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Cryptosporidium parvum infection in experimentally infected mice: infection dynamics and effect of immunosuppression

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Abstract. The effect of mouse strain, age, sex, and the size of infective dose on the susceptibility to infection with the coccidium *Cryptosporidium parvum* Tyzzer, 1912 was determined using several murine models. Mice were infected with *C. parvum* oocysts originally of cervine origin, maintained by repeat passage in calves. All mice in the experimental groups proved susceptible to infection, though this resulted asymptomatic in all cases. *C. parvum* infection in BALB/c and Porton mice exhibited some variation. BALB/c mice demonstrated a longer prepatent period than Porton mice. They also produced a greater oocyst output over the patent period, though the differences were not statistically significant. Differences were observed between mice infected at either 3 or 4 weeks of age. Prepatent period was shorter in those mice infected at 3 weeks of age, reaching 100% infection rate by day 7 post-inoculation. The patent period was longer in younger mice showing that age at time of infection can modify the oocyst shedding profile. However, no sex related differences in the course of infection were observed. The effect of different infective doses of oocysts was analysed. The three doses used (10^4, 10^5, 10^6) proved infective for all mice, there were no statistical differences in either prepatent or patent periods, or in the oocyst shedding profiles. Experimental cryptosporidiosis was also induced in cyclophosphamide-immunosuppressed mice. Cyclophosphamide was orally administered by stomach tube at a dose of 50 mg/kg/day starting 10 days before the intragastric inoculation of 10^6 oocysts of *C. parvum* per mouse and continuing until the end of the experiment. Immunosuppressed mice had a shorter prepatent period, remained infected longer and shed more oocysts than immunocompetent mice. Immunosuppression produced high mortality rates; during the course of the experiment 44% of immunosuppressed-infected and 30% of immunosuppressed-uninfected mice died. There were no deaths in the untreated groups. Differences in the clinical course of the infection were also observed between immunosuppressed and immunocompetent mice; however, some mice recovered without immunosuppression withdrawal.

Cryptosporidium coccidia were first described in the mouse by Tyzzer (1907) at the beginning of the twentieth century. Since then *Cryptosporidium parvum* Tyzzer, 1912 has been diagnosed as the cause of diarrhoeal illness in many mammalian species, including man, on a worldwise basis (Fayer et al. 1990). Infection has been reported in wild mice, and the absence of species specificity shown by this parasite suggests the mouse may be considered as a potential reservoir of cryptosporidial infection (Klesius et al. 1986).

Although different mouse strains are susceptible to infection when challenged with *C. parvum* oocysts (Tzipori et al. 1980, Sherwood et al. 1982, Heine et al. 1984, Current and Reese 1986, Ernst et al. 1986, Klesius et al. 1986, Enriquez and Sterling 1991), the extent of that susceptibility appears to be age related (Sherwood et al. 1982, Enriquez and Sterling 1991, Novak and Sterling 1991, Upton and Gillock 1996) and no sex related differences have been reported.

The necessity of a competent immune system for the resistance to the infection has been clearly established in natural and experimental infections (Current and Garcia 1991, Mead et al. 1991, McDonald and Bancroft 1994). A great deal is now known about specific factors which appear to influence the immune response, including lymphocytes groups, IFN- and presence of gut flora (Harp et al. 1992). Cellular immunity plays an important role in the protection and defence against *C. parvum*. However, the severe clinical course observed in hosts with selective IgA depletion suggests humoral immunity is also necessary to combat cryptosporidial infection (Heyworth 1990, Jacyna et al. 1990).

Most experimental studies on cryptosporidiosis have been undertaken using rodent models because of their wide availability. Mice appear susceptible to inocula derived from other hosts, and in the absence of appropriate tissue culture systems provide a convenient model for testing therapeutic and prophylactic drugs (Current and Garcia 1991). The use of a murine model may promote understanding of immunopathogenic mechanisms and the development of active and passive

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immunotherapy against *Cryptosporidium* infections (Riggs and Perryman 1987, Arrowood et al. 1989, Fayer et al. 1989, Riggs et al. 1989, Perryman 1990).

This study analyses the effect of different factors (mouse strain, age, sex, infective dose and immunodepression) on the susceptibility of the mouse to *C. parvum* infection, and demonstrates the high infectivity rates obtainable using this model.

MATERIALS AND METHODS

Inocula

Cryptosporidium parvum oocysts were obtained from the faeces of a red deer (*Cervus elaphus* L.) calf, and maintained by repeat passage in newborn bovine calves.

Oocyst extraction was performed by sedimentation and differential centrifugation (Hill et al. 1990). Faeces were diluted 1 : 5 in water, mildly acidified with 2% sulphuric acid and then allowed to sediment for 1 hour. The clear liquid phase was reduced in volume, washed with 1% sodium dodecyl sulphate (SDS) for 1 hour, then pelleted by centrifugation. The resulting pellet was washed with water several times to remove the SDS, after which the remaining debris flocculated and the oocysts were removed in the remaining clear aqueous phase. Oocysts were stored at 4°C in Hank's balanced salt solution with penicillin and streptomycin added.

Experimental animals

Susceptibility to *C. parvum* infection was studied using Porton and BALB/c male mice infected at 3 or 4 weeks of age with 10^6 *C. parvum* oocysts. Mice were maintained in separate cages and provided with pelleted food and water *ad libitum*. Prepatent and patent periods were observed, and mean oocyst shedding was calculated. Porton mice were infected at 4 weeks of age and studied to determine whether any sex-related differences in the course of infection occurred. Infective dose size was titrated in BALB/c mice infected at 4 weeks of age with either 10^4, 10^5, or 10^6 oocysts.

Finally, cyclophosphamide was administered as immunodepressor to 3-week-old BALB/c mice and the effect of this treatment on the course of cryptosporidiosis was analysed.

Mice number was between 9 and 11 mice per group and non-infected control mice were observed in parallel with all these experiments

Collection and preparation of samples

Mice were infected with *C. parvum* oocysts by gastric intubation. Over the 28 day experimental period, faeces were collected from each mouse every two days, weighted and a 0.5 g subsample used to concentrate the oocysts for counting.

Oocyst concentration

Half a gram of faeces was homogenised in 4 ml of water, then mixed with 6 ml of 45% sucrose solution. The mixture was centrifuged at 400 *g* for 20 minutes, the meniscus (approx. 2 ml) was removed into a conical centrifuge tube, diluted to 10 ml with water, and centrifuged for a further 10 minutes at 400 *g*. The pellet was resuspended in 0.1 ml of water and the oocysts counted in an improved Neubauer haemocytometer after further dilution in an aqueous solution containing 0.16% malachite green/1% SDS.

Induction of immunosuppression

It was initially intended to administer the drug with the drinking water, so for 7 days 10 individually housed mice were monitored for daily water consumption. Statistical analysis of the data showed that the differences were very significant ($p < 0.0001$). Because of these variations, it was decided to dose the mice daily using a stomach tube in order to ensure uniform dosing.

Cyclophosphamide was administered at a dosage of 50 mg/kg/day, commencing 10 days before inoculation with *C. parvum* oocysts. Immunossuppression was continued until the end of the experiment, 6 weeks later. The immunosuppressive activity of cyclophosphamide was evaluated by assaying the IgA secretory immune response in immunosuppressed and immunocompetent mice. Extraction of faecal IgA was as described by Hill et al. (1990). Faeces, 0.3 g, were mixed with 0.9 ml of PBS containing 0.05% of Tween 20 and then centrifuged at 10,000 *g* for 20 minutes. Supernatants were decanted and stored at –20°C until tested. Serial dilutions of faecal extracts were made to establish a semiquantification of IgA secretion by a double immunodiffussion assay (Ouchterlony test) using standardized techniques (Hudson and Hay 1989).

Statistical analysis

Differences in prepatent and patent periods, and in mean oocyst shedding, were analysed for statistical significance using Student's *t*-test, a significance level of 5% being set for each test. Non-parametric data were evaluated with the Mann-Whitney U test or the Kruskal-Wallis test.

RESULTS

Cryptosporidium parvum infection in Porton and BALB/c mice

Mice were infected at 4 weeks of age. All mice proved susceptible to infection with *C. parvum*, though the infections were asymptomatic. BALB/c mice demonstrated a significantly longer prepatent period (11.7 ± 3.2 days) than Porton mice (7.0 ± 1.6 days) ($p = 0.001$). Total numbers of oocysts shed was small in both strains of mice without statistically significant difference. Shedding profiles are shown in Fig. 1A,B.

Comparison between ages at infection

The prepatent period was shorter in mice infected at 3 weeks of age (5.7 ± 2.3 days) than at 4 weeks (11.7 ± 3.2 days) ($p = 0.01$). Shedding profiles are shown in Fig. 2A. The patent period was shorter in the older mice (Fig. 2B), and they shed significantly fewer oocysts than the mice infected at 3 weeks of age ($p = 0.001$).

Infection rate and host sex

There were no significant differences between male and female mice in either oocyst shedding profile or total numbers of oocysts shed (data not shown).

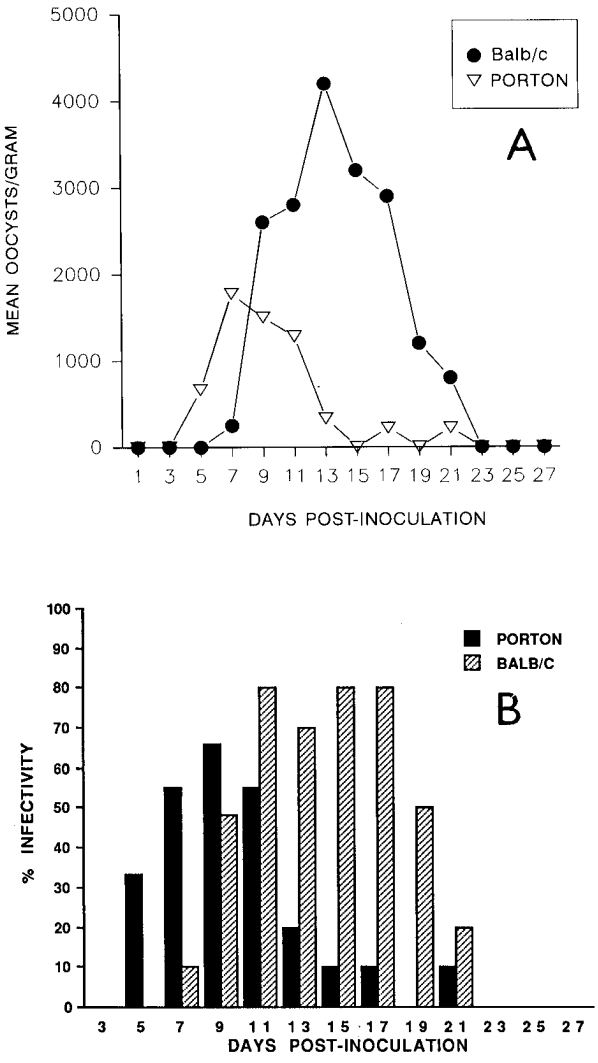

Fig. 1. Susceptibility to *Cryptosporidium parvum* in BALB/c and Porton mice. **A** – kinetics of mean oocyst shedding after infection with 10^6 oocysts; **B** – percentage of infectivity during the experimental period.

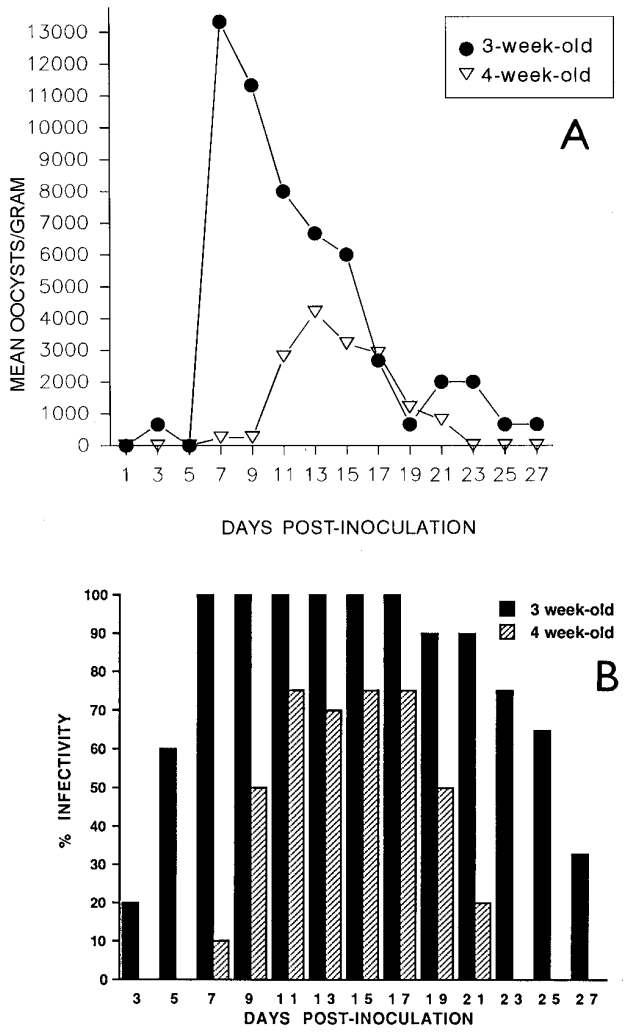

Fig. 2. Susceptibility to *Cryptosporidium parvum* in mice at different age. **A** – kinetics of mean oocyst shedding in mice after infection with 10^6 oocysts. Comparison between mice infected at 3 or 4 weeks of age. **B** – percentage of infectivity during the experimental period.

Infective dose size

The three assayed doses (10^4, 10^5, 10^6) were effective to produce cryptosporidial infection in mice. There were no statistically significant differences in the infections produced by the different infective doses. Figure 3 shows oocyst shedding profiles.

Effect of cyclophosphamide on cryptosporidial infection

Cyclophosphamide provoked a marked depletion of IgA in gut secretion (data not shown) and a more severe clinical course of cryptosporidiosis. Figure 4A shows the oocyst shedding profile for the infected groups during the study. Prepatent period was marginally longer in immunocompetent mice (11.7 ± 3.2 days) than in immunosuppressed mice (9.2 ± 2.8 days), but these differences were not statistically significant ($p > 0.1$). Figure 4B shows the percentage of mice shedding oocysts during the experiment.

The mean oocyst count was higher in immunosuppressed mice than in immunocompetent mice, the difference being statistically significant ($p = 0.025$). Patent period was longer in the immunosuppressed mice (14.7 ± 3.4 days) than in non-immunosuppressed mice (9.2 ± 5 days) ($p = 0.05$).

Immunocompetent mice showed no clinical symptoms. However, immunosuppressed mice (infected and uninfected) showed a gradual impairment, more severe in infected mice. Weight gain was lower in immunosuppressed mice (Fig. 5). Differences between immunosuppressed-infected and immunocompetent infected mice were statistically significant ($p = 0.001$).

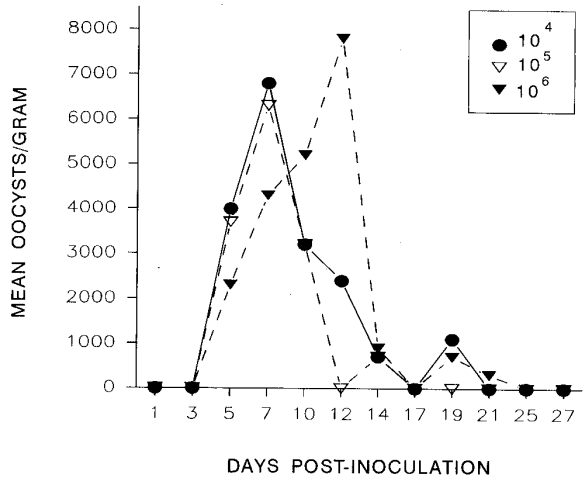

Fig. 3. Kinetics of mean oocyst shedding in mice after infection with 10^4, 10^5, or 10^6 *Cryptosporidium parvum* oocysts.

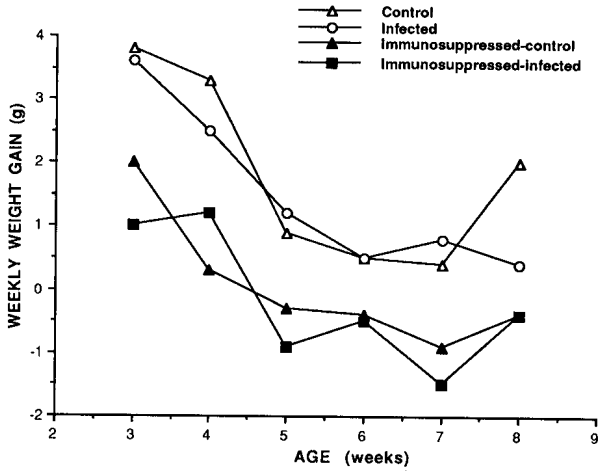

Fig. 5. Weekly weight gain in immunocompetent and cyclophosphamide immunosuppressed mice infected with 10^6 *Cryptosporidium parvum* oocysts, and in uninfected controls.

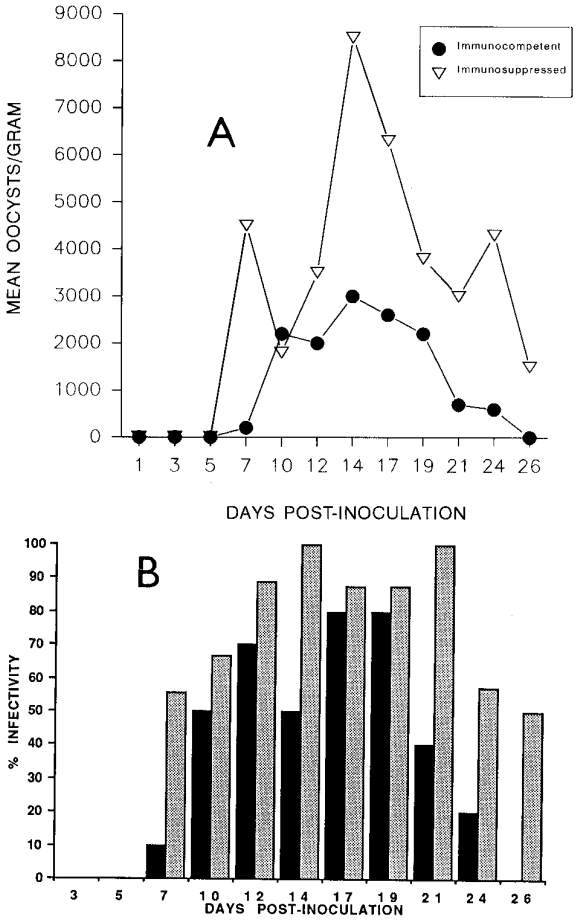

Fig. 4. *Cryptosporidium parvum* infection in immunocompetent and cyclophosphamide-immunosuppressed mice. **A** – kinetics of mean oocyst shedding after infection with 10^6 oocysts. **B** – percentage of infectivity during the experimental period. Black bars: Immunocompetent. Shaded bars: Immunosuppressed

and also between uninfected control mice and immunosuppressed uninfected mice ($p = 0.001$). No differences were found between uninfected and infected either in the group of immunosuppressed mice or in immunocompetent mice. The 44.4% of immunosuppressed infected mice (4 out of 9), and 30.0% of immunosuppressed uninfected mice (3 out of 10) died. The differences were not statistically significant ($p > 0.1$). None immunocompetent mice died during the experiment.

DISCUSSION

The studies undertaken in mice thus far published indicate that most strains can be infected with *Cryptosporidium parvum*, with varying degrees of success (Tzipori et al. 1980, Sherwood et al. 1982, Heine et al. 1984, Current and Reese 1986, Ernst et al. 1986, Harp et al. 1988, Moon et al. 1988, Tarazona-Lafarga and Dominguez-Carmona 1988, Enriquez and Sterling 1991). However, variations in oocyst shedding profiles are less well established.

This study demonstrates that, while both BALB/c and Porton mice are susceptible to infection, prepatent period was significantly longer in BALB/c mice, though the mean oocyst output was greater for BALB/c mice the difference was not statistically significant. This difference in behaviour may be due to a variety of factors, including nutritional status, stress and genetic background as was already established in wild mice with greater severity of cryptosporidial infection compared to laboratory mice (Klesius et al. 1986).

Age-related resistance to infection has been demonstrated in several species, including mice, calves, and lambs (Sherwood et al. 1982, Harp et al. 1990, Ortega and Wright 1994, Upton and Gillock 1996). This

study demonstrates a difference between mice infected at 3 and 4 weeks of age; all became infected, but those infected at 3 weeks exhibited a shorter prepatent period, and shed oocysts for longer, than those infected at 4 weeks. Since IgA production in murine intestine is age dependant (Van der Heijden et al. 1988), this may be one of the reasons that make older mice more refractory to infection, the parasite being controlled by the development of specific local immunity. Harp et al. (1992) reported differences in susceptibility to infection between germ-free mice and those with a developed gut flora, possibly due to stimulation of the immune system to produce IFN by the microflora.

In the present study, there were no apparent differences in the susceptibilities of male and female mice to infection. We also analyse the effect of different infective doses on cryptosporidial infection. Opinions vary as to whether infection are dose-dependant or independant (Current and Snyder 1988, Tarazona-Lafarga and Dominguez-Carmona 1988). The fact that there were no significant differences in the infection produced by inocula ranging from 10^4 to 10^6 oocysts per mouse may be a reflection of the size of dose-chosen. ID_{50} values have been established for some mouse strains, and are substantially lower than the lowest of our chosen doses. Very low infective doses have been reported to extend prepatent period (Blewett et al. 1993).

In this report we used cyclophosphamide as an immunosuppressive drug and it induced a marked depletion of IgA in gut secretion and a more severe clinical course of cryptosporidiosis. Cyclophosphamide provokes a partial depletion of T cells and a marked B lymphopenia (Kerckhaert et al. 1974). In the present study, cyclophosphamide was administered orally by gastric tube to ensure the correct dosage. The dose of cyclophosphamide selected after a dose-response experiment (data not shown) was similar to that used in other experimental models (Rehg et al. 1987). Administration of the immunosuppressive protocol 10 days before inoculation with the parasite was sufficient to block IgA secretion. Mean oocyst counts were significantly higher in immunosuppressed mice, as was the patent period.

In previous studies, cyclophosphamide treated mice did not become more susceptible to *Cryptosporidium parvum* infection (Sherwood et al. 1982), while immunosuppressed rats developed chronic cryptosporidiosis (Regh et al. 1987). These differences may depend on animal species; however, our results in mice were similar to those reported in rats. It is known that some strains of mice are genetically resistant to cyclophosphamide (Hurme et al. 1980) which may explain the results obtained by Sherwood et al. (1982).

Our mice did not develop diarrhea unlike other reports in SCID mice (Mead et al. 1991, McDonald and Bancroft 1994), prednisolone treated mice (Tarazona - Lafarga and Dominguez-Carmona 1988) and nude mice (Heine et al. 1984, Ungar et al. 1990). Unlike reports in which *C. parvum* produces a severe chronic infection when cyclophosphamide is administered continuously in rats (Regh et al. 1987), our results showed that some mice could recover before the end of the experiment. Mortality in our study was linked to immunosuppression rather than to infection by *C. parvum*.

Local immunoglobulin secretion into intestine may play an important role in defending against the parasite. A relationship between intestinal IgA production and the course of cryptosporidial infection has been reported previously in other animals (Williams and Burden 1987, Hill et al. 1990, Peeters et al. 1992), and in mice (Tarazona et al. 1997). Human beings, as well as animals, infected with *C. parvum* develop a specific humoral immune response, both systemic and local. It remains unclear if these antibodies contribute to the elimination of infection or are necessary to protecting against reinfection. However, in our study, protective IgA immunity was not essential, since some mice were able to recover before cyclophosphamide-treatment was withdrawn.

We have shown here that mice are susceptible to infection by *C. parvum* and diferent aspects of infectivity have been analysed. Mice, because of their cost, availability, rapid rate of development, and the ready availability of immunological reagents constitute a useful model for cryptosporidial infection, particularly for drug efficacy screening. Further studies about cryptosporidiosis in immunosuppressed mice, reinoculation of immunosuppressed mice, and use of passive immunity, or immunomodulatory agents, will help in the development of an alternative therapy by improving local immune response against *Cryptosporidium*.

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