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Lymphocyte Proliferation in Peyer's Patches of *Giardia muris*-Infected Mice

DAVID R. HILL

Division of Infectious Diseases, Department of Medicine, University of Connecticut School of Medicine, Farmington, Connecticut 06032

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Gastrointestinal immune events in giardiasis are important in controlling infection. In this study, Peyer's patch lymphocytes from mice infected with *Giardia muris* developed specific, proliferative responses to *G. muris* antigen. This proliferation correlated with clearance of infection. Further understanding of the gut immune response will be helpful in developing immunoprophylactic strategies in the control of giardiasis.

In the common intestinal infection giardiasis, host immunity plays an important role in clearance of *Giardia* spp. (12, 24, 30), in protection from reinfection (14, 21, 25), and in production of disease, especially in individuals who present with spruelike lesions, as determined by intestinal biopsy (7, 23). In the murine model of *Giardia muris* infection, data support the importance of both T and B lymphocytes in this immune response (6). Athymic nude mice cannot clear infection until they have been reconstituted with lymphocytes (24). After reconstitution, it is likely that these mice clear their infections because they have been supplied with helper-inducer T lymphocytes, which are required in the production of intestinal antibody (4, 5, 8, 12, 16). Intestinal anti-*Giardia* immunoglobulin G (IgG) and secretory IgA have been implicated in both protection against and clearance of *G. muris* (11, 12, 30).

Because of the luminal location of this enteric protozoan, the gut-associated lymphoid tissue is likely to play the major role in the development of any immune response (2, 6, 28, 29). Intestinal Peyer's patches (PP) are the site of antigen recognition and processing. One of the first steps in this process may be the ingestion of *Giardia* antigen by PP macrophages (2, 8, 20; D. R. Hill, submitted for publication). This is likely to be followed by antigen processing by macrophages and sensitization of a population of T cells which function to help PP B cells differentiate and switch to produce *Giardia* sp.-specific secretory IgA (4, 8, 12, 15, 16, 29). In this study, we have examined the development of antigen-specific lymphocyte proliferation in the PP of *G. muris*-infected mice.

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For these studies, the murine model of *G. muris* infection was used (26). Six- to eight-week-old female BALB/c mice (specific pathogen free; Charles River Breeding Laboratories, Portage, Mich.) were screened for the presence of intestinal parasites and found to be negative. Mice were fed 10^3 *G. muris* cysts by esophageal cannula, and at multiple time points after infection, stool cyst counts per eight fecal pellets were determined with a hemacytometer (26) after sedimentation on a 1 M sucrose gradient. Uninfected mice were studied simultaneously as controls.

PP cells were obtained by previously established methods (17, 19, 22). Mice were anesthetized by ether and sacrificed by cervical dislocation. By using sterile techniques, the intestines were removed and flushed with cold (4°C) Hank's balanced salt solution. PP were identified, removed, and placed in cold RPMI 1640 with 5% (vol/vol) fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), penicillin (100 U/ml), and gentamicin (50 μg/ml). The PP were teased apart, the debris was allowed to settle, and the cells in the supernatant were obtained. The debris was treated with 200 U of collagenase (type IA; Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C for 60 min to further dissociate adherent cells (10, 19, 22). These cells were washed and combined with the previous fraction. All cells were enumerated with a hemacytometer, tested for viability by trypan blue (0.1%) exclusion, and differentiated by May-Grunwald staining of cytocentrifuge preparations. By this method, $2.9 \times 10^7 \pm 0.9 \times 10^7$ cells per mouse were obtained, of which 93.4% ± 4.6% were lymphocytes with greater than 90% viability (values are means ± standard deviations).

G. muris antigen was obtained by harvesting trophozoites from female CF1 mice 10 days after infection. The small bowels were opened longitudinally, placed in cold phosphate-buffered saline, and shaken vigorously for 10 s. Trophozoites were washed and then purified twice on a metrizamide gradient (specific gravity, 1.10) (1). The purified trophozoite preparation contained less than 400 CFU of bacteria per 10^8 trophozoites per ml. Trophozoites were then concentrated to 5×10^7 to 10×10^7 cells per ml with 1 mM phenylmethylsulfonyl fluoride, sonicated, and clarified by centrifugation (5,000 × *g*, 20 min). Protein concentrations were determined by the method of Lowry et al. (18). Approximately 3 mg of protein was obtained per 5×10^7 to 10×10^7 trophozoites.

At days 0, 3, 7, 10, 14, 21, 28, 35, 49, and 63 after infection, PP cells from two infected and two control mice were concentrated to 10^6 cells per ml of RPMI 1640 with 2 mM glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 100 U of penicillin per ml, 10 μg of gentamicin per ml, 100 μg of streptomycin per ml, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum. Cells (10^5) were added to triplicate wells of 96-well tissue culture plates with irradiated splenic accessory cells (3×10^5 cells per well irradiated at 2,500 rads for 25 min in a Gamma Cell 40, ^{137}Cs source). For all experiments, spleen cells were obtained from uninfected BALB/c mice. The optimal *G. muris* protein concentration was determined in preliminary experiments to be 50 μg/ml. *G. muris* antigen, ovalbumin as a control antigen (50 μg/ml), or concanavalin A (5 μg/ml) was added to a total volume of 250 μl per well, and the plate was incubated in humidified 95% room air–5% CO_2 at 37°C. After

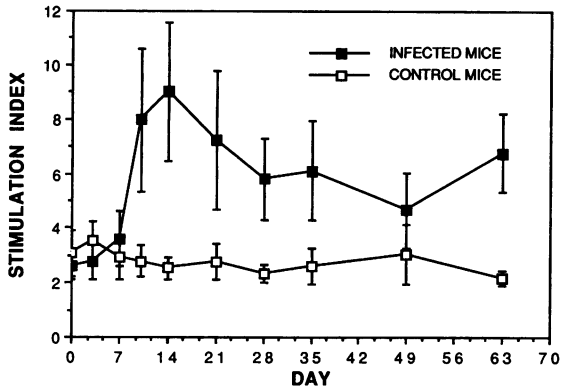

FIG. 1. SI of PP cells from *G. muris*-infected and control, uninfected mice. PP cells were incubated with *G. muris* antigen (50 μg/ml) plus irradiated, splenic accessory cells from uninfected mice, and [^{3}H]thymidine incorporation was measured after 3 days. The SI is the result of dividing the counts per minute of antigen-stimulated cells by those of cells without antigen. Bars represent standard errors.

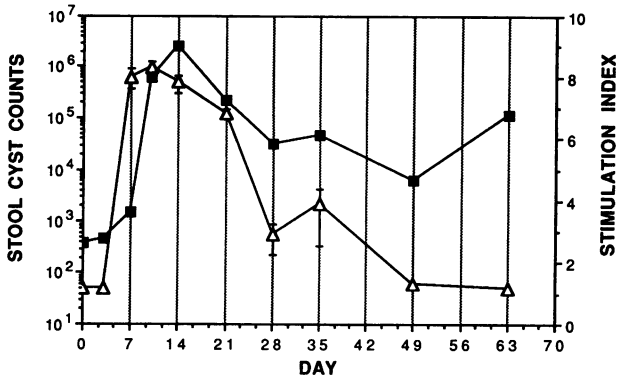

FIG. 2. A double plot with *G. muris* cyst excretion (△) and the SI of PP cells (■) from *G. muris*-infected mice. Cyst counts were obtained from eight fecal pellets, and the lower limit of cyst detection was 50. Bars represent standard errors.

72 h, wells were pulsed with [^{3}H]thymidine (1 μCi per well) for 4 to 6 h and then harvested, and incorporated radioactivity was measured.

Data are expressed as the stimulation index (SI), in which the counts per minute of PP cells (with splenic accessory cells) plus antigen are divided by the counts per minute of PP cells plus spleen cells alone. Comparisons between groups were evaluated by Student's *t* test. All data are expressed as the mean ± the standard error of the mean of results from five complete experiments, with at least three datum points for each time period.

PP cells obtained from mice infected with *G. muris* demonstrated blastogenic responses to *G. muris* antigen. Figure 1 illustrates the *G. muris*-specific SI of PP cells from infected and control mice during the course of the study. Control mice remained free of infection with *G. muris*. From day 0 to 7 there were no differences in the SIs of infected and control mice. However, the SI of cells from infected mice rose after day 7, peaked at 9.0 between days 10 and 21, and then gradually declined, but it remained significantly higher than the SI from control mice throughout the study (i.e., to day 63). The mean SI ± the standard error for PP cells from infected mice from days 28 through 63 was 5.83 ± 0.44, compared with 2.54 ± 0.19 for control cells ($P < 0.01$). The latter SI did not differ significantly from the mean SI of control cells for all days (0 through 63), which was 2.78 ± 0.13 ($P = 0.33$). The mean SI of control cells never rose above 3.6. When PP cells from infected mice were incubated with ovalbumin as a control antigen, there was no response; the mean SI for days 0 through 63 was 1.52 ± 0.12. The mean, baseline counts per minute ± the standard error in the absence of exogenous antigen for PP cells from infected mice (plus irradiated spleen cells) for all days was 2,768 ± 338. The mean counts per minute for these PP cells (plus spleen cells) incubated with concanavalin A was 229,000 ± 41,000.

Figure 2 correlates cyst excretion in infected mice with the SI of their PP cells. The stool cyst counts began to rise at day 7, remained elevated through day 21, and declined by 3 logs on day 28 and to below detection (<50 cysts) by day 63. The SI reached maximum levels 3 to 7 days after cyst counts peaked. After the maximum SI was reached (at 10 to 21 days), cyst excretion began to decline and the infection was cleared.

Increasing evidence points to important gastrointestinal immune events in the control of infection with *Giardia* spp. (6). This study demonstrates that during murine infection with *G. muris*, there is a specific lymphocyte response to *Giardia* antigen, the development of which correlates with clearance of infection. This finding supports other work which has examined immune events in PP of *G. muris*-infected mice. Carlson and colleagues (3) demonstrated that the total number of lymphocytes in PP began to rise at 7 to 10 days, remained elevated through 21 days, and gradually declined to control levels as infection was cleared. Associated with this increase was a switch in surface immunoglobulin markers of B cells from the IgM isotype to IgA (4). Serum and gut anti-*Giardia* antibodies followed this same course by beginning to rise 1 to 2 weeks following infection (6, 11, 12, 27). Taken together with previous work, the results from this study suggest a probable sequence in the host response to *Giardia* spp. There is initial antigen processing with a subsequent blastogenic response in which T cells sensitized to *Giardia* antigen act as help for B cells in immunoglobulin switching and maturation for the production of specific anti-*Giardia* antibody (3–6, 10, 12, 13, 16).

In our study, the blastogenic response peaked from days 10 through 21 and remained elevated for the duration of the study, suggesting that even though mice had cleared their infection, their gut immune system remained primed to *Giardia* antigen. This finding is consistent with the resistance to reinfection that mice demonstrate (25). Although the specific contribution of B and T cells to the proliferative response was not measured, it is likely that both are being stimulated, on the basis of the sequence of immune events in PP.

Lastly, the *Giardia* antigen preparation itself induced weak thymidine uptake in cells from uninfected mice (Fig. 1) compared with the response by cells from infected mice incubated with ovalbumin. This suggests a minor mitogenic effect of the preparation, possibly from *Giardia* proteins or from contamination with enteric contents such as endotoxin.

As the immune events in the host response to *Giardia* spp. are elucidated, the system used in the present study should prove valuable in defining the specific antigens to which this response is directed. This should yield additional information in understanding giardiasis and in developing immunoprophylactic strategies to control this common infection.

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