Immunology 1986 **59** 269–275

Killing of *Giardia muris* trophozoites *in vitro* by spleen, mesenteric lymph node and peritoneal cells from susceptible and resistant mice

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Accepted for publication 3 June 1986

SUMMARY

The ability of spleen, mesenteric lymph node (MLN) and peritoneal exudate (PEC) cells from susceptible (A/J) and resistant (B10.A) mice to kill trophozoites *in vitro* was determined. Both duration of incubation and cell density influenced giardicidal activity. Maximal killing was observed after 6 hr of incubation at the effector to target ratios of 30:1 and 50:1. Cells isolated from A/J and B10.A mice during the elimination phase of the infection killed more trophozoites than those isolated from mice during the acute phase of the infection. Cells isolated from mucosal sites (MLN) of donors infected for 15 days killed more trophozoites *in vitro* than those isolated from systemic sites (spleen, PEC). There were no differences in the giardicidal activity of cells from susceptible and resistant mice. Killing of trophozoites was mediated by plastic-adherent cells with macrophage properties. Non-specific stimulation with thioglycollate and the presence of immune mouse serum enhanced the capacity of macrophages to kill parasites. There was no apparent relationship between the capacity of A/J and B10.A mice to mount cell-mediated effector responses and their ability to control the infection with *Giardia muris*.

INTRODUCTION

Host response to *Giardia muris*, a common enteric protozoan parasite of mice, may be modulated by immune factors. This was initially suggested by observations that prior exposure of mice to *G. muris* resulted in a long-lasting resistance to reinfection (Roberts-Thomson *et al.*, 1976a; Belosevic & Faubert, 1983a). In addition, immunodeficient nude (nu/nu) mice and mice treated with immunosuppressive drugs exhibited a characteristic inability to control the infection with *G. muris* (Stevens, Frank & Mahmoud, 1978; Roberts-Thomson & Mitchell, 1978; Nair, Gillon & Ferguson, 1981; Belosevic, Faubert & MacLean, 1986).

Macrophages have been identified as one of the effector cells in animal and human giardiasis. For example, Radulescu & Meyer (1981) showed that rabbit peritoneal macrophages have the capacity of engulfing opsonized trophozoites of *G. lamblia in vitro*. Smith *et al.* (1982) reported that human peripheral blood monocytes exhibit spontaneous cytotoxicity for *in vitro* grown trophozoites of *G. lamblia*. Using a mouse–*G. muris* model, Owen, Allen & Stevens (1981) have demonstrated that mucosal macrophages are capable of ingesting trophozoites *in vivo*. Recently, Kaplan *et al.* (1985) reported that mouse peritoneal macrophages killed *G. muris* trophozoites *in vitro*. They showed that phagocytosis and/or adherence to trophozoites by macrophages was enhanced in the presence of immune serum or immune milk containing anti-parasite IgG and IgA.

The objectives of this study were three-fold: (i) to compare cell-mediated effector responses of susceptible (A/J) and resistant (B10.A) mice and determine whether these responses could be related to the ability of these mouse strains to control the infection; (ii) since *G. muris* is a gut-dwelling parasite, we compared the efficacy of cells isolated from mucosa-associated lymphoid tissues (MLN) and those from systemic sites (spleen, PEC) to kill parasites *in vitro*; and (iii) to assess giardicidal activity of cells isolated from donors during the acute and elimination phases of the infection.

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MATERIALS AND METHODS

Mice

Eight-week-old female A/J and B10.A/SgSnJ (B10.A) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The procedures for the maintenance of a *Giardia*-free mouse colony have been described previously (Belosevic *et al.*, 1984).

Parasite

Giardia muris used in this study was originally passaged by Roberts-Thomson *et al.* (1976b) and was obtained from B. J. Underdown, McMaster University, Hamilton, Ontario. The parasite was maintained by 20-day passages in mice.

Isolation of cysts and infection of mice

Cysts of *G. muris* were isolated using sucrose gradient centrifugation technique and were enumerated using procedures described previously (Belosevic & Faubert, 1983b). The infection dose in all experiments was 10^3 cysts/mouse administered orally in 0·2 ml of 0·85% saline to unanaesthetized mice.

Isolation of trophozoites

Mice infected with *G. muris* for 7–14 days were killed and the entire small intestine was removed and stretched over a dissecting board. The intestine was cut into four equal sections and each segment was slit longitudinally and placed in 12 × 125 mm glass tubes containing 6 ml of phosphate-buffered saline (PBS, 0·01 M, pH 7·2). The tubes containing the segments were incubated for 1 hr at 37° in a shaker bath (100 cycles/min). After incubation, each segment was removed from the tube using wood applicator sticks and washed in cold PBS. The original suspension containing the trophozoites and the solution in which each segment was washed were combined. The tubes were chilled on ice for 10 min to detach trophozoites, which adhered to the wall of the tube. Trophozoites were purified by two passages through nylon-fibre columns. After purification, trophozoites were suspended in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, 25 mM HEPES, 2 g/litre sodium bicarbonate and 100 units/ml of penicillin–streptomycin. This mixture will be referred to as 'medium'. The total number of trophozoites was determined using a haemocytometer and the number of trophozoites adjusted to 1×10^5/ml.

Isolation of cells

Peritoneal exudate cells were harvested aseptically from individual mice by lavage of the peritoneal cavity using 10 ml of RPMI-1640 medium. The cells were washed once by centrifugation at 200 ***g*** for 10 min. The pellet was resuspended in a known volume of medium and the total number of viable cells/mouse was determined using the Trypan blue exclusion method of Hudson & Hay (1981). Differential counts were done on 300 cells from Giemsa-stained cytocentrifuge smears.

Spleen and mesenteric lymph node cells were isolated aseptically by gently sieving the organs through 80-mesh stainless steel screens. The cells were filtered through 30-mesh nylon screens and collected in RPMI-1640 medium. After isolation, cells were washed three times at 200 ***g*** for 10 min. The pellet was resuspended in a known volume of RPMI-1640 medium and the total number of viable nucleated cells was determined using a haemocytometer.

Separation of cells

Cells were separated into non-adherent and adherent subpopulations by adherence to plastic. After isolation, the cell number was adjusted to 2×10^6/ml. Two millilitres of the cell suspension were inoculated into sterile six-well flat-bottomed plates (Flow Laboratories, Toronto, Ontario). The plates were incubated for 120 min in 5% CO_2 atmosphere, and after incubation non-adherent cells were removed by washing three times with RPMI-1640 medium. The adherent cells were harvested using a rubber policeman and were washed once by centrifugation at 200 ***g*** for 10 min. The pellet was resuspended in known volume of medium and the total number of viable adherent cells was determined using a haemocytometer.

In vitro *killing assay*

An assay was developed in order to assess the mortality of trophozoites in the presence of different lymphoid cell populations. The assay was done in 24-well Linbro polystyrene plates (Flow). To each well, 1×10^5 trophozoites/0·5 ml and a known number of cells suspended in 0·5 ml were added. On each plate, 12 control wells contained 1×10^5 trophozoites in 1 ml of medium without cells. The plates were incubated for 6 hr at 37° in 5% CO_2 atmosphere. After incubation, the plates were chilled on ice for 10 min to detach trophozoites, which adhered to the walls of the culture vessel. Flagellar movement, as determined by bright field microscopy, was used as a measure of trophozoite viability. The number of live trophozoites in each well was determined using a haemocytometer. The percentage of killed trophozoites was determined by subtracting the number of trophozoites in each well containing the parasites and cells from the mean number of trophozoites in 12 control wells for each plate, divided by the mean number of trophozoites in 12 control wells × 100.

Experimental protocol

The course of infection in inbred mice. The pattern of cyst release was studied in 10 female A/J and 10 B10.A mice, inoculated orally with 10^3 cysts of *G. muris*. Cyst release in individual mice was monitored daily for 20 days, on alternate days until Day 30, and at 5-day intervals until Day 70 of infection. The numbers of cysts released by individual mice/g faeces were transformed into $\log_{10}$ values, and the geometric mean numbers of cysts released/g faeces/mouse were calculated for each day. On Day 80 after infection, all mice were killed and their small intestine examined for trophozoites.

The effects of the number of cells and duration of incubation. In order to determine optimal conditions for assaying cell–parasite interactions *in vitro*, the effects of both the number of lymphoid cells and the duration of incubation were examined. Different numbers of spleen, MLN or PEC cells from A/J and B10.A donors infected with *G. muris* for 30 days were incubated with trophozoites. Cells were isolated from four mice per strain, and known number of cells (from 1×10^5 to 5×10^6) were inoculated into individual wells containing 1×10^5 trophozoites. In order to assess the effects of the duration of incubation on the survival of parasites, 1×10^5 trophozoites were incubated with 3×10^6 cells/well (effector to target ratio of 30:1) for 1, 2, 4, 6, 8 or 10 hr. These experiments were repeated twice.

Killing of trophozoites by lymphoid cells from A/J and B10.A mice. The objectives of this experiment were three-fold: firstly, to compare the ability of lymphoid cells obtained from A/J and B10.A mice during the acute (Days 7 and 15) and elimination (Days 30 and 60) phases of the infection to kill trophozoites *in vitro*; secondly, to determine whether there were differences in the ability of cells from mucosal and systemic sites to kill trophozoites; and thirdly, to assess the capacity of adherent and non-adherent spleen and PEC cells to kill trophozoites *in vitro*.

In the first group of experiments, spleen, MLN and PEC cells were isolated from five mice of each strain per test period on Days 0, 7, 15, 30 and 60 after infection. The ability of cells to kill trophozoites *in vitro* was determined using the effector to target ratio of 30:1. These experiments were repeated twice.

In the second group of experiments, spleen and PEC cells were obtained from 10 A/J and 10 B10.A mice on Day 60 of infection. Cells from Day 60 infected animals were used because

in preliminary experiments they exhibited highest giardicidal activity. The cells were separated into adherent and non-adherent subpopulations by adherence to plastic, and the ability of each cell subpopulation to kill trophozoites *in vitro* was assessed. This experiment was repeated twice.

Killing of trophozoites by peritoneal macrophages from A/J and B10.A mice. The objectives of this experiment were to quantify the killing of trophozoites by resident and thioglycollate-stimulated (elicited) peritoneal macrophages from A/J and B10.A mice, and to assess the capacity of macrophages from susceptible and resistant mice to kill trophozoites *in vitro* in the presence of immune mouse serum. Peritoneal cells were obtained from non-injected and thioglycollate-injected *Giardia*-free A/J and B10.A mice (n = four donor mice of each strain/experiment). The cells were separated into adherent and non-adherent subpopulations by adherence to plastic. Prior to each experiment, the percentage of macrophages in the adherent cell population was determined by counting cells in Giemsa-stained cytocentrifuge smears. In experiments where the killing of trophozoites by macrophages in the presence of immune mouse serum was studied, 0·25 ml of heat-inactivated (56°, 30 min) immune or normal mouse serum was added to each well. Immune serum was obtained from 20 mice of each strain, which were inoculated with 10^3 *G. muris* cysts 120 days previously. The sera for each strain of mice were pooled and checked for the presence of anti-parasite IgG and IgA by indirect immunofluorescence (end titres were 128 and 256, respectively). These experiments were repeated twice.

RESULTS

The course of infection in inbred mice

The course of infection with *G. muris* in A/J and B10.A mice was markedly different. The results presented in Fig. 1 show that the latent period was shorter in susceptible A/J mice (4 days) when compared to the resistant B10.A mice (6 days). Throughout the acute and elimination phases of the infection, the geometric mean number of cysts released/g faeces/mouse was significantly higher in A/J mice. B10.A mice had the ability to eliminate the infection with *G. muris* faster than A/J animals. On Day 40 after infection, B10.A mice did not have cysts in the faeces but all A/J mice were passing cysts on that day (Fig. 1). By Day 70 of infection, 30% of A/J mice had a detectable level of cysts in the faeces, but by Day 80 after infection trophozoites were not detected in the small intestine of these mice. This indicates that the susceptible A/J mice also had the ability to eliminate the infection, but at a slower rate when compared to the resistant B10.A mice.

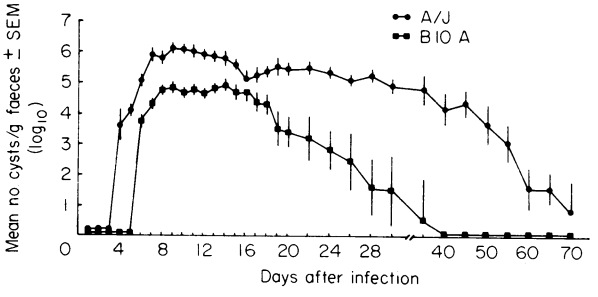

Figure 1. The pattern of cyst release in female A/J and B10.A mice infected with *Giardia muris*. Each point on the graph represents the geometric mean number of cysts/g faeces/mouse ± standard error of the mean (n = 10 mice/strain). Mice were inoculated with 10^3 cysts each.

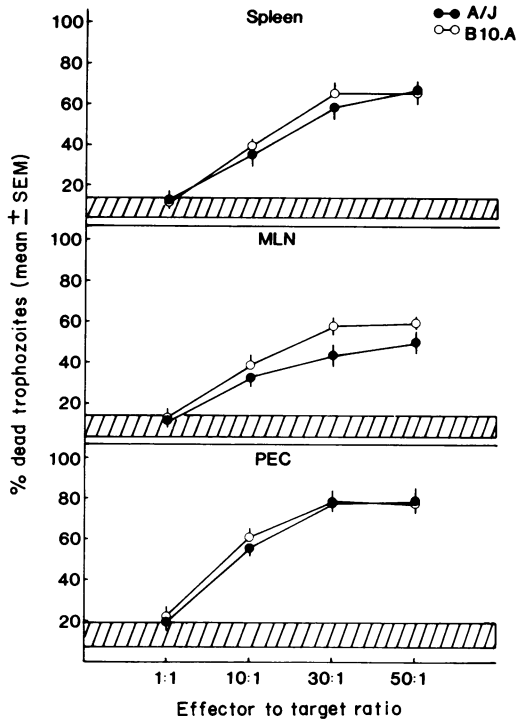

Figure 2. Mortality of trophozoites of *Giardia muris* incubated with different numbers of spleen, mesenteric lymph node (MLN) and peritoneal exudate (PEC) cells from A/J and B10.A donors infected for 30 days. The infection dose was 10^3 cysts/mouse. Each point on the graph represents the percentage of dead trophozoites (mean ± SEM) by pooled cell populations from four mice per strain. Hatched area represents the percentage of dead trophozoites after incubation in medium without cells.

The effects of the number of cells and duration of incubation

Cell density had a significant influence on the mortality of trophozoites *in vitro* (Fig. 2). The incubation of trophozoites with an equal number of cells (1:1 ratio) resulted in relatively low mortality of trophozoites (10–25%) for spleen, MLN or PEC cells. Maximal killing of trophozoites (60–80%) was observed when the number of cells was 30 and 50 times higher than the number of trophozoites. There were no significant differences in the ability of lymphoid cells from A/J and B10.A mice to kill trophozoites at all effector to target ratios tested (Fig. 2).

The extent of killing of trophozoites by cells was also dependent on the duration of incubation (Fig. 3). The percentage specific killing increased from about 5–20% after 1 hr of incubation to 50–80% after 6 hr of incubation. However, the percentage of killed trophozoites reached a plateau between 6 hr and 10 hr of incubation. This was observed for spleen, MLN and PEC cells from both A/J and B10.A mice. There were no significant differences in the capacity of cells from the susceptible and resistant mice to kill trophozoites *in vitro*.

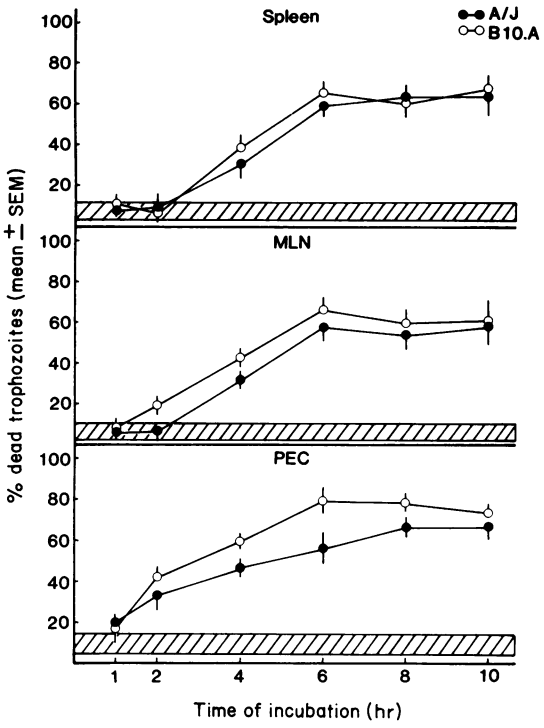

Figure 3. Mortality of trophozoites of *Giardia muris* incubated for various periods with spleen, MLN or PEC cells from A/J and B10.A donors infected for 30 days. The infection dose was 10^3 cysts/mouse. Each point on the graph represents the percentage of dead trophozoites (mean ± SEM) by pooled cell populations from four mice per strain. Hatched area represents the percentage of dead trophozoites after incubation in medium without cells.

Killing of trophozoites by lymphoid cells from A/J and B10.A mice

The data presented in Fig. 4 show that the incubation of trophozoites with spleen, MLN or PEC cells from A/J and B10.A mice resulted in significant mortality of parasites. However, there were no significant differences in the capacity of spleen, MLN and PEC cells from susceptible and resistant mice to kill trophozoites *in vitro*.

The percentage specific killing of trophozoites by spleen and PEC cells from non-infected A/J and B10.A mice was similar, and it ranged from 32 to 47%. A 20–50% increase in giardicidal activity was observed for spleen and PEC cells obtained from A/J and B10.A mice during the elimination phase of the infection (Fig. 4). For example, spleen and PEC cells obtained from donors infected for 30 or 60 days killed 50–75% and 78–90% of trophozoites, respectively.

The incubation of trophozoites with cells from mucosal sites (MLN) of non-infected A/J and B10.A mice resulted in similar mortality of organisms (Fig. 4). However, MLN cells from mice infected for 15, 30 and 60 days killed a significantly higher number of trophozoites, suggesting that increased giardicidal activity of cells from mucosal sites was expressed earlier (Day 15 of infection) when compared to cells from the systemic sites (Day 30 of infection).

Most of the giardicidal activity was mediated by the adherent cell subpopulation (Table 1). For example, adherent cells from the spleen of A/J and B10.A donors infected for 60 days killed 48·5% and 53·4% of trophozoites, respectively. The giardicidal activity of PEC adherent cells was of higher magnitude than that of spleen adherent cells. As much as 68·7% and 77·9% of trophozoites died after incubation with PEC adherent cells from A/J and B10.A mice, respectively. On the other hand, non-adherent cells from the spleen and PEC killed

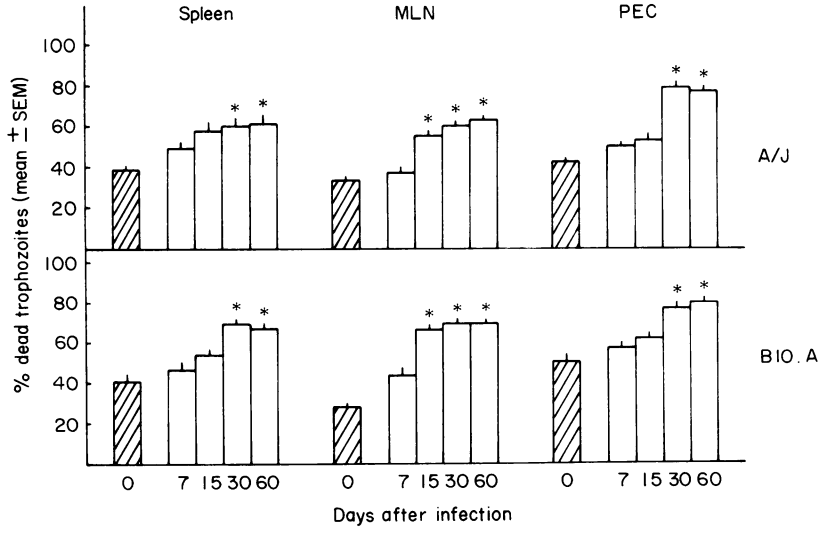

Figure 4. Mortality of trophozoites of *Giardia muris* after incubation with spleen, MLN or PEC cells isolated from A/J and B10.A mice during the acute (Days 7 and 15) and elimination (Days 30 and 60) phases of the infection. Infection dose was 10^3 cysts/mouse. Each bar represents the percentage of dead trophozoites (mean ± SEM) by cell populations from individual mice (n = five mice per strain per test period). Hatched bars represent killing by cells from uninfected mice. The cell to parasite ratio was 30:1 and the incubation time 6 hr. (*) Significantly different when compared with control (hatched bars) values by Student's t-test.

Table 1. Killing of trophozoites of *Giardia muris* by non-adherent and adherent spleen and peritoneal exudate cells from infected A/J and B10.A mice*

Donor strain	Cell subpopulation	% dead trophozoites (mean ± SEM) Spleen	PEC
A/J	Adherent	48·5 ± 1·3†	68·7 ± 3·2†
	Non-adherent	18·2 ± 1·6	8·3 ± 1·3
B10.A	Adherent	53·4 ± 2·7†	77·9 ± 3·7†
	Non-adherent	22·5 ± 2·3	3·5 ± 1·1

* Cells were obtained from donors inoculated 60 days previously with 10^3 cysts of *Giardia muris*. 1×10^5 trophozoites were incubated with 3×10^6 adherent or non-adherent cells from 10 mice per strain.

† Significantly different ($P < 0{\cdot}001$) adherent compared to non-adherent (Student's *t*-test).

significantly ($P < 0{\cdot}001$) fewer trophozoites than the adherent cells. Interestingly, giardicidal activity of spleen non-adherent cells (18·2% and 22·5% for cells from A/J and B10.A mice, respectively), was higher than that of PEC non-adherent cells (Table 1).

Killing of trophozoites by peritoneal macrophages from A/J and B10.A mice

The results of the preceeding experiment showed that the killing of trophozoites by adherent cells was significantly higher when compared to the non-adherent cells. Since more than 90% of the PEC adherent cell population were macrophages, we elected to study the macrophage-mediated killing of trophozoites *in vitro*.

The results presented in Table 2 show that when trophozoites were incubated with resident macrophages from A/J and B10.A mice, 42·5% and 45·4% of parasites were killed, respectively. The percentage of killed trophozoites was higher when the parasites were incubated with elicited macrophages, where 57·2% and 55·1% of trophozoites were killed by macrophages from A/J and B10.A mice, respectively. There were no significant differences in the ability of resident or elicited macrophages from A/J and B10.A mice to kill trophozoites *in vitro* (Table 2).

The killing of trophozoites by macrophages was enhanced in the presence of immune mouse serum. For example, giardicidal activity of resident macrophages increased from 57·2 to 79·0% in the presence of immune mouse serum (Table 2). A further increase in giardicidal activity of macrophages in the presence of immune serum was observed if the macrophages were previously stimulated with thioglycollate. In this case, more than 97% of trophozoites were killed by elicited macrophages in the presence of the immune serum. On the other hand, the killing of trophozoites by resident or elicited macrophages and normal mouse serum was lower (40–60%), and it was similar to that induced by macrophages incubated with trophozoites without serum (Table 2).

DISCUSSION

This is the first report to suggest that murine spleen and MLN cells exhibit toxicity for trophozoites of *G. muris in vitro*. Cells isolated from mice exposed to *G. muris* had greater capacity to kill trophozoites when compared to cells obtained from uninfected mice. This was particularly evident for cells isolated from mice during the elimination phase of the infection (Days 30 and

Table 2. Killing of trophozoites of *G. muris* by resident and thioglycollate-stimulated peritoneal macrophages from *Giardia*-free A/J and B10.A mice

Incubation conditions*	Thioglycollate†	% dead trophozoites (mean ± SEM) A/J	B10.A
RPMI-1640		9·2 ± 1·4	
Macrophages	−	42·5 ± 1·7	45·4 ± 2·0
	+	57·2 ± 1·4	55·1 ± 1·8
Macrophages plus normal serum‡	−	41·5 ± 2·3	42·7 ± 1·8
	+	58·1 ± 2·1	57·2 + 1·9
Macrophages plus immune serum‡	−	79·0 ± 3·7§	82·3 ± 1·5§
	+	97·2 ± 1·8§	98·1 ± 0·9§

* 3×10^6 macrophages were incubated with 1×10^5 trophozoites for 6 hr at 37° in 5% CO_2, except for the RPMI-1640 group where trophozoites were incubated in medium without cells. Data from three separate experiments (n = four thioglycollate-injected and four non-injected mice of each strain/experiment).

† Cells were obtained from mice injected i.p. with 1 ml of 3% Brewer's thioglycollate 3 days prior to assay.

‡ Serum was heat-inactivated at 56° for 30 min.

§ Significantly different ($P < 0{\cdot}01$) compared to macrophages plus normal serum groups by Student's *t*-test.

60), suggesting that longer exposure to antigens of trophozoites may have enhanced the ability of cells to kill *G. muris*. Interestingly, the killing of trophozoites by cells from susceptible A/J and resistant B10.A mice was similar, indicating that there was no correlation between the ability of A/J and B10.A mice to eliminate *G. muris*, and the capacity of their lymphoid cells to kill trophozoites *in vitro*.

The mortality of trophozoites was higher after incubation with adherent cells from the spleen or PEC when compared to mortality after incubation with non-adherent cells. Since most of the adherent cells were macrophages, it appears that giardicidal activity is mediated primarily by this cell population. However, this does not exclude the contribution of other cell types in the killing of trophozoites. In fact, 18·2% and 22·5% of trophozoites were killed by non-adherent spleen cells, suggesting that other cells such as neutrophils, mast cells and natural killer (NK) cells may contribute to the killing of parasites *in vitro*. Increased splenic NK-cell activity in mice infected with *G. muris* has been reported (Tagliabue *et al.*, 1982), which may explain the higher mortality of trophozoites after incubation with non-adherent spleen cells.

The incubation of resident or elicited macrophages with trophozoites in the presence of heat-inactivated immune mouse serum resulted in high mortality of trophozoites. On the other hand, the killing of trophozoites by macrophages in the presence of normal mouse serum or without serum was significantly lower. These results indicate that the killing of trophozoites by macrophages was enhanced in the presence of specific anti-parasite antibodies. The contribution of anti-*G. muris* IgG or IgA in the enhancement of macrophage-mediated killing remains to be determined. However, it is likely that the enhancement of killing of trophozoites by macrophages was primarely due to the IgG isotype, which has been shown to mediate antibody-dependent cellular cytotoxicity (Gale & Zinghelboim, 1975). Our results confirm those of Kaplan *et al.* (1985) who showed that adherence to and phagocytosis of *G. muris* trophozoites by macrophages and neutrophils were enhanced in the presence of opsonic antibodies from immune serum and immune milk.

Smith *et al.* (1984) reported that murine peritoneal macrophages exhibit both spontaneous and antibody-dependent cellular cytotoxicity (ADCC) for axenically cultured trophozoites of *G. lamblia*. These authors also reported that macrophages from mice that are susceptible to infection, C3H/HeJ, have defective spontaneous cytotoxicity for trophozoites when compared to macrophages from resistant C3H/HeN mice. This was not confirmed in this study, since both spontaneous and antibody-mediated cytotoxicity for trophozoites by macrophages from susceptible and resistant mice were similar. The apparent discrepency in the findings could be due to differences in the experimental protocol. For example, in this study immune serum was obtained from mice experimentally infected with *G. muris*, while Smith *et al.* (1984) used serum from mice hyperimmunized with suspensions of cultured trophozoites. Anti-parasite antibodies produced after the hyperimmunization of mice may be directed against different antigens of the parasite, as compared to those obtained during the natural course of the infection. In addition, we employed trophozoites of *G. muris* as targets in the cytotoxicity assay, whereas Smith *et al.* (1984) used axenically cultured trophozoites of the human parasite *G. lamblia*.

The macrophage is an important participant in host resistance to various intracellular and extracellular organisms. Studies *in vitro* have emphasized its capacity to kill, damage or participate in the killing of *Toxoplasma gondii* (Murray & Cohn, 1979), *Leishmania major* (Nacy *et al.*, 1982) and *Schistosoma mansoni* (Ellner & Mahmoud, 1979). Owen *et al.* (1981) have shown that macrophages from the Peyer's patches have the capacity of engulfing trophozoites of *G. muris in vivo*. ADCC has been widely investigated as an important host defence mechanism against viral, bacterial and parasitic infections (Gale & Zinghelboim, 1975; Lowell *et al.*, 1980; Kassis, Aikawa & Mahmoud, 1979; Smith *et al.*, 1984). The co-operation between host effector cells and antibodies may contribute to clearance of *G. muris* infection.

ACKNOWLEDGMENTS

The technical assistance of Miss Rebecca Guy is gratefully acknowledged. M. Belosevic is a Fellow of the McConnell Memorial Foundation. This work was supported by grants from the Medical Research Council of Canada (MRC) and the Natural Sciences and Engineering Council of Canada (NSERC) to G. M. Faubert. Research at the Institute of Parasitology is supported by NSERC and Fonds FCAR pour l'aide et soutien a la recherche.

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