite. Immune serum was shown to have antibody activity, as determined by the indirect fluorescent antibody reaction, and the volume of the serum given to the mice was comparable (per unit of body weight) to those given to rats and chickens, where some degree of protection was obtained with passive immunization (24, 25; Wittchow, Dr. Med. Vet. thesis). The infective dose given in the present study, however, may have been too high to reveal a slight degree of protection. Significant protection with immune serum was found only when birds were infected with 50 but not with 500 or more oocysts of *E. maxima* (24). Several investigators were unable to protect animals against coccidiosis by passive immunization with immune serum (1, 4, 23). Considerable evidence supports the view that both avian and mammalian coccidia evoke cell-mediated responses (8–11, 20, 26); however, it has yet to be established that any specific cell-mediated mechanism mediates protective immunity to coccidial parasites. In fact, rabbits showing a positive delayed hypersensitivity reaction to antigens of *E. steidae* were found to be susceptible to reinfection with the same coccidium (8). A clearer definition of the mechanism(s) whereby T-lymphocytes mediate immunity to coccidia may be obtained by studying the effects of lymphocyte subpopulations and mediators released from activated lymphocytes on specific parasitic stages grown in tissue culture.

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