Effects of Acquired Resistance on Infection with *Eimeria falciformis* var. *pragensis* in Mice

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Mice immunized with infections of 500, 5,000, or 20,000 oocysts of *E. falciformis* var. *pragensis* were reinfected with 20,000 and 100,000 oocysts at 20 and 38 days, respectively, after the initial infection. After the first challenge infection, none of the immunized mice showed clinical signs of coccidiosis; a few mice passed very low numbers of oocysts, and oocyst discharge seemed to correlate negatively with immunizing dose. None of the mice immunized twice passed oocysts after challenge. Mice immunized with three infections were completely immune to challenge for 4 months. The effect of the immune response on the life cycle of the coccidium was determined by histological examination of the intestines of immune and nonimmune mice infected with the parasite. In both the immune and nonimmune groups, sporozoites penetrated absorptive epithelial cells and migrated to crypt epithelial cells during the first 6 to 24 h postinfection. At 48 to 72 h postinfection, the sporozoites developed into mature first-generation schizonts in the nonimmune mouse, whereas the developing first-generation schizonts degenerated within the crypt epithelial cells of the immune mouse. In nonimmune mice, third-generation merozoites, inoculated intracecally, developed into mature fourth-generation schizonts, whereas in immune mice the developing fourth-generation schizonts degenerated before maturing. The possibility that a cell-mediated immune mechanism is responsible for the arrest in schizogony is discussed.

Both humoral and cellular immunities are thought to play a role in resistance to coccidia (26), but the precise mechanisms involved are poorly understood. Coccidia have a complex life cycle. Sporozoites enter host cells and develop into successive generations of schizonts which release merozoites that invade other host cells. The final generation of merozoites develops into gametes. Microgametes and macrogametes combine to form oocysts, which are passed from the host into the environment. Knowledge of the specific event in the pathogenesis or stage of development which is affected by the immune response could give some indication of the mechanisms responsible for the immunity. Opinions about which stage is affected vary considerably. It has been reported that sporozoites of *Eimeria nieschulzi* and *E. tenella* failed to penetrate intestinal epithelial cells of immune rats and chickens, respectively (22; S. A. Edgar, Ph.D. thesis, University of Wisconsin, Madison, 1944). Several studies with avian coccidia suggested that sporozoites can penetrate epithelial cells of immune birds but fail to develop into mature 1st generation schizonts (1, 12, 18, 33). Similar results were observed in rabbits infected with *E. magna* (25). The coccidial stages affected by the immune response have not been determined for any of the murine coccidia.

The aims of this study were to determine the following in mice by using *E. falciformis* var. *pragensis*: (i) the effects of the immune response on the life cycle of coccidia, (ii) the relative immunizing ability of different doses of oocysts, and (iii) the duration of acquired resistance to coccidia.

**MATERIALS AND METHODS**

**Experimental animals.** Swiss white mice, obtained from the Animal Resources Center, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, were used in this study. Mice used to determine oocyst discharge and weight changes were kept in individual wire-netted cages. The others were kept in groups of four to five in plastic cages.

**Oocysts and merozoites.** *E. falciformis* var. *pragensis* (4), established from a single oocyst infection, was maintained by frequent passage through coccidia-

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free mice, sporulated in 2.5% potassium dichromate, and stored at 4°C for not more than 8 weeks before it was used. A suspension of third-generation merozoites was obtained from mice killed 5 days after oral inoculation with 50,000 oocysts of E. falciformis var. pra-gensis. To reduce the intestinal bacterial population, the donor mice were given neomycin at 10 mg/kg of body weight for 4 days before they were killed. After the colonic contents were washed away with water, the colonic mucosa was completely removed with a scalpel and suspended in phosphate-buffered saline (pH 7.2). The merozoites were released from the epithelial cells by gently crushing and teasing the mucosal scrapings. The suspension was filtered through a 1-mm wire mesh, and the filtrate was centrifuged. The sediment was suspended in a small volume of phosphate-buffered saline and maintained at 37°C until used within 1 to 3 h. The number of merozoites given to each mouse was estimated with a Fuchs-Rosenthal hemocytometer.

Infection. Mice, randomly assigned to three test groups, were infected orally with 500, 5,000 or 20,000 oocysts. On the 20th day postinfection (p.i.), previously infected mice and uninfected mice were infected with 20,000 oocysts. Mice immunized with two oral infections of 20,000 oocysts at 18- to 20-day intervals and challenged with 100 oocysts were used to study the life cycle of the coccidium in immune mice. The third immunizing dose of 100,000 oocysts was given on the 18th day after the second infection. Mice were infected with merozoites by inoculating approximately 4 × 10⁶ third-generation merozoites into the cecum exposed surgically. Mice were kept under halothane anesthesia during the surgery.

Clinical observations and oocyst counting procedures. Feed intake and consistency of feces were visually evaluated daily. The day of inoculation was designated as day 0 and weights were recorded on days 0, 3, 6, 7, 8, 9, 10, 12, 14, and 18 during the first challenge and on days 0, 3, 6, 8, 10, 12, and 16 during the second challenge to compare weight changes between test and control groups. Daily oocyst output was determined by the method of Long and Rowell (20). If there were too few oocysts to count using a McMaster chamber, feces were qualitatively examined for oocysts by the sugar flotation method.

Determination of duration of immunity. The immune status of mice immunized with 3 infections of E. falciformis var. pra-gensis was evaluated by determining the oocyst discharge in mice infected with 100,000 oocysts at 2, 3, 4, 5 and 6 months after the last immunizing dose. Test mice were killed on the 15th day after the challenge infection and the cecum and colon were examined histologically.

Histological methods. Three pieces of colon at approximately 2, 4, and 5 cm distal to the cecocolic junction were obtained from mice killed by atlantooccipital dislocation. Tissues were fixed in Bouin’s fluid for 24 h, postfixed in 70% alcohol, dehydrated, paraffin embedded, sectioned at 5 to 6 μm, and stained with hematoxylin—eosin. Selected tissues were stained with Heidenhain’s iron hematoxylin and Warthin-Faulkner silver stain. The comparative numbers of coccidial stages developing in the colonic mucosa of immune and nonimmune mice were determined by counting all of the sporozoites, trophozoites, and schizonts, in one histological section (6 μm) of colon from each mouse taken at each of 6, 12, 24, 48, 72, 96, and 120 h p.i.

Statistical methods. The t test was used to compare the total number of coccidial stages found in the immune and nonimmune mice. The mean weights of mice immunized with different doses of the coccidium were compared by using an analysis of variance and Duncan’s multiple range test (32).

RESULTS

Effects of immunity on clinical signs and oocyst discharge. For convenience of expression, the term "immune" will be used to refer to mice that have been previously infected with the coccidium and have subsequently recovered from the disease.

The clinical signs exhibited by the mice after the primary infection with the different doses of oocysts were similar to those observed previously (G. M. Mesfin, J. E. C. Bellamy, and P. H. G. Stockdale, Can. J. Comp. Med., in press), namely, dehydration, weight loss, diarrhea, and dysentery. These signs were not seen in immune mice; the feces of a few of the immune mice were less well formed for 1 or 2 days, but diarrhea was never evident. The weight changes observed in the immune and nonimmune mice after challenge with 20,000 oocysts are shown in Fig. 1. Significant weight loss occurred in the suscepti-
ble (nonimmune) group, especially from 8 to 10 days p.i. The mice in this group showed a compensatory weight gain from 12 to 16 days p.i. No weight loss occurred in the immune mice; their growth curve was similar to that of the uninfected controls. Similarly, when the groups were challenged again with 100,000 oocysts, only the nonimmune mice lost weight.

The effects of immunity on oocyst discharge are indicated in Table 1. Immunization greatly reduced oocyst production after a challenge infection with 20,000 oocysts. Every mouse in the nonimmune group passed more than a total of $5 \times 10^6$ oocysts, whereas none of the immune mice passed even one-tenth that number. In the immune group, the numbers of discharged oocysts were so low that usually the counts could not be done with a McMaster chamber. Only 29% of the mice immunized with 20,000 oocysts passed oocysts after the challenge infection, whereas 57 and 72% of the two groups immunized with fewer oocysts passed oocysts after challenge. The prepatent period of the infection was extended by 24 h in some of the immunized mice. In the same immune mice, the patent period was shortened by 3 to 5 days. Immune mice did not pass any oocysts when rechallenged with 100,000 oocysts.

**Effects of immunity on the life cycle and pathological changes.** The effects of immunity on the total number of coccidia that developed in specific areas of the colonic mucosa are summarized in Table 2. The number and location of sporozoites during the first 24 h of the infection were similar in both the immune and nonimmune mice. Sporozoites penetrated the absorptive epithelium and migrated to the crypt epithelium apparently between the basement membrane and the basal edge of the epithelial cells. The pathological changes observed at 6 and 12 h were similar in both the immune and nonimmune mice. They consisted of submucosal edema and neutrophil migration through submucosal venules. At 24 h p.i., the sporozoites and trophozoites in the nonimmune group appeared normal. Most of the trophozoites in the immune mice were normal as well, but a few appeared degenerate (Fig. 2A). Sporozoites were occasionally found in the lamina propria of both immune and nonimmune mice, and some appeared degenerate. Desquamation of epithelial cells into the crypt lumen was more prominent in the immune group, but coccidial stages were not as prominent.

**Table 1.** Total oocyst discharge from immune and nonimmune mice infected with 20,000 oocysts of *E. falciformis var. pragensis*

<table>
<thead>
<tr>
<th>Immunizing dose (oocysts)</th>
<th>Oocyst discharge ($\times 10^6$) in mouse no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>5,000</td>
<td>1</td>
</tr>
<tr>
<td>20,000</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>593</td>
</tr>
</tbody>
</table>

<sup>a</sup> When there were too few oocysts to count with a McMaster chamber, oocyst production was assessed qualitatively with the sugar flotation method and, if detected, they were assigned the value <1 $\times 10^6$.  
<sup>b</sup> ND, Not determined.

**Table 2.** Number and location of coccidial stages in the colonic mucosa of immune and nonimmune mice infected with $10^6$ oocysts of *E. falciformis var. pragensis*

<table>
<thead>
<tr>
<th>Time (h p.i.)</th>
<th>Total</th>
<th>Absorptive epithelium</th>
<th>Crypt epithelium</th>
<th>Lamina propria</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>81±28</td>
<td>58±28</td>
<td>14±9</td>
<td>11±6</td>
</tr>
<tr>
<td>12</td>
<td>63±32</td>
<td>17±12</td>
<td>42±18</td>
<td>9±6</td>
</tr>
<tr>
<td>24</td>
<td>67±30</td>
<td>3±3</td>
<td>60±27</td>
<td>4±4</td>
</tr>
<tr>
<td>48</td>
<td>151±44</td>
<td>5±4</td>
<td>152±44</td>
<td>4±4</td>
</tr>
<tr>
<td>72</td>
<td>473±121</td>
<td>14±12</td>
<td>457±119</td>
<td>3±2</td>
</tr>
<tr>
<td>96</td>
<td>500&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>500&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<table>
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<tr>
<th>Time (h p.i.)</th>
<th>Total</th>
<th>Absorptive epithelium</th>
<th>Crypt epithelium</th>
<th>Lamina propria</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>72±34</td>
<td>51±22</td>
<td>12±6</td>
<td>9±7</td>
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<td>12</td>
<td>52±27</td>
<td>15±7</td>
<td>36±24</td>
<td>5±3</td>
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<td>24</td>
<td>57±29</td>
<td>5±3</td>
<td>47±24</td>
<td>5±4</td>
</tr>
<tr>
<td>48</td>
<td>47±41&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2±2</td>
<td>43±39</td>
<td>2±2</td>
</tr>
<tr>
<td>72</td>
<td>22±28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>21±27</td>
<td>1±1</td>
</tr>
<tr>
<td>96</td>
<td>6±6</td>
<td>1±1</td>
<td>5±4</td>
<td>1±1</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent the total number of sporozoites, trophozoites, and immature and mature schizonts found in a complete histological transverse section (6 μm) at one specific site in the colon. The values are expressed as the mean ± standard deviation for 5 mice.

<sup>b</sup> Significantly fewer than in the corresponding nonimmune mice (P < 0.01).

<sup>c</sup> Significantly fewer than in the corresponding nonimmune mice (P < 0.001).

<sup>d</sup> Not counted; but since almost every crypt epithelial cell contained one or more schizonts, the total count would be greater than 500.
not seen in any of the exfoliated cells. The submucosal edema that was prominent at 12 h p.i. was not very apparent in either group of mice at 24 h.

By 48 h p.i., obvious differences became apparent between the immune and nonimmune mice. The number of coccidia developing in the colonic mucosa of the immune mice was significantly lower than in the nonimmune group (Table 2). Numerous immature and a few mature first-generation schizonts were observed within the epithelial cells of the nonimmune mice. In contrast, the coccidial stages in the immune mice had not matured and were degenerate (Fig. 3A). Most of the degenerate immature schizonts had coarse granular cytoplasm and poor nuclear differentiation, which rendered their appearance distinctly different from the normal schizonts seen in the nonimmune mice. Often, the degenerate coccidial stages could be positively identified by the remains of their refractile globules. Many degenerate cells that could not be identified as coccidial remnants or degenerate inflammatory cells were frequently observed within crypt epithelial cells. A few similar degenerate cells were also found in the nonimmune mice. Occasionally, unidentified degenerate eosinophilic globules, similar to those in the epithelium, were observed in the lamina propria. Lymphocytes, neutrophils, and globule leukocytes were randomly scattered throughout the crypt epithelium of the colon in both the immune and nonimmune mice. The morphology of epithelial cells that contained coccidial stages was similar in both the immune and nonimmune mice, regardless of whether the coccidia were degenerate or not. The host cell nucleus was often indented and usually displaced to one edge of the cell; otherwise, the cell appeared normal.

At 72 h p.i., the number of parasites seen in the nonimmune group greatly exceeded the number seen in the immune group (Table 2). The mature schizonts seen in the nonimmune mice did not develop in the immunized mice; with the rare exception, only degenerate coccidial stages were observed in the immune group. By 96 h p.i., even the shrunken remnants of coccidia were few in number in the immune mice. A second phase of submucosal edema was again observed but only in the nonimmune group. By 120 h p.i., the crypt epithelial cells of the nonimmune group were filled with normally developing schizonts whereas, in the immune mice, coccidial stages were almost completely absent. The coccidia had failed to develop in the immunized mice. The pathological changes seen in the nonimmune mice were similar but more marked than those described previously (Mesfin et al., in press). In the immune mice, the colonic mucosa appeared normal. A few retained oocysts, and accompanying focal granulomatous tissue, as a result of the primary infection, were seen in all immune mice.

Mice infected intraceccally with merozoites. Mature fourth-generation schizonts, immature gamonts, and oocysts were found in two of the three nonimmune mice killed at 24, 48, and 72 h p.i., respectively. Coccidial stages were not seen in any of the immune mice killed at 12, 48, and 72 h p.i.; however, at 24 h a few degenerate and developmentally arrested fourth-generation schizonts were found (Fig. 4A). Most mice in both groups had a mild localized peritonitis due to the surgery.

Duration of immunity. Mice immunized with three infections of E. falciformis var. praegensis had complete immunity (as measured by oocyst discharge) for 4 months. By 5 and 6 months after immunization, immunity began to wane in some of the mice. However, although

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**Fig. 2.** Trophozoites (arrows) in the crypt epithelial cells of mice 24 h after infection with E. falciformis var. praegensis. The trophozoite in the immune mouse (A) has degenerated, compared to the developing trophozoite with four nuclei in the nonimmune mouse (B). Stained with hematoxylin—eosin; ×930.

**Fig. 3.** Immature schizonts (arrows) in the crypt epithelial cells of mice 48 h after infection with E. falciformis var. praegensis. The schizont in the immune mouse (A) is shrunken and lacks nuclear differentiation, compared to the well differentiated multinucleated schizont in the nonimmune mouse (B). Stained with hematoxylin—eosin; ×930.
FIG. 4. Fourth-generation schizonts in the crypt epithelial cells of mice 24 h after infection with third-generation merozoites of E. falciformis var. pragensis. The schizont in the immune mouse (A) has a few marginated nuclear remnants compared to the mature schizont with numerous merozoites in the nonimmune mouse (B). Stained with hematoxylin—eosin; ×930.

four of the seven mice challenged 6 months after immunization did pass a few oocysts, they did not become clinically ill. Histological examination showed that the number of oocysts retained in the colonic mucosa from the primary infection progressively decreased from the second to the sixth month after immunization.

DISCUSSION

Infection with E. falciformis var. pragensis induced good immunity in mice. The number of mice completely immune increased in proportion with the immunizing dose, suggesting that the degree of immunity was at least partly dependent on the immunizing dose. Every mouse that was immunized twice failed to discharge any oocysts after a challenge. Immunity to E. falciformis var. pragensis, therefore, may relate to both the concentration of antigen and the immunizing schedule. Dose-dependent resistance to coccidia has been reported with E. bovis in cattle (9, 31) and E. maxima and E. acervulina in chickens (11).

Complete immunity to E. falciformis var. pragensis lasted for 4 months after immunization. Mice immunized with one or two infections of E. pragensis (E. falciformis var. pragensis) were resistant to reinfection at 3 months after immunization (3). In the present study, although some of the mice challenged at 6 months after immunization passed a few oocysts, clinical signs of coccidiosis were not seen in any of the mice. Immunity to coccidia has been reported to last from a few weeks to up to 4 months, presumably in the absence of reinfection (26). It is difficult to reach a general consensus as to the duration of immunity to coccidial parasites because of the differences in the mode of immunization and the criteria used to assess immunity in the different studies. The complete resistance of rabbits to E. steidae infection for up to 2 years was suggested to have been due to persistent antigenic stimulus provided by oocysts retained within the liver of recovered rabbits (13). Such an association between oocyst retention and duration of immunity was not apparent in the present study. Mice discharged a few oocysts when challenged at 5 and 6 months after immunization despite the presence of oocysts retained within the mucosa of the colon.

The immune response to E. falciformis var. pragensis did not prevent penetration of the absorptive epithelium by sporozoites, nor did it interfere with sporozoite migration to crypt epithelium, but it prevented the complete development of sporozoites into mature first-generation schizonts. To determine whether developing first-generation schizonts were unique in this respect, immune mice were infected intracereally with third-generation merozoites of the coccidium. The result was similar to that in oocyst-infected immune mice. It seems that the immune response to E. falciformis var. pragensis acts to specifically block merozoite production at every stage in merogony. In the natural situation, the immune response probably blocks the maturation of most first-generation merozoites.

Local mucosal antibodies, which play such an important role in immunity to viral and bacterial enteric infections by blocking adherence to or penetration of the epithelium (2), seem to have little influence on the motile stages of the coccidium. Both sporozoites and merozoites were able to penetrate intestinal epithelial cells of immune mice. Although this result is at variance with that of Morehouse (22) and Edgar (Ph.D. thesis), it is consistent with the observations of a number of investigators (1, 12, 13, 18, 33) working with other avian and mammalian coccidia. Although a limited degree of protection has been conferred through passive transfer of immune serum (26, 27; W. Wittchow, doctoral dissertation, University of Berlin, Berlin, West Germany, 1972), there is no evidence as yet to support the idea that local antibodies acting within the intestinal lumen have any influence on the infection of the epithelium by the motile forms of the parasite. Because intestinal mucosal antibody is transported primarily through crypt epithelial cells (34) in which degeneration of the developing schizonts occurred, it is conceivable that local antibody could act within the epithelial cells to somehow mediate the effect. However, the isolation of infective sporozoites of E. tenella and E. maxima after exposure to the "immune intestine" for up to 24 and 88 h, respectively (18, 29), does not support such a mechanism, unless immunity in birds operates...
in a manner different from that in mammals.

Acquired resistance to a variety of obligate and facultative intracellular pathogens is dependent on cell-mediated immunity (21, 24). Cell-mediated immunity to *Eimeria* has been demonstrated by using transfer factor (14, 15, 19), the macrophage migration inhibition test (23), the delayed hypersensitivity reaction (15, 16, 28), and in vitro lymphocyte blastogenesis (16). Since the immune response to *E. falciformis* var. *pragensis* and to several other coccidia acts to cause degeneration of schizonts that are developing within an epithelial cell, a cell-mediated immune mechanism would appear to be a likely mediator of immunity.

The possibility that cell-mediated cytotoxicity of coccidia-bearing cells is responsible for immunity deserves consideration. Because epithelial desquamation was more apparent in immune birds than in nonimmune birds challenged with *E. acervulina*, Kouwenhoven and Van Der Horst (17) suggested that immunity to the coccidium was directed against the parasitized epithelial cells. Rose et al. (30), however, considered that the epithelial loss was due to inflammation resulting from a hypersensitivity reaction rather than as a result of an immune response acting specifically against parasitized epithelial cells. Neither in the previous (17) nor in the present study were parasitic stages seen within the desquamated epithelial cells to suggest that the coccidial stages were expelled in this manner. Injured parasite-containing epithelial cells, an indicator of cell-mediated cytotoxicity (5) were not observed in immune mice infected with *E. falciformis* var. *pragensis*.

The possibility that mediators released from immune T cells mediate degeneration of the developing coccidial stages warrants investigation. *Toxoplasma gondii* and Besnoitia jelli-soni, parasites closely related to the *Eimeria* (10), failed to proliferate in fibroblasts and kidney cells treated with mediators released from antigen-stimulated immune lymphocytes (6). A non-antibody-soluble substance released from sensitized lymphocytes was also considered to have mediated intra-erythrocytic degeneration of *Babesia microti* (7, 8).

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