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Lymphocytes and not IFN γ mediate expression of iNOS by intestinal epithelium in murine cryptosporidiosis

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Abstract

We hypothesized that unrecognized differences in epithelial expression of inducible nitric oxide synthase (iNOS), resulting from engineered immunodeficiency, could explain the contradictory findings of prior studies regarding the importance of nitric oxide (NO) in murine models of *C. parvum* infection. Severe combined immunodeficient mice (SCID) failed to constitutively or inducibly express epithelial iNOS or increase NO synthesis in response to *C. parvum* infection. In contrast, mice lacking IFN γ alone induced both epithelial iNOS expression and NO synthesis in response to infection. Accordingly, lymphocytes mediate epithelial expression of iNOS and NO synthesis independent of IFN γ in response to *C. parvum* infection. These findings in large part explain the contradictory conclusions of prior studies regarding the role of iNOS in *C. parvum* infection.

Keywords

Cryptosporidium; nitric oxide; IFN γ ; mouse

Introduction

The small intestine is lined by a single layer of epithelial cells that are responsible for nutrient and water absorption while serving as the first line of defense against a hostile intraluminal environment. *Cryptosporidium parvum* is a minimally invasive protozoan parasite that resides and multiplies within small intestinal epithelial cells. The infection results in loss of epithelial cells, profound villous atrophy, nutrient malabsorption, and severe diarrhea.

Infectious enteritis of a variety of causes is associated with an increase in synthesis of nitric oxide (NO) and the intestinal epithelium has been identified as an important source of this NO synthesis (Gookin et al. 2002; Hoffman et al. 1997). Nitric oxide has been demonstrated to play an important role in epithelial defense against *C. parvum* in some models of the infection (Gookin et al. 2005; Gookin et al. 2006; Hoffman et al. 1997; Lacroix et al. 2001; Leitch and He 1994; Leitch and He 1999; Waters et al. 1997) while other models have failed to demonstrate similar effects (Hayward et al. 2000; Kuhls et al. 1994; Lean et al. 2003; McDonald et al. 1996). A confounding factor in interpreting these studies is the use of mice with an assortment of engineered immune deficiencies that are required to infect these animals with *C. parvum*. Consequently, susceptibility of severe combined immunodeficient (SCID) and IFN γ deficient mice to *C. parvum* infection has identified CD4⁺ T cells and IFN γ as key mediators of host defense. Both mediators are able to stimulate iNOS

expression by intestinal epithelial cells (IEC) in culture; however the means by which they promote epithelial defense in vivo remain unclear. This study was designed to investigate the independent role of lymphocytes and IFN γ on expression of IEC iNOS in *C. parvum*-infected mice and to determine whether failure of iNOS expression could explain the differences in disease severity between SCID and IFN γ deficient mice or the contradictory findings of prior studies regarding the role of iNOS in *C. parvum* infection.

Materials and methods

Mice

Female BALB/c wild-type, IFN γ deficient (GKO; Jackson laboratories) and SCID mice (Taconic Laboratories) treated with or without neutralizing anti-IFN γ antibodies (100,000 neutralizing units/mouse; clone R4-6A2 from ATCC) administered into the peritoneal cavity 24 hours before infection, the day of infection, and once a week until euthanasia were studied. Mice were infected at 6-weeks of age with 1×10^7 *C. parvum* oocysts (Bunch Grass Farms, White City ID) or left as uninfected controls. All studies were approved by the Institutional Animal Care and Use Committee.

Expression and immunolocalization of iNOS

Mice were heparinized (200U intraperitoneal) immediately prior to euthanasia at peak infection for each genotype (day 14 for GKO; day 28 for SCID $\pm$ anti-IFN γ). Filtered plasma was prepared from cardiac blood and assayed for total NO $_2$ + NO $_3$ (Griess Assay; Cayman Chemical Company). Immunoblots for quantification of iNOS expression by the intestinal epithelium were performed using epithelial cells exfoliated from the distal ileum following immersion in oxygenated citrate (27 mmol/L) and calcium-chelating (EDTA, 1.5 mmol/L) PBS buffers at 37°C. Epithelial protein lysates (40 μ g each) were separated on 6% SDS-polyacrylamide gels, transferred to nitrocellulose, blocked with 5% powdered milk and immunoblotted overnight with 1:5000 polyclonal rabbit anti-iNOS (Transduction Laboratories, Lexington, KY). Membranes were incubated for 1-hr with 1:7000 HRP-conjugated secondary antibody and visualized by enhanced chemiluminescence. Densitometric analysis of immunoblots was performed using commercially available software (Scanalytics, Fairfax, VA). Immunohistochemistry for iNOS was performed using formalin-fixed, paraffin-embedded sections of ileal mucosa. Tissue sections were deparaffinized, rehydrated, boiled in 10mM citrate buffer, incubated in 0.3% H $_2$ O $_2$, and blocked for endogenous avidin and biotin binding. Sections were incubated with polyclonal rabbit anti-mouse iNOS (1:100 in blocking serum), immunostained using a secondary antibody streptavidin-biotin-peroxidase method with NovaRED substrate (Vector Laboratories) and counterstained with hematoxylin. Positive control slides containing sections of small intestine and colon from LPS and *Salmonella*-infected BALB/c mice were included as positive controls for iNOS expression. Negative sections were treated with isotype control or omission of primary antibodies.

Assessment of infection severity

Fecal oocysts were counted after separation in a 25%-50% discontinuous sucrose gradient. Sections of ileum, cecum and colon were stained with hematoxylin and eosin and examined for *Cryptosporidium* parasites by an observer blinded to mouse genotype. Infection severity was graded on a 0-4 scale where 0 = no organisms; 1 = occasional organisms within crypts; 2 = 1-2 crypts with multiple organisms; 3 = organisms consistently present in majority of crypts; and 4 = widespread infection of villus and crypts.

Statistical analysis

Data are reported as mean $\pm$ SE. For all analyses, $p < 0.05$ was considered significant. Data were tested for normality and equal variance using a statistical software package (SigmaStat, Jandel Scientific, San Rafael, CA). Normally distributed data were analyzed using an ANOVA while non-parametric data were analyzed using a Mann-Whitney rank sum test. Number of mice = n.

Results and discussion

High constitutive levels of iNOS were expressed by ileal epithelium of uninfected wild type and IFN γ deficient mice. In contrast, epithelial expression of iNOS was not observed in the ileum of SCID mice (Fig. 1a). Plasma NO concentrations were significantly reduced in both uninfected SCID and IFN γ deficient mice compared to wild type mice (Fig. 1b). In response to infection with *C. parvum*, SCID mice failed to mount an increase in epithelial expression of iNOS or plasma NO concentration. In contrast, deficiency of IFN γ did not impair iNOS expression or the capacity to increase NO synthesis in response to the infection (Fig. 1c-e). To determine whether differences in epithelial iNOS expression or capacity for NO synthesis could account for differences in severity of *C. parvum* infection between SCID and IFN γ deficient mice, epithelial infection and oocyst excretion were quantified at the time of peak infection for each experimental model (2-wks post-infection for IFN γ deficient and 4-wks post-infection for SCID mice $\pm$ anti-IFN γ). Consistent with the relative resistance of immunocompetent adult mice to *C. parvum* infection, low numbers of oocysts were excreted by only a subset of experimentally infected wild type BALB/c mice when enumerated on weeks 2 (17/24 mice) and 4 (6/12 mice) of infection (Fig. 2a). In only 1 of 12 experimentally infected wild type mice were epithelial parasites observed on histological examination of the ileum, cecum, and colon (Fig. 2b). All experimentally infected SCID, SCID + anti-IFN γ , and IFN γ deficient mice (18/18 mice each) excreted significantly more oocysts into the feces than wild type BALB/c mice. The number of mice in which parasites were observed (1/12 SCID mice; 7/12 IFN γ deficient mice; 6/12 SCID + anti-IFN γ mice) and the infection severity scores were greatest for those mice deficient in or depleted of IFN γ despite preservation of iNOS expression and NO synthesis.

Results of the present study identify lymphocytes as mediating both constitutive expression of iNOS by the intestinal epithelium and induction of NO synthesis in response to *C. parvum* infection. Failure of iNOS expression or induced NO synthesis by SCID mice could not be attributed to lack of IFN γ production because mice with selective deficiency of IFN γ expressed intestinal epithelial iNOS at levels equivalent to that of immunocompetent wild type mice and significantly increased synthesis of NO in response to the infection.

Although we have not defined the specific lymphocyte phenotype responsible for iNOS expression or NO synthesis in response to *C. parvum* infection, it is probable that CD4+ intraepithelial lymphocytes (IEL) are responsible for this effect. Using lymphocyte subsets and intestinal epithelial cells (IEC-18) in co-culture, intraepithelial $\gamma\delta$ CD4+ lymphocytes and not splenic or mesenteric lymph node lymphocytes were demonstrated to be specifically required for iNOS expression by IEC (Hoffman 2000). Further, in nude mice which do not possess thymus-derived T cells or conventional $\alpha\beta$ CD4+ IEL, $\gamma\delta$ CD4+ IEL develop extrathymically and are associated with levels of iNOS expression by intestinal epithelium that are comparable to wild type mice (Hoffman et al. 1997). The mechanism of IEL-induced expression of iNOS by IEC, as studied in vitro, is reported to require cognate interaction between IEL and IEC in addition to the presence of a soluble mediator (Hoffman 2000). Although the identity of the ligand-receptor interaction is yet to be identified, ICAM-1, LFA-1, CD4, CD8, MHCI, and MHCII do not appear to be involved (Hoffman 2000).

A number of lymphocyte-derived cytokines are reported to stimulate iNOS expression by IEC in vitro including IFN γ , IL-1 β , and TNF α (Hoffman 2000). Their role in induction of iNOS by IEC in vivo is largely unknown. In *C. parvum* infection, CD4⁺ IEL induce immunity through a mechanism that requires IFN γ production (Culshaw et al. 1997; McDonald et al. 1996). Accordingly, IFN γ has been largely assumed to be the driving force for induction of iNOS and synthesis of NO in the infection. For this first time, the present study demonstrates that IFN γ does not mediate the stimulatory effect of lymphocytes on expression of iNOS by the intestinal epithelium or synthesis of NO in response to *C. parvum* infection in vivo. Whether IFN γ deficiency attenuates a potentially more robust NO response to infection in adult mice is difficult to ascertain, because immunocompetent adult mice do not increase iNOS expression or NO synthesis in response to *C. parvum* (Lacroix et al. 2001) presumably due to their low susceptibility to infection. Along these lines, prior studies in which adult IFN γ deficient mice failed to increase ileum iNOS mRNA in response to *C. parvum* infection cannot be used to conclude that IFN γ mediates iNOS expression, because iNOS mRNA was not concurrently measured in wild type mice (Lacroix et al. 2001). In contrast, studies in neonatal mice have demonstrated smaller fold increases in iNOS mRNA expression by the ileum of IFN γ deficient versus wild type neonatal mice following infection with *C. parvum*, however, actual iNOS protein or NO concentrations were not measured (Lacroix et al. 2001). Our finding that IFN γ does not mediate the effect of lymphocytes on expression of epithelial iNOS or NO synthesis in *C. parvum* infection in vivo is supported by in vitro studies demonstrating that IEL co-stimulate IEC to induce iNOS in the presence of IL-1 β and not IFN γ (Hoffman 2000).

Our findings that lymphocytes mediate epithelial expression of iNOS independent of IFN γ , in large part explains the contradictory findings of prior studies regarding the role of iNOS in *C. parvum* infection. For example, studies reporting a significant role for iNOS or NO in control of *C. parvum* were performed using immunocompetent neonates or athymic nude mice for which expression of iNOS protein by intestinal epithelium has been documented (Gookin et al. 2005; Gookin et al. 2006; Hoffman et al. 1997; Lacroix et al. 2001; Leitch and He 1994; Leitch and He 1999; Waters et al. 1997). In these animals, experimental infection with *C. parvum* induces epithelial iNOS expression (Gookin et al. 2006; Leitch and He 1999) and knockout or pharmacological inhibition of iNOS activity results in significant increases in epithelial parasitism and oocyst excretion (Gookin et al. 2006; Leitch and He 1994; Leitch and He 1999). Additionally, treatment with L-arginine or a nitric oxide donor decreases epithelial infection and oocyst shedding (Leitch and He 1994; Leitch and He 1999). In contrast, studies using immunocompetent adult mice conclude no significant role for iNOS (Hayward et al. 2000; Kuhls et al. 1994) perhaps because these mice are resistant to *C. parvum* and fail to induce iNOS expression or NO synthesis in response to infection as shown here and elsewhere (Lacroix et al. 2001). Along these lines, inhibition of NOS was demonstrated to extend the age to which neonatal mice remain vulnerable to *C. parvum* infection (Leitch and He 1999) but was not sufficient to confer susceptibility to adult mice (Hayward et al. 2000; Kuhls et al. 1994). Other studies concluding an absent role for iNOS in *C. parvum* infection were performed using SCID mice (Kuhls et al. 1994) or cell culture models (Lean et al. 2003; McDonald et al. 1996) where absence of lymphocyte-mediated iNOS expression is a likely explanation for the negative findings. Although major differences in iNOS expression were documented between SCID and IFN γ deficient mice, they did not account for differences in infection severity. This finding is in keeping with the relatively modest role iNOS appears to play in defense of *C. parvum* infection primarily in the neonate (Gookin et al. 2005; Gookin et al. 2006; Leitch and He 1999) and the growing number of mechanisms attributed to the protective effects of IFN γ (Lean et al. 2003; Pollok et al. 2001). The significance of NO-independent IFN γ effects on epithelial defense are providing in the present study by exacerbation of infection in SCID mice simultaneously treated with anti-IFN γ neutralizing antibodies.

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Fig. 1. Constitutive (a, b) and induced NO responses to *C. parvum* infection (c, d, e, f) are absent in mice lacking lymphocytes but not IFN γ . Immunohistochemistry for iNOS expression by ileal epithelium and plasma NO concentrations were measured in uninfected mice and at peak infection in wild-type BALB/c, SCID, IFN γ deficient (GKO), and SCID mice treated with anti-IFN γ antibodies (SCID + anti- IFN γ). Numbers of mice are indicated at the base of each bar. Differences in iNOS expression were quantified by densitometric analysis of Western blots from 3 mice of each genotype (e, f). *+p<0.05, ** p<0.01 one-way ANOVA versus wild-type BALB/c within the corresponding time period.

Fig. 2. Number of oocysts excreted (a) and histological severity of *C. parvum* infection (b) is greatest in mice lacking IFN γ . Infection severity scores (scale 1-4) were assigned to the ileum, cecum and colon of wild-type BALB/c, SCID, IFN γ deficient (GKO), and SCID mice treated with anti-IFN γ antibodies (SCID + anti-IFN γ) at the time of peak infection (2 –vs- 4-weeks). Numbers of mice are indicated at the base of each bar. * p <0.05, ** p <0.01, *** p <0.001 one-way ANOVA versus wild-type BALB/c within the corresponding time period. *** p <0.001 Mann-Whitney Rank Sum between scores obtained from the ileum and cecum versus wild type BALB/c mice.