

Local Peroxynitrite Formation Contributes to Early Control of *Cryptosporidium parvum* Infection

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In intestinal inflammation, mucosal injury is often exacerbated by the reaction of NO with neutrophil-derived superoxide to form the potent oxidant peroxynitrite. Peroxynitrite also has antimicrobial properties that aid in the killing mechanism of macrophages and neutrophils. *Cryptosporidium parvum* parasitizes intestinal epithelium, resulting in loss of epithelial cells and mucosal inflammation. Synthesis of NO is significantly increased and arises from the induced expression of inducible nitric oxide synthase (iNOS) by the infected epithelium. Inhibition of iNOS results in intensified epithelial parasitism and oocyst excretion. We hypothesized that formation of peroxynitrite is restricted to sites of iNOS expression by the epithelium and contributes to host defense in *C. parvum* infection. Accordingly, the location and biological effects of peroxynitrite formation were examined in neonatal piglets infected with *C. parvum*. Infected piglets were treated daily with a selective iNOS inhibitor [L-N⁶-(1-iminoethyl)-lysine] or one of two peroxynitrite scavengers [5,10,15,20-tetrakis(*N*-methyl-4'-pyridyl)porphyrinato iron(III) or uric acid] or received vehicle. At peak infection, peroxynitrite formation was restricted to sites of iNOS expression by parasitized epithelium and lamina propria of the apical villi. Peroxynitrite formation was dependent on iNOS activity and was inhibited by treatment with peroxynitrite scavengers. Scavengers increased the number of intracellular parasites and the number of infected epithelial cells present per villus and significantly exacerbated oocyst excretion. Recovery from infection was not delayed by ongoing treatment with scavenger. The present results are the first to demonstrate an *in vivo* role for peroxynitrite formation in acute mucosal defense against a noninvasive intestinal epithelial pathogen.

Cryptosporidium spp. are noninvasive protozoan parasites that replicate within parasitophorous vacuoles that are located intracellularly, but extracytoplasmically in absorptive epithelial cells lining the small intestine. Infection causes accelerated loss of absorptive intestinal epithelial cells and mucosal inflammation that result in nutrient malabsorption, fluid secretion, and debilitating diarrhea (16). *Cryptosporidiosis* is a prevalent cause of diarrhea in the young and in humans with poorly controlled human immunodeficiency virus infection-AIDS (1, 11). The agent is responsible for epidemics of diarrhea in immunocompetent humans infected via contamination of municipal water supplies and swimming pools (2, 6, 13, 14, 18, 24, 25, 32, 38, 39, 43, 44). Despite intensive effort, a consistently effective antimicrobial therapy for *Cryptosporidium* infection has yet to be identified. Little is known regarding the mechanisms and mediators of mucosal defense that predominate at the time of acute epithelial infection and diarrhea in the naturally susceptible host. Such information may provide insights into rational pharmacological or nutritional therapies to promote epithelial defense and repair of the infection.

In intestinal inflammation, reactive oxygen and nitrogen intermediates are commonly involved in the initiation or exacer-

beration of mucosal injury. In particular, NO may react with O₂⁻ to produce a highly reactive and toxic nitrogen intermediary, peroxynitrite (ONOO⁻). In addition to mediating lipid peroxidation injury, peroxynitrite can nitrate tyrosine residues on mucosal proteins to produce nitrotyrosine (NT; so-called “footprints” of peroxynitrite formation). Significant nitrotyrosine formation has been demonstrated in ulcerative colitis, Crohn’s disease, diverticulitis, celiac disease, and necrotizing enterocolitis (8, 12, 20, 21, 37, 41). Further, scavengers of peroxynitrite can ameliorate the severity of ongoing intestinal injury and inflammation (7, 34, 47). In contrast, in intestinal infection, the inflammatory response is intended to neutralize or destroy invading microorganisms, remove irritants, and set the stage for tissue repair (30). In particular, peroxynitrite has bactericidal properties that have been shown to aid in the killing mechanisms of macrophages and neutrophils (26, 30).

We and others have shown that NO synthesis is significantly increased in *C. parvum* infection and that it arises from the induced expression of inducible nitric oxide synthase (iNOS) by the infected epithelium (15, 23). In the absence or inhibition of iNOS, epithelial infection and oocyst shedding are significantly exacerbated (22, 23; J. Gookin, J. Allen, S. Chiang, and M. Armstrong, abstract from the American Gastroenterology Association Digestive Disease Week, Gastroenterology **126** [Suppl. 2]:A-575, 2004). Furthermore, in contrast to noninfectious inflammatory bowel disease, administration of antioxidants has been consistently shown to exacerbate *C. parvum*

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infection (19, 23, 45). We hypothesized that the anticryptosporidial effect of iNOS is mediated, in part, by the localized formation of peroxynitrite that serves to promote mucosal defense against epithelial infection.

MATERIALS AND METHODS

***C. parvum* infection in piglets.** One-day-old crossbred piglets were removed from the sow and housed in infected and control isolation units. The piglets were fed a liquid diet hourly (Express Milk Replacer; Hubbard Feeds, Mankato, MN) by an automated delivery system (Baby Pig Saver; Gillis Agricultural Systems, Wilmore, MN). At 3 days of age, 10^8 *C. parvum* oocysts (Bunch Grass Farms, Deary, ID) were administered by orogastric intubation. The piglets were studied over days 0 to 11 postinoculation, a time span inclusive of the course of *C. parvum* infection and recovery in this model (3). The piglets were euthanized with sodium pentobarbital given intravenously (i.v.), and their ilea were collected for histology, immunohistochemistry, and immunoblotting. All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee.

In vivo inhibitor and scavenger studies. Piglets were paired by body weight and treated daily from the time of infection (day zero) until the time of euthanasia. All treatments were adjusted to a pH of 6.8 to 7.2 and sterile filtered (0.22 μ m) prior to administration.

(i) **Inhibition of iNOS.** Piglets were injected daily with a selective iNOS inhibitor, L-NIL [L-N⁶-(1-*iminoethyl*)-lysine; Cayman Chemical, Ann Arbor, MI; 3 mg/kg of body weight intraperitoneally (i.p.) every 24 h; $n = 3$] or an equivalent volume of phosphate-buffered saline vehicle (2 ml i.p. every 24 h; $n = 4$). The dose of L-NIL used in these studies has been shown to significantly inhibit NO synthesis when given to *C. parvum*-infected piglets (Gookin et al., *Gastroenterology* **126**[Suppl. 2]:A-575, 2004).

(ii) **Scavenging of peroxynitrite.** Piglets were sedated by intramuscular injection of ketamine (10 mg/kg) and xylazine (1 mg/kg) prior to i.v. injection using a lateral ear vein. Two scavengers of peroxynitrite were evaluated. The first scavenger, 5,10,15,20-tetrakis(*N*-methyl-4'-pyridyl)porphyrinato iron(III) (FeTMPyP; Mid-Century Chemicals, Posen, IL; $n = 7$) was dosed at 5 mg/kg i.v. in a 1-ml volume of phosphate-buffered saline. The second scavenger was uric acid sodium salt (Sigma Chemical Company, St. Louis, MO; $n = 3$) and was dosed at 10 mg/kg i.v. (~256 milliosmols) in a 5-ml volume of phosphate-buffered saline. Infected littermate control piglets were treated identically but using equivalent volumes of phosphate-buffered saline vehicle.

(iii) **Treatment effects on control piglets.** Uninfected piglets were treated identically to infected piglets with L-NIL, FeTMPyP, uric acid, or phosphate-buffered saline ($n = 2$ each) as described above for 11 consecutive days.

Enumeration of fecal oocyst excretion. Thin fecal smears were prepared daily for each piglet using a cotton-tipped applicator inserted per rectum. The smears were stained with Auramine-O (Sigma Chemical Company, St. Louis, MO) for 15 min at room temperature (RT), rinsed with tap water, decolorized for 2 min with 0.5% HCl in 70% ethanol, rinsed in tap water, and counterstained for 3 min with 0.5% aqueous potassium permanganate. After a final tap water rinse, the slides were stored in the dark. Oocysts were counted within a 64-mm² grid using a fluorescence microscope (Zeiss, Welwyn Garden City, United Kingdom). Due to the cyclic and unsynchronized shedding of oocysts in feces, oocyst excretion was reported as the average daily fecal oocyst count (by smear) of each piglet over the entire period of infection.

Histopathology. Sections of ileum were fixed in formalin, paraffin embedded, sectioned at 5 μ m, and stained with hematoxylin and eosin for examination by light microscopy. Three sections from each tissue were examined. Because parasites may not be present on every villus of infected piglets, three to five villi were selected without bias on the basis of orientation, not severity of infection, by an examiner blinded to the treatment category. Villi were considered well oriented if the adjacent crypt lumen was patent to the level of the muscularis mucosa. The average villus height (from the crypt opening to the villus tip) and crypt depth were measured using an ocular micrometer. For each of the selected villi, the percentage of epithelialized villus surface was calculated from linear measurements of epithelialized versus denuded villus perimeter. The total number of villous epithelial cells, number of infected epithelial cells, and total number of intracellular parasites along the perimeter of each of the selected villi were determined.

Immunohistochemistry for iNOS and nitrotyrosine. Immunohistochemistry for iNOS (1:100 polyclonal rabbit anti-NOS II; Transduction Laboratories, Lexington, KY) or nitrotyrosine (1:100 polyclonal rabbit antinitrotyrosine; Cayman Chemical Co., Ann Arbor, MI) was performed using formalin-fixed 5- μ m sec-

tions of ileal mucosa removed from control and infected piglets on days 1, 3, 5, 8, and 11 postinfection. The tissues were deparaffinized by immersion in xylene, rehydrated in a graded series of ethanol (100, 95, 70, and 50%), and hydrated to buffer (phosphate-buffered saline, pH 7.4). The tissues were treated with 3% H₂O₂ in methanol for 10 min at 4°C to quench endogenous peroxidase and blocked for 30 min at room temperature with nonimmune goat serum. A commercial kit was used for blocking endogenous avidin and biotin activities (Avidin/Biotin Blocking kit; Zymed Laboratories, Inc., San Francisco, CA). Tissue sections were incubated for 1 h at RT or overnight at 4°C. Sections were immunostained using a commercially available broad-spectrum streptavidin-biotin-peroxidase system with 3,3'-diaminobenzidine as the chromogen (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Sections were counterstained with hematoxylin and eosin or methyl green. Negative control sections were treated with isotype control primary antibody, or the primary antibody was omitted.

Immunoblotting. (i) Protein preparation. The distal ileum was removed from each piglet, opened lengthwise, and rinsed in cold Ringer's solution. The epithelium and lamina propria were scraped from the seromuscular layers over ice using a glass slide and then frozen in liquid nitrogen and stored at -80°C. Mucosal samples were thawed on ice in RIPA buffer (0.15 M NaCl, 50 mM Tris [pH 7.2], 1% deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS]) containing bestatin, leupeptin, aprotinin, sodium orthovanadate, and phenylmethylsulfonyl fluoride. This mixture was sonicated and then centrifuged at 10,000 \times g for 10 min at 4°C. The supernatants were saved, and their protein concentrations were determined (D_c protein assay; Bio-Rad, Hercules, CA).

(ii) **Immunoprecipitation.** Equal concentrations of soluble total mucosal protein (500 μ g) were incubated for 1 h at 4°C with 2.5 μ g of mouse anti-NT monoclonal antibody (Zymed, San Francisco, CA), followed by incubation with agarose A/G conjugate (Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4°C. Immunoprecipitated NT-containing proteins were washed three times using ice-cold phosphate-buffered saline and pelleted by centrifugation at 1,000 \times g for 5 min at 4°C. Samples were resuspended in 40 μ l of 2 \times SDS-polyacrylamide gel electrophoresis sample buffer, boiled for 4 min, and loaded in their entirety in a 10% SDS-polyacrylamide gel. For detection of iNOS, soluble mucosal protein samples at equal concentrations were suspended in 2 \times SDS-polyacrylamide gel electrophoresis sample buffer, boiled for 4 min, and loaded in a 6% SDS-polyacrylamide gel.

(iii) **Immunoblotting.** Electrophoresis was carried out, and proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Science, Birmingham, United Kingdom) using an electroblotting mini-transfer apparatus. The membranes were blocked at RT for 1 h in Tris-buffered saline plus 0.05% Tween 20 (TBST) and 5% powdered milk. The membranes were incubated for 4 h in primary antibody (rabbit anti-iNOS polyclonal antibody [pAb], 1:10,000, or goat anti-actin pAb, 1:500 [Santa Cruz Biotechnology Inc., Santa Cruz, CA], or rabbit anti-NT pAb, 1:1,000 [Cayman Chemical Co., Ann Arbor, MI]). After being washed three times each with TBST, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:5,000; Santa Cruz Biotechnology Inc.). After being washed three additional times for 5 min each time with TBST, the membranes were developed for visualization of protein by addition of enhanced chemiluminescence reagent according to the manufacturer's instructions (Amersham, Princeton, NJ).

Measurement of lipid peroxidation. Thiobarbituric-acid-reactive substances were measured in homogenates of ileal mucosa on the basis of the formation of a colored adduct of malondialdehyde with 2-thiobarbituric acid. An 800- μ l aliquot of homogenate was added to a reagent solution containing 20% acetic acid (1.5 ml), 8.1% SDS (200 μ l), and 0.8% 2-thiobarbituric acid and 0.05% butylated hydroxytoluene (1.5 ml). The mixture was boiled for 1 h in a water bath. After cooling, the malondialdehyde products were extracted with 5 ml of *n*-butanol and pyridine (15:1). The absorbance of the butanol layer was measured at a wavelength of 532 nm. A calibration curve was constructed by using malonaldehyde bis(dimethyl acetal) as a standard.

Statistics. Data are reported as mean \pm standard error (SE). Parametric data were compared using one-way analysis of variance (ANOVA) and a posthoc Tukey's test. Nonparametric data were compared using Kruskal-Wallis one-way ANOVA on ranks and a posthoc Dunn's test (SigmaStat; Jandel Scientific, San Rafael, CA). n is the number of pigs receiving each treatment. For all analyses, a P value of <0.05 was considered significant.

RESULTS

Localization of iNOS and protein tyrosine nitration in *C. parvum* infection. At peak infection with *C. parvum* (days 3 to 5 postinoculation), all infected piglets had marked villous at-

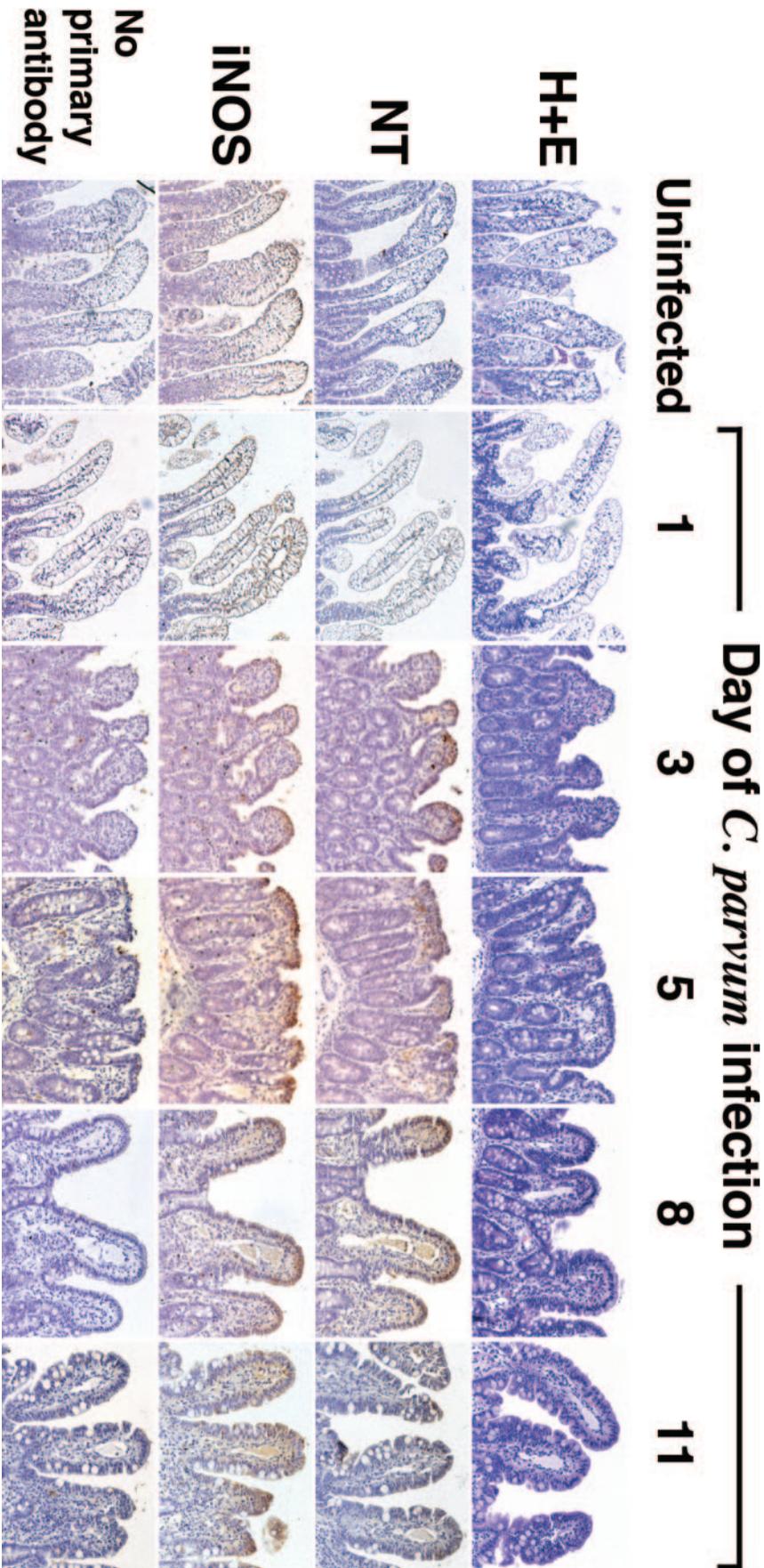


FIG. 1. Immunohistochemistry for NT and iNOS expression in ileal mucosa from uninfected and *C. parvum*-infected piglets on days 1, 3, 5, 8, and 11 of experimental infection. Immunohistochemistry for control piglets is shown for day 5 only and was similar at all time points. H+E, hematoxylin and eosin.

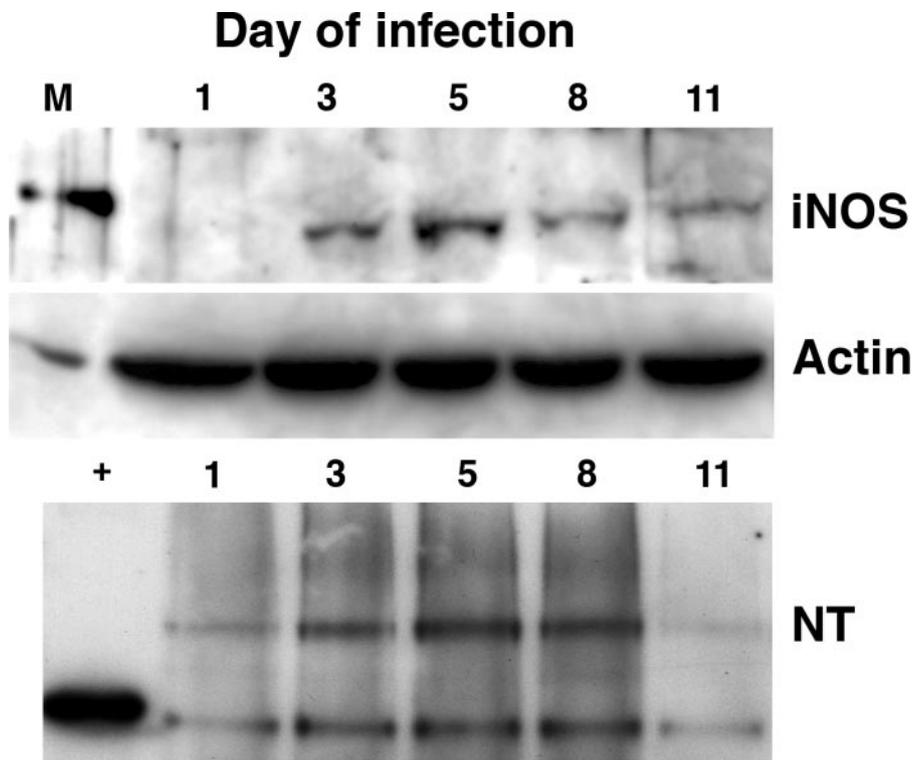


FIG. 2. Immunoblot results for iNOS (130 kDa) and immunoreactive bands of NT (a stable “footprint” of peroxynitrite action) performed using ileal mucosal protein obtained from piglets at the onset (day 1), peak (days 3 to 5), and recovery (days 8 to 11) stages of infection with *C. parvum*. Equal protein loading was demonstrated by immunoblotting for actin (40 kDa). Tyrosine-nitrated proteins were immunoprecipitated from equal starting concentrations of total mucosal protein (500 μ g). +, nitrotyrosine-labeled bovine serum albumin. M, molecular mass marker (marker shown for iNOS = 132 kDa; marker for actin = 43 kDa).

rophy of the ileal mucosa, excreted oocysts in the feces, and had organisms present within villus epithelial cells. In contrast, uninfected piglets had normal villous architecture, did not excrete oocysts in the feces, and had no evidence of intestinal epithelial infection (Fig. 1). We have previously shown that iNOS is expressed by parasitized intestinal epithelium in *C. parvum*-infected piglets (15; Gookin et al., *Gastroenterology* 126[Suppl. 2]: A-575, 2004). The potent oxidant peroxynitrite is generated in vivo by the diffusion-limited reaction of nitric oxide with superoxide radical (28). To determine whether peroxynitrite is generated in *C. parvum* infection and in proximity to the parasitized enterocytes expressing iNOS, immunohistochemistry for NT (a stable “footprint” of peroxynitrite action) was performed using ileal mucosa from control and infected piglets. The results were compared to serial sections on which immunohistochemistry for iNOS protein expression was performed. In uninfected piglets and during early infection (day 1), NT and iNOS were minimally detected. With the onset of peak infection (days 3 to 5), NT colocalized with iNOS to parasitized epithelium and lamina propria of the apical villi. With recovery from infection and the return of normal villus architecture (days 8 to 11), iNOS and NT expression both diminished (Fig. 1). The temporal changes observed by immunohistochemistry were validated by immunoblotting protein lysates of ileal mucosa at corresponding time periods for immunoreactive bands of tyrosine-nitrated proteins and iNOS (Fig. 2). Both approaches revealed earlier resolution of NT

formation relative to iNOS expression, which was temporally consistent with resolving neutrophilic inflammation in this model (3).

Dependence of peroxynitrite formation on iNOS. To determine if peroxynitrite formation in *C. parvum* infection is dependent on iNOS, piglets were infected with *C. parvum* (day zero) and treated daily with a selective iNOS inhibitor (L-NIL, 3 mg/kg i.p.) or an equivalent volume of phosphate-buffered saline (2 ml i.p.). Protein tyrosine nitration was examined at peak infection (day 4) by immunohistochemistry and immunoblotting of mucosal protein lysates for NT. Inhibition of iNOS prevented peroxynitrite formation in *C. parvum* infection, as evidenced by the elimination of tyrosine-nitrated mucosal proteins (Fig. 3).

Effects of peroxynitrite scavengers on protein tyrosine nitration. Recent studies suggest that NT can be formed by oxidation of NO_2^- and tyrosine without the intermediate formation of peroxynitrite (ONOO^-) (9, 31, 40). To determine if peroxynitrite mediates NT formation in *C. parvum* infection, piglets were infected with *C. parvum* (day zero) and treated daily with the peroxynitrite scavengers FeTMPyP (5 mg/kg i.v.) or uric acid (10 mg/kg i.v.) or an equivalent volume of phosphate-buffered saline. Treatment with either peroxynitrite scavenger greatly diminished the NT content of the ileal mucosa, as determined by immunohistochemistry and immunoblotting, compared to infected piglets treated with saline alone (Fig. 3).

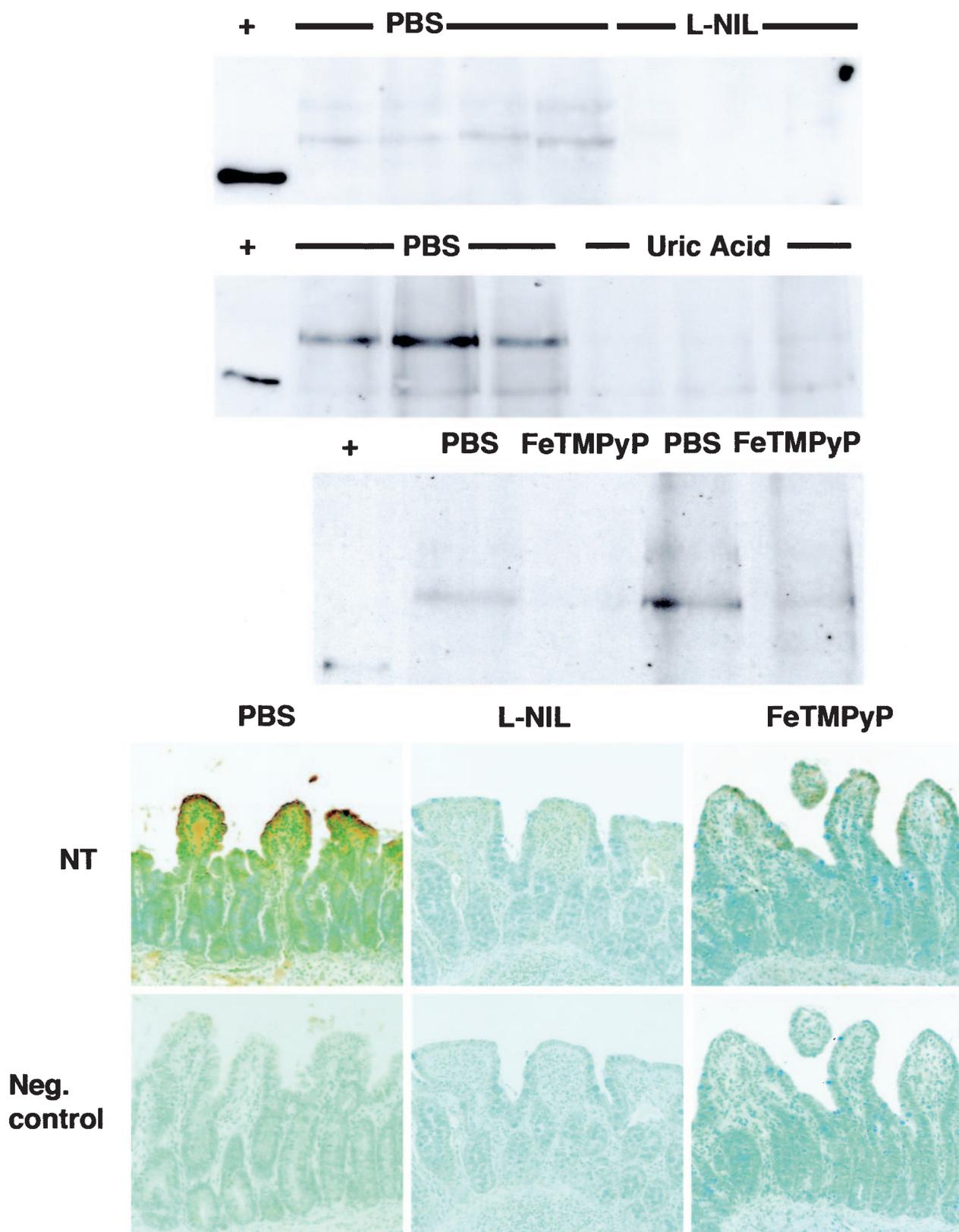


FIG. 3. Immunohistochemistry and immunoblots for detection of immunoreactive bands of tyrosine-nitrated proteins in ileal mucosa from piglets infected with *C. parvum*. Immunoblots are shown from piglets treated daily with phosphate-buffered saline (PBS; 2 ml i.p.; $n = 4$) or the selective iNOS inhibitor L-NIL (3 mg/kg i.p.; $n = 3$) (day 4 postinfection) and piglets treated daily with PBS (5 ml i.v.; $n = 3$) or the peroxynitrite scavenger uric acid (10 mg/kg i.v.; $n = 3$) (day 11 postinfection). Two piglets treated with the peroxynitrite scavenger FeTMPyP (5 mg/kg i.v.) (day 4 postinfection) are shown paired with their PBS-treated littermate (1 ml i.v.). Tyrosine-nitrated proteins were immunoprecipitated from equal starting concentrations of total mucosal protein (500 μ g). +, nitrotyrosine-labeled bovine serum albumin. Negative (Neg.) control, primary antibody omitted.

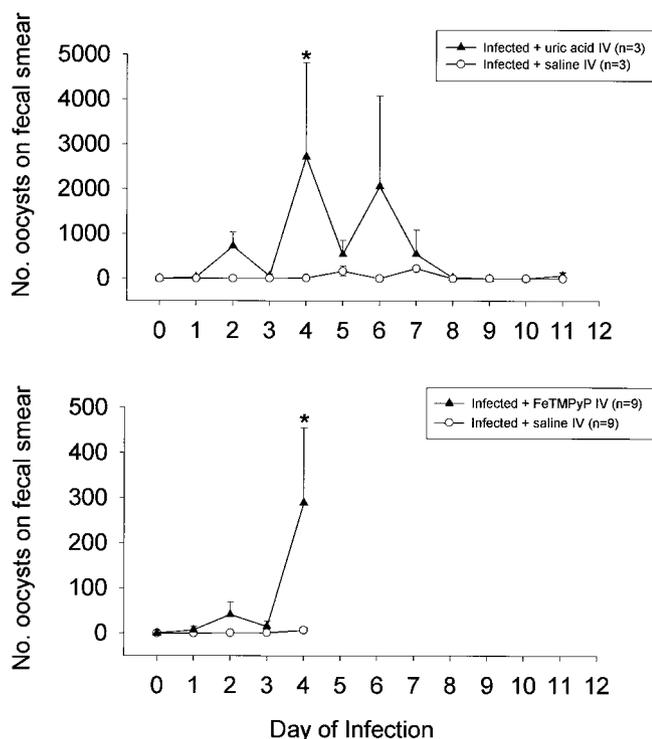


FIG. 4. Numbers of fecal oocysts excreted by *C. parvum*-infected piglets. Piglets were treated daily for 11 days with phosphate-buffered saline (5 ml i.v.) or the peroxynitrite scavenger uric acid (10 mg/kg i.v.). In the lower graph, piglets were treated daily with phosphate-buffered saline (1 ml i.v.) or the peroxynitrite scavenger FeTMPyP (5 mg/kg i.v.) and euthanized on day 4 for histologic studies (shown in Table 1). *n*, number of piglets. The data are mean \pm SE. *, significantly different ($P < 0.05$) from the value for saline-treated piglets as calculated by Student's *t* test.

Contribution of peroxynitrite to control of *C. parvum* infection. We have previously shown that selective inhibition of iNOS significantly increases oocyst excretion, epithelial parasitism, and villous atrophy in *C. parvum*-infected piglets (Gookin et al., *Gastroenterology* 126[Suppl. 2]: A-575, 2004). The localization of NT to sites of iNOS expression by infected epithelium suggested that peroxynitrite formation may mediate, in part, the anticryptosporidial effects of iNOS. To establish a preliminary time course of the effect of peroxynitrite scavenging on the sever-

ity of *C. parvum* infection, we first treated infected littermate piglets with uric acid or phosphate-buffered saline ($n = 3$ each) daily for 11 days, a time frame previously shown to be inclusive of peak infection and recovery in this model (3). Waves of fecal oocyst excretion, consistent with cycles of replication of *C. parvum* within the small intestine, were amplified in piglets treated with uric acid. Both groups of piglets ceased excreting oocysts and cleared the ileal mucosa of epithelial parasites by day 11 of the infection (Fig. 4). Because of the large peripheral injection volume of uric acid required to maintain drug solubility at physiologic pH and osmolarity (5 ml), additional studies were performed using FeTMPyP. Piglets treated with FeTMPyP also excreted significantly ($P < 0.05$) greater numbers of oocysts in the feces than infected littermates treated with saline alone (Fig. 4 and Table 1). Light microscopic examination of ileal mucosa from infected piglets treated with FeTMPyP revealed greater numbers of epithelial parasites that could be attributed to an increase in the number of infected enterocytes present per histologic section of villus (Table 1). There was no difference in the severity of epithelial disruption or diarrhea between piglets treated with FeTMPyP and those treated with saline alone. When uninfected piglets were treated intravenously with FeTMPyP, uric acid, or phosphate-buffered saline, no adverse drug effects were observed over 11 days of administration.

Contribution of peroxynitrite to oxidative injury. Lipid peroxidation injury, as determined by the ileal mucosal concentration of malondialdehyde, was significantly increased in *C. parvum*-infected piglets. Lipid peroxidation was not attenuated by treatment of infected piglets with L-NIL or FeTMPyP, suggesting that peroxynitrite formation does not significantly contribute to mucosal oxidative injury in *C. parvum* infection (nMol/mg protein [*n*]: uninfected = 17.4 ± 1.8 [5] [$P < 0.05$], *C. parvum* = 36.2 ± 6.4 [6], *C. parvum* plus FeTMPyP = 41.6 ± 4.6 [6], *C. parvum* plus L-NIL = 45.5 ± 4 [8]).

DISCUSSION

Synthesis of NO is significantly increased in *C. parvum* infection and arises from the induced expression of iNOS by the infected epithelium (15, 23). *C. parvum* infection is also accompanied by significant mucosal inflammation (3). Whether these inflammatory responses culminate in the formation of peroxynitrite has not been examined for *C. parvum* infection or

TABLE 1. Effect of the peroxynitrite scavenger FeTMPyP on severity of *C. parvum* infection in neonatal piglets

| Parameter | Value for treatment group ^a (<i>n</i>) | | |
|---|---|--------------------------------|---------------------------------|
| | Uninfected control | <i>C. parvum</i> + saline i.v. | <i>C. parvum</i> + FeTMPyP i.v. |
| Avg no. of oocysts/fecal smear per day | 0 (10) | 19 ± 13 (9) | 149 ± 59^b (9) |
| Villus ht (μ m) | 638 ± 39 (10) | 182 ± 38^c (6) | 267 ± 65^c (6) |
| Crypt depth (μ m) | 133 ± 8 (10) | 131 ± 6 (6) | 140 ± 4 (6) |
| No. of epithelial cells/villus | 208 ± 14 (10) | 75 ± 7^c (6) | 85 ± 14^c (6) |
| No. of parasites/villus | 0 (10) | 18 ± 10 (6) | 33 ± 10 (6) |
| No. of infected epithelial cells/villus | 0 (10) | 11 ± 4 (6) | 20 ± 6 (6) |
| % Villus epithelial cells infected | 0 (10) | 16 ± 6 (6) | 30 ± 2 (6) |
| No. of parasites/infected epithelial cell | 0 (10) | 1.6 ± 0.3 (6) | 1.7 ± 0.2 (6) |

^a *n*, number of piglets. Epithelial cell and parasite counts were performed along the perimeters of well-oriented villi sectioned at a thickness of 5 μ m. Values are mean \pm SE.

^b Significantly different ($P < 0.001$) from value for *C. parvum* plus saline i.v.-treated piglets as calculated by Student's *t* test.

^c Significantly different ($P < 0.001$) from value for uninfected control piglets as calculated by one-way ANOVA.

for any intestinal pathogen restricted to the intestinal epithelium. For invasive pathogens, such as *Salmonella enterica* serovar Typhimurium, iNOS and NT colocalize within macrophages and neutrophils, where peroxynitrite exerts antibacterial effects (5, 42). In the present study of *C. parvum* infection, iNOS and NT colocalized to the infected epithelium, suggesting that peroxynitrite formation was restricted to sites of iNOS expression. Nitrotyrosine can also be formed by oxidation of NO_2^- and tyrosine without the intermediate formation of peroxynitrite (9, 31, 40). Our results using pharmacological agents specific for peroxynitrite decomposition clearly indicate that peroxynitrite is the major cause of nitrotyrosine formation in *C. parvum* infection. Likewise, administration of L-NIL had a pronounced effect on reducing the amount of nitrotyrosine, suggesting that NO derived from iNOS activity directly contributed to peroxynitrite formation.

In *C. parvum* infection, the absence or inhibition of iNOS significantly exacerbates epithelial infection and oocyst shedding (22, 23; Gookin et al., *Gastroenterology* 126[Suppl. 2]: A-575, 2004). Furthermore, administration of antioxidants has been consistently shown to exacerbate *C. parvum* infection (19, 23, 45). Thus, we hypothesized that the anticryptosporidial effect of iNOS is mediated, in part, by the localized formation of peroxynitrite, which serves to promote mucosal defense against epithelial infection. To explore directly the role of peroxynitrite in mucosal defense in *C. parvum* infection, we used a class of porphyrin-containing compounds that catalyze the isomerization of peroxynitrite to nitrate (29, 35). These catalysts do not react with either O_2^- or NO and therefore can be used to assess the direct contributions of peroxynitrite. Likewise, uric acid selectively binds to and inactivates peroxynitrite (17).

Peroxyntirite scavengers had no untoward effects on uninfected piglets. In infected piglets, scavengers of peroxynitrite significantly increased the excretion of *C. parvum* oocysts. As evaluated in histologic sections of mucosa, this was associated with an increase in the number of parasites per villus. While the number of parasites per epithelial cell remained unaltered, piglets treated with scavengers had a larger number of their total villus epithelial cells infected. These data suggest that in vivo, peroxynitrite contributes to the elimination of infected epithelial cells from the villus as opposed to promoting defense of the enterocytes against infection by the parasite. In activated macrophages, phagocytosis of bacteria is followed by the contained generation of peroxynitrite that selectively kills the bacterium. In contrast, generation of peroxynitrite by epithelial cells may result in self-destruction as a means to control the spread of infection, particularly as *C. parvum* resides in a privileged location that is intracellular but extracytoplasmic. Accordingly, peroxynitrite has a number of cytopathic activities, including peroxidation of membrane lipids, disruption of the cytoskeleton, promotion of single-stranded DNA breakage, oxidation of sulfhydryl residues, inhibition of the mitochondrial enzyme aconitase, and initiation of apoptosis (4, 27, 36, 46). Direct enteric administration of peroxynitrite induces edema, inflammation, and necrosis (33). Because *C. parvum* oocysts have minimal antioxidant capability (10, 45), a direct effect of peroxynitrite on oocyst viability is also possible.

Our observation that piglets treated with either an iNOS inhibitor or a peroxynitrite scavenger are capable of normal

recovery from *C. parvum* infection suggests that reactive nitrogen intermediates may serve as an early and innate defense against intestinal epithelial infection. Their propensity to also damage uninfected cells, while mitigated by their local formation in *C. parvum* infection, likely reflects a strategy of innate immune responses to sacrifice part to save the whole (30). There was a significant increase in lipid peroxidation injury in *C. parvum*-infected piglets that was not attenuated by iNOS inhibition or scavenging of peroxynitrite. Thus, the focal nature of iNOS expression and peroxynitrite formation in the infection may result in their negligible contribution to total mucosal oxidation injury. In contrast, a role for neutrophil-derived reactive oxygen intermediates in lipid peroxidation injury in *C. parvum* infection will require further examination.

While peroxynitrite clearly mediated early control of *C. parvum* infection rather than mucosal injury, exacerbation of *C. parvum* infection in scavenger-treated piglets was not as severe as that previously observed in piglets treated with iNOS inhibitors (Gookin et al., *Gastroenterology* 126[Suppl. 2]: A-575, 2004.) Specifically, while both iNOS inhibition and scavenging of peroxynitrite significantly exacerbated oocyst excretion, epithelial parasitism was significantly increased in the former but not the latter group of piglets. This suggests that peroxynitrite is not the exclusive effector of iNOS-mediated mucosal defense in *C. parvum* infection or that the available scavengers of peroxynitrite are incapable of fully neutralizing this highly reactive metabolite. Evidence for the latter is suggested by the presence of some residual NT formation by the infected epithelium despite treatment with scavenger. The present results are the first to demonstrate an in vivo role for peroxynitrite formation in acute mucosal defense against a noninvasive intestinal epithelial pathogen.

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