Response to re-infection with *Brachylaima cribbi* in immunocompetent and immunodeficient mice

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Abstract

The course of infection in C57BL/6J mice re-infected with *Brachylaima cribbi* was assessed by comparing faecal egg excretion of re-infected mice with age- and sex-matched mice receiving a primary infection only. For both male and female mice there was a significant reduction in the mean number of eggs per gram of faeces at the peak of infection 4 weeks after the challenge infection compared with mice receiving a primary infection only. There was no significant difference in the duration of the infection. This experiment was repeated using age-matched male mice but on this occasion all mice were killed and dissected 4 weeks after the challenge infection and mean eggs per gram of faeces, worm burden and fecundity determined. There was no significant difference in the worm burdens of the re-infected mice compared with age-matched animals receiving a primary infection only. However, there were significant differences in the mean faecal eggs per gram and worm fecundity with the challenge infection group having lower egg counts and reduced fecundity. An enzyme-linked immunosorbent assay using whole worm antigens was developed and used to determine mouse anti-*B. cribbi* serum antibody levels during the course of infection. Anti-*B. cribbi* serum antibody absorbance ratios increased six- to sevenfold by 4 weeks after a primary infection beyond which a constant level was maintained. The course of challenge infection in non-obese diabetic severe combined immunodeficient mice showed no significant differences in egg excretion, worm burden or fecundity when primary and challenge infections were compared. These results indicate that the immune response invoked by a previous *B. cribbi* infection in immunocompetent mice affects fecundity but does not affect the establishment or duration of infection.

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1. Introduction

*Brachylaima cribbi* (Digenea: Brachylaimidae) is a terrestrial fluke worm infecting mammals and birds in South Australia [1–3]. Humans have also been reported as incidental hosts with both children and adults being infected by eating helicid and hygromiid land snails harbouring metacercariae of *B. cribbi* ([4,5], unpublished observations). The clinical report of two *B. cribbi* infections in children from the Yorke Peninsula in South Australia showed an infection which persisted in one child...
for 8 months [4]. The second child was reported to have gastro-intestinal symptoms of 2 weeks duration prior to being hospitalised for congestive cardiac failure. These two cases showed that *B. cribbi* infections could result in a chronic infection of a child who was continually eating infected snails or produce an acute infection in a child who appeared to have only a limited exposure to infected snails. To our knowledge, since this report and that of an infection in an adult [5], there have been a further nine human infections consisting of two adults and seven children (unpublished observations). In the majority of these cases, symptoms, predominantly diarrhoea and abdominal discomfort, persisted for greater than 4 months before diagnosis (unpublished data). As *B. cribbi* is a relatively recently described infection in humans, the effects of continual exposure and ingestion of infected snails are unknown.

In our studies of the life cycle and host--parasite relationships of *B. cribbi* we have found that C57BL/6J mice were the most susceptible laboratory mouse strain and provide a suitable definitive host model [6]. In these mice, egg counts in *B. cribbi* infections peaked after 4 weeks then resolved spontaneously by 10 weeks or so. Also, older mature female C57BL/6J mice were more resistant to a primary *B. cribbi* infection and showed reduced worm burden, fecundity and egg fertility when compared with mature males and adolescent females or males [7]. On the other hand, we have also shown that infections persist indefinitely in non-obese diabetic severe combined immunodeficient (NOD SCID) mice with no significant differences in egg excretion, worm burden or fecundity among mice infected for 4, 8 or 18 weeks [7]. These results indicated that worm expulsion in C57BL/6J mice was mediated by an immune response but it is unknown if this is a protective response that can prevent or modulate secondary and subsequent infections.

We have now used these immunocompetent and immunodeficient mice to study the course of a challenge infection in order to confirm that prior exposure stimulates the development of specific, acquired immunity in immunocompetent but not immunodeficient animals previously infected with *B. cribbi*. To our knowledge, there are no other reports of studies of this nature with brachylaimids. Examination of this host--parasite response may help us understand the potential effects of re-infection in humans, especially in those young children who have a habit of eating live land snails.

### 2. Materials and methods

#### 2.1. Parasite, animals and inoculation procedure

*B. cribbi* metacercariae were obtained from experimentally infected laboratory-reared *Helix aspersa* from the laboratory life cycle established by Butcher and Grove [3]. For each experiment, metacercariae were dissected from the kidneys of infected snails, washed and placed in a single pool in distilled water. Seven-week-old male and female C57BL/6J mice were obtained from the University of Adelaide Laboratory Animal Services, Adelaide, South Australia. Mice were housed in standard animal house conditions with water and food available on demand. Seven-week-old female non-obese diabetic severe combined immunodeficient NOD/Lt-Prkd<sup>scid</sup> (NOD SCID) mice were obtained from the Animal Resource Centre, Perth, Western Australia. These mice were barrier housed in clean room conditions with water and food available on demand.

For each experiment, mice were inoculated on the same day from the same pool of metacercariae. Fifteen metacercariae were given to each mouse by intra-oesophageal inoculation using a blunt lumbar puncture needle. After inoculation the syringe and needle were rinsed with distilled water and the water examined microscopically for any residual metacercariae to ensure each mouse received 14–15 metacercariae. Any shortfall in inoculation for any of the mice was made-up by a second inoculation with the required number of metacercariae.

#### 2.2. Faecal egg counts, worm burden and fecundity

Counts of eggs per gram of faeces were determined at weekly intervals using the filter/sedimentation/diethyl ether sedimentation technique described elsewhere [6]. At the conclusion of each
experiment mice were killed by cervical dislocation and the intestinal tract removed and opened longitudinally. *B. cribbi* worms were located macroscopically or with the aid of a dissecting microscope and counted. Worm fecundity was calculated as the mean number of eggs per gram of faeces per worm.

2.3. *B. cribbi* mouse serum antibody assay

An enzyme-linked immunosorbent assay (ELISA) for mouse serum antibodies against *B. cribbi* was developed by modifying the method reported by Brunet et al. [8] for *Echinostoma caproni* in mice. Briefly, whole worm antigen (WWA) was prepared using 4–7 week old *B. cribbi* worms collected from laboratory-infected C57BL/6J mice. Worms were washed in sterile phosphate buffered saline (PBS) pH 7.2 and placed in RPMI-1640 cell culture media (Gibco, Paisley, UK) supplemented with 10 g/l glucose, 25 mmol/l HEPES (ICN, Aurora, OH), 30 mg/l penicillin, 100 mg/l gentamicin and 25 mg/l amphotericin B and incubated at 35 °C in 5% CO₂ for 24 h. Worms were recovered from RPMI-1640 culture medium, washed twice with sterile PBS and stored at −70 °C in groups of 30–40 worms. A total of 147 worms were thawed and homogenised in sterile PBS then centrifuged at 10 000×g for 1 h at 4 °C. Supernatant fluid was recovered, centrifuged again for 30 min and then sterilised by passage through a 22 μm filter. An aliquot was removed and the total protein level determined. The filter-sterilised PBS-soluble WWA was then diluted with sterile PBS to a total protein level of 100 μg/ml and stored in 1 ml aliquots at −20 °C.

Stock 100 mg/ml WWA was thawed, diluted to 10 μg/ml in 0.5 M sodium carbonate buffer (pH 9.6), then 100 μl was added to each well of the required number of Nunc PolySorb U16 microtitre plate strips (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The plates were then washed four times with 0.05% Tween–PBS (T–PBS) and then blocked with 1% casein–PBS (C–PBS) for 30 min at 35 °C. Following incubation, plates were washed four times with T–PBS, then 100 μl of 1:150 dilution of test or negative pooled mouse serum diluted in C–PBS was added to the appropriate wells and incubated at room temperature for 90 min. After four further washes with T–PBS, 100 μl of peroxidase-conjugated affinity-purified goat anti-mouse IgG, IgA, IgM heavy and light chain conjugate (Rockland Inc., Gilbertsville, PA) diluted 1:14 000 in C–PBS was added to all wells and incubated for 30 min at 35 °C. Assay wells were then developed for 15 min at room temperature with the substrate tetramethylbenzidine (Sigma, St. Louis, MN) and read at 450 nm using an ETI-Lab immunoassay analyser (Sorin Biomedica, Saluggia, Italy). The means of duplicate test and quadruplicate negative control sera were used to calculate the absorbance ratio of test sera compared with the negative control mouse serum.

3. Results

3.1. Re-infection of male and female C57BL/6J mice

Two groups of 6 male or 6 female C57BL/6J mice were each infected with a primary *B. cribbi* inoculation (infection A). A further two groups of 6 uninfected age- and gender-matched control mice were maintained in standard animal house conditions. Faecal egg counts were determined weekly. Nine weeks after infection when all mice receiving a primary infection were excreting no or very low numbers of eggs, these mice received a second inoculation (infection A+B) and the uninfected age- and gender-matched control mice were given a primary inoculation (infection B). Egg counts were then determined for 10 weeks after the second infection. Both male and female C57BL/6J mice showed a similar response when primary and secondary infections were compared (Fig. 1). There were no significant differences between the peak egg counts 4 weeks following primary infection at time points A or B or in the duration of infections. However, both male and female mice receiving a secondary infection challenge had significantly lower peak egg excretion levels compared to mice receiving a primary infection only at time point B (P <0.05 Mann–Whitney U-test). There were no significant differences in the num-
Fig. 1. Faecal egg counts as mean eggs per gram ± S.E.M. in (a) male and (b) female C57BL/6J mice at weekly intervals after receiving primary and secondary infection [A+B] or primary infection only [B] with 15 *B. cribbi* metacercariae per mouse per infection.
ber of worms recovered at dissection from either primary or secondary infected mice 10 weeks after infection B; only one worm was recovered from each of the male and female groups receiving the challenge infection and 2 worms from each of the male and female groups receiving the primary control infection.

In a second experiment the same procedure described above was repeated but on this occasion only male mice (6 per group) were infected and the animals were killed and dissected 4 weeks after the challenge infection. The mean faecal eggs per gram, worm burden and fecundity were determined for all mice. As was observed in the first challenge infection those mice receiving a secondary infection had a significantly lower faecal egg count ($P < 0.05$ Mann–Whitney $U$-test) compared with the age-matched mice receiving only a primary infection (Fig. 2). However, there was no significant difference in the worm burden but there was a significant difference in fecundity. Mice receiving a re-infection challenge produced $83 \pm 103$ S.D. eggs per gram of faeces per worm compared with $296 \pm 127$ S.D. for mice receiving a primary infection only ($P < 0.05$ Mann–Whitney $U$-test).

During the course of infection, tail vein blood was collected at weekly intervals from all infected mice and $B. cribbi$ serum antibody levels were determined. There was a rapid response to a primary infection with an increase of two- to threefold in absorbance ratios at 2 wpi (Fig. 3).
Fig. 3. Mean anti-*B. cribbi* serum antibody absorbance ratios of male C57BL/6J mice at weekly intervals after primary plus secondary infections [A + B] or primary infection only [B] with 15 *B. cribbi* metacercariae per mouse per infection. Antibodies detected by ELISA using crude PBS-soluble *B. cribbi* WWA.

At 4 wpi absorbance ratios reached levels six- to sevenfold higher than that of uninfected mice and these levels were maintained for the duration of the infection. No further increase in titre was produced by a challenge infection.

3.2. Re-infection of NOD SCID mice

Two groups of 10 age-matched female NOD SCID mice were inoculated with 15 *B. cribbi* metacercariae (infection A). Ten weeks later, one of these groups plus a third age-matched control group were inoculated with 15 *B. cribbi* metacercariae (infection B). Faecal egg counts were determined at weekly intervals for all surviving mice. During the course of infection 2 of the 10 mice from each of the groups receiving infection A only or infection A + B and 1 of the 10 mice receiving infection B only died. Post-mortem examinations of these mice showed that they died from overwhelming tumour growth and not from the effects of *B. cribbi* infection. All remaining mice were killed and dissected at 7 weeks after the challenge infection (infection B) and the mean number of eggs per gram of faeces, worm burden and fecundity were determined. Egg counts fluctuated greatly during the course of both primary and secondary infection (Fig. 4). There were no significant dif-

Fig. 4. Weekly faecal egg counts of NOD SCID mice after receiving primary infection only [A], primary infection only [B] or primary plus secondary infection [A + B] with 15 *B. cribbi* metacercariae per mouse per infection.
Fig. 5. (a) Mean worm burden and (b) fecundity in NOD SCID mice dissected 17 weeks after receiving primary infection only [A] or 7 weeks after receiving either a primary infection only [B] or primary plus secondary infection [A+B] with 15 B. cribbi metacercariae per mouse per infection. Bars = ± S.E.M.

ferences in egg counts among any of the groups (one-way ANOVA with Tukey’s multiple comparison post-test). There were no clear peaks in egg counts although there was a general increase in egg excretion from 2 to 8 wpi before the counts stabilised. Animals receiving a secondary infection at 10 weeks after infection A showed a slight boost in egg counts but they were not significantly different when compared to age-matched animals receiving primary infections at times A or B. Dissection of surviving mice 7 weeks after challenge infection showed higher worm burdens in those animals receiving a secondary infection although this was not statistically significant, nor was there a significant difference with the worm burdens of those mice receiving a primary infection only (Fig. 5). Finally, there were no significant differences among the three groups in worm fecundity.

4. Discussion

There have been few studies investigating the response of definitive host animals to challenge infections with intestinal trematodes. The majority of work in this field over the past three decades has focused on various species of Echinostoma [8–12]. The driving forces behind these studies are that echinostomiasis is a food-borne zoonotic disease which can infect humans and that the life cycle of these parasites can be maintained in the laboratory. For similar reasons we have investigated aspects of the kinetics of primary and secondary infections with B. cribbi in mice.

The first experiment, investigating the response to primary and secondary infections in male and female C57BL/6J mice, showed that the course of infection followed a similar pattern in both sexes. However, at all time points during the infection when age-matched mice receiving a primary or secondary infection were compared, male mice always had a higher, although not statistically significant, level of egg excretion. This supports our previous observations that male mice are more susceptible to B. cribbi infection than are female mice [7]. Comparison of age-matched mice receiving a primary vs. a secondary infection showed significantly lower egg excretion levels in reinfected animals for both males and females, indicating that the immune response has a similar effect on the course of a secondary infection in both sexes.

The reduction in egg counts after a secondary infection could be due to one or a combination of factors such as: the inability of metacercariae to establish an infection; the enhanced expulsion from the gut of adult worms capable of producing eggs; and/or a reduction in the number of eggs released by adult worms in immune animals. In male mice at least, we have shown that reduced faecal egg counts are mainly due to a reduction in fecundity rather than a reduction in worm burden or expul-
sion of worms early in the course of infection. Later in the infection, adult worms are expelled but the speed of that expulsion does not appear to be accelerated by a prior infection. Inhibition of fecundity after challenge infections has been demonstrated in various nematode, cestode and trematode infections [13–17]. Vaccine studies in sheep with purified cysteine proteinase complex which is present in the regurgitant fluids of adult Fasciola hepatica significantly decreases fecundity in challenged sheep but had no effect on worm burden [18]. Chauvin et al. [19] investigated the humoral and cellular immune responses to F. hepatica primary and challenge infections in sheep. They reported that the animals receiving a secondary infection demonstrated a reduction in humoral response, especially against ‘early recognised antigens’. There was no anamnestic response in animals receiving a secondary infection with IgM levels remaining high, as in animals infected only once but that the IgG levels decreased to below that of animals receiving a primary infection only. The authors surmised that modulation of the immune response after a second infection could allow flukes to escape specific immune mechanisms allowing quicker growth and development of worms compared with a primary infection. Such an altered immune response could be responsible for the change in fecundity of F. hepatica in immunised sheep reported by Wijffels et al. [18]. Studies of schistosomes have also demonstrated anti-fecundity immunity in response to secondary infection or vaccination. Studies of human immune responses to S. mansoni recombinant 28 kDa glutathione-S-transferase (Sm28GST) antigen showed that IgA antibodies to this antigen impaired fecundity [20]. Other studies in mice have also shown similar anti-fecundity responses to S. bovis 28 kDa GST monoclonal antibodies with impairment of both fecundity and egg viability [21]. Similarly, pigs vaccinated with a recombinant Schistosoma japonicum antigen displayed impaired fecundity [22]. Likewise, monoclonal antibodies raised against S. mansoni Sm28GST transferred passively to mice infected with S. mansoni impaired fecundity as a result of inactivation of the enzyme [23,24]. The similar anti-fecundity effects observed among the various classes of helminths suggest that the reduced fecundity observed in B. cribbi challenge infections is a general immunological response to common helminth antigens.

Further studies are required to elucidate the mechanisms involved in B. cribbi anti-fecundity effects after a secondary infection in mice but it is likely that IgA-mediated humoral immunity at the mucosal surface is important. In addition, further studies of cell-mediated immune responses following infection are required. For example, Brunet et al. [8] reported that establishment of a chronic infection in mice with the intestinal trematode E. caproni is associated with a Th1 immune response during the early stages of infection. Further delineation of the mechanisms of the immune responses could be achieved by observing the effects of transfer of immune and non-immune lymphocyte subsets in NOD SCID mice.

The observation of an anti-fecundity response in secondary B. cribbi infections in mice has potential implications for human infections and laboratory diagnosis. If a similar response is invoked in human infections and the patients are continuously re-infecting themselves with B. cribbi, as could be the case in young children who have a habit of eating live land snails, worms could still establish infections and cause clinical symptoms but egg excretion levels may be below the level of laboratory detection. We may have observed this phenomenon in our diagnostic laboratory in patients who have presented with typical symptoms and a history of eating land snails but laboratory testing was unable to confirm any infection (unpublished data). It is in these patients that a sero-diagnostic test would have great utility. The present studies have shown that infections with B. cribbi in mice induce antibodies, which can be detected in an ELISA. However, it must be recognised that this preliminary assay was developed in mice that had no other helminth infections. It, therefore, is a measure of sensitivity rather than specificity. Further studies need to be carried out with mice infected with various trematodes, cestodes and nematodes in order to delineate specificity. In terms of human infections in our population, cross-reactivity with other helminths is unlikely to be a problem as helminth infections in this area.
with the exception of enterobiasis, are most uncommon [25]. Nevertheless, antigen purification, optimisation and assessment of cross-reactivity with other human helminths would be required before any ELISA could be considered for human sero-diagnosis.

Clearly, humoral and/or cell-mediated immunity are central in mediating resistance, for when infections in immunodefinient NOD SCID mice were compared with immunocompetent C57BL/6J mice there was a failure to show inhibition of egg excretion after challenge infections. NOD SCID mice demonstrated erratic egg excretion over the course of infection with no clear peaks and the infection persisting for the life of the animal. This was not observed in C57BL/6J mice as egg excretion always peaks at 4–5 wpi followed by a decline in egg counts until worms are expelled at approximately 10–17 wpi [6,7]. The fluctuation in faecal egg counts observed in NOD SCID mice could be related to the natural variation in egg release, the influence of the animals’ physiology on egg production as well as faecal consistency and excretion volume. Fluctuation in egg excretion in immunocompetent animals is well recognised. For example, comparisons of egg counts and stability of egg excretion of Clonorchis sinensis in six strains of mice showed that there is significant variation in egg counts among the different strains of mice [26]. Similarly, marked fluctuations were noted, even on the same day, in egg counts from pigs infected with S. japonicum [27]. Studies of S. japonicum and S. mansoni infections in SCID mice showed that there is a delay in the initial production of eggs when compared with fecundity in immunocompetent mice; this is independent of TNF-alpha [28]. In our NOD SCID mice there were no observed differences in faecal consistency during the experiment with stool samples collected at the same time each week. As NOD SCID mice produce no humoral or cellular-mediated immune response, the fluctuations in faecal egg counts most likely represent the natural variation in egg excretion of B. cribbi in these animals. There was a trend towards a higher worm burden in NOD SCID mice given two infections but this was not statistically significant; it is likely that this is a reflection of the small number of mice in each group. Although there were no significant differences in fecundity among the three groups of NOD SCID mice, there was a trend towards a higher fecundity in mice given infections at the second time point (infection B), suggesting that egg production may have waned somewhat after the worms had aged a few months.

In conclusion, this study has shown that a challenge infection with B. cribbi in immunocompetent mice but not in immunodeficient mice significantly affects the fecundity of the worms but does not affect the duration or establishment of infection. This has potential implications in the diagnosis of human infections where there is a reliance on the detection of B. cribbi eggs in faecal specimens.

References


