Giardia induces proliferation and interferon γ production by intestinal lymphocytes

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Abstract

***Background*—Murine intraepithelial lymphocytes kill *Giardia lambia*; responses of human intestinal lymphocytes to this parasite are unknown.**

***Aims*—To examine giardia induced proliferation, interferon γ production, migration, and cytotoxicity by lymphocytes from the human intestine and peripheral blood.**

***Methods*—Giardia were added to intraepithelial lymphocytes, lamina propria lymphocytes, and peripheral blood lymphocytes, obtained from jejunal mucosa and blood of otherwise healthy patients undergoing gastric bypass surgery for morbid obesity. Proliferation was measured by ^{3}H-thymidine incorporation; frequency of proliferation precursors, by limiting dilution analysis; interferon γ production, by ELISA; cytotoxicity, by ^{51}Cr release of radiolabelled giardia and by release of serine esterases by effector lymphocytes that mediate cytotoxicity.**

***Results*—The CD4+ T lymphocytes from intestine and blood proliferated in response to giardia. The stimulus by the parasite was mitogenic rather than antigenic due to the fact that the peak response was on day 3 rather than day 6, and the large number of precursors was in the range of that for mitogens. CD4+ T lymphocytes from both sites produced interferon γ in response to giardia. Lymphocytes did not migrate towards or kill the parasite.**

***Conclusions*—Giardia induced the same degree of proliferation and interferon γ production by CD4+ T lymphocytes in intestine and blood, but did not trigger cytotoxicity or migration.**

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Keywords: intraepithelial lymphocytes; lamina propria lymphocytes; parasite; chemotaxis; T cell mediated cytotoxicity; giardia

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The protozoan *Giardia lamblia* attaches to jejunal mucosa with its sucking disk whose lectins adhere to oligosaccharides on the microvillus membrane of epithelial cells.[1] Giardia infection can lead to diffuse loss of the microvillus surface area, resulting in malabsorption. With continued disease, the villi shorten, the crypts become hyperplastic, and the number of lymphocytes increases. The small bowel develops this generic "flattening" in response to other insults, such as cryptosporidium, bacterial overgrowth, coeliac sprue, and graft versus host disease, indicating that a similar mechanism may occur in several situations.

The role of T cells in eradicating giardia is unclear. Evidence in favour of T cell targeting of giardia is the demonstration that murine intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) have greater cytotoxic activity against giardia than do splenic lymphocytes, particularly after infection, presumably due to the sensitising phase.[2] Murine IEL, however, are predominantly TCRγδ+, a phenotype that can have greater cytotoxic activity than the TCRαβ+ T cells, which comprise the majority of human IEL. This lytic activity in rodents, then, may not be found in humans. The fact that T cell depleted and nude mice do not clear the organism indicates the importance of T cells,[3] although this may be due to concomitant loss of immunoglobulin that requires T cell help. In contrast, the presence of cytolytic granules containing granzyme B in the IEL in coeliac disease but not giardiasis suggests that these lymphocytes are not armed to lyse this pathogen.[4]

Little is known regarding the T cell mediated response to giardia, particularly by human intestinal lymphocytes. In addition to the direct cytotoxic activity of murine IEL against giardia, antibody dependent cellular cytotoxicity has been shown with murine LPL and human neutrophils.[5] However, only 5% of human IEL and LPL express Fc receptors, and, accordingly, they were unable to carry out antibody dependent cellular cytotoxicity using other indicator systems.[6]

In the present study, IEL and LPL retrieved from proximal jejunum of healthy individuals were evaluated for reactivity to giardia. The organism preferentially adheres to this same area of intestine. As the phenotype and function of intestinal lymphocytes vary with location, matching the area of lymphocyte procurement with the area that is prone to disease may be important.

Methods

LYMPHOCYTE ISOLATION AND GIARDIA CULTURES

IEL were isolated from jejunal mucosa from healthy individuals undergoing gastric bypass operations for morbid obesity. In brief, the minced mucosa was treated for 30 minutes at 37°C with 1 mM dithiothreitol followed by

Abbreviations used in this paper: CTL, cytotoxic T lymphocyte; IEL, intraepithelial lymphocyte; IFN, interferon; IL, interleukin; LAK, lymphokine activated killer; LPL, lamina propria lymphocyte; PBL, peripheral blood lymphocyte; SC, spontaneous cytotoxicity; TGF, transforming growth factor; TNF, tumour necrosis factor; PHA, phytohaemagglutinin.

three 45 minute incubations in a shaking water bath with 0.75 mM EDTA, and the supernatant cells were collected. After purification by Percoll density gradient centrifugation, IEL preparations were comprised of over 90% lymphocytes that were 94 (5)% CD2+ and 89 (2)% CD8+.

To isolate LPL, the treated tissue received three further 45 minute incubations with EDTA, and the released cells were discarded. The remaining tissue was digested for three hours at 37°C in 20 U/ml collagenase, then pressed through a wire mesh sieve to disperse the cells. LPL, purified using a Percoll density gradient, were 55 (10)% CD4+ and 35 (11)% CD8+. PBL were isolated by Ficoll density gradient centrifugation.[7]

T lymphocytes were separated into CD4+ and CD8+ subsets by negative selection using antibodies to CD8 and CD4, respectively, followed by goat antimouse IgG attached to magnetic beads as detailed elsewhere.[8] After separation, lymphocytes were less than 1% positive for the depleted phenotype.

Giardia lamblia (American Type Culture Collection (ATCC), Rockville, Maryland, USA) were grown in TYI-S-33 medium (ATCC) with tightly capped tubes to maintain a microaerophilic environment. The organisms were used in log growth when over 80% of them were moving. Parasites coated with TY medium were washed before being placed in RPMI-1640 with the lymphocytes.

CYTOTOXICITY ASSAY

Cytotoxic cells were generated with lymphocytes in three situations: just after isolation (spontaneous cytotoxicity, SC), after a three day culture with interleukin 2 (IL-2) or IL-15 (100 ng/ml) (lymphokine activated killer (LAK) activity), or after a five day culture with giardia (cytotoxic T lymphocyte (CTL) activity). The cells were then washed and incubated for four hours at various ratios with ^{51}Cr labelled giardia. Alternatively, labelled parasites were incubated with tumour necrosis factor α (TNFα) and/or interferon γ (IFNγ) (0.1 μg/ml). The percentage of cytotoxicity was calculated in relation to the spontaneous and maximal releases of target cells in medium and 0.1% Triton X-100, respectively, as detailed previously.[9] Giardia, as targets loaded with ^{51}Cr-sodium chromate, released 30% of label after four hours with medium (spontaneous release) and 80% of the incorporated radioactivity with Triton X-100 (maximal release).

SERINE ESTERASE RELEASE

Lymphocytes, prepared as described above, were lysed by freeze thaws, and the supernatants tested for the amount of granule secretion using the N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) assay.[10]

MIGRATION

The multiwell chemotaxis chamber of Boyden was used to measure lymphocyte migration in response to various factors. Intestinal lymphocytes and PBL were activated with IL-2 for 48 hours as they do not migrate to chemokines without stimulation.[7] Conditioned medium, tested for its ability to attract lymphocytes, consisted of RPMI-1640 with 10% fetal calf serum (FCS) that was incubated for 24 hours with giardia (1×10^5/ml) and then filtered. IL-2 activated IEL, LPL, and PBL (1×10^5/0.05 ml) were placed in the upper wells of the Boyden chamber, and giardia (1×10^4/0.05 ml) or RPMI-1640 medium (conditioned or not) was placed in the lower wells. After four hours at 37°C, the apparatus was kept at 4°C overnight to release lymphocytes bound to the underside of the membrane. After disassembly of the apparatus, the numbers of lymphocytes in each lower chamber were counted.

PROLIFERATION AND IFNγ PRODUCTION

Lymphocytes (2×10^5/0.2 ml) were cultured with one or two of the following: giardia (1×10^4/0.2 ml), KD fibroblasts (ATCC), IL-10, transforming growth factor β (TGFβ), neutralising antibody to TGFβ (R&D Systems, Minneapolis, Minnesota, USA), and phytohaemagglutinin (PHA) (Murex Diagnostics, Norcross, Georgia, USA). All assays were carried out in RPMI-1640 medium with 10% fetal bovine serum, 1% antibiotic-antimycotic, and 1% glutamine (Sigma). Proliferation was determined by ^{3}H-thymidine incorporation.

To measure IFNγ production, supernatants of lymphocyte cultures were collected after 72 hours and tested by an enzyme linked immunosorbent assay (ELISA) for IFNγ (Immunotech, Westbrook, Maine, USA).

LIMITING DILUTION ANALYSIS

The responder frequency was determined by culturing various numbers of CD4+ T lymphocytes from the lamina propria and peripheral blood with giardia (1×10^4/0.2 ml) and irradiated (3000 rads) autologous PBL (1×10^4/0.2 ml) in round bottomed wells for 10 days. Proliferation was determined by ^{3}H-thymidine incorporation. Wells were scored as negative if ^{3}H-thymidine incorporation did not exceed the mean of the control wells (medium alone) by more than three standard deviations or positive if it did. The percentage of negative wells for each cell number was plotted against the number of cells added, resulting in a linear regression from which the 37% negative cell number could be interpolated. The frequency of precursors was determined by the reciprocal of the number of cells yielding 37% negative wells.[11]

STATISTICAL ANALYSIS

For each set of data, the mean (SEM) was calculated. Pairs of data sets were analysed by Student's *t* test for paired or independent variables.

Results

CYTOTOXICITY

Lymphocyte SC, LAK, and CTL activity was tested against giardia as the target cells. For SC activity, IEL, LPL, and PBL were tested just after isolation or after an 18 hour incubation in medium to permit recovery from the isolation procedure. Before the LAK assay, IEL, LPL, or

PBL were stimulated for 72 hours with IL-2 or IL-15 (each at 100 ng/ml). For CTL activity, IEL, LPL, or PBL (2×10^5/0.2 ml) were cultured with giardia (1×10^4/0.2 ml) for five days before the lytic assay. Sensitivities of the organisms to TNFα and IFNγ were tested in a four hour assay. The parasites were added live without irradiation to all assays; most ceased moving after about 36 hours in RPM1–1640 medium. As giardia move constantly and cytotoxicity does not occur without effector-target cell binding, the cells were placed in V bottomed microwells and spun down before the four hour incubation. The cell pellet remained grossly intact at the conclusion of the experiment. No lysis could be shown using any of these methods at a 50:1 effector to target cell ratio (n=4 for each type of lysis). Serine esterase content in lymphocytes after a four hour coculture with giardia was measured and found to be the same as lymphocytes in medium alone (not shown).

One possible reason for lack of lysis is the absence of binding of effector to target cells. After incubating the mixed cell pellet for 10, 30, and 60 minutes and analysing under the microscope, there was no binding of giardia to IEL, LPL, or PBL (n=3, not shown).

MIGRATION

The possibility that giardia release chemokines that attract IEL was tested using the Boyden transwell apparatus. Live giardia (1×10^4/well) or RPMI with 10% FCS conditioned for 24 hours by giardia were placed in the lower wells with IL-2 activated IEL, LPL, and PBL (1×10^5/0.05 ml) in the upper wells. The number of lymphocytes that migrated through the pores in the membrane separating the upper from lower chambers was counted. The same number of IEL migrated in response to giardia or conditioned medium as to control serum supplemented RPMI-1640 medium (8 (1) $\times 10^4$, 7 (2) $\times 10^4$, 7 (1) $\times 10^4$, respectively, n=3), indicating that IEL do not preferentially migrate towards live giardia or their secreted products. The same results were obtained with LPL and PBL (not shown).

PROLIFERATION

LPL, PBL, and to a lesser extent, IEL, proliferated when cultured with live giardia, responding maximally in three days, as documented by ^{3}H-thymidine incorporation: 15 045 (2381) cpm with LPL, 13 235 (3220) cpm with PBL, and 2133 (233) cpm with IEL. Maximal LPL blastogenesis occurred at a trophozoite concentration of 1×10^4/0.2 ml. Testing proliferation of CD4+ and CD8+ subsets revealed that the giardia response was due entirely to CD4+ T cells (table 1). Several modulators were tested for their effect on giardia stimulated proliferation. There was no enhancement of cell division by IEL or LPL in response to the pathogen using an irradiated (3000 rads) fibroblast (KD) monolayer (n=5, not shown), which enhances IEL blastogenesis to other stimuli.[12] The inhibitory cytokines, IL-10 and TGFβ, as well as the blocking antibody to CD2 reduced giardia induced blastogenesis of LPL by 87 (5)%, 85 (6)%, and 87 (5)%, respectively (n=3). This was similar to the blocking effect of these agents on PHA induced proliferation of LPL: 88 (7)%, 89 (9)%, and 83 (6)%, respectively (n=3). Neutralising antibody to TGFβ, in contrast, increased spontaneous and PHA induced proliferation of LPL by 188 (22)% and 188 (18)%, respectively (n=3), but had no effect on giardia induced proliferation (98 (25)%, n=3), suggesting that the responsiveness of LPL to TGFβ increases with PHA but not with giardia.

Table 1 Giardia induced proliferation of LPL T cell subsets

	Stimulus	
Lymphocyte type	*Medium*	*Giardia*
Experiment 1		
LPL	4.7	14.3
CD4+ LPL	4.1	14.1
CD8+ LPL	0.8	0.9
Experiment 2		
LPL	3.3	15.1
CD4+ LPL	2.9	14.8
CD8+ LPL	0.5	1.1

Unseparated LPL or subsets, selected by negative magnetic immunoselection, were cultured at 2×10^5/0.2 ml for three days with giardia (1×10^4/0.2 ml); proliferation was measured by ^{3}H-thymidine incorporation. Results expressed as cpm $\times 10^3$.

To determine whether giardia was acting as a mitogen or an antigen, the characteristics that differentiate between the two were investigated: peak response on day 3 for mitogens and day 6 for antigens; and a much higher frequency of precursor cells in response to mitogens compared with antigens. The blastogenesis of IEL, LPL, and PBL to giardia peaked on day 3, similar to mitogens (not shown). Limiting dilution analysis was performed with CD4+ T lymphocytes derived from autologous LPL and PBL (fig 1). The precursor frequency, similar for the two lymphocyte types, was in the range for mitogens. Thus, using both criteria, the giardial stimulus has characteristics of a mitogen rather than an antigen.

CYTOKINE PRODUCTION

Production of IFNγ by giardia stimulated LPL was measured as this cytokine is secreted by LPL[13] and it has myriad immunological functions relevant to giardia infection, including increased expression of MHC class II on

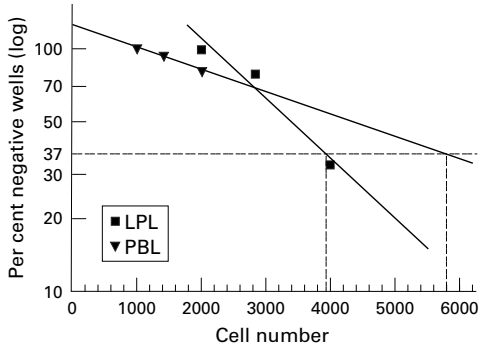

Figure 1 CD4+ T lymphocytes from LPL and PBL were combined with giardia (1×10^4) and irradiated autologous PBL (1×10^4) for 10 days. Linear regression analysis allowed an interpolation of the cell number (reciprocal of the precursor frequency) yielding 37% negative wells.

Table 2 Interferon γ production by LPL and PBL in response to giardia

	Stimulus	
Lymphocyte type	*Giardia*	*Control*
LPL	8300 (2200)	2750 (2100)
PBL	132 (92)	60 (40)

Lymphocytes (2×10^5/0.2 ml) were cultured with giardia (1×10^4 /0.2 ml) in serum supplemented RPMI medium for 72 hours and the supernate filtered and tested by ELISA for IFNγ content. Results expressed in pg/ml (mean (SEM)).

epithelial cells and reduction in the barrier function of the epithelium.[14] LPL (2×10^5/0.2 ml) were cultured with live giardia (1×10^4/0.2 ml) for 72 hours (optimal time for IFNγ production).[13] The supernatants were collected and measured for cytokine levels by ELISA. LPL and PBL, but not IEL, produced IFNγ in response to giardia (table 2).

In summary, giardia cause CD4+ T cells in the intestine to proliferate and produce IFNγ, with similar precursor frequencies in LPL and PBL The reactive components on giardia stimulate lymphocytes in a mitogenic rather than an antigenic manner, as shown by the response peaking on day 3 rather than day 6 and by the high precursor frequency in the range of that for mitogens. Giardia do not induce lymphocyte chemotaxis, and are not susceptible to lysis by lymphocytes, probably deterred by their continuous motion that interferes with lymphocyte-parasite binding.

Discussion

The human T cell mediated immune response to giardia by intestinal lymphocytes is largely unstudied. This response is important, not only because giardia is a common pathogen, but because similar damage to the small bowel is seen with a variety of chronic insults, such as coeliac sprue, bacterial overgrowth, and graft versus host disease. Whether T lymphocytes aid in destruction of the parasite and in damage to the intestine is unclear.

The exact role of T cells in mucosal damage is unknown. Similar morphology can be induced in vitro with fetal intestine cultured with TNF, staphylococcal enterotoxin B, or antibody to CD3, while antibody to IFNγ reverses the mucosal destruction induced by gliadin in coeliac patients.[15–17] These studies suggest that T cells mediate at least some of the structural damage in these jejunal diseases. Further evidence for T cell mediated destruction is the less severe abnormality in the villus architecture of athymic compared with euthymic mice infected with giardia. Also, transfer of lymphocytes from normal mice into athymic mice increases the damage.[18] In man, however, mucosal disease is more pronounced in immunocompromised hosts.[19]

What causes the expansion in IEL and LPL numbers in this disease is unclear. Trophozoites can invade the mucosa and are occasionally found in the lamina propria; this occurs with certain strains and high pathogen loads, especially with host malnourishment, immunodeficiency, and breaks in the epithelial cell barrier.[20] Alternatively, IEL may move into the lumen to contact the organism in the unstirred water layer above the epithelium protected from turbulence. Giardia also release 70% of cell surface antigens over each 24 hour period[21]; these antigens may block secretory antibody or stimulate T cell mediated immune events. The inflammatory infiltrate may be formed from proliferative responses to the pathogen or from migration towards chemokines produced in response to giardia. Epithelial cells, for example, secrete various chemokines, such as IL-8, in response to lipopolysaccharide, TNF, and invasive pathogens.[22] IEL, and to a lesser extent, LPL, migrate in response to chemokines of both the α and β families.[7]

No cytotoxic activity by IEL, LPL, or PBL could be shown against giardia, including SC, LAK activity, and CTL activity. In contrast to the present study, murine IEL lyse giardia.[2] However, these lymphocytes are predominantly TCRγδ as opposed to human IEL which are mainly TCRαβ. Human IEL lack cytotoxic granules in giardiasis but not in coeliac sprue, substantiating the low lytic activity of IEL against these parasites.[4] Antibody dependent cellular cytotoxicity has been shown using human neutrophils and rabbit peritoneal exudate cells with giardia specific antibody.[2 19] Antibodies may be major host defences against this parasite as they agglutinate the trophozoites, perhaps reducing their motility and attachment to epithelial cells.

T cell mediated killing has been shown with other parasites. Murine IEL (mainly of the TCRαβ type) kill macrophages and epithelial cells infected with *Toxoplasma gondii*.[23] Similar genetically restricted CTL activity as well as IFNγ production has been shown with murine IEL against rotavirus.[24] IFNγ stimulation of the IEC-6 line inhibits the intracellular replication of *T gondii*,[25] showing an interaction between host derived cytokines and the parasite.

The increased numbers of intestinal lymphocytes, particularly IEL, with giardia infection could be due to enhanced migration or proliferation. Using the transwell assay, there was no migration of IEL, LPL, or PBL towards live giardia or RPMI medium conditioned by giardia. There was, however, heightened proliferation of the CD4+ lymphocytes in response to live trophozoites. This agrees with a study showing that *Giardia muris* infection was prolonged in mice treated with a monoclonal antibody that depletes CD4+ lymphocytes.[26] If this was the sole mechanism accounting for the lymphocyte infiltrate in this disease, the excess IEL in infected jejunum should be of the CD4+ phenotype. There are no detailed studies of the lymphocyte phenotypes in giardiasis.

The proliferation experiments are limited by the growth conditions of the parasites, which are facultative anaerobes that utilise large molecules, such as bile, casein, and yeast. Mammalian cells, on the other hand, are aerobes that utilise sugars and amino acids. It is difficult, then, to coculture these two cell types finding an environment that is satisfactory to both. While reports of such cocultures exist, not all of them show that each cell type retains optimal

growth and metabolism. The assays used in the present study required that the giardia only be viable during the first few hours of culture with lymphocytes; RPMI medium, favouring lymphocyte growth, was used as lymphocyte responses were being measured. The best lymphocyte responses occurred when most of the parasites were viable and moving; viability alone is not a sufficient criterion of parasite health.

The positive findings in this study are the proliferation and IFNγ production by CD4+ intestinal lymphocytes, found mainly in the lamina propria. Giardial antigens or whole organisms may contact CD4+ IEL or LPL, triggering IFNγ production. This, in turn, would increase epithelial cell permeability, permitting more giardia components to enter the mucosa and further sensitise the lymphocytes.

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