Clin Exp Immunol 1994; 95:485–489

Antimicrobial action of antibodies against *Giardia muris* trophozoites

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(Accepted for publication 4 November 1993)

SUMMARY

The activities of immune serum and trophozoite-specific MoAb were examined *in vitro* and *in vivo*. Immune serum and anti-*Giardia muris* MoAb caused immobilization of the trophozoites *in vitro* and were cytotoxic for trophozoites in the presence of exogenous complement. Both immune serum obtained from experimentally infected mice and anti-*G. muris* MoAb administered directly into the duodenum of mice significantly reduced the number of trophozoites in the small intestine during the acute phase of the infection. These results suggest that serum antibodies play a central role in the elimination of the primary *Giardia* infection.

Keywords *Giardia muris* polyclonal and monoclonal antibodies immobilization lysis

INTRODUCTION

Original observations that hypogammaglobulinaemia predisposes patients to chronic giardiasis indicate that humoral immunity contributes to protection against this parasite [1,2]. Since then, evidence has accumulated supporting an integral role of antibodies in the clearance of primary infection and resistance to subsequent exposure to *Giardia* (reviewed by denHollander *et al.* [1]). We know that *Giardia*-specific immunoglobulins are present in intestinal secretions and on trophozoites [3–7]. Chronic infections occur in patients suffering from hypogammaglobulinaemia [2] and in experimentally infected mice treated from birth with anti-IgM and in *xid* mice [3,8].

Circulating and secretory anti-*Giardia* antibodies have been identified in animals and humans [3,5,9–12]. Snider *et al.* [3] detected anti-parasite serum IgG and IgA, but no IgM in BALB/c and C57Bl/6 mice during the course of the infection. Of the three immunoglobulins, only anti-*G. muris* IgA was present in gut secretions. They concluded that IgA antibody may be the dominant and possibly only effector antibody active in intestinal secretions of mice. In contrast, Heyworth [5] detected IgA and IgG, but not IgM, bound to the surface of trophozoites harvested from the intestinal lumen of immunocompetent BALB/c mice on day 10 after infection. The percentage of trophozoites with either IgA or IgG bound to their surface was significantly higher in immunocompetent mice than in hypothymic (nude) mice [5]. The presence of monomeric IgG on the surface of trophozoites suggests that this immunoglobulin must gain entry into the small intestine during the course of *G. muris* infection. Gillon *et al.* [13] and Butscher & Faubert [14] suggested, but have not established, that serum antibodies gain access to the intestinal lumen by leakage caused by the damage to the intestinal mucosa during the acute and elimination phases of the infection.

MoAbs against *Giardia* kill trophozoites in the absence [15] or presence of complement [14]. A number of studies demonstrate the protective effects of passive administration of anti-parasite MoAbs to experimental animals [16–19].

To date there has been only one study on the *in vivo* effects of MoAbs in giardiasis. Intraperitoneal injection of a cytotoxic MoAb against *G. muris* resulted in nearly three-fold fewer trophozoites on day 8 of infection in mice [14]. We examined the activity of anti-*G. muris* MoAb *in vitro* and *in vivo*. This anti-*Giardia* MoAb reduced the viability of trophozoites of *G. muris* *in vitro*, and significantly reduced the trophozoite burden in the small intestine of infected hosts when administered intraduodenally.

MATERIALS AND METHODS

Parasite

Giardia muris was originally isolated by Roberts-Thomson *et al.* [20]. The parasite was maintained by 20-day passages in mice.

Animals

Inbred 8–10-week-old BALB/c and C3H/HeN mice were purchased from Charles River Breeding Labs (St. Constant, Quebec, Canada). Upon arrival from the animal breeders, the animals were housed in a specific pathogen-free facility (P-2 level) of the Department of Zoology, University of Alberta, Canada. The protocols and procedures for the maintenance of a *Giardia*-free animal colony have been described previously [21,22].

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Isolation of cysts and infection of mice

Cysts were isolated from the faeces using a sucrose gradient centrifugation technique and were enumerated using procedures described previously [22]. The infection dose in all experiments was 10^4 cysts/mouse suspended in PBS and administered orally to unanaesthetized mice.

Isolation and purification of G. muris *trophozoites*

Trophozoites used in the *in vitro* assays and immunization of mice were isolated from mice infected with *G. muris* 7 days previously. Mice were killed by cervical dislocation and the upper 60% of the small intestine was removed and cut into 2–3 cm pieces. Each piece of the intestine was cut longitudinally, placed in an Erlenmeyer flask containing 100 ml of PBS and the solution was incubated at room temperature for 30 min with stirring. After the incubation, the solution containing the trophozoites was passed in succession through 20 layers of moist cheese cloth, 180-μm, 100-μm and 50-μm standard brass sieves (Endecotts Ltd., London, UK). The trophozoites that passed through the sieves were transferred to glass Petri dishes containing PBS and incubated for 1–2 h at 37°C in 5% CO_2 atmosphere to allow for trophozoite attachment to the glass. After the incubation, the supernatant was removed and the parasites washed once with warm PBS. Petri dishes containing attached trophozoites were placed on ice for 15 min to detach the organisms, and the material from several vessels was pooled. The total number of trophozoites was determined using a haemocytometer and the number of trophozoites adjusted to 1×10^5/ml.

Preparation of immune serum

BALB/c mice were infected orally with 1×10^4 cysts each of *G. muris*. Sixty days after infection, 0·3 ml of *G. muris* trophozoite-soluble extract containing 0·1 mg of protein was administered to each mouse intravenously into the tail vein. Ten days after the administration of the trophozoite extract, mice were bled by cardiac puncture and the serum from 10 mice was pooled and placed at −80°C before use in the experiments. The titre of the anti-*G. muris* antiserum was 1:3200, and was determined by immunofluorescence assay (IFA).

Preparation of monoclonal antibody

Parasite-specific MoAb was produced by Butscher & Faubert [14]. This IgG1 MoAb binds to the flagella, flagellar insertions as well as the surface of the trophozoites. The main targets for this MoAb is a Triton-soluble glycoprotein of an approximate mol. wt of 35000 D. The control MoAb used in the *in vivo* experiments was an anti-digoxin IgG1 designated as 159.4F.1.1. This MoAb did not bind to the trophozoites of *G. muris* as indicated by IFA and ELISA.

In vitro *assays*

The assays were done in 24-well tissue culture plates (GIBCO, Burlington, Ontario, Canada). One ml of PBS containing 1×10^5 trophozoites was added to each well, followed by known amounts of anti-*Giardia* MoAb or irrelevant control antibody. In certain experiments, 1:100 dilution of guinea pig serum (the source of complement) (Cedarlane, Hornby, Ontario, Canada) was added to the wells. The total volume in each well was adjusted to 1·5 ml by adding an appropriate amount of PBS. The plates were incubated for 1 h at 37°C in 5% CO_2 atmosphere. After incubation, the plates were chilled on ice for 15 min to detach the parasites from the walls of the culture vessels. The number of live trophozoites, as measured by microscopical examination for flagellar activity, was determined by counting two aliquots from each well using a haemocytometer.

In vivo *assay*

*Single intraduodenal administration of anti-*G. muris *antiserum and MoAb.* On day 7 after inoculation with *G. muris*, 4–6 mice/experimental group were anaesthetized by i.p. injection of sodium pentobarbital solution (0·06 mg/g body weight). The abdominal wall was cut along the linea alba and the duodenal portion of the small intestine elevated, for injection, with a surgical hook. For experiments where immune serum was used, 0·2 ml of immune serum or normal serum was injected directly into the duodenum. In other experiments, different concentrations of anti-*G. muris* or anti-digoxin IgG1 MoAb (0·1–1 mg/ml), suspended in 0·2 ml PBS, were injected directly into the lumen of the duodenum. The muscle layer of the body wall and the skin were closed with black braided 3-0 silk (Ethicon Inc., Somerville, NY).

To enumerate the number of trophozoites after the intraduodenal injection of MoAb, mice were killed 16–20 h after injection of MoAb, the entire small intestine was removed and placed on a dissecting board, and the intestine was cut in four equal sections. Each section was slit longitudinally and placed in 12 × 100 mm plastic tubes containing 6 ml of PBS (sections 1 and 2) or 3 ml of PBS (sections 3 and 4). The tubes containing the sections were incubated at 37°C in a shaker bath (100 cycles/min) for 2 h. After incubation, the tubes were chilled on ice for 15 min to detach the trophozoites which adhered to the wall of the tube. Intestinal sections were then removed from each tube using wooden applicator sticks, and the tubes containing the trophozoites kept on ice until the total number of trophozoites was determined using a haemocytometer. The lower limit of detection of trophozoites in each section of the small intestine was 5×10^3 organisms.

Multiple intraduodenal injections of MoAb. The MoAb was administered using a cannulation procedure. On day 7 after infection, mice were anaesthetized with sodium pentobarbital (0·06 mg/g body weight) and the duodenum was removed as described above. Medical-Grade Tubing (6 mm i.d. × 8 mm o.d.; Dow Corning Silastic tubing) was inserted to 1 cm into a small cut made approximately 1 cm below the pyloric sphincter in the wall of the duodenum. The tube was anchored with black braided 6-0 silk (Ethicon) to the intestinal wall by a collar made of a 1 mm length of tubing. Exiting the abdominal cavity through the abdominal incision, the tube was guided under the skin and through a small incision made at the nape of the neck. Anchors were also made to the abdominal wall and skin. The MoAb at a concentration of 1 mg/ml suspended in 0·2 ml of PBS was injected via the cannula every 12 h (9:00 p.m. and 9:00 a.m.) starting after recovery from surgery on day 7 until day 9 after infection. Four injections of the antibody were given to each mouse. An evaluation of the total number of trophozoites isolated from each section of the small intestine was done as described above.

Statistical analysis

The data were analysed by one-way ANOVA and Student's *t*-test using Statview SE + Graphics software (Abacus Concepts)

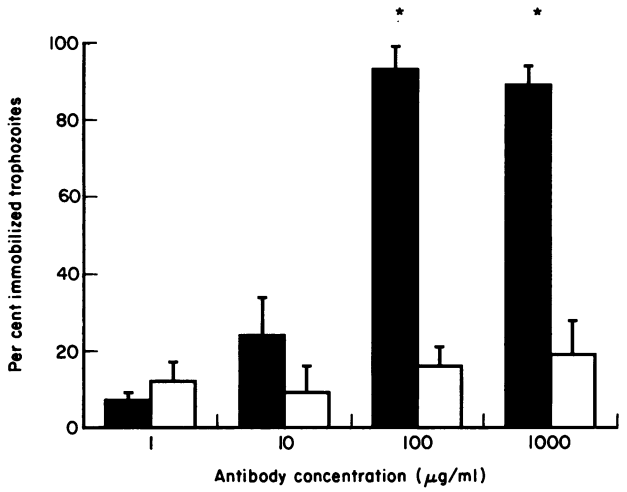

Fig. 1. Immobilization of *Giardia muris* trophozoites *in vitro* by parasite-specific F623.A20 IgG1 MoAb. Each bar represents the per cent immobilized trophozoites (mean ± s.e.m.) of three replicates per group. The data are from a representative experiment out of six. Student's *t*-test (*$P < 0.05$ or lower). ■, F623.A20 IgG; □, anti-digoxin IgG.

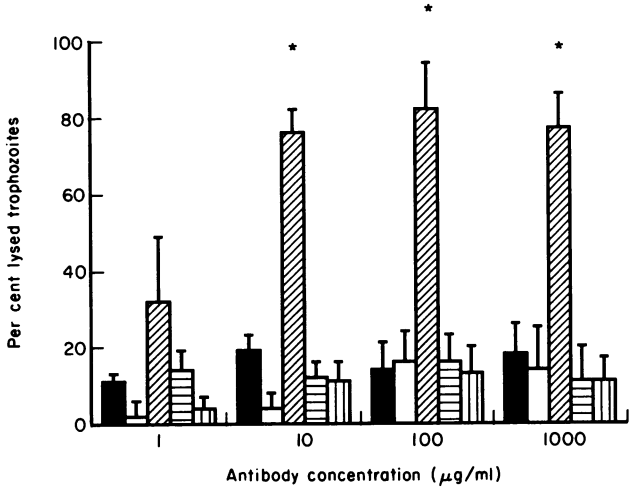

Fig. 2. Complement-dependent lysis of *Giardia muris* trophozoites *in vitro* by parasite-specific F623.A20 IgG1 MoAb. Each bar represents the per cent lysed trophozoites (mean ± s.e.m.) of three replicates per group. The data are from a representative experiment out of four. Student's *t*-test (*$P < 0.05$ or lower). ■, F623.A20 IgG; □, anti-digoxin IgG; ■, F623.A20 IgG + complement; ■, anti-digoxin IgG + complement; ■, complement.

on a Macintosh computer. Probability level of $P < 0.05$ was considered significant.

RESULTS

In vitro *anti-parasite effects of anti-*G. muris *MoAb*

We have previously reported that serum obtained from mice naturally infected with *G. muris* immobilized trophozoites, and in the presence of complement lysed the parasites *in vitro* [23]. To determine whether F623.A20 MoAb immobilized trophozoites *in vitro*, we incubated them in medium containing different amounts of the anti-*G. muris* MoAb and control anti-digoxin MoAb (Fig. 1). At concentrations from 100 μg/ml to 1000 μg/ml, we observed significant agglutinating activity of F623.A20 MoAb. This anti-*G. muris* MoAb also exhibited significant cytotoxic activity when complement was added to the cultures (Fig. 2). These data indicate that F623.A20 had both immobilizing and cytotoxic activity for *G. muris* trophozoites *in vitro*, similar to that observed for serum from naturally infected mice.

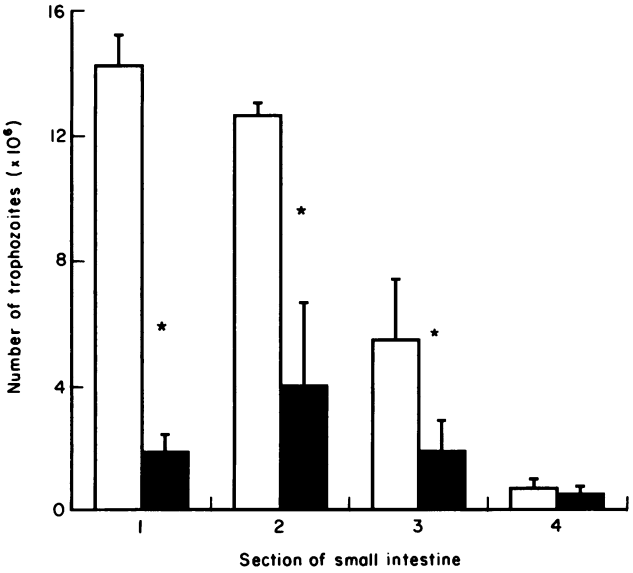

Fig. 3. The number of trophozoites in four equal sections of the small intestine of C3H/HeN mice infected with *Giardia muris* and treated on day 7 after infection with parasite-specific F623.A20 MoAb (■) or an irrelevant antibody (anti-digoxin, □) of the same isotype (IgG1). Each bar represents the mean number of trophozoites of 4–6 mice per group ± s.e.m. This experiment was done three times with similar results. Student's *t*-test (*$P < 0.05$ or lower).

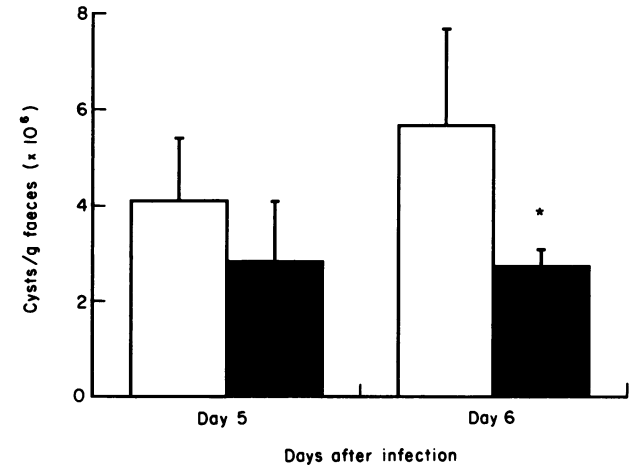

Fig. 4. Cyst output of C3H/HeN mice infected with 1×10^4 cysts each of *Giardia muris* and injected intraduodenally with either F623.A20 (■) or anti-digoxin MoAb (□) 24 h after infection. Each bar represents the mean number of cysts released ± s.e.m. of four mice per group. Student's *t*-test (*$P < 0.05$).

In vivo *anti-parasite effects of MoAb*

*The effects of anti-*G. muris *anti-serum on the course of infection in mice.* Preliminary experiments showed that the oral

administration of the immune serum or anti-*G. muris* MoAbs did not affect the course of infection as judged by the trophozoite load in the small intestine 12–24 h after treatment. For this reason we decided to administer anti-parasite antibodies by intraduodenal injection. Single intraduodenal administration of immune serum obtained from infected and immunized mice resulted in a significant decrease in the number of trophozoites 20 h after injection, compared with the number of parasites in the small intestines of mice injected with normal serum. For example, the overall mean total number of trophozoites/mouse was $18{\cdot}8 \pm 3{\cdot}4 \times 10^6$ and $4{\cdot}6 \pm 2{\cdot}4 \times 10^6$ for the resistant BALB/c mice treated with normal and immune mouse serum, respectively. A decrease in the number of trophozoites was observed in 5/6 experiments. Typically, the reduction in the trophozoite number was 60–90% following the administration of the immune serum, and was similar in both resistant BALB/c and the susceptible C3H/HeN mice. To ascertain whether the reduction of the trophozoite load in the small intestine was due to the anti-parasite antibodies and not due to non-specific components found in the serum, we examined the activity of anti-*Giardia* monospecific antibody administered intraduodenally to mice during the acute phase of the infection.

*The effects of anti-*G. muris *MoAb on the course of infection in mice.* Single administration of 1 mg/mouse of anti-*G. muris* MoAb into the lumen of the small intestine resulted in a statistically significant reduction in the total trophozoite burden in the small intestine. A 68% reduction in the total number of trophozoites was observed in mice given the F623.A20 MoAb, compared with that in animals injected with the irrelevant anti-digoxin MoAb. Mice treated with F623.A20 MoAb had $4{\cdot}6 \pm 0{\cdot}7 \times 10^6$/mouse, and those treated with the control anti-digoxin IgG had $14{\cdot}4 \pm 3{\cdot}9 \times 10^6$/mouse ($P < 0{\cdot}01$, one way ANOVA).

Multiple injections of F623.A20 MoAb resulted in a significant decrease (75%) in the mean number of trophozoites per mouse, which was $33{\cdot}1 \pm 2{\cdot}8$ and $8{\cdot}2 \pm 3{\cdot}5 \times 10^6$ for anti-digoxin IgG and F623.A20 IgG, groups, respectively. Control mice in this experiment had more than twice the number of trophozoites compared with that of control mice in single injection experiments. The reason for this may be stress and possible immunosuppression caused by the trauma associated with the surgical procedure to implant the cannula and with carrying the cannula for 48 h.

F623.A20-treated mice had significantly lower numbers of trophozoites in the first three sections of the small intestines compared with that in animals treated with the irrelevant MoAb (Fig. 3). No significant difference was observed between F623.A20 MoAb and anti-digoxin MoAb treatments on the number of trophozoites in the ileum of the small intestine (section 4).

The administration of F623.A20 MoAb 1 day after inoculation of *G. muris* cysts to mice also resulted in a significant decrease in cyst release during the acute phase of the infection (Fig. 4). On day 6 after infection, mice treated with F623.A20 released significantly fewer cysts/g faeces than those treated with control anti-digoxin MoAb, $2{\cdot}8 \pm 0{\cdot}3 \times 10^6$ and $5{\cdot}9 \pm 2{\cdot}1 \times 10^6$, respectively.

DISCUSSION

In the present study, we examined the activity of parasite-specific MoAb *in vitro* and *in vivo* against *G. muris*. In general, the results suggest a participatory role of parasite-specific antibodies in elimination of the trophozoites of *G. muris* from the small intestine. Of particular interest is the finding of antimicrobial activity of serum antibodies and parasite-specific MoAb in the lumen of the small intestine in murine giardiasis.

We have previously shown [23] that immune serum obtained from mice experimentally infected with *G. muris* has the ability to immobilize trophozoites *in vitro*, and in the presence of complement also lyses the parasites. In the present study we document that the immune serum has antimicrobial activity *in vivo*, when administered intraduodenally. These results indicate that serum antibodies that develop during giardiasis in animals [3,4,10,11], and possibly in humans [9,12], may participate in host defence provided that they can reach the lumen of the small intestine during the course of the infection. IgG has been identified on the surface of *G. muris* trophozoites during the acute phase of infection [5], suggesting that the antibodies can gain entry into the lumen of the small intestine. It should be noted, however, that other studies [3,4] have failed to detect serum immunoglobulins in the lumen of the small intestine during acute murine giardiasis. This discrepancy may be due to different strains of mice and methods used to measure immunoglobulins in the gut lumen.

To examine the antibody–parasite interactions and to eliminate the possible effects of serum components other than antibodies, we assessed the *in vitro* and *in vivo* effects of a parasite-specific MoAb against *G. muris* trophozoites using an established animal model system [20,22]. F623.A20 was very effective in immobilizing trophozoites of *G. muris*, but only in the presence of complement was cytotoxic for the trophozoites *in vitro*. Complement is not always required for the lysis of protozoans. For example, trophozoites of *G. lamblia* were killed by a MoAb within 7 min of exposure to the MoAb [15]. Killing has also been demonstrated against *Trichomonas vaginalis* trophozoites by parasite-specific MoAb in the absence of complement [24]. The precise mechanism of killing by parasite-specific MoAbs in the absence of complement is unknown, although it is known that the MoAbs against *Trypanosoma cruzi* affect viability by inhibiting the incorporation of nucleic acid precursors into culture forms of the parasite [25]. The agglutination of the flagella by MoAbs may kill parasites by affecting their attachment to critical substrates. More research is needed to define precisely the mechanism(s) of the cytotoxicity of MoAbs in the absence of complement.

To determine whether anti-*Giardia* antibodies influenced the course of the infection in animals, we administered them directly into their duodenum, because preliminary experiments showed that they lost activity if administered orally. When administered during the early course of the infection (within 24 h following parasite inoculation), the MoAbs decreased the number of trophozoites and cyst output in infected animals. This is the first report that immune serum or an anti-parasite MoAb can significantly reduce the intestinal burden of a lumen-dwelling protozoan parasite. However, MoAbs against *Escherichia coli*, administered orally into newborn calves before inoculation with bacteria, do cause a significant decrease in diarrhoea and mortality rate [26].

Although MoAbs will control *G. muris* infection, it is perhaps more relevant that immune serum obtained from mice infected with *G. muris* also possesses antimicrobial activity when injected into the intestine. In murine giardiasis, the susceptibility to a primary *G. muris* infection is consistently related to the

ability to mount an appropriate IgG serum antibody response, rather than a secretory IgA response [3,4,11]. For example, susceptible C3H/HeN mice, which suffer from chronic giardiasis, have a significantly higher secretory IgA level than resistant BALB/c animals, which eliminate the infection in 4–5 weeks [11]. Heyworth [5] demonstrated the presence of IgG on the surface of the trophozoites from the small intestine of mice, indicating that serum antibodies can breach the intestinal barrier. The intestinal barrier may be compromised if an inflammatory response accompanies the highest trophozoite burden in the small intestine during acute giardiasis. If inflammation and subsequent leakage of serum antibodies into the lumen of the small intestine occur during the acute phase of infection with *Giardia*, then one would expect mice that are resistant to *Giardia* to exhibit more robust inflammatory responses. This hypothesis is consistent with our previous observations that mice that are resistant to *G. muris* have a greater ability to mount *in vivo* inflammatory responses during the infection than animals which are susceptible to the infection [27]. The decrease in the trophozoite burden in the small intestine of mice treated intraperitoneally with anti-*G. muris* MoAb observed by Butscher & Faubert [14] is also consistent with this hypothesis. Cells involved in the inflammatory response may also play a part. For example, Erlich *et al.* [28] reported that mast cell-deficient W^f/W^f mice are susceptible to chronic giardiasis. It remains to be determined whether hosts that are resistant to *Giardia* have higher levels of parasite-specific serum antibodies in the lumen of the small intestine, a task that must await the development of more precise assays for the determination of antibody levels in the digestive tract.

ACKNOWLEDGMENTS

We thank Mrs Feng Wong and Mrs Cathie McDougall for expert technical assistance. This work was supported by grants from Natural Sciences and Engineering Council of Canada (NSERC) to G.M.F. and to M.B. and Alberta Heritage Foundation for Medical Research (AHFMR) grant to M.B. Research at the Institute of Parasitology is supported by NSERC and Fonds FCAR pour l'aide a la recherche.

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