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Gut Intraepithelial Lymphocytes Induce Immunity against *Cryptosporidium* Infection through a Mechanism Involving Gamma Interferon Production

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Immunological control of infection with cryptosporidia in mice is dependent on $CD4^+$ T cells and the production of gamma interferon (IFN-γ), but to date, the mucosal T cells which produce IFN-γ local to the infection have not been characterized. We previously showed that immunity against the gastric parasite *Cryptosporidium muris* could be adoptively transferred to adult SCID (severe combined immunodeficiency) mice with small intestinal intraepithelial lymphocytes (IEL) from previously infected immunocompetent mice, but only if the donor $CD4^+$ T cells were intact. The present investigation examined whether IFN-γ was important in the effector mechanisms mediated by immune IEL in SCID mice. The development of resistance against *C. muris* infection in SCID mice given immune IEL was prevented by treatment with a hamster anti-mouse IFN-γ-neutralizing monoclonal antibody, but following cessation of antibody treatment, the mice recovered from infection. In further experiments, an enzyme-linked immunospot (ELISPOT) technique was used to compare frequencies of IFN-γ-producing cells in activated T-cell populations from *C. muris*-immune and naive donor mice. Stimulation with concanavalin A or a rat anti-mouse CD3 monoclonal antibody resulted in detection of greater numbers of cells producing IFN-γ from immune than naive IEL populations. Small numbers of IEL from *C. muris*-immune mice, but not from naive mice, also produced IFN-γ when cultured with soluble oocyst antigen, but this occurred only if γ-irradiated spleen cells were cocultured with the immune IEL. These results suggested that IEL were important in the generation of immunity to *Cryptosporidium* and that one of their crucial functions was to produce IFN-γ at the site of infection.

Cryptosporidiosis is an infectious disease affecting mucosal surfaces in different types of vertebrates (8). In domestic mammals and humans, the infection usually affects the gastrointestinal tract, and the most prominent clinical feature is an unpleasant watery diarrhea which may be accompanied by vomiting and abdominal pain. The illness normally lasts for a number of days if there are no complicating circumstances but may be chronic and potentially fatal in immunocompromised hosts, including patients with AIDS. Cryptosporidiosis is common in neonatal cattle and sheep, and these animals may play an important role in zoonotic transmission. In humans, the disease is found in all age groups and is responsible for 1 to 4% of reported cases of diarrhea in developed countries; in tropical regions, there is a particularly high incidence in infants. The infectious agents are members of the genus *Cryptosporidium*, parasitic protozoans belonging to the subclass *Coccidia* which develop in mucosal epithelial cells. The parasite completes its life cycle in a single host and is transmitted in a fecal-oral manner by oocysts. The enteric disease of mammals is caused by *Cryptosporidium parvum*, while a less common parasite, *C. muris*, infects the gastric mucosa of cattle and rodents and may produce lesions and weight loss in cattle (2, 31).

In murine models of cryptosporidiosis involving *C. parvum* or *C. muris*, control of infection has been shown to require the presence of T cells (12, 17), particularly subpopulations which are T-cell receptor (TCR) $\alpha\beta^+$ (37) or $CD4^+$ (6, 16, 18, 22, 32, 33). In addition, evidence has been obtained that gamma interferon (IFN-γ) activity is important in expression of a partial innate immunity against the parasite and in the T-cell-dependent elimination of the infection. Thus, cryptosporidial infections in immunocompromised or immunocompetent mice were exacerbated by treatment with anti-IFN-γ antibodies (6, 7, 16, 17, 33, 34), and the development of T-cell-mediated control of infection correlated with increased production of IFN-γ in cells from lymphoid tissues identified in the spleen as predominantly $CD4^+$ (9, 11, 13, 30, 33). There is a need, however, to characterize the mucosal T cells involved in the immunological control of cryptosporidial infection and to elucidate the effector mechanisms requiring IFN-γ activity induced by these cells. Recent work in this laboratory has been directed at characterizing the role of mucosal lymphocytes and, specifically, intraepithelial lymphocytes (IEL) in the development of immunity against cryptosporidia.

IEL form one of three discrete immune compartments of the mucosal tissues, the others being organized lymphoid follicles such as Peyer's patches in the small intestine and the lamina propria (26). Intestinal IEL are predominantly T cells, but there is evidence that these are distinct from other peripheral T cells. For example, at least some subpopulations of T cells in IEL appear to mature locally in the gut independent of thymic influence (15). In addition, in the small intestine, the majority of the T cells are $CD8^+$, with expression of either a heterodimeric CD8αβ or homodimeric CD8αα molecule, whereas at other peripheral sites, $CD8^+$ cells form a minority of T cells and the heterodimeric form of the CD8 molecule is normally expressed (15, 26). IEL also have an unusually high percentage of TCR $\gamma\delta^+$ cells but few or no B cells and may demonstrate T-cell activation characteristics in vitro, although

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generally they are significantly less susceptible to activation than other T-cell populations (26, 28).

The biological functions of IEL in vivo are unclear, although their location suggests that they may be important in the regulation of local immune responses and in the protection of mucosal surfaces against pathogenic microorganisms. Shields and Parrott (25) observed that IEL demonstrated immunological memory in vivo, as the delayed hypersensitivity response to a protein antigen was adoptively transferred between mice. Results from in vitro studies have demonstrated that IEL could positively or negatively regulate immune responses, produce T-cell cytokines, demonstrate NK cell activity, or specifically lyse parasite- or virus-infected cells in a major histocompatibility complex-restricted manner (5, 20, 21, 26, 29, 36).

Models of *Cryptosporidium* infection will be valuable in the elucidation of the protective role of IEL against local intracellular infection, since the parasite's development is confined to the epithelium. Recently, we reported that immunity against *C. muris* infection could be transferred adoptively to immunocompromised SCID (severe combined immunodeficiency) mice from previously infected BALB/c mice, using as donor cells IEL isolated from the small intestine (19). Protection against the infection was associated with the $CD4^+$ T-cell subpopulation, since immunity was abrogated by depleting these cells from the donor IEL population, whereas depletion of $CD8^+$ cells reduced the level of immunity only slightly. IEL-reconstituted SCID mice which had recovered from infection were shown to have $CD4^+$ cells located in the gut epithelium, suggesting these cells may have played a direct role in the elimination of the parasite.

The present study was designed to examine whether this protective role of IEL against *C. muris* involved production of IFN-γ. Experiments were performed to measure the ability of IEL-reconstituted SCID mice to control *C. muris* infection following treatment with an anti-IFN-γ-neutralizing antibody. In addition, an enzyme-linked immunospot (ELISPOT) technique for in vitro detection of IFN-γ-secreting cells was used to compare the numbers of IEL from immune and naive animals producing this cytokine after stimulation with concanavalin A (ConA), anti-mouse CD3 antibody, or *C. muris* antigen. The results demonstrated that immune IEL elicited control of infection through a mechanism involving IFN-γ activity. In addition, in in vitro experiments, it was shown that immune IEL had an increased potential to produce IFN-γ following mitogenic or TCR-mediated nonspecific activation and that immune IEL, but not naive IEL, could be stimulated by parasite antigen in vitro to release IFN-γ. These results suggested that an important protective role for IEL in immunity to cryptosporidial infection was to provide a local source of IFN-γ production required for inducing parasite killing.

MATERIALS AND METHODS

Animals. C.B-17 female SCID mice (4) were obtained at 12 weeks of age from a colony maintained under aseptic conditions in this institute. Mice were housed individually in groups of five in wire-floored cages with filter lids and supplied with food sterilized by radiation and autoclaved water. BALB/c female mice, 8 to 12 weeks old, were obtained from Charles River U.K. Ltd.

Parasite. The RN 66 strain of *C. muris* was maintained and stored as previously described (17). Mice were infected following oral inoculation with 2×10^5 oocysts in sterile water or RPMI 1640 medium, and infections were monitored by measurement of oocyst production in feces as reported previously (17).

Preparation of lymphoid cell suspensions. Spleen and mesenteric lymph node (MLN) cells were obtained from BALB/c mice which had recovered 2 to 5 weeks previously from *C. muris* infection or were uninfected. Single-cell suspensions were prepared in RPMI 1640 medium supplemented with 25 mM HEPES, 200 U of penicillin per ml, 200 μg of streptomycin per ml, and 10% heat-inactivated fetal calf serum (all from GIBCO) (18). IEL were obtained from the small intestines of the same or similarly treated BALB/c mice, using a method described in detail elsewhere (18). In brief, small segments of the intestines of four to five mice were incubated in Ca^{2+}- and Mg^{2+}-free medium at 37°C and then shaken to release cells from the epithelial layer into the medium. IEL were then separated from epithelial cells and cellular debris by centrifugation through 40 and 70% Percoll in RPMI 1640 medium, collecting the lymphocytes at the interface between the two Percoll layers. The IEL were washed in supplemented medium (see above), and the viability, examined by trypan blue exclusion, was >90%. Approximately 4×10^6 purified IEL were obtained from each immune or naive mouse, and in adoptive transfer of immunity experiments, SCID mice were injected with 10^6 cells by the intravenous route.

Depletion of T-cell subsets. $Thy.1^+$, $CD4^+$, and $CD8^+$ cells were depleted from IEL by incubation of cells in 1 ml of medium with 1:200 dilution of rat monoclonal antibody YTS.154.7 (anti-Thy.1), YTS191.1 (anti-CD4), or YTS169.4 (anti-CD8α) plus 1:15 reconstituted rabbit complement (all from SeraLab) at 37°C for 30 min. The cells were then washed twice in supplemented medium by centrifugation at $250 \times g$ for 10 min.

In vivo treatment with anti-IFN-γ. Hamster immunoglobulin G monoclonal antibody specific for mouse IFN-γ (H22) was kindly provided by R. D. Schreiber, Department of Pathology, Washington University School of Medicine, St. Louis, Mo. Mice were injected by the intraperitoneal route with 200 μg of antibody in sterile saline.

ELISPOT for detection of IFN-γ-secreting cells. The ELISPOT method used for measuring numbers of cells producing IFN-γ was based on that described by Taguchi et al. (29), with some modifications. Standard parameters of the technique were established in studies with spleen and MLN cell cultures, and the system was then adapted for use with IEL. A 96-well microtiter plate with a nitrocellulose base (Millititer HA; Millipore Corp.) was incubated overnight at 4°C with 100 μl of rat anti-mouse IFN-γ monoclonal antibody R4-6A2 (5 μg/ml; Pharmingen) in enzyme-linked immunosorbent assay (ELISA) coating buffer (pH 9.6). Following washing of the plate with phosphate-buffered saline, pH 7.3 (PBS), the wells were blocked by incubation for 2 h at room temperature with supplemented medium and washed again with PBS. Cells were cultured in the wells in a volume of 200 μl, and the number of lymphoid cells added to wells varied from 0.1×10^6 to 1.0×10^6. Unless otherwise stated, 10^5 BALB/c spleen cells which received 900-rad γ-irradiation were also added to cultures containing IEL. The T cells were stimulated nonspecifically by addition of ConA (Sigma) or rat anti-mouse CD3 monoclonal antibody (Pharmingen); alternatively, *C. muris* soluble antigen, prepared as described by Tilley et al. (30), was added to wells. The plate was incubated for 20 h at 37°C in 5% CO_2 and air and washed thoroughly with PBS, and then PBS–0.005% Tween 20 (Sigma) was placed in each well for 5 min. After removal of PBS-Tween, 100 μl of 1:10,000 rabbit anti-mouse IFN-γ (3) in supplemented medium was added, and the plate left overnight at 4°C. The plate was washed with PBS-Tween, 100 μl of 1:20,000 goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma) was added to wells, and the plate was again incubated overnight at 4°C. Following a final wash with PBS-Tween, 100 μl of alkaline phosphatase substrate prepared with SigmaFast BCIP/NBT tablets (Sigma) was added; 30 to 60 min later, when maximal spot development had occurred, the plate was washed with distilled water. After allowance of 2 h for the nitrocellulose base to dry, the spots were counted in a dissection microscope.

Statistics. Standard errors are shown in the figures, and data were analyzed with Student's *t* test.

RESULTS

Role of IFN-γ in immunity to infection conferred by IEL. In an earlier study, we showed that SCID mice injected with IEL, isolated from BALB/c mice that had recovered from *C. muris* infection, developed immunity against this parasite (19). In the present study, we measured the effect on the development of this adoptive immunity of treatment with hamster anti-IFN-γ-neutralizing antibody. Groups of SCID mice received IEL from immune or naive mice prior to infection on day 0 with *C. muris* and were injected with the hamster anti-mouse IFN-γ neutralizing antibody H22 diluted in saline, or saline alone, on days 0, 7, 14, and 21. In a previous study, we found that treatment of mice with a control hamster antibody of the same isotype as H22 had no effect on the course of *C. muris* infection (17). Figure 1 shows that oocyst shedding from each group was first observed on day 13. The levels of oocyst excretion from control SCID mice (i.e., animals which received no IEL) and mice which received naive IEL then rose continuously until day 20 to reach a mean of $\geq 5 \times 10^6$ oocysts per day. Subsequently, chronic infections with this level of oocyst shedding were maintained in both groups throughout the remainder of the experiment. This demonstration that naive IEL were incapable of establishing an effective immunity was in

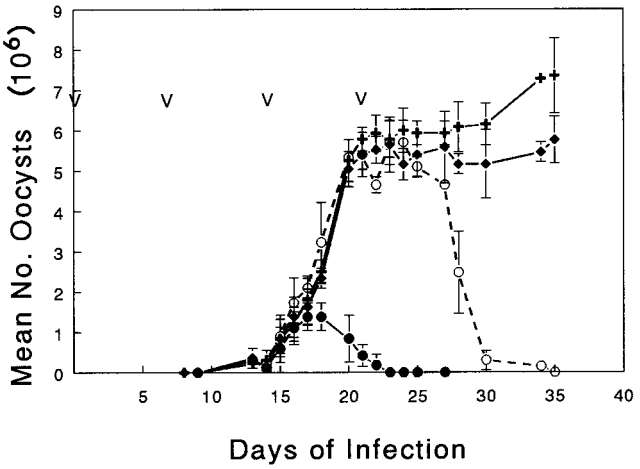

FIG. 1. Patterns of oocyst shedding in groups of IEL-reconstituted C.B-17 SCID mice infected with *C. muris* and the role of IFN-γ in recovery. SCID mice were injected intravenously with IEL from *C. muris*-immune or naive BALB/c mice prior to infection on day 0. The mice were also injected intraperitoneally with H22 hamster anti-mouse IFN-γ-neutralizing antibodies in saline or saline alone at intervals of 7 days at points indicated by arrowheads. The values on the graph represent the mean numbers of oocysts shed over a 24-h period. The groups of infected mice observed had received saline but no IEL (+), IEL from naive immunocompetent mice and saline (◆), IEL from immune mice and saline (●), or IEL from immune mice and anti-IFN-γ antibodies (○).

agreement with results of similar experiments in our previous study (19). Mice which received immune IEL, however, developed protective immunity early in the patent infection. The peak of infection in these mice occurred on day 17, when $(1.4 \pm 0.4) \times 10^6$ oocysts were excreted, and the infection had become subpatent by day 23.

In contrast, mice which received immune IEL plus anti-IFN-γ antibodies until day 21 postinfection failed to demonstrate immunity to infection during this period; indeed, a chronic pattern of infection similar to that observed in the controls was obtained. Starting from day 28 (i.e., 7 days after cessation of antibody treatment), however, these animals which had received immune IEL demonstrated a progressive decrease in levels of oocyst shedding; by day 35, the infections were subpatent. Similar results were obtained in a repetition of this experiment. These results indicated, therefore, that the adoptive transfer of immunity against *C. muris* infection obtained with immune IEL was inhibited by anti-IFN-γ neutralizing antibodies.

Quantification of IFN-γ-secreting cells in immune and naive mice following T-cell activation in vitro. An ELISPOT technique was used to measure the frequency of IFN-γ-producing cells in cultures of lymphoid cells from mice immune to *C. muris* infection and naive mice after stimulation with either a rat anti-mouse CD3 monoclonal antibody or ConA. In the initial experiments, we examined the effect of stimulation of spleen cells with anti-CD3 antibody. A notable finding with our ELISPOT system was that cytokine-producing cells were rarely detected when cells were cultured in medium alone; i.e., there was little evidence of spontaneous IFN-γ production. When either immune or naive spleen cells were stimulated with anti-CD3 (4 μg/ml), however, cells producing the cytokine were readily detected, but greater numbers of the IFN-γ-positive cells were obtained in the immune compared with naive populations; e.g., in one experiment, the mean numbers of cytokine-releasing cells measured in 10^5 cells were 400 ± 10 for the immune population and 168 ± 39 for the naive population. Similar results were also obtained when immune and naive splenocytes were nonspecifically activated with ConA (data not presented). These data indicated, therefore, that the spleen cell population from mice recovered from *C. muris* infection had a greater propensity to release IFN-γ after nonspecific activation than similar cells from naive animals.

Similar experiments were carried out to measure numbers of IFN-γ-producing cells in BALB/c IEL which came from naive or immune animals following coculture with anti-CD3 antibody or ConA. In preliminary experiments, we found that the presence of γ-irradiated spleen cells (iSp) in culture with IEL enhanced the frequency of cells observed in ELISPOT. In one experiment, for example, stimulation of IEL plus iSp with anti-CD3 antibody resulted in the detection of 1,075 ± 176 IFN-γ-positive cells in 10^6 cells, compared with 570 ± 70 when iSp were not present; no cytokine production was detected when iSp were cultured in the absence of IEL. In the experiments described below, therefore, iSp were routinely added to cultures of IEL unless otherwise stated. Figure 2 shows that no spontaneous production of IFN-γ was observed with IEL from either immune or naive mice when IEL were cultured in the absence of anti-CD3 or ConA. In the presence of different concentrations (0.5 to 8.0 μg/ml) of anti-CD3, IFN-γ-releasing

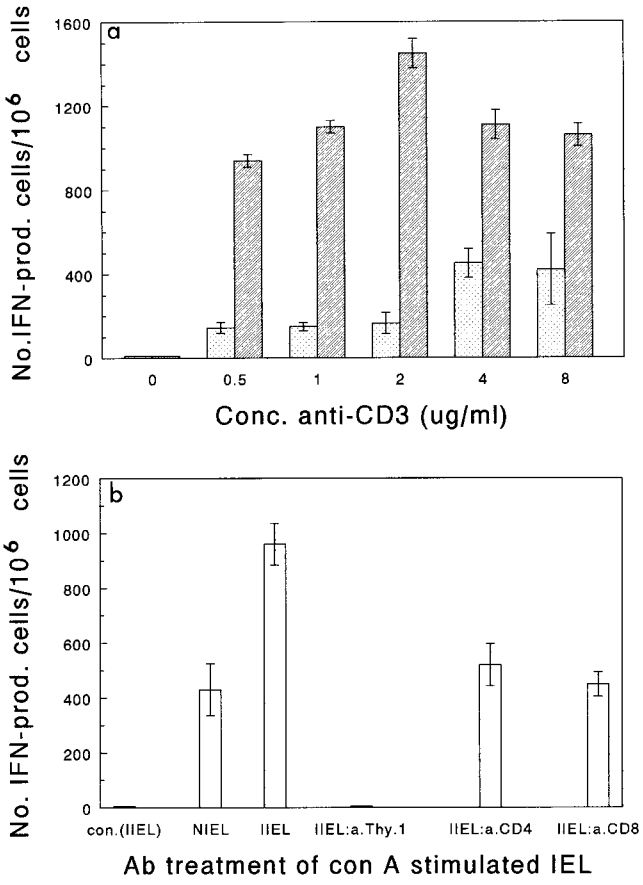

FIG. 2. Measurement by ELISPOT of the frequencies of IFN-γ-producing (IFN-prod.) cells in nonspecifically activated IEL from BALB/c mice recovered from *C. muris* infection (immune) or from uninfected (naive) mice. (a) Effects of the addition of various concentrations of rat anti-mouse CD3 monoclonal antibody on cytokine production of IEL from immune (grey bars) or naive (stippled bars) mice. (b) Measurement of the frequency of IFN-γ-producing cells in IEL from immune (IIEL) or naive (NIEL) mice stimulated with ConA (5 μg/ml) and the effect on cytokine production of depleting T-cell subsets. T cells were depleted by incubation of cells with antibodies (Ab) against T-cell surface markers (a.Thy.1, a.CD4, and a.CD8) and rabbit complement. con., control.

cells were readily detected (Fig. 2a) but significantly greater numbers were present in the immune than in the naive IEL population. For example, in cultures with 2 μg of anti-CD3 per ml, 1,445 ± 71 and 165 ± 49 IFN-γ-positive cells were found in the immune and naive IEL populations, respectively.

Similar results were obtained when IEL from immune and naive mice were stimulated with ConA (Fig. 2b). In addition, it was found that Thy.1 cells were responsible for production of IFN-γ following stimulation with this mitogen, since following depletion of Thy.1 cells from IEL, no production of IFN-γ could be detected after culture with ConA. This observation was in agreement with results from studies by other groups showing that the Thy.1$^+$ subpopulation present in IEL was more susceptible to activation in vitro with ConA (or anti-CD3) than a Thy.1$^-$ T-cell subpopulation also present (36). Depletions of either CD4$^+$ or CD8$^+$ cells resulted, in each case, in comparable (>40%) reductions in the frequency of cells detected. The results of T-cell depletion experiments with naive IEL provided similar results (data not presented). The data from these experiments, therefore, demonstrated an increased potential of Thy.1$^+$ cells from gut epithelium of *C. muris*-immune mice to produce IFN-γ following nonspecific activation.

Antigen-specific stimulation of IFN-γ production. The ELISPOT system was used to compare parasite antigen stimulation of IFN-γ production by cells from lymphoid tissues of *C. muris*-immune and naive animals. In the initial experiments, we measured the responses of immune or naive spleen and MLN cells to parasite (oocyst) soluble antigen. No production of IFN-γ was observed if cells were cultured in the absence of antigen or if cells from naive mice were cultured with antigen (data not shown). When immune spleen or MLN cells were cultured with antigen, however, IFN-γ production could be readily demonstrated, although spleen cells contained higher numbers of cytokine-secreting cells than MLN cells. In one experiment, the immune spleen and MLN cell populations had 317 ± 24 and 130 ± 32, respectively, antigen-specific IFN-γ-secreting cells per 10^6 cells. This observation was in accord with results of an earlier study in which we found that spleen cells from *C. muris*-infected mice produced higher amounts of IFN-γ, measured by ELISA, than MLN cells (9). Using the ELISPOT system, therefore, we demonstrated that spleen and MLN cells of *C. muris*-immune mice were specifically activated to release IFN-γ in the presence of parasite antigen.

Finally, we examined the capability of immune and naive IEL to produce IFN-γ when IEL were cultured with parasite antigen (Fig. 3). Stimulation of IFN-γ production by IEL was obtained following incubation with *C. muris* oocyst antigen, but cytokine release was detectable with IEL from *C. muris*-immune mice and not with IEL from naive mice. Relatively few IFN-γ-producing cells, however, were observed as a result of antigen stimulation (62 ± 2 in 10^6 cells in this experiment), and to detect antigen-specific activation of IEL, it was necessary to place at least 10^6 of these cells in a well (data not shown). Furthermore, production of the cytokine was detected only when the IEL were cultured in the presence of iSp. Nevertheless, in five experiments, parasite antigen was consistently shown to stimulate IFN-γ release by cells in immune but not naive IEL populations. Thus, the results of these experiments demonstrated that IEL from *C. muris*-immune mice were specifically activated by parasite antigen to produce IFN-γ.

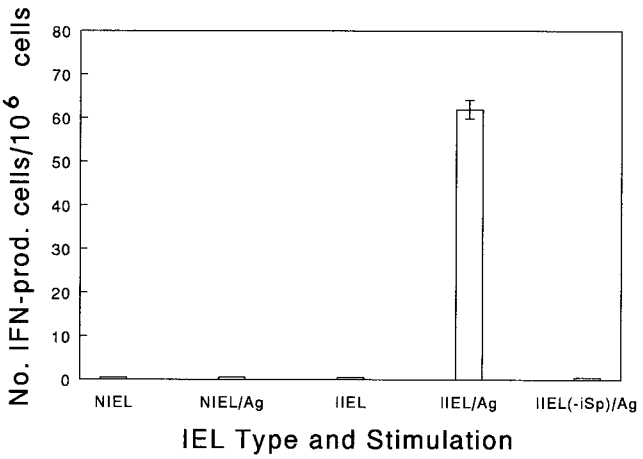

FIG. 3. Measurement of numbers of IFN-γ-producing (IFN-prod.) IEL from BALB/c mice stimulated with *C. muris* antigen. The IEL were isolated from mice which had recovered from *C. muris* infection (IIEL) or had been uninfected (NIEL) and cultured with soluble oocyst antigen (Ag; 5 μg/ml). The IEL were cultured with iSp except where indicated (-iSp).

DISCUSSION

Results presented here indicated that IEL could generate an immune response in vivo leading to elimination of cryptosporidial infection and that an important role for these cells was to serve as a mucosal source of IFN-γ production. We obtained evidence confirming our earlier finding that SCID mice, which normally develop chronic cryptosporidial infections, recovered from *C. muris* infection if injected with IEL from immunocompetent mice previously infected with this parasite (19). In addition, the results from the present study showed that the mechanism of immunity expressed by IEL involved IFN-γ activity. Thus, SCID mice given immune IEL failed to control infection while undergoing treatment with anti-IFN-γ-neutralizing antibody and could recover only after cessation of the antibody treatment. This observation was consistent with previous findings by a number of workers that T-cell-mediated control of *C. muris* or *C. parvum* infection required IFN-γ activity (6, 17, 33, 34). Immunity to these parasites was also associated with increased production of IFN-γ by spleen or MLN cells (9, 11, 30). Furthermore, two recent reports indicated that the development of immunity to *C. parvum* correlated with the production of IFN-γ in the intestine (13, 34), but the cellular source of the cytokine was not identified in either investigation. The present results suggested that IEL may be an important mucosal source of IFN-γ in the immune response against *Cryptosporidium*, particularly as we showed previously that in IEL-reconstituted SCID mice which had recovered from *C. muris* infection, protective donor cells had migrated to the gut epithelium (19).

To measure IFN-γ production by cells from *C. muris*-immune and naive mice, we used a sensitive ELISPOT method to detect cytokine-producing cells. Following nonspecific stimulation with ConA or anti-CD3 antibody, greater numbers of IFN-γ-producing cells were evident in IEL or spleen cells from immune compared with naive donors. These results were unlikely to have been due to differences between the ratios of major lymphocyte subpopulations present in immune and naive cell populations, as we found no significant changes in these ratios in either IEL or spleen populations as a result of infection (9, 19). The bias toward production of IFN-γ by immune IEL could have reflected the development of a mucosal T-cell inflammatory (TH1) response (1) which may be required for elimination of cryptosporidial infection (34). A similar finding was recently made in the study of IEL from intestinal infection with *Listeria monocytogenes*, which, like *Cryptosporidium*, in-

duces protective cell-mediated immunity via mechanisms involving IFN-γ (38).

In the study of in vitro stimulation of lymphoid cells by cryptosporidial antigen, cells from the spleen, MLN, and IEL populations of *C. muris*-immune mice, but not naive mice, released IFN-γ in the presence of soluble oocyst antigen. The observation of cells from the mucosa responding in this way to antigen was particularly important, as it implied that cryptosporidial infection could activate IFN-γ production by lymphocytes within the gut epithelium. The presence of iSp was necessary for detection of antigenic stimulation of IFN-γ production, and it is possible that the iSp provided macrophages, which are uncommon in intestinal IEL populations (26), for antigen processing; alternatively, the iSp may have provided a soluble factor(s) or direct cell-to-cell signalling with IEL necessary for the IFN-γ response. The antigen-specific response of IEL was relatively small, however, and the conditions for successful detection were more stringent than for spleen cells (e.g., a minimum of 10^6 IEL were required in a well). The reasons for this are unclear, but T cells in IEL and nonmucosal T cells have different activation signals (26), and the former may be less suited to the conditions of culture used. (Certainly, the amounts of IFN-γ produced in supernatants from IEL cultured with antigen [or ConA] were usually undetectable by ELISA [results not presented], whereas under similar culture conditions, large amounts of IFN-γ were found in supernatants of similar cultures of *C. muris*-immune spleen cells [9, 30]). Furthermore, although both immune $CD4^+$ and $CD8^+$ IEL responded to nonspecific activation with ConA (Fig. 2b), it was possible that only a small subset of T cells in IEL responded specifically to parasite antigen. In this regard, we have shown in adoptive immunization experiments, using small intestinal IEL as donor cells, that the $CD4^+$ cell subset, representing less than 10% of these cells (26), was required for protection against *C. muris*, whereas the $CD8^+$ cells, forming the majority of the cells (26), were of minor importance (18). Other studies have also indicated that $CD4^+$, and not $CD8^+$, cells were necessary for control of cryptosporidial infections (6, 18, 22, 32); in support of this observation, we and others have reported that $CD4^+$ cells of immune splenocytes were predominantly or entirely responsible for IFN-γ production following stimulation with parasite antigen (11, 30). Future studies in this area will attempt to improve the sensitivity for detecting cytokine production by IEL and also characterize the IEL which produce IFN-γ in response to infection.

With respect to mucosal infections, there have been few other reports showing antigen-specific induction of cytokine production by IEL in vitro; two examples involved studies of mice recovered from infection with either *Trichinella* or *Listeria* species (10, 38). The finding in our infection experiments that IEL from *C. muris*-immune mice promoted resistance to infection with this parasite through IFN-γ activity correlated with the in vitro findings that immune IEL had an increased capability to produce IFN-γ following nonspecific activation and that cells in this immune population could release IFN-γ when stimulated with parasite antigen. These results indicated, therefore, that in the local immune response to cryptosporidial infection, IEL were probably an important source of the increased mucosal production of IFN-γ associated with elimination of the parasite.

Finally, the role of IFN-γ in the effector phase of immunity to cryptosporidial infection remains to be established. Epithelial cells have been shown to possess IFN-γ receptors (35), and the cytokine has been shown to inhibit development of other coccidia (24, 27) by mechanisms which include macrophage activation, inhibition of host cell invasion (14), and alteration of host cell metabolism (23).

In conclusion, the data shown here indicate that IEL play a major role in the development of the mucosal immune response for elimination of *C. muris* infection and that a crucial function of these cells is to provide IFN-γ required for effecting parasite killing.

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