

# Direct and antibody dependent cell mediated cytotoxicity against *Giardia lamblia* by splenic and intestinal lymphoid cells in mice

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**SUMMARY** Direct cytotoxicity and antibody dependent cell mediated cytotoxicity against *Giardia lamblia* trophozoites exhibited by splenic, intraepithelial and lamina propria lymphocyte populations isolated from *G lamblia* infected mice were studied. Different patterns of cytotoxicity were found. Intraepithelial lymphocytes showed a direct cytotoxic activity of  $20.6 \pm 5.6\%$  before infection. It was significantly higher on the 20th ( $p < 0.01$ ) and 30th ( $p < 0.05$ ) day postinfection. Lamina propria lymphocytes showed a significantly augmented level of both direct cytotoxicity and antibody dependent cell mediated cytotoxicity on the 20th and 30th postinfection days. Direct cytotoxicity by splenic lymphocytes remained unchanged during infection but antibody dependent cell mediated cytotoxicity was significantly increased.

The host response to *G lamblia* involves the immune system. Previous exposure to this infection is known to increase resistance to a second challenge in both man and animals.<sup>1,2</sup> Smith *et al*<sup>3</sup> reported that human peripheral blood monocytes/macrophages are spontaneously cytotoxic for *G lamblia* trophozoites and this cytotoxicity is reduced in chronic giardiasis.<sup>4</sup> In another study they reported defective spontaneous cytotoxicity but normal antibody dependent cytotoxicity by resident macrophages isolated from C3H/Hej mice.<sup>5</sup>

*Giardia lamblia* has been shown to infect mice.<sup>6-8</sup> Using a mouse model, we have examined the role of cytotoxic effector cells in resistance of mice to this infection. Direct cytotoxicity of lymphocytes and antibody dependent cell mediated cytotoxicity against *G lamblia* are likely to be most relevant in mucosae. We have therefore investigated the direct and antibody mediated cytotoxicity of mucosal lymphocytes (intraepithelial lymphocytes and lamina propria lymphocytes) against *G lamblia*.

## Methods

### ANIMALS AND MODE OF INFECTION

Parasite free, 2-3 weeks old Swiss albino mice,

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weighing 10-12 g were used in this study. *G lamblia* cysts were obtained from the stool of a patient and a fixed inoculum of 10 000 cysts/0.2 ml was prepared on a sucrose gradient<sup>9</sup> and fed to the animals.<sup>10</sup> Five animals were killed on each of the days 0, 10, 20, and 30 postinfection.

### QUANTITATION OF CYST AND TROPHOZOITE

#### (a) Cyst count

Two hour stool samples from each animal were collected, homogenised in normal saline and layered on sucrose gradient.<sup>9</sup> The results were expressed as number of cysts/g of stool.

#### (b) Trophozoite count

The small intestine was flushed with a fixed amount of normal saline, and the trophozoites in the intestinal perfusate counted in a haemocytometer.<sup>6</sup>

### PREPARATION OF EFFECTOR CELLS

#### (a) Splenic lymphocytes

These were removed and harvested according to the method described earlier.<sup>6</sup> They were purified by Ficoll-isopaque density gradient.

#### (b) Intestinal lymphocytes

Gut lymphocytes were isolated by a modified method of Davies and Parrott<sup>11</sup> and Taglibue *et al*.<sup>12</sup> Briefly, the small intestine was washed with calcium

and magnesium free Hank's balanced salt solution. The mesentery, adherent connective tissue and fat were removed from the intestine and Peyer's patches were dissected. The intestine was opened longitudinally and cut into 1–2 cm long pieces and washed thoroughly in calcium and magnesium free Hank's balanced salt solution. To isolate intraepithelial lymphocytes, gut pieces were incubated at 37°C for 15 minutes in calcium and magnesium free Hank's balanced salt solution containing 5 mmol EDTA with constant stirring. This process was repeated three times. The supernatants were pooled, centrifuged, resuspended in RPMI-1640, filtered through glass wool column and washed. Most of the lymphocytes obtained by this method were from the epithelial layer.<sup>11</sup>

For isolation of lymphocytes from the lamina propria, gut pieces were washed with calcium and magnesium free Hank's balanced salt solution and incubated for 20 minutes in 25 ml RPMI-1640 containing 5% heat inactivated fetal calf serum (RPMI-FCS) and 2 mmol L-glutamine with stirring. These pieces were then incubated in 15 ml RPMI-FCS containing 25 U/ml collagenase (Boehringer, W. Germany) for 15 minutes. The whole procedure was repeated three times. The supernatants were pooled, filtered through a glass wool column and washed in RPMI-FCS.

The lymphocyte rich preparations of intraepithelial lymphocytes and lamina propria lymphocytes were loaded on a percoll density gradient (Pharmacia, Sweden) and the lymphocytes recovered from the 1.050/1.085 g/ml density interface. Their viability was checked by the 0.2% trypan blue dye exclusion test. The yield of intraepithelial lymphocytes obtained was 0.6–2.1 × 10<sup>7</sup>.

#### ANTISERUM AGAINST *G lamblia*

Antiserum to *G lamblia* trophozoites was raised in rabbits<sup>13</sup> and titrated in a cytotoxicity assay. The serum was used at a dilution of 1:3000 in all experiments. At this concentration, the antiserum itself had no giardicidal activity. Normal rabbit serum was used at the same dilution. Both normal and immune sera were heated at 56°C for 30 minutes before use.

#### CYTOTOXICITY ASSAY

*Giardi lamblia* trophozoites were purified by the Feely and Erlandsen<sup>14</sup> method of sticking them to polystyrene petri dishes, and washed with RPMI-1640 containing 25 mM HEPES, 10% heat inactivated fetal calf serum and 50 µg/ml gentamicin. The cytotoxicity assay was a modified technique of Smith *et al.*<sup>5</sup> Trophozoites were labelled with sodium<sup>51</sup> chromate using 70 µCi/5 × 10<sup>6</sup> parasite/ml for 1½

hours at 37°C in a shaking water bath. 0.1 ml of labelled trophozoites (5 × 10<sup>5</sup>),<sup>5</sup> suspended in incubation medium, were placed in sterile small test tubes. 0.1 ml of effector cells were also added (effector cell density was adjusted to achieve 25:1, 50:1 and 100:1 E:T ratios). To assay antibody dependent cell mediated cytotoxicity, 0.1 ml of immune serum (1:3000 diluted) was added. The effector cells, labelled trophozoites and antiserum were mixed and tubes were centrifuged at 100 g for five minutes. These were then incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere for two hours. After incubation, the tubes were centrifuged and the amount of radio-activity in the supernatant was measured in a gamma counter. The maximum release was obtained by treating the labelled trophozoites with 10% Triton-x100 followed by processing as outlined above. The spontaneous release varied from 10–15% of the maximal release. The percentage specific cytotoxicity was calculated by the following formula: % specific cytotoxicity = experimental release (cpm) - spontaneous release (cpm) × 100 / maximal release (cpm) - spontaneous release (cpm).

#### STATISTICAL ANALYSIS

Student's *t* test was used to analyse all the data.

#### Results

Figure 1 shows the kinetics of cyst excretion and trophozoite counts in the intestine during the 30 day period of study. The peak cyst excretion and peak trophozoite count occurred on the 10th postinfection day. By the 30th day, cyst excretion ceased in all the animals, although a few trophozoites could still be detected in 30–40% of infected animals in the intestinal washings.

Different effector:target cell (E:T) ratios were used (25:1, 50:1 and 100:1) to test for direct cytotoxicity and antibody dependent cell mediated cytotoxicity. There was a slight but consistent increase in direct cytotoxicity at E:T ratios of 50:1 and 100:1. A very low level of direct cytotoxicity (6.34 ± 2.15%) was observed at E:T ratios of 25:1 with splenic lymphocytes, while with intraepithelial lymphocytes and lamina propria lymphocytes, the respective values were 19.03 ± 2.08% and 18.30 ± 3.92%. With lamina propria lymphocytes, the antibody dependent cell mediated cytotoxicity was much higher (44.98%), in comparison to the values obtained with intraepithelial lymphocytes and splenic lymphocytes at E:T ratios of 100:1 (Fig. 2).

Intraepithelial lymphocytes showed a cytotoxic activity of 20.6 ± 5.63% before infection. On the 20th postinfection day, the values were increased to 39.82 ± 5.87% (p < 0.01), whereas antibody depen-

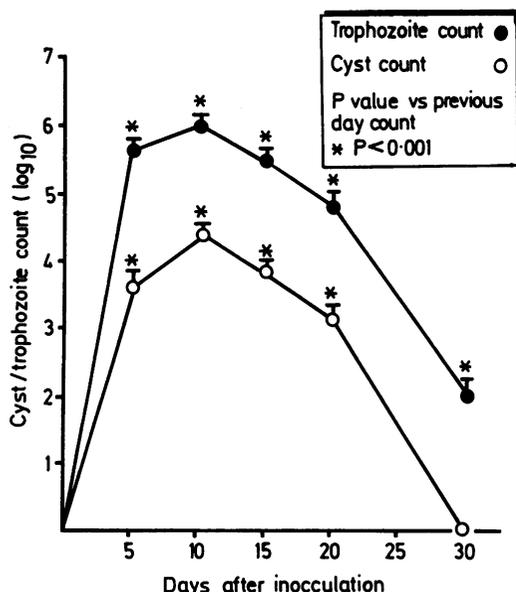


Fig. 1 *Giardia lamblia* cyst and trophozoite counts ( $\log_{10}$ ) on different postinfection days. Each point represents the mean  $\pm$ SD of five animals.

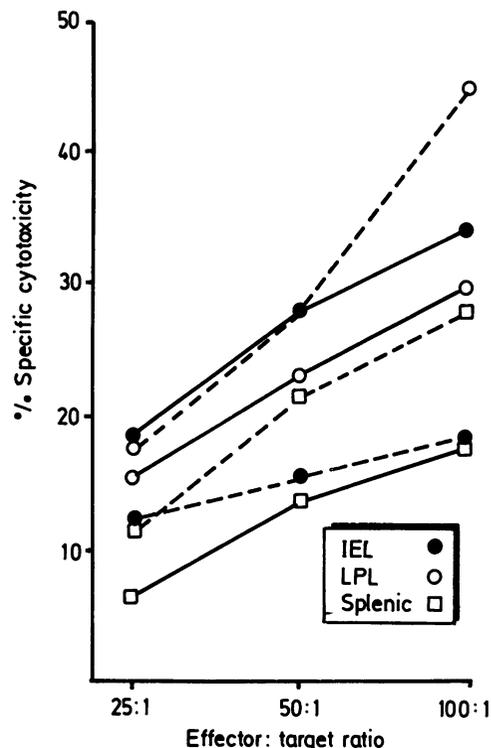


Fig. 2 Direct (continuous line) and antibody dependent (dotted line) cell mediated cytotoxic activity exhibited by splenic (□), IEL (●) and LPL (○) at different E:T ratios against *G lamblia* trophozoites. Values represent the mean of three experiments before infection.

dent cell mediated cytotoxicity for intraepithelial lymphocytes showed no significant change from the preinfection value. In contrast, lamina propria lymphocytes showed a significantly higher direct cytotoxicity and antibody dependent cell mediated cytotoxicity on day 20 and 30 (Table 1).

The level of direct cytotoxicity exhibited by splenic lymphocytes at E:T ratios of 50:1 before infection was  $12.9 \pm 5.4\%$ ; this remained unchanged after infection (Table 2). In the presence of immune serum, cytotoxic activity was augmented to a significant level ( $p < 0.01$  and  $p < 0.02$  respectively on 20th and 30th day).

### Discussion

In our mouse model, *G lamblia* infection persisted for 30 days. The self-resolution of the infection appears to be immune mediated. We have already

Table 1 Level of cytotoxicity shown by intraepithelial lymphocytes and lamina propria lymphocytes at E:T ratios of 25:1 using radio labelled giardia trophozoites as target cells (Mean  $\pm$ SD)

Effector cells		Postinfection days			
		0	10	20	30
IEL	Direct	20.6 $\pm$ 5.63	25.84 $\pm$ 6.42	39.82 $\pm$ 5.87 ( $< 0.01$ )	32.01 $\pm$ 5.35 ( $< 0.05$ )
	ADCC	15.11 $\pm$ 5.11	19.49 $\pm$ 4.69	20.56 $\pm$ 4.09	21.9 $\pm$ 9.0
LPL	Direct	15.75 $\pm$ 4.85	22.3 $\pm$ 5.49	31.92 $\pm$ 7.23 ( $< 0.05$ )	29.02 $\pm$ 7.02 ( $< 0.05$ )
	ADCC	14.00 $\pm$ 4.83	20.10 $\pm$ 6.06	38.82 $\pm$ 4.21 ( $< 0.01$ )	33.97 $\pm$ 4.42 ( $< 0.01$ )

\* p value vs 0 day value.

Table 2 Level of cytotoxicity shown by splenic lymphocytes at E:T ratios of 50:1 using radio labelled *G lamblia* trophozoites as target cells

	Postinfection days			
	0	10	20	30
Direct	12.92±5.4	18.20±6.60	24.98±7.2	23.93±10.42
ADCC	21.29±5.07	28.06±7.05	43.72±7.04 (<0.01)	37.88± 8.47 (<0.02)

p values vs 0 day value.

reported the appearance of antibodies in these animals during the course of primary and secondary infection.<sup>15, 2</sup> In this study, we have shown that intraepithelial lymphocytes populations exhibit only direct cytotoxicity, whereas lamina propria lymphocytes exhibit both direct and antibody dependent cell mediated cytotoxicity. The augmented level of cytotoxicity of these two lymphocyte populations coincides with the decline of parasite numbers in the intestine. *Giardia lamblia* infection in mice has been shown to increase the intraepithelial lymphocyte count.<sup>16, 17</sup> The intraepithelial lymphocytes population is dominated by T cells,<sup>18</sup> and in *G lamblia* infected mice they respond by blast transformation with *G lamblia* antigen.<sup>17</sup> The presence of cytotoxic T cells in the intraepithelial lymphocytes and lamina propria lymphocytes population has also been shown.<sup>19</sup> Owen *et al*<sup>20</sup> showed the attachment of lymphocytes to *G muris* trophozoites in the lumen, and thus the higher level of direct cytotoxicity exhibited by intraepithelial lymphocytes may be because of their continuous presence in close vicinity to the parasite. Different patterns of cytotoxicity between the two intestinal lymphocyte populations may be explained either by population heterogeneity or by rigorous selection of homogeneous population at the basement layer of the epithelium (by the failure of antibody dependent cell mediated cytotoxicity to penetrate the epithelium). Alternatively a specialised micro-environment might produce phenotypic changes.

In the human host, Smith *et al*<sup>21</sup> have reported that the peripheral blood granulocytes, but not lymphocytes, were cytotoxic for *G lamblia*. The predominant isotype of the antibodies in antibody dependent cell mediated cytotoxicity was IgG, but when IgG depleted serum was used, there was still some cytotoxic activity. This raises the possibility that other immunoglobulin isotypes may also mediate antibody dependent cell mediated cytotoxicity. In mucosal secretions, secretory IgA is the predominant isotype. It has been recently reported by Taglibue *et al*<sup>22</sup> that S-IgA in mice can mediate

antibacterial antibody dependent cell mediated cytotoxicity when bound to lymphocytes from gut lymphoid tissue. It is possible that specific S-IgA may be more effective in augmenting antibody dependent cell mediated cytotoxicity by lamina propria lymphocytes, or even by intraepithelial lymphocytes, in our system.

No increase in direct cytotoxicity occurred with the splenic lymphocytes but the antibody dependent cell mediated cytotoxicity was significantly augmented. Taglibue *et al*,<sup>12</sup> in a preliminary study, reported an increase in splenic NK activity when mice were infected with *Nippostrongylus brasiliensis* or *Giardia muris*. These workers did not give details of their observations thus making comparisons difficult.

We conclude that intraepithelial lymphocytes exhibit direct cytotoxicity, whereas lamina propria lymphocytes exhibit both direct and antibody dependent cell mediated cytotoxicity against *G lamblia*. In contrast, splenic lymphocytes exhibit only antibody dependent cell mediated cytotoxicity. These findings may help in explaining the mechanism of mucosal defence against *G lamblia* infection.

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