Experimental Cryptosporidiosis in Laboratory Mice

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Eight strains of laboratory mice were susceptible to subclinical infections with Cryptosporidium sp. at 1 to 4 days of age, but only a transient infection could be established at 21 days of age or older. Immunosuppression of 21-day-old mice failed to render them more susceptible to infection. Laboratory storage conditions for Cryptosporidium sp. were investigated by titration in 1- to 4-day-old mice. Storage by freezing with a variety of cryoprotectants was unsuccessful, but storage at 4°C in phosphate-buffered saline or 2.5% potassium dichromate was possible for 4 to 6 months.

Cryptosporidium sp. is a member of the family Cryptosporididae, suborder Eimeriorina, but it differs from most other enteric coccidia in that the organisms are smaller (diameter, 3 to 4 μm) and only invade the microvillous border of gut enterocytes.

Cryptosporidium sp. infections were described first in tamed wild mice (16, 17) and subsequently in C57 mice (7). Infections associated with diarrhea have also been reported in a variety of animals and in humans (2, 5, 10, 13, 18, 20, 21, 23, 24, 28).

Studies in guinea pigs (26, 27) suggested that Cryptosporidium sp. is species specific, but recently, subclinical infections were recorded in 1-day-old specific-pathogen-free (SPF) suckling mice and rats inoculated with feces containing Cryptosporidium sp. taken from calves, lambs, and humans (19).

This paper describes the use of laboratory mice as models for the study of several aspects of Cryptosporidium sp. infections: susceptibility of mouse strains, age-related susceptibility, infections in immunosuppressed mice, and the effect of multiple passage through mice on the pathogenicity of the organisms for lambs. Storage in laboratory media was also investigated.

MATERIALS AND METHODS

Animals. All animals were bred as SPF at the Moredun Research Institute and were maintained in plastic isolators. Two random-bred strains (Schneider Swiss White and Porton) and six inbred strains (CBA, CBA Nude, C57 Black, BALB/c, Porton [derived from the random Porton stock], and Hairless [HR/HR-ADR]) were used. Inbred Porton mice were used except where stated otherwise. Two gnotobiotic lambs were also used.

Administration of inocula to mice. Mice were inoculated orally with 0.1 ml of fecal suspension or gut homogenate with a 23-gauge needle tipped with plastic tubing. The dose was increased to 0.2 ml for mice 21 days or more in age.

Preparation of inocula. Cryptosporidium sp., isolated from the feces of a diarrheic calf, was passaged in SPF rats, gnotobiotic lambs (22), and SPF mice and was used to prepare inocula 1 through 4 (Fig. 1).

All inocula were prepared as 20% (wt/vol) homogenates of feces (inoculum 1) or whole gut (inocula 2 through 4) in 5% bovine serum albumin (BSA). The fecal preparation used in the storage experiment contained a calf Cryptosporidium sp. isolate that had been passaged four times in gnotobiotic lambs (D. R. Snodgrass, unpublished data) and had an initial starting 50% mouse infective dose of 3.08 (log10).

Susceptibility of mouse strains. Two litters each of eight strains of mice (Table 1) were inoculated between 1 and 4 days of age. Mice were killed and examined daily from 2 to 7 days post inoculation (p.i.) for evidence of infection.

Age-related susceptibility. Four litters of suckling mice were inoculated with inoculum 2 at 4 days of age, and 24 weaned mice were inoculated with inoculum 3 at 21 days of age. Two mice from each group were killed and examined for infection on 11 occasions from 2 to 16 days p.i. In addition, mice of the Hairless, Swiss White, and CBA Nude strains (14 mice from each strain) were inoculated at 21 to 42 days of age with inoculum 1, and 1 mouse from each strain was examined daily for evidence of infection for 14 days p.i.

Immunosuppressed mice. A total of 24 mice 21 days old were treated with cyclophosphamide at doses of 70 mg/kg, with two doses given intraperitoneally 7 days apart (1). Of these mice, 12 were inoculated with inoculum 4 at 2 days after the second cyclophosphamide injection. The immunosuppressive activity of cyclophosphamide was evaluated by assaysing the serological response to the injection of lumpy-ill virus vaccine, as was done before with respect to TAB vaccine (1). Lumpy-ill virus vaccine (Moredun type vaccine, Wellcome) was given to all cyclophosphamide-treated mice and to 12 control mice.

Pairs of mice from each group were killed daily on days 3 to 8 p.i. and were examined for cryptosporidial
TABLE 1. Strains of mice inoculated with Cryptosporidium sp. at 1 to 4 days of age

<table>
<thead>
<tr>
<th>Mice</th>
<th>Inoculum no.</th>
<th>Infectiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random Porton</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Schneider Swiss White</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Inbred Porton</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>BALB/c</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>CBA Nude</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Hairless</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>(HR/HR-ADR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57 Black</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>CBA</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>None</td>
<td>-</td>
</tr>
</tbody>
</table>

a+, Cryptosporidium sp. detected by histology and fecal examination; -, no Cryptosporidium sp. detected.

**RESULTS**

**Susceptibility of mouse strains.** All eight strains of mice inoculated between 1 and 4 days of age were susceptible to Cryptosporidium sp. infection (Table 1), although none of the mice had clinical illness at any time. Fecal cryptosporidia were detected from 3 to 7 days p.i.

When examined by light microscopy, villi in the terminal ilea and the luminal surfaces of the ceca were extensively covered with cryptosporidia, but the duodena were only sparsely infected. Significant pathological changes were not observed at any intestinal site in mice infected with Cryptosporidium sp.

Histological and fecal examination did not detect infection in uninoculated age-matched mice from the same isolators as inoculated animals.

**Age-related susceptibility.** Mice inoculated at 4 days of age became heavily infected in the ilea, but only mild infections were established in 21-day-old mice (Table 3). Histological examination correlated well with fecal detection of the organism, but infected areas of gut could be detected histologically after fecal shedding of the organism had ceased (Table 3).

Hairless (HR/HR-ADR) and Swiss White mice inoculated at 21 to 42 days of age did not...
become infected (total of 28 mice examined), and infection was detected in only 1 CBA Nude mouse 9 days p.i. (total of 14 mice examined).

**Immunosuppressed mice.** The cyclophosphamide treatment was effective in reducing the louping-ill antibody response from a value of 160 to 320 in normal mice to one of 10 to 40 in immunosuppressed mice. However, no mice in any group became infected with *Cryptosporidium* sp.

**Effect of multiple passage on pathogenicity.** The calf *Cryptosporidium* sp. isolate remained infective for mice after 16 mouse passages over a 3-month period. Two gnotobiotic lambs 2 days old that were given inoculum 3 developed diarrhea and pathological lesions typical of those described for cryptosporidiosis in experimental lambs (22).

**Storage of Cryptosporidium sp.** Freezing destroyed the activity of *Cryptosporidium* sp. irrespective of the cryopreservation method used (Table 2).

There was a progressive loss of infectivity in all media at 4°C (Fig. 2). No infectivity was detectable after 2 months of storage in distilled water; the most stable preparation was in 2.5% potassium dichromate, in which infectivity lasted 4 to 6 months. Complete loss of infectivity occurred at 15 to 20°C within 2 weeks and at 37°C within 5 days.

**DISCUSSION**

These experiments demonstrate that *Cryptosporidium* sp. isolates can subclinically infect eight different strains of SPF mice 1 to 4 days old, but the same strains at 21 days of age or older have a lower susceptibility to infection. This age-related susceptibility could explain why some workers using *Cryptosporidium* sp. isolated from guinea pigs (26) and cats (9) failed

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**TABLE 2. Freezing and freeze-drying methods used to store Cryptosporidium sp.**

<table>
<thead>
<tr>
<th>Cryopreservative</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 5% PVP</td>
<td>Rapid cooling in liquid nitrogen for 10 min, then quick thaw at 37°C</td>
</tr>
<tr>
<td>2. 5% Glycerol</td>
<td>Rapid cooling in liquid nitrogen for 10 min, then quick thaw at 37°C</td>
</tr>
<tr>
<td>3. 10% DMSO</td>
<td>Rapid cooling in liquid nitrogen for 10 min, then quick thaw at 37°C</td>
</tr>
<tr>
<td>4. 10% DMSO + 20% BSA</td>
<td>Rapid cooling in liquid nitrogen for 10 min, then quick thaw at 37°C</td>
</tr>
<tr>
<td>5. SPGA + DMSO to 8% (vol/vol)</td>
<td>Rapid cooling in liquid nitrogen, then slow thaw at 15-20°C</td>
</tr>
<tr>
<td>6. 20% NRS + 10% glycerol (equilibrated at 15 to 20°C for 20 h)</td>
<td>Slow cooling to ~70°C, then slow thaw at 15-20°C</td>
</tr>
<tr>
<td>7. 10% DMSO</td>
<td>-20°C for 3 days, slow thaw</td>
</tr>
<tr>
<td>8. 20% Glycerol</td>
<td>-20°C for 3 days, slow thaw</td>
</tr>
<tr>
<td>9. PBS</td>
<td>-20°C for 14 days, slow thaw</td>
</tr>
<tr>
<td>10. PBS</td>
<td>-70°C for 14 days, slow thaw</td>
</tr>
<tr>
<td>11. 10% Glycerol</td>
<td>Freeze-drying for 2 days at 4°C</td>
</tr>
</tbody>
</table>

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**TABLE 3. Age-related susceptibility to infection by Cryptosporidium sp.**

<table>
<thead>
<tr>
<th>Age at inoculation (days)</th>
<th>Infection at day p.i.*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
</tr>
</tbody>
</table>

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*Abbreviations: PVP, Polyvinylpyrrolidone (molecular weight, 44,000); DMSO, dimethyl sulfoxide; SPGA, per liter 74.6 g of sucrose, 0.52 g of KH₂PO₄, 1.25 g of K₂HPO₄, 0.91 g of L-glutaric acid, 10 g of BSA; NRS, normal rabbit serum.

*Two mice were killed on each day from each age group. Symbols: -, No infection as detected by fecal examination or histology; NT, not tested; +, mild infection as detected by histology; ++, mild infection as detected by histology plus fecal shedding of *Cryptosporidium* sp.; ++++, moderate to heavy infection as detected by histology plus fecal shedding of *Cryptosporidium* sp.*
to infect weanling mice or 7-week-old ICR mice, respectively. This effect has been recognized before (8, 19), but recently adult mice have been infected with Cryptosporidium sp. of human and calf origin (15), and hence, the infectivity of the inoculum is important.

When considering the extent of infection present in the ilea and ceca of 1- to 4-day-old mice, it is surprising that no pathological changes occur and that only subclinical infections can be established. This situation is in contrast to the severe clinical disease and pathological changes observed in young lambs infected with the organism (22).

Cryptosporidium sp. infections have been observed in immunosuppressed humans (5, 10, 28), but no enhanced susceptibility could be shown in mice treated with cyclophosphamide, a procedure which impaired the antibody response to louping-ill virus vaccine. The reduction in antibody response to virus served as an indicator of general antibody depression, but this apparently failed to increase the susceptibility of weanling mice to Cryptosporidium sp. infections. Presumably, factors other than antibody-mediated responses are involved in resistance to these infections. With one exception, all CBA Nude mice inoculated at 28 days of age were apparently not susceptible to Cryptosporidium sp. infection.

The calf Cryptosporidium sp. isolate used was passaged 12 times in mice and still remained pathogenic for gnotobiotic lambs. It is not known whether this capability applies to all Cryptosporidium sp. isolates, but its presence could be advantageous in maintaining and storing field isolates and in providing a biological filtration system to remove enteric viruses (e.g., rotavirus and coronavirus) or pathogenic bacteria (e.g., enterotoxigenic Escherichia coli). Hence, an inoculum devoid of any other known enteropathogens could be established.

Continuous biological passage is time consuming and costly, and there would be many advantages in being able to store strains provided their pathogenicity could be maintained. The various cryopreservatives used failed to preserve the organism at low temperatures, although two of the methods used (Table 2, numbers 5 and 6) have been successful in long-term storage of rickettsiae (3) and Toxoplasma gondii (6), respectively. Storage of the Cryptosporidium sp. isolate at 15 to 20 or at 37°C in PBS resulted in inactivation within 2 weeks, but at 4°C the organism remained viable for at least 3 to 4 months. Heine and Boch (8) also reported that calf feces containing Cryptosporidium sp. stored at 4°C remain viable for 3 to 6 months. These findings may have epidemiological implications in that the organisms may survive in feces for a considerable period in a temperate climate.

The use of the mouse titration method to quantitate a Cryptosporidium sp. inoculum was preferred to the flotation method described by Iseki (9), which in our experience yields only a small percentage of the cryptosporidia in feces. Moreover, it is impossible to assess the percentage of viability in the sample since it has been shown that flotation can affect viability (8). With the mouse titration method, an overall viability, measured as the 50% mouse infective dose, is obtained for each inoculum, so that comparisons between inocula can be made.

The sensitivity of the mouse titration method was improved by employing histological examination of the gut as an index of infection rather than relying on Giemsa staining of fecal smears. The histological findings in selected titrations correlated with smear results (data not shown), but in general the percentage of infected animals detected on histological examination was higher.

In conclusion, laboratory mice are a very convenient animal model which may facilitate the study of many aspects of Cryptosporidium sp. infections which are not clearly understood, such as the immunology and the life cycle of the organism.

ACKNOWLEDGMENTS

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LITERATURE CITED

