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Role of CD4+ TH1- and TH2-cell-secreted cytokines in cryptosporidiosis

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Abstract. *Cryptosporidium parvum* is now recognized as an important gastrointestinal pathogen around the world. Unfortunately, control measures for cryptosporidiosis are currently not well defined and very little is known about the immunological events relevant to the control of infection. Cytokine depletion experiments were conducted by injecting adult BALB/c mice with monoclonal antibodies directed to IL-2, IL-4, and IL-5, prior and during *C. parvum* infection. Faecal oocyst excretion and intestinal parasite number were monitored from day 4 to day 31 post-infection. Mice injected with monoclonal antibodies to IL-5 or IL-4/IL-5, but not IL-4 alone, harboured significantly higher numbers of both intestinal parasites and excreted oocysts. It is concluded that IL-5 complemented with IL-4 appears to be an important TH2-dependent mechanism for controlling cryptosporidiosis. Nevertheless, we suggest that cytokines secreted by both TH1 and TH2 cells may operate in concert in controlling cryptosporidiosis, triggering different functional mechanisms in a dynamic and simultaneous up- and down-regulatory fashion.

Cryptosporidium parvum Tyzzer, an enteric dwelling coccidian parasite, has emerged recently as a leading cause of diarrhoeal illness worldwide, targeting primarily the immunologically naive and the immunocompromised, particularly AIDS patients (Grant 1983, Ungar 1990). Severe illness may predispose immunocompromised individuals to life threatening situations, and is exacerbated by the absence of effective chemotherapy. This is in part due to the shortage of appropriate *in vitro* and *in vivo* laboratory models. Animal studies of cryptosporidiosis have been largely confined to neonates due to an "inherent lack of susceptibility" in older animals (Tzipori 1988, Novak and Sterling 1991) and to severely immunodeficient murine models such as T cell deficient athymic nude mice, SCID mice, NIH-III mice (bg/nu/xid), dexamethasone-treated C57BL/6 mice, and LP-BM5 B cell leukemia virus-infected C57BL/6 mice (Darban et al. 1991; Mead et al. 1991a,b; Rasmussen and Healy 1992; Kuhls et al. 1992). Unfortunately, the lack of immunocompetent laboratory animal models has hampered work to define the functional immunoresponses operative in cryptosporidiosis. One recent approach to unravelling the immunological mechanisms influencing susceptibility to *C. parvum* infections has been to treat mice with monoclonal antibodies (MAbs) directed to specific surface markers such as CD8 and CD4, as well as specific cytokines such as IFNγ (Ungar et al. 1991).

In an attempt to identify specific cytokines secreted by $CD4^+$ TH cells that may influence susceptibility to infection, BALB/c mice were treated with MAbs specific to IL-2, IL-4, and IL-5, and the course of cryptosporidiosis was followed by determining the number of parasites in the intestine and the number of oocysts excreted in faecal material. The influence of TH1 and TH2-secreted cytokines on susceptibility to infection is discussed.

MATERIALS AND METHODS

Animals. Male BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Maine) at 6 weeks of age. All mice underwent a 2-week quarantine and were checked for common pathogens prior to experimentation. The animals were maintained at the University of Arizona Animal Care Facility in filter-topped plastic cages with wood chip bedding. Animals were maintained at 12 hr photoperiod cycles and food and water were provided *ad libitum*.

***Cryptosporidium parvum* oocysts.** An Iowa isolate of *Cryptosporidium parvum* was originally obtained from Dr. Harley Moon. This isolate was maintained by passage of infection using ~10^8 oocysts given *per os* to 2-day-old Holstein bull calves. Faecal material was filtered through stainless steel screens having diminishing pore sizes (final mesh size was 230:63 μm). Oocysts were purified by sequential centrifugation procedures involving discontinuous sucrose (1.064/1.103 g/ml) and isopycnic Percoll (1.091 g/ml) gradients and stored in potassium dichromate at 4°C until used (Arrowood and Sterling 1987).

Ascites production. Groups of 10 male BALB/c mice were primed with 500 μl of pristane (Sigma) and 10 days later all mice received 525 rads. This radiation dose was found to be optimal for growth of xenogeneic hybridomas and ascites production in mice (Enriquez, unpublished observations). Twenty-four hours later, each group of mice was injected intraperitoneally with $2x10^6$ cells of the respective rat anti-mouse cytokine hybridomas, namely rat anti-murine IL-4 11B11, kindly supplied by the Biological

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Response Modifiers Program at the National Cancer Institute, rat anti-murine IL-5 TRFK 5, generously donated by the DNAX Research Institute of Molecular and Cell Biology; rat anti-murine IL-2 G4G11 developed in our laboratory (Ragland et al. 1992); and the control MAb H4H9 directed against *Heligmosomoides polygyrus* Dujardin (Monroy and Enriquez 1992) developed in our laboratory (unpublished data). Ascites was collected by tapping the mice with an 18 gauge needle for 1–2 weeks after hybridoma injection. Ascites from each group of mice receiving the same hybridoma were pooled, centrifuged at 3,000 rpm for 15 minutes, and the respective MAbs were purified by column and affinity chromatography (BioRad and Cole Palmer, respectively), and stored in cryovials (3 mg/vial) at -80°C. The MAb protein concentration was calculated using the BioRad kit and by rat Ig capture ELISA determined for each sample according to the O.D. obtained from known concentrations of MAbs (Harlan Bioproducts for Science, Indianapolis, IN) run for each ELISA plate separately. The specificity of each MAb was also confirmed using recombinant IL-2 (rIL-2; Genzyme Corp., Boston, MA), rIL-4 and rIL-5 (kindly donated by the Biological Response Modifiers Program at the National Cancer Institute) by ELISA using peroxidase-labelled goat anti-rat antiserum (Kirkgaard and Perry).

Experimental design. Five groups of 6 mice per treatment group were injected on day -2 and on day 5 post-*C. parvum* infection with 1 mg of the respective MAb (anti-IL-2, anti-IL-4, anti-IL-5, both anti-IL-4/IL-5, and *H. polygyrus* control) intravenously in the lateral tail vein using a 30 gauge needle. On day 0 all mice received $5x10^5$ *C. parvum* oocysts *per os* using a blunted 18 gauge needle. Faecal pellets were collected from all mice on days 4, 7, 12, 18, 25, 28, and 31, and stored in 10% formalin. These faecal samples were used to determine the number of oocysts by immunofluorescence using the anti-oocyst MAb OW50 (Arrowood and Sterling 1989). Mice from each group were sacrificed by cervical dislocation following ether sedation on days 4, 12, and 31 to examine the number of parasites in the terminal ileum. Briefly, the entire small intestine was dissected out and one-inch sections of the terminal ileum were cut and transferred into a vial containing 10% formalin. Histological cuts of the intestinal fractions in paraffin were stained with hematoxylin and eosin. The slides were observed at 400x using a microscope and the number of parasites per intestinal villus were counted and recorded. A minimum of 25 villi were counted from each sample. Significance was determined by the Student's *t*-test (Snedecor and Cochran 1980).

RESULTS

As seen in Fig. 1, treatment with anti-IL-2 MAb caused an infection in mice with significant faecal oocyst excretion from days 4 to 7 as compared to control MAb-treated mice ($p < .0001$). However, no parasites were found in the terminal ileum (Fig. 2). Anti-IL-4-treated mice showed similar oocyst shedding but marginally lower than anti-IL-2-treated mice with no evidence of parasites in terminal ileum. Conversely, anti-IL-5-treated mice shed a consistent number of oocysts that persisted until day 31 post-infection, peaking on day 7 post-infection. Intestinal parasites in anti-IL-5-treated mice were present in high numbers between days 12 and 31 post-infection that differed significantly from MAb control-treated mice ($p < .001$). Mice treated with MAb to both IL-4 and IL-5 exhibited the highest and more consistent numbers of both intestinal parasites and faecally-excreted oocysts (Figs. 1, 2).

DISCUSSION

Immune response studies to *C. parvum* infection have largely been confined to immunodeficient hosts (Ungar 1990). The majority have been documented as case reports of individuals suffering from the acquired immune deficiency syndrome (AIDS). Cryptosporidiosis in murine laboratory hosts has been demonstrated in neonatal mice, severely immunodeficient inbred strains (nude, SCID, NIH-III), chemically-immunosuppressed mice, or mice immunodeficient following an AIDS-like viral infection (Tzipori 1988; Novak and Sterling 1991; Darban et al. 1991; Mead et al. 1991a,b; Rasmussen and Healy 1992; Kuhls et al. 1992). A systematic screening of inbred and congenic mice of different H-2 haplotypes for susceptibility to *C. parvum* infection failed to identify a useful immunocompetent model (Enriquez and Sterling 1991). Even mast cell deficient (W/Wv) and NK cell deficient C57BL/6bgJ (beige) mice have proven only marginally susceptible to infection (Enriquez and Sterling 1991, Harp and Moon 1991). Thus, immunological studies following cryptosporidiosis in immunocompetent hosts are not yet feasible. Nevertheless, based on clinical studies and in passive immunotherapy it seems that both humoral and cellular immunity are essential to control infection effectively (Zu et al. 1992).

The central component of the immune response cascade is the $CD4^+$ T helper cell (TH). The heterogeneity of cloned TH cells permitted identification of at least two major subsets of TH cells based on their lymphokine secretion patterns. IL-2 and IFNγ-producing TH1 cells induce IgG2a (to a lesser extent IgM and IgG1) among other efferent immune mechanisms and are associated primarily with responses to intracellular pathogens (Mossman and Coffman 1989, Sher and Coffman 1992). Conversely, IL-4, IL-5, IL-9, and IL-10-secreting TH2 cells are associated with responses to multicellular gut-dwelling helminths (Coffman et al. 1989, Finkelman et al. 1991). Both IL-4 and IL-5 act to enhance the growth and differentiation of activated B cells leading to the production of mucosal IgA (McGhee et al. 1992). Antibodies, particularly at gut-associated lymphoid tissue (GALT) level, may be important in the control of cryptosporidiosis. For example, antibodies within hyperimmune bovine colostrum and anti-*C. parvum* MAbs have proven effective in reducing infection burdens in immunodeficient animal models and AIDS patients (Riggs and Perryman 1987, Tzipori et al. 1987, Arrowood et al. 1989,

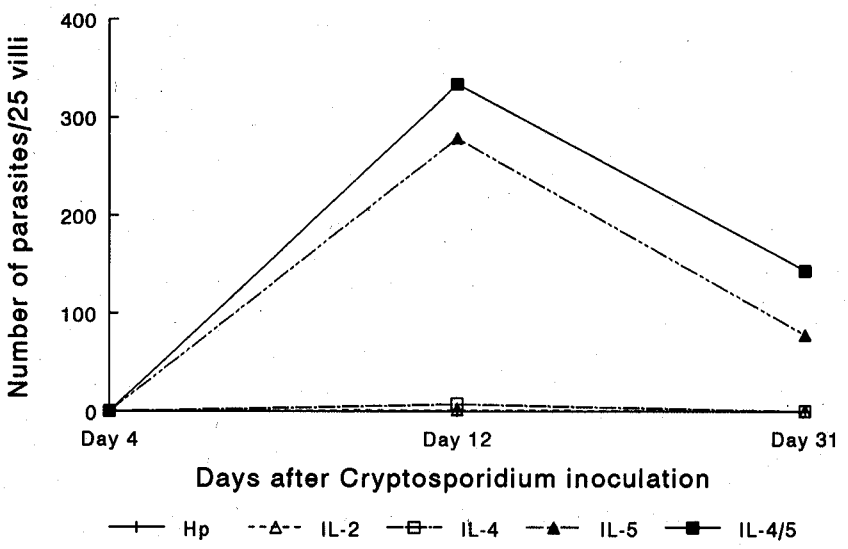

Fig. 1. Pattern of *Cryptosporidium parvum* oocyst faecal excretion in adult BALB/c mice depleted of IL-2, IL-4, IL-5, or both IL-4 and IL-5. Mice were injected with 1 mg of the respective anti-cytokine MAbs or control anti-*Heligmosomoides polygyrus* MAb iv 2 days prior to and 5 days post-infection with $5x10^5$ oocysts. The number of excreted oocysts represents the mean number of oocysts per microscopic field.

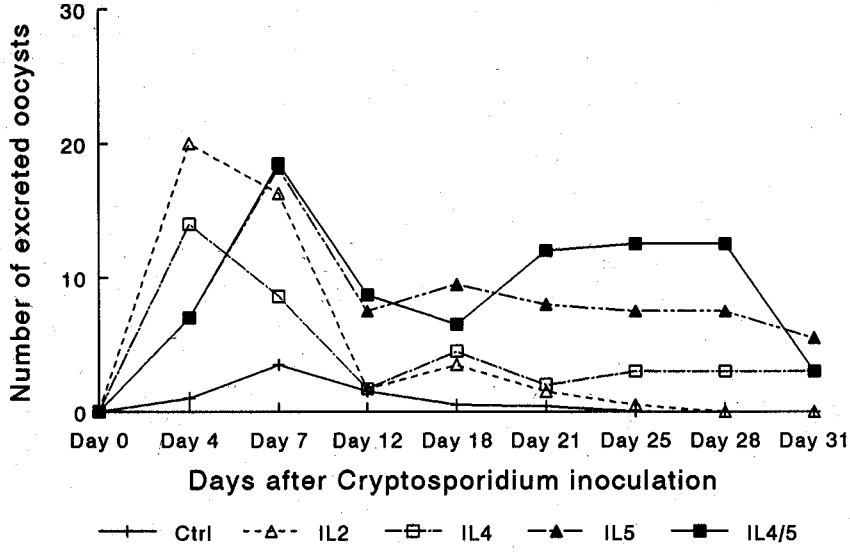

Fig. 2. Pattern of *Cryptosporidium parvum* intestinal infection in adult BALB/c mice depleted of IL-2, IL-4, IL-5, or both IL-4 and IL-5. Mice were injected with 1 mg of the respective anti-cytokine MAbs or control anti-*Heligmosomoides polygyrus* MAb iv 2 days prior to and 5 days post infection with $5x10^5$ oocysts. On the respective day post-infection the number of parasites were counted at 400x in hematoxylin/eosin stained histological sections of terminal ileum. Data are expressed as number of parasites per intestinal villus. A minimum of 25 intestinal villi were counted from each sample.

Ungar et al. 1990, Perryman and Bjorneby 1991). However, virtually nothing is known about the identification and localization of interacting cell types of the immune network, particularly within GALT in response to this mucosal infection. During *C. parvum* infections in calves, IgA and IgM coproantibodies were increased during infection and decreased during recovery (Peeters et al. 1992). Additionally, both IgA and IgG antibodies increase in intestinal secretion and appear to recognize a 17 kDa molecule (Reperant et al. 1992). IgA constitutes >80% of all antibodies produced in mucosa-associated tissues and this production is TH2-dependent.

In virtually all intracellular parasitic infections there is clear association of TH1 cell responses such as observed in *Leishmania, Trypanosoma*, and *Plasmodium* (Chatelain et al. 1992, Sher and Coffman 1992, Silva et al. 1992). *Eimeria vermiformis* infections in resistant BALB/c and susceptible C57BL/6 mice resulted in an increase of both IFNγ and IL-5 in antigen-stimulated mesenteric lymph nodes of resistant but not susceptible mice. In addition, there was no evidence of downregulation of TH2-secreted cytokines on production of IFNγ. This pattern however, was suggested to be a kinetic rather than a qualitative ability to mount a protective

TH cell subpopulation response (Wakelin et al. 1993). This infection, as opposed to *C. parvum* infections, is self-limited in both susceptible and resistant strains.

In many enteric parasitic infection models (e.g. *Heligmosomoides polygyrus*, *Trichuris muris*, *Nippostrongylus brasiliensis*) responses are predominantly TH2 as demonstrated by cytokine depletion studies (Coffman et al. 1989, Pearce et al. 1991, Finkelman et al. 1991, Sher and Coffman 1992), with differential TH1/TH2 induction between susceptible and resistant strains (Else et al. 1992). In some cases like in *Trichinella spiralis* infections however, delayed expulsion of intestinal worms are TH2-mediated while rapid worm expulsion responses are TH1-mediated, suggesting that TH responses are compartmentalized (Pond et al. 1989).

While an overall immunodeficient state, along with a high infective dose, appear to influence the chronicity and severity of *C. parvum* infections, the actual immunological mechanisms are not well understood. It is clear that $CD4^+$ and $CD4^+/CD8^+$ T cells, as well as IFNγ have a role in development of cryptosporidiosis as observed by *in vivo* cytokine depletion studies following injection with specific MAbs (Ungar et al. 1991). This suggests that susceptibility to *C. parvum* infections may be controlled by $CD4^+$ TH1-mediated responses. Data presented herein suggest that both TH1 and TH2 cells may be associated in response to infection and that the TH1/TH2 cell regulation of cryptosporidiosis appears to be a dynamic one. While IL-2 and IL-4 seem to play a role during the initial stages of *C. parvum* infection, IL-5 as well as IL-5 complemented by IL-4, appear to control the chronicity of infection. It is probable that after a few days following cytokine depletion, cytokine levels would approach pretreatment levels and thus, the infection could be then controlled. However, anti-IL-5 as well as anti-IL-5/IL-4-treated mice exhibited intestinal infection and excreted oocysts until the end of the experiment (day 31 post-infection). The TH1/TH2 dichotomy in infection control seen in other protozoan and helminth infections seems not to operate in the same fashion as in this intestinal intracellular coccidian infection. Rather, a multisignal TH1/TH2 mechanism may control or exert susceptibility to *C. parvum* infection. IL-2 (TH1-secreted) and IL-4 (TH2-secreted) may play a role at the initial stages of infection, which then it may be taken over by IL-5 (TH2-secreted) and in turn complemented by IL-4. Further studies involving the role of other cytokines in cryptosporidiosis are necessary before firm conclusions can be drawn. However, we suggest that both TH1 and TH2 cells may operate in concert in controlling cryptosporidiosis, triggering different functional mechanisms in a dynamic and simultaneous up- and down-regulatory game.

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