Expression of Inducible Nitric Oxide Synthase and Interleukin-12 in Experimental Necrotizing Enterocolitis

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Background. Previous investigators have relied on administration of pro-inflammatory cytokines or invasive surgical procedures to reproduce the morphologic changes of necrotizing enterocolitis (NEC) in rats. However, these artificial insults do not mimic the human disease. We developed a reproducible model of NEC in rats that more closely resembles human NEC and determined the pattern of inflammatory cytokine expression in this model.

Materials and methods. Newborn rats were randomized into four groups. Groups 1 and 2 were breast-fed, while Groups 3 and 4 were gavaged with formula thrice daily. In addition, Groups 2 and 4 were subjected to 3 min of hypoxia thrice daily, prior to each feeding. The rats were killed on day 4 and the distal 2 cm of terminal ileum was harvested for morphological studies and analysis of inflammatory cytokine mRNA expression.

Results. Nearly 70% of formula-fed neonatal rats displayed moderate or severe morphological abnormalities resembling human NEC. Breast-fed pups had normal histology. The terminal ileum from rats with abnormal histology demonstrated increased inducible nitric oxide synthase (iNOS) expression, decreased interleukin-12 (IL-12) mRNA expression, and enterocyte apoptosis. There was a trend toward upregulation of IFN-γ mRNA, but no difference in expression of TNF-α mRNA. Hypoxia did not significantly alter intestinal morphology or mRNA expression.

Conclusions. Formula-fed neonatal rats, with or without hypoxia, exhibit morphological changes in the intestinal epithelium similar to those seen in patients with acute NEC. The mechanism likely involves upregulation of iNOS mRNA, enterocyte apoptosis, and decreased IL-12 production in the intestinal epithelium.
taneous injection of Pitocin (1 U). Immediately after birth, the neonates were weighed and randomized into one of four treatment groups. Group 1 consisted of neonatal rats that were left with their mother and then were breast-fed. Newborn rats in Group 2 were also left with their mothers, but were subjected to 3 min of hypoxia (5% O₂, 95% N₂) (Praxair, Pittsburgh, PA) thrice daily in a Modular Incubator Chamber (Billups-Rothenberg Inc., Del Mar, CA). The fraction of inspired oxygen was monitored using an Oxychek oxygen monitor (McNeil Lab Inc., Irvine, CA). Group 3 consisted of neonates that were separated from their mothers, housed in an incubator (Ohio Medical Products, Madison, WI), and gavaged with a special rodent formula three times daily. Rats in Group 4 were treated in a similar fashion to those in Group III; however, they were subjected to 3 min of hypoxia prior to each feeding. The formula consisted of 15 g Similac 60/40 (Ross Pediatrics, Columbus, OH) in 75 ml of Esbilac canine milk replacement (Pet-Ag Inc., Hampshire, IL) as described by Barlow et al. [9] and was designed to approximate the protein and caloric content of rat breast milk. The neonatal rats were killed on day 4; the last 2 cm of terminal ileum was harvested for morphological studies and detection of messenger RNA for various pro-inflammatory cytokines by Northern blotting or reverse transcriptase-polymerase chain reaction (RT-PCR). A dose-response curve was generated for both the frequency and the duration of hypoxic insult, as well as the concentration of inspired O₂, before the regimen used in our experiments was selected.

Morphologic studies. Intestinal specimens were harvested as described previously. For light microscopy, hematoxylin and eosin (H&E) slides were prepared as per standard protocol [10]. The morphologic changes in the intestinal epithelium were graded as normal, mild, moderate, or severe by a Children's Hospital of Pittsburgh pathologist (A.K.) who was blinded to the treatment groups. The definition for each histological grade was as follows: (1) mild, separation of the villus core, without other abnormalities; (2) moderate, villus core separation, submucosal edema, and epithelial sloughing; (3) severe, denudation of epithelium with loss of villi, full thickness necrosis, or perforation. For data analysis, only specimens that displayed moderate or severe histologic abnormalities were considered to have experimental NEC.

For scanning electron microscopy (SEM), samples were fixed in 2.5% glutaraldehyde in phosphate-buffered solution (PBS) and pinned flat onto dental wax using minutin pins. Samples were then fixed overnight at 4°C. Samples were washed three times in PBS, postfixed for 1 h in aqueous 1% osmium tetroxide, and then washed three times in PBS. Samples were dehydrated using a series of graded ethanol solutions (30–100%), further dehydrated by three additional 15-min washes with absolute ethanol, and then critical point dried (EmScope CPD 750, Ashford, Kent, UK). Samples were removed from dental wax, mounted onto aluminum stubs, and then sputter coated with platinum/palladium (Hummer VI, Technics West, San Jose, CA). Samples were viewed in a Jeol JSM-T300 scanning electron microscope (Peabody, MA) at 20 kV. The SEM pictures were reviewed by two investigators (D.B.S. and S.C.W.) who were blinded to the treatment groups.

RNA preparation and reverse transcriptase-polymerase chain reaction. Our methods for RNA preparation and RT-PCR have been described in detail previously [8]. Briefly, a 2-cm section of terminal ileum, starting 0.5 cm from the ileo-cecal valve and extending proximally, was harvested. The specimens were homogenized in guanidium isothiocyanate using a Polytron homogenizer (Kinematic, Switzerland), and total RNA was extracted according to the method of Chomczynski and Sacchi [11]. The total amount of RNA was determined spectrophotometrically. Two micrograms of RNA from each sample was subjected to first-strand cDNA synthesis using oligo(dT) primers and 100 μl murine Maloney leukemia virus (MMLV). Samples were incubated at 37°C for 60 min. To test the efficacy of reverse transcriptase, RT-PCR was performed for β-actin mRNA. Amplification was performed for 18–32 cycles, each of which consisted of a 1-min denaturation at 94°C, a 1-min annealing at 58°C, and a 1-min primer extension at 72°C. PCR products were visualized on 2% agarose gel containing ethidium bromide. Semi-quantitation was performed using γ-32P-end-labeled 5′ primer. Fifteen microfilters of the PCR was separated on 10% polyacrylamide gel. The gel was dried and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) and the relative radioactivity of the bands was determined by band density volume integration using laser scanning densitometry and expressed as arbitrary units [12]. Primers for iNOS, tumor necrosis factor (TNF-α), interleukin (IL)-12, and IFN-γ were synthesized by the University of Pittsburgh DNA Synthesis Facility. The primers for iNOS were as follows: 5′-3′ TGGCTGCCCCTGAAGCTT and 3′-5′ CGAACGGGGACCTTCAAA. The primers for TNF-α were as follows: 5′-3′ TGTCATGGCTGAGGCTGGGTTG and 3′-5′ ATGGTCTCTTTTACGCCGAG. The primers for β-actin were as follows: 5′-3′ CAAGGCACACTCATTTGAAAGC and 3′-5′ TACTGTAGCCGTTCAGGCT.

Northern blot analysis. Our methods for RNA preparation and Northern blot analysis have been described in detail previously [8]. Briefly, intestinal specimens were homogenized and total cellular RNA was extracted using RNAzol. Twenty micrograms of RNA was then electrophoresed in 1% agarose containing 3% formaldehyde. The gels were then blot-transferred to gene screen membranes (NEN Life Science Products, Boston, MA) and ultraviolet cross-linked. Membranes were hybridized with a human iNOS cDNA probe (4.5 kb) overnight at 43°C. The cDNA probe was labeled with [32P]dCTP using a random priming kit (Boehringer Manheim, Indianapolis, IN). After appropriate washing, autoradiography was performed by exposure to Kodak X-Omat film at 70°C for 3 or 5 days in the presence of intensifying screens.

Statistical analysis. Morphologic changes among the groups were compared using χ² analysis and Fisher's exact test. Continuous data are presented as means ± SD. Multiple group comparison of continuous data was performed using Fisher's Least Significant Difference procedure. A statistical significance was presumed for a P value <0.05.

RESULTS

Effect of Formula Feeding and Hypoxia on Weight Gain and Intestinal Morphology

Newborn rats were fed either breast milk or a conventional formula for 3 days, with or without a 3-min hypoxic insult thrice daily. On day 4, the animals were killed and the last 2 cm of terminal ileum was harvested for morphological analysis. Table 1 shows that formula-fed animals had a significant weight loss compared to breast milk-fed rats, which gained weight. The hypoxic insults did not affect weight change in either breast-fed or formula-fed neonatal rats. Morphologic analysis of ileal segments from formula-fed rat pups revealed various degrees of inflammatory changes ranging from villous core separation or epithelial sloughing to frank bowel necrosis (Fig. 1). In contrast, breast-fed pups had normal intestinal architecture and rarely showed any inflammatory changes (Fig. 1). Nearly two-thirds (64.3%) of formula-fed hypoxic neonatal rats and three-quarters (75%) of formula-fed normoxic rats displayed moderate or severe histologic abnormalities (Table 2). One animal in...
These findings were confirmed by Northern blot, which mRNA expression for any of the cytokines examined. either feeding regimen did not significantly alter weighed at birth and just prior to sacrifice. The data represent the birth weight subjected to 3 min of hypoxia thrice daily. Group 3 was formula-fed, and Group 4 was formula-fed and exposed to hypoxia. The animals were 5 groups, although IFN-\(\gamma\) expression in neonatal rats (Table 3). There was no difference in IL-12 mRNA expression in the intestinal epithelium, while expres-
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<table>
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<th>Treatment group</th>
<th>Group 1 ((n = 8))</th>
<th>Group 2 ((n = 9))</th>
<th>Group 3 ((n = 12))</th>
<th>Group 4 ((n = 14))</th>
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<tr>
<td>Birth weight (g)</td>
<td>(6.85 \pm 0.35)</td>
<td>(6.81 \pm 0.39)</td>
<td>(5.5 \pm 0.71^*)</td>
<td>(6.13 \pm 0.51^{<em>,</em>})</td>
</tr>
<tr>
<td>Weight change (g)</td>
<td>(7.21 \pm 1.56)</td>
<td>(6.67 \pm 1.22)</td>
<td>(-1.12 \pm 1.02^*)</td>
<td>(-0.65 \pm 0.96^*)</td>
</tr>
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</table>

Note. Newborn rats were randomized into one of four groups. Group 1 was left with their mothers. Group 2 was breast-fed and also subjected to 3 min of hypoxia thrice daily. Group 3 was formula-fed, and Group 4 was formula-fed and exposed to hypoxia. The animals were weighed at birth and just prior to sacrifice. The data represent the birth weight \(\pm SD\) and the change in weight \(\pm SD\).

* \(P < 0.05\) vs Groups 1 and 2, Fisher's Least Significant Difference procedure.
** \(P < 0.05\) vs Group 3, Fisher's Least Significant Difference procedure.

Group 3 showed evidence of subserosal gas or pneuma-
tosis (not shown). There was no gross perforation in any of the rats. None of the breast-fed pups showed significant histologic abnormalities. Hypoxia did not appre
cably affect the morphological changes in either breast-fed or formula-fed neonatal rats. Scanning EM confirmed loss of epithelial integrity in formula-fed neonatal rats with or without hypoxic insults. These animals had a significant number of apoptotic entero-
cytes in their terminal ilea compared to the breast milk-fed rats (Fig. 2). Apoptotic enterocytes were shed into the intestinal lumen, which resulted in denuda-
tion of the epithelium and exposure of the basement membrane (Figs. 1 and 2). Ileal sections from breast-
fed pups showed no evidence of enterocyte apopto

Effect of Formula Feeding and Hypoxia on Cytokine Expression in the Intestine

We have previously shown that acute NEC is asso-
ciated with upregulation of iNOS and IFN-\(\gamma\) mRNA expression in the intestinal epithelium, while expres-
sion of TNF-\(\alpha\), IL-6, and transforming growth factor-\(\beta\) (TGF-\(\beta\)) mRNA was unaffected [8]. Furthermore, pre-
liminary evidence from our laboratory suggests that IL-12 mRNA expression in the intestinal epithelium is decreased in patients with acute NEC (Ford et al., unpublished observations). To determine the cytokine profile in our model of experimental NEC, we evalu-
ated iNOS, TNF-\(\alpha\), IL-12, and IFN-\(\gamma\) mRNA expression using RT-PCR. Formula feeding, with or without hypoxia, resulted in a significant increase in INOS mRNA and in a concomitant decrease in IL-12 mRNA expres-
sion in neonatal rats (Table 3). There was no difference in the expression of TNF-\(\alpha\) or IFN-\(\gamma\) mRNA among the groups, although IFN-\(\gamma\) mRNA expression was signif-
ically higher in the formula-fed pups as a whole com-
pared to breast-fed pups (46.3 \(\pm 28.1\) vs 26.3 \(\pm 17.7\); \(P = 0.03\), Student’s t test). The addition of hypoxia to either feeding regimen did not significantly alter mRNA expression for any of the cytokines examined. These findings were confirmed by Northern blot, which

DISCUSSION

NEC is the most frequent and most lethal disease that affects the gastrointestinal tract of premature in-
fants. Its incidence has steadily increased over the past 30 years [7]. The overall mortality rate for patients with NEC ranges from 10 to 50% and approaches 100% for patients with the most severe form of the disease characterized by involvement of the entire bowel (pan-
necrosis) [13, 14]. Although various risk factors have been implicated in the pathogenesis of NEC, the etiol-
ology of the disease is still unknown. Hypoxia and for-

mula feeding are some of the putative factors that have been shown to consistently play a role in the develop-
ment of NEC [15].

Barlow et al. [9] described an animal model of NEC in which newborn rats were subjected to brief periods of hypoxia daily for 7 days, using a plastic bag. The animals were fed either formula or breast milk. Ileal sections from breast milk-fed rats displayed normal intestinal architecture; however, sections from formula-fed rats revealed atrophic villi, ulcerations of the bowel wall, and, in some cases, full-thickness necrosis. These authors later demonstrated that repeated episodes of hypoxia correlated with increased incidence of NEC and that cold-stress was as effective as hypoxia in inducing experimental NEC [16]. However, in these studies the fraction of inspired oxygen was unknown since hypoxia was induced by placing the animals in a plastic bag. Furthermore, the only parameter assessed by the authors was intestinal morphology. We have previously shown that acute NEC is associated with upregulation of iNOS and IFN-\(\gamma\) mRNA expression, increased nitrosative stress, and enterocyte apoptosis in the intestinal epithelium [8]. In the present study, we attempted to modify Barlow’s experimental model using a hypoxic chamber that allows precise measurement of the concentration of inspired oxygen. In addi-
Our data show that formula-fed rats lost weight over the study period and displayed morphological changes in the intestinal epithelium that resemble those seen in patients with NEC. Breast-milk fed rats nearly doubled their birth weights and showed preservation of the intestinal architecture. Addition of a hypoxic insult to either feeding regimen had no effect on weight gain or on the pattern of histologic abnormalities. In contrast, in the report by Barlow et al., formula-fed rats gained nearly the same amount of weight as breast milk-fed rats, even though the former group showed morphologic abnormalities similar to those exhibited by our formula-fed rats. It is possible that the pups in our study were relatively more ill than those in Barlow's study and thus did not gain weight even though they were fed formula that was isocaloric with breast milk. A second possibility is that the volume of formula fed to these neonatal rats by gavage was less than that received by breast-fed rats and thus the development of intestinal inflammation or experimental NEC in the rats was due to malnutrition. However, we and others have previously reported that malnutrition alone does not cause gut-barrier failure [17, 18]. The fact that the hypoxic insult did not exacerbate the inflammatory changes seen in formula-fed rats suggests that the combination of malnutrition and formula feeding may simply render the neonatal rat more susceptible to gut

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Group 1 (n = 8)</th>
<th>Group 2 (n = 9)</th>
<th>Group 3 (n = 12)</th>
<th>Group 4 (n = 14)</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>8 (100%)</td>
<td>8 (88.9%)</td>
<td>1* (8.3%)</td>
<td>1* (7.1%)</td>
</tr>
<tr>
<td>Mild</td>
<td>0 (0%)</td>
<td>1 (11.1%)</td>
<td>2 (16.7%)</td>
<td>4 (28.6%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>5 (41.7%)</td>
<td>4 (28.6%)</td>
</tr>
<tr>
<td>Severe</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4 (33.3%)</td>
<td>5 (35.7%)</td>
</tr>
<tr>
<td>Moderate or severe</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>9* (75%)</td>
<td>9* (64.3%)</td>
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</table>

Note. Newborn rats were randomized into one of four groups. Group 1 was left with their mothers. Group 2 was breast-fed and also subjected to 3 min of hypoxia thrice daily. Group 3 was formula-fed, and Group 4 was formula-fed and exposed to hypoxia. The terminal ileum of each rat was harvested on day 4, fixed in formalin, and stained with hematoxylin and eosin. Morphological changes were graded by a Children's Hospital of Pittsburgh pathologist blinded to the treatment groups. The definition for each histological grade was as follows: (1) mild, separation of the villus core, without other abnormalities; (2) moderate, villus core separation, submucosal edema, and significant epithelial sloughing; (3) severe, denudation of epithelium with loss of villi, full thickness necrosis, or perforation. The data presented are the number and percentage of rats in each group with the specified degree of intestinal damage.

* *P* < 0.01 vs Groups 1 and 2, Fisher's Exact test.

**FIG. 1.** Effect of formula feeding and hypoxia on intestinal morphology. Newborn rats were randomized into one of four groups as described. On day 4, the terminal ileum was harvested, fixed in formalin, and stained with hematoxylin and eosin. Panel A, which represents an intestinal segment from a breast-fed animal, shows normal histology. The villi are tall and healthy. B, which represents an intestinal segment from a formula-fed animal without hypoxic insult, shows mild inflammatory changes. There is villus core separation (arrowheads) and minimal separation of the mucosa from the basement membrane (arrows). Panels C and D, which represent an intestinal segment from a formula-fed rat subjected to hypoxia, display moderate histologic abnormalities. There is villus core separation, submucosal edema, and significant epithelial sloughing (arrowheads). Panels E and F, which represent intestinal segments from a formula-fed rat subjected to hypoxia, show severe morphologic changes consisting of denudation of epithelium with loss of villi (arrowheads) and full thickness necrosis. The segment depicted in F shows significant epithelial sloughing in the intestinal lumen. Original magnification 120× (A, B, and D); original magnification 60× (C, E, and F).
Since rat breast milk is difficult to collect, we could not gavage the pups with diluted breast milk to determine whether there are intrinsic factors within the breast milk that may enhance gut barrier function and prevent the development of inflammatory changes in the epithelium as we have previously reported in neonatal rabbits [10].

An unexpected finding was that despite our attempt at randomization, pups in the formula-fed groups had a significantly lower birth weight than the breast-fed pups. Thus, it is possible that induction of experimental NEC by formula-feeding may be due at least in part to the lower birth weight of the formula-fed rats. This theory is supported by Sibbons et al., who showed that the intestine in low-birth-weight piglets may be particularly susceptible to mesenteric ischemia [19]. The birth weight of the neonatal rats in Group 4 in our study was the same as that of breast-fed rats in Barlow’s study, yet the pups in Barlow’s study did not develop NEC, which suggests that breast milk prevents experimental NEC even in low-birth-weight rats. However, we can only speculate that this hypothesis is correct since the birth weights differed between formula-fed and breast-fed rats.

Increased iNOS expression has been implicated in the pathogenesis of gut-barrier failure in inflammatory bowel disease [22], experimental ileitis [23], and endotoxic shock [24]. We and others have shown that upregulation of iNOS mRNA is associated with increased

**FIG. 2.** The effect of formula feeding and hypoxia on intestinal architecture. Newborn rats were randomized into one of four groups as described. On day 4, the animals were killed and the terminal ileum was evaluated by scanning electron microscopy. Panel A, which represents an intestinal segment from a breast-fed pup, displays smooth, well-preserved villi without any apparent abnormalities. In contrast, in B, the villi from a formula-fed pup subjected to hypoxia show loss of epithelial integrity with cytoplasmic blebbing and apical shedding indicative of enterocyte apoptosis (arrows). (Bar, 100 μm).

### TABLE 3

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Group 1 (n = 8)</th>
<th>Group 2 (n = 9)</th>
<th>Group 3 (n = 7)</th>
<th>Group 4 (n = 9)</th>
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<tr>
<td>iNOS</td>
<td>10.2 ± 9.1</td>
<td>10.4 ± 5.6</td>
<td>51.6 ± 20.2*</td>
<td>44.8 ± 13.8*</td>
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<tr>
<td>IL-12</td>
<td>48.8 ± 17.3</td>
<td>49.2 ± 21.7</td>
<td>9.0 ± 3.7*</td>
<td>7.1 ± 6.9*</td>
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<tr>
<td>IFN-γ</td>
<td>27.2 ± 24.8</td>
<td>25.3 ± 7.9</td>
<td>44.1 ± 29.1</td>
<td>47.9 ± 28.8</td>
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<tr>
<td>TNF-α</td>
<td>37.1 ± 22.3</td>
<td>41.2 ± 21.1</td>
<td>36.9 ± 40.9</td>
<td>25.5 ± 15.7</td>
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* P < 0.05 vs Groups 1 and 2, Fisher’s Least Significant Difference procedure.
nitrosative stress in the intestinal epithelium, presumably as a result of the formation of peroxynitrite. Peroxynitrite leads to enterocyte apoptosis and increased intestinal permeability [25, 26]. Accelerated enterocyte apoptosis at the apices of the villi results in denuded areas of the basement membrane that facilitate bacterial adherence and penetration [27]. We have previously shown that administration of a NO scavenger, NOX, or a competitive NOS inhibitor that is relatively more specific for iNOS, aminoguanidine, can abrogate bacterial translocation and intestinal hyperpermeability in rats challenged with LPS by decreasing nitrosative stress and enterocyte apoptosis [26, 28].

The decrease in IL-12 expression may be a byproduct of NO production since NO has been shown to inhibit T-cell proliferation by decreasing IL-12 expression [29]. Decreased T-cell proliferation in the intestinal epithelium or lamina propria may impair intestinal immune function, thus allowing bacteria to escape intrinsic host defense mechanisms in the lamina propria. Furthermore, IL-12 has been shown to be directly involved in bacterial clearance; thus decreased mucosal IL-12 production may directly contribute to the pathogenesis of NEC by allowing microbial escape [30, 31]. In fact, Hensler et al. reported that humans with postoperative sepsis produced significantly less IL-12 than patients who recovered from surgery uneventfully [32]. Thus, decreased intestinal mucosal IL-12 production may render neonatal rats more susceptible to bacterial invasion or serve as a marker for advanced disease.

IL-12 is a potent inducer of IFN-γ expression and is necessary for IFN-γ production in some models of endotoxemic shock [33]. However, in our model we found decreased IL-12 mRNA and increased IFN-γ mRNA expression in the intestinal epithelium. Similar findings have been reported by Jansen et al., who detected lower serum levels of IL-12 in baboons after lethal challenge with live Escherichia coli when compared to sublethal challenge [34]. The level of IFN-γ in lethally challenged baboons was threefold that of the sublethally challenged group. IFN-γ can increase intestinal permeability directly by dilating interepithelial tight junctions [35, 36] or indirectly by increasing NO production in the intestinal epithelium [37, 38]. Either pathway may contribute to the pathogenesis of NEC in our model. Increased paracellular permeability may allow bacteria to traverse the epithelium and exacerbate the initial injury by releasing LPS, which in turn can further stimulate NO production. The fact that TNF-α mRNA production was unaffected in our model (and in human NEC) may be due to the fact that the morphologic changes in experimental NEC and in human NEC are typically “bland infarcts” with a paucity of acute inflammatory cell infiltration [5, 39].

In conclusion, we have shown that formula-fed neonatal rats, with or without hypoxia, exhibit morphological changes in the intestinal epithelium similar to those seen in patients with acute NEC. The mechanism appears to involve upregulation of iNOS mRNA, enterocyte apoptosis, and decreased IL-12 production in the intestinal epithelium. These changes may lead to increased intestinal permeability that may facilitate bacterial invasion and further promote gut-barrier failure. This model may be a simple reproducible method for inducing experimental NEC.

REFERENCES


