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ORIGINAL PAPER

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Kinetics of mucosal ileal gamma-interferon response during cryptosporidiosis in immunocompetent neonatal mice

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Abstract The kinetics of serum and ileal interferon-gamma (IFN-γ) content were determined during recovery from cryptosporidiosis in NMRI suckling mice. A total of 60 mice aged 4 days were inoculated by intragastric gavage with 10^4 cryptosporidia (n = 30) or phosphate-buffered saline (n = 30). Six animals per group were killed on days 0, 3, 6, 9 and 13 postinoculation. Blood samples and ileum were collected. Experimental infection was followed by a rise in parasite load in the ileum starting on day 3 postinfection, which peaked at day 6 postinoculation. Ileal IFN-γ levels increased rapidly in parasitized mice from day 3 to day 6, then fell rapidly. These levels were significantly higher than the control values (day 3 $P<0.05$, days 6 and 9 $P<0.001$). IFN-γ secretion began before parasite excretion, but the curves of these two parameters correlated positively. Recovery from cryptosporidiosis in immunocompetent neonatal mice is thus associated with an early and marked increase in ileal IFN-γ content.

Introduction

Cryptosporidium parvum is a coccidial protozoan that infects the gastrointestinal epithelial cells of humans and other mammals. The infection can involve all the gastrointestinal segments but is mainly located in the ileum (Current and Garcia 1991). In immunocompetent individuals, *C. parvum* infection is uncommon and causes only self-limited diarrhea. In contrast, in patients with acquired immunodeficiency syndrome (AIDS), cryptosporidiosis is a frequent opportunistic infection causing unremitting and, frequently, life-threatening diarrhea (Current and Garcia 1991; Current et al. 1983; Tzipori 1988). This points to an important role of host immune factors in controlling the infection.

The intestinal immune system is characterized by the secretion of large amounts of immunoglobulins onto the mucosal surfaces. An altered secretory immune response has been suggested to explain the frequent and chronic mucosal infections encountered in AIDS patients (Hill et al. 1990; Laxer et al. 1990; Peeters et al. 1992). However, although they mount a pathogen-specific mucosal antibody response, AIDS patients with cryptosporidiosis fail to clear the parasite (Benhamou et al. 1995b).

Cell-mediated immunity might play an important role in the host defenses against *C. parvum*. Experimental studies have shown that immunocompetent mice can overcome cryptosporidial infection, but CD4+ lymphocyte-deficient animals develop a chronic infection (Heine et al. 1984; McDonald et al. 1992; Sherwood et al. 1982; Ungar et al. 1990, 1991). Moreover, chronic enteric cryptosporidiosis is observed in patients infected with human immunodeficiency virus (HIV) when the CD4+ lymphocyte count is under 140/μl (Flanigan et al. 1992). Gamma-interferon (IFN-γ) might be a key cytokine in resistance against *Cryptosporidium* as both immunocompetent and immunodeficient mice become more susceptible to *Cryptosporidium* infection when treated with anti-IFN-γ monoclonal antibodies (Chen et al. 1993a,b; McDonald and Bancroft 1994; Ungar et al. 1991). However, intestinal mucosal IFN-γ production has never been studied during cryptosporidiosis. The purpose of this study was to measure the kinetics of the intestinal mucosal IFN-γ content during cryptosporidiosis in an immunocompetent suckling mouse model.

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Materials and methods

Mice

Six NMRI dams with 1-day-old litters were purchased from IFFA-CREDO (L'Arbresle, France). A single dam with litter was housed individually in a cage, and the size of each litter was adjusted to ten individuals.

Parasites

Cryptosporidium parvum were produced in a high-yield outbred suckling mouse model (Buraud et al. 1995). In brief, *C. parvum* oocysts obtained from HIV-infected patients were first purified by serial filtration, then inoculated by intragastric gavage into 4-day-old suckling NMRI mice. On day 6 postinoculation the mice were killed and the ileum was removed, split lengthwise, and homogenized for 1 min in 1 ml of 0.025 *M* phosphate-buffered saline (PBS, pH 7.2) using a glass tissue-grinder. An aliquot of 10 µl was smeared onto a glass slide and stained with the Ziehl-Neelsen reagent according to Henriksen's method (Henriksen and Pohlenz 1981) to quantify the infection. The cryptosporidia in the aliquot were counted by microscopic examination (X200). The ileal homogenates were kept for 7–15 days at 4°C after the addition of penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml), until the next inoculation.

Inoculation

A total of 30 NMRI mice aged 4 days were inoculated by gastric intubation with 50 µl of a day-6 ileal homogenate containing 10^5 cryptosporidia. Six animals were killed at days 0, 3, 6, 9, and 13 postinoculation, respectively. Blood samples were obtained by intracardiac puncture and serum samples were immediately stored individually at –20°C. The ileum was removed and homogenized as described above. The parasite load was determined by counting of cryptosporidia in a 10-µl aliquot of the ileal homogenate. A 25-µl volume of aprotinin (Sigma, St. Louis, USA) was added to each sample before storage at –20°C. A group of 30 NMRI mice aged 4 days were inoculated by gastric intubation with 50 µl of 0.025 *M* PBS and used as controls.

IFN-γ assay

Prior to assay, cells in the ileal homogenates were broken by ultrasound treatment in a 50-W Vibracell 72434 ultrasonic processor (Sonics and Materials Inc., Danbury, USA) for 30 s in an ice bath. IFN-γ levels in serum and sonicated ileal homogenates were measured in duplicate by means of a sandwich enzyme-linked immunosorbent assay (ELISA) method (InterTest-γ, Mouse IFN-γ ELISA kit; Genzyme, Cambridge, USA). The sensitivity of the ELISA was 500 pg/ml in serum (dilution 1:4) and 250 pg/ml in ileal homogenate (dilution 1:2).

Nitrogen determination

The amount of nitrogen contained in the ileal homogenate was determined using an Elemental Analyser EA 1108 (Carlo Erba Strumentazione, Rodano, Italy). The method is based on the complete and instantaneous oxidation of the sample by flash combustion, which converts all organic and inorganic substances into combustion products. The resulting combustion gases pass through a reduction furnace and are swept by the carrier gas (helium) into the chromatography column, where they are separated and detected by a thermal conductivity detector that gives an output signal proportional to the concentration of the nitrogen in the sample.

Presentation of results and statistical analysis

The number of cryptosporidia counted in 10 µl of ileal homogenate (*n*) served as an index of the parasite load (*N*) in the whole ileal homogenate (1,000 µl), as follows: $N = n \times 100$. Serum IFN-γ concentrations are expressed in picograms per milliliter. IFN-γ levels in ileal homogenates are expressed in picograms per milligram of nitrogen. Data are expressed as mean values ± SD and were compared by analysis of variance (ANOVA). Results were considered to be statistically different when $P<0.05$. Correlations were identified with the Spearman rank test.

Results

Parasite load

In infected neonatal mice the parasite load in the ileum was very low on day 3 postinfection, peaked on day 6 and fell from day 9 to day 13 postinfection (Fig. 1).

Serum

IFN-γ was never detected (< 500 pg/ml) in the serum of control mice between day 0 and day 13 after intragastric gavage with PBS. In *Cryptosporidium*- infected mice, IFN-γ was detected in serum only on day 3 (2,189 ± 2,137 pg/ml) and day 6 (890 ± 389 pg/ml) postinoculation.

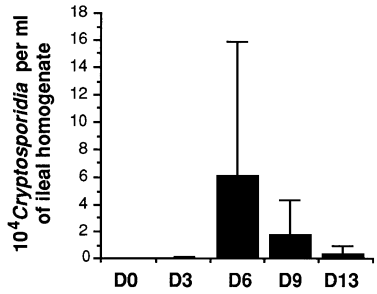

Fig. 1 Kinetics of the *Cryptosporidium parvum* load (cryptosporidia per ml of ileal homogenate; mean values ± SD) in suckling NMRI mice (6 animals per point) inoculated with 10^4 cryptosporidia at 4 days of age (*D* Day postinoculation)

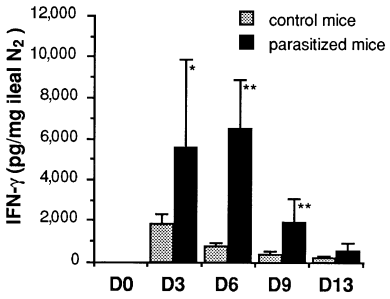

Fig. 2 Kinetics of ileal IFN-γ levels (pg/mg ileal N_2; mean values ± SD) in control and parasitized mice (6 animals per point). * $P<0.05$ as compared with controls; ** $P<0.001$ as compared with controls (*D* Day postinoculation)

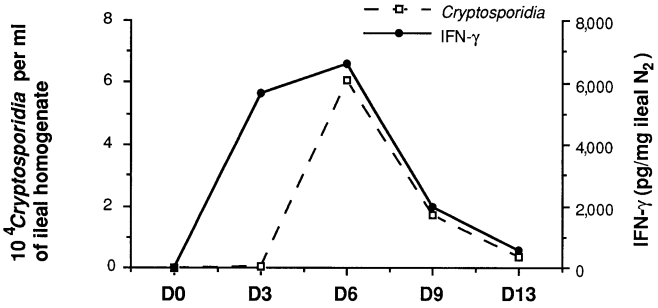

Fig. 3 Kinetics of mean cryptosporidial loads and mean levels of ileal IFN-γ after experimental infection of 4-day-old NMRI mice (6 animals per point). Cryptosporidial loads and ileal IFN-γ levels are positively correlated ($P<0.05$; *D* Days postinoculation)

Ileal homogenate

Patterns of ileal nitrogen levels did not significantly differ in control versus parasitized mice at any experimental time point (day 0 to day 13; data not shown). In control mice, ileal IFN-γ levels fell from 1,884 ± 439 pg/mg ileal N_2 on day 3 to 259 ± 70 pg/mg ileal N_2 on day 13. Experimental infection was followed by a rise in ileal IFN-γ levels that started on day 3, peaked on day 6 (6,613 ± 2,374 pg/mg ileal N_2) and then fell rapidly (Fig. 2). Despite large interindividual variations, these levels were significantly higher in parasitized mice than in controls (day 3 $P<0.05$, days 6 and 9 $P<0.001$). No correlation between the serum and the ileal IFN-γ content was found in controls or parasitized mice. Ileal IFN-γ secretion correlated positively ($P<0.05$) with the cryptosporidial load. However, peak ileal levels of IFN-γ (day 3) began before the peak parasite load (day 6; Fig. 3).

Discussion

Neonatal animals are particularly susceptible to cryptosporidiosis (Current and Garcia 1991; Ernest et al. 1986). However, immunocompetent mice usually clear *Cryptosporidium parvum* from the gastrointestinal tract by 2–3 weeks after inoculation (Buraud et al. 1995), suggesting that immune responses are rapidly effective. In the present study we found that recovery from infection in immunocompetent neonatal mice was associated with an early and marked increase in the ileal IFN-γ content. This increase might have involved the entire gastrointestinal tract, since we did not measure the IFN-γ content in other regions of the intestine. The ileal IFN-γ increase may have been related to peripheral activation, since IFN-γ was detectable in serum soon after infestation (day 3); however, mucosal secretion is more likely to account for this finding, as the kinetics of serum and ileal IFN-γ levels did not correlate. The increase in ileal IFN-γ levels might have been related to nonspecific activation of the mucosal immune system secondary to the gavage in a nonsterile environment and might therefore play no role in parasite clearance. Our data suggest the contrary, since oocyst shedding and ileal IFN-γ levels were closely correlated. In addition, our data confirm the study of Urban et al. (1996) who described an increase in mRNA for IFN-γ in the mesenteric lymph node and ileum at 3 days after the inoculation of suckling mice with *C. parvum.* Enterocyte invasion by *C. parvum* is likely to lead to immunocyte activation and cytokine secretion in the intestinal mucosa rather than in the blood circulation.

Secretion might have been induced by parasite inoculation, since the ileal IFN-γ level rose before the emergence of detectable *C. parvum*in in the ileal homogenate. After peaking, cryptosporidia production and mucosal IFN-γ secretion followed similar patterns. This close correlation suggests that ileal IFN-γ production is an early step in the development of mucosal cellular immune responses that lead to parasite eradication. The time lag between the beginning of ileal IFN-γ secretion (day 3) and the beginning of important parasite shedding (day 6) might correspond to the time required for an effective immune response to develop. In addition, IFN-γ has been found to inhibit the development of other protozoa, including *Toxoplasma*, *Plasmodium*, and *Eimeria* (Pfefferkorn 1984; Rose et al. 1991; Schofield et al. 1987), via macrophage activation, inhibition of host-cell invasion, or modification of host-cell metabolism (Kogut and Lange 1989; Schreiber et al. 1985). However, the way in which IFN-γ intervenes in the clearance of *C. parvum*, which multiplies only in epithelial cells, remains to be determined. Recent identification of IFN-γ receptors on human intestinal cells raises the possibility of a direct action at the site of parasite replication (Valente et al. 1992). However, factors independent of IFN-γ are probably also involved in parasite eradication, as treatment of newborn severe combined immunodeficient (SCID) mice with pharmacological doses of IFN-γ fails to clear the parasite (Kuhls et al. 1994).

IFN-γ intestinal secretion might be altered in patients with AIDS. Reinecker et al. (1992) found a decrease in IFN-γ secretion by stimulated lamina propria mononuclear cells isolated from the colon of such patients. In addition, we found no increase in IFN-γ content in the intestinal lamina propria of patients with AIDS (even in those with chronic cryptosporidiosis) relative to healthy subjects. This finding might be related to an impairment of IFN-γ synthesis by mucosal lymphocytes (Benhamou et al. 1995a).

Our results suggest a key role of IFN-γ in *C. parvum* eradication in a model of acute intestinal cryptosporidiosis, a finding that remains to be confirmed during chronic human cryptosporidiosis.

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