Colonic production and expression of IL-4, IL-6, and IL-10 in neonatal suckling rats after LPS challenge

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Adams, Jodi K., and Barry L. Tepperman. Colonic production and expression of IL-4, IL-6, and IL-10 in neonatal suckling rats after LPS challenge. Am J Physiol Gastrointest Liver Physiol 280: G755–G762, 2001.—It has been demonstrated that the neonatal suckling rat is more susceptible to endotoxin [lipopolysaccharide (LPS)]-induced colonic damage compared with weaned littermates. There is evidence to suggest that differences in the production of certain cytokines, including interleukin (IL)-4, IL-6, and IL-10, are associated with intestinal inflammation in children. We have examined the production, localization, and mRNA detection of these cytokines in suckling and weaned rat colons after bacterial LPS challenge. Suckling (10 day old) and weaned (25 day old) rats were injected with LPS (3 mg/kg ip). Colon samples were taken up to 4 h after treatment, and cytokines were measured by ELISA. LPS-induced cytokine levels were significantly different in suckling rats compared with weaned rats. Cytokine localization to the colonic mucosa was evident in suckling rats up to 4 h after LPS administration but was not consistently seen in weaned rats. The mRNA for cytokines examined were detected by RT-PCR in suckling but not in weaned rat colons after LPS treatment. Induction of neutropenia via anti-neutrophil serum (ANS) administration did not affect cytokine mRNA detection in neonates after LPS treatment. Weaned animals displayed positive detection of all cytokines examined after ANS. Therefore, we have shown that the suckling rat displays a different production and expression of colonic IL-4, IL-6, and IL-10 compared with weaned littermates after LPS challenge. Furthermore, neutrophils may be implicated in colonic cytokine expression after LPS challenge in rats.

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sion of these cytokines in the intestinal mucosa of the pre- and postweaned rat after LPS treatment. These findings suggest that the observed differences in colonic production of IL-4, IL-6, and IL-10 are associated with and may contribute to the enhanced susceptibility to LPS-induced colonic injury in neonatal rats.

MATERIALS AND METHODS

Animals. Male and female Sprague-Dawley rat pups aged 10 or 25 days postpartum were purchased from Canada Breeding Laboratories (St. Constant, Quebec) for all experiments. Animals were maintained in a temperature-controlled environment (22 ± 1°C) with a 12:12-h light-dark cycle and were used 3 days after arrival in the animal care quarters at the University of Western Ontario. Pups were reared with their mother, who was allowed chow and water ad libitum. All studies were approved by the University of Western Ontario Animal Care Committee, and all animals were treated according to the guidelines set out by the Canadian Council on Animal Care.

Treatments. Pups of either sex from various litters of equal age were randomized to three experimental groups and received the following treatments: 1) control (sterile 0.9% saline ip, ~50 μl for 10-day-old rats and 150 μl for 25-day-old rats); 2) Escherichia coli LPS [serotype 0111:B4 from Sigma Chemical (St. Louis, MO); 3 mg/kg ip in sterile 0.9% saline]; 3) anti-neutrophil serum [ANS; Accurate Chemical & Scientific (Westbury, NY); 10 μl ip] 2 h before administration of LPS. Animals were killed by cervical dislocation 0–4 h after injection of LPS. A midline incision was made to expose the peritoneal contents, and whole-thickness samples of colon were rapidly removed, flushed in ice-cold sterile saline, and placed on ice.

ELISA. Whole colon tissue samples from 10- and 25-day-old rats were cultured in RPMI 1640 culture medium (GIBCO Canada) supplemented with 10% FCS (Sigma), 100 U/ml penicillin, and 50 μg/ml streptomycin solution. Samples were cultured in 24-well plates (Costar) for 24 h in a humid 5% CO₂ atmosphere. After 24 h, supernatants were collected, centrifuged, and stored at −80°C until determination of cytokine levels. The concentrations of IL-4, IL-6, and IL-10 in the supernatants were assessed using a specific sandwich ELISA immunoassay kit (Biosource, Camarillo, CA). All samples were analyzed in duplicate. The level of each cytokine in mucosal specimens was calculated as the amount per milligram of dry tissue weight. Sensitivity levels were between 2 and 500 pg/ml for IL-4, 8 and 2,000 pg/ml for IL-6, and 5 and 1,000 pg/ml for IL-10.

Histological assessment of mucosal damage. Whole colon sections isolated from 0 to 4 h after LPS treatment were harvested and fixed in 4% paraformaldehyde (Sigma), processed routinely, embedded in paraffin, and sectioned at an 8 μm thickness. To visualize the intestinal tissue, sections were stained with hematoxylin and eosin. Sections were examined by light microscopy by a naive observer utilizing a grading system to assign tissue damage developed by Wallace (37). A damage score of one indicated epithelial cell damage; a score of two indicated glandular disruption, vasocongestion, or edema in the upper mucosa; a score of three indicated hemorrhagic damage in the mid to lower mucosa; and a score of four indicated deep necrosis and ulceration. Each section was evaluated on a cumulative basis to give the histological index of injury with a maximum score of 10.

Myeloperoxidase assay. Whole colon myeloperoxidase (MPO) levels were measured to provide an index of polymorphonuclear leukocyte infiltration. MPO activity was determined as described by Wallace (37). Samples of whole colon were suspended in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0; Sigma) at a tissue concentration of 50 mg/ml. Samples were homogenized for 15 s using a Polytron homogenizer, freeze-thawed three times in liquid nitrogen, and centrifuged at 2,000 g for 2 min. MPO activity in the supernatant was determined by adding 100 μl of the supernatant to 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine hydrochloride (Sigma) and 0.0005% (wt/vol) hydrogen peroxide. The change in absorbance at 460 nm over a 3-min period was measured. One unit of MPO activity was defined as that which would convert 1 μmol of hydrogen peroxide to water in 1 min at 22°C.

Immunocytochemistry. All immunocytochemistry was performed using Vectastain ABC kits (Vector Laboratories, Burlingame, CA). Whole colon sections (8 μm) were deparaffinized in three xylene washes and dehydrated in a series of ethanol (EtOH) washes (100, 95, and 70% EtOH). Slides were also incubated in 0.3% hydrogen peroxide in methanol for 30 min to quench any endogenous peroxidase activity. Slides were then incubated with normal serum provided in the kit to block nonspecific binding. Anti-rat IL-4 (4 μg/ml), IL-6 (3 μg/ml), and IL-10 (1 μg/ml) polyclonal antibodies and control normal rabbit serum (1:500 dilution) were applied, and slides were allowed to incubate overnight at 4°C in a humidified chamber. Secondary antibody treatment and peroxidase staining were performed as specified by the kit protocol. Peroxidase staining was visualized with diaminobenzidine tablet sets (Sigma) and yielded a brown end product. Sections were counterstained with hematoxylin and dehydrated by a series of EtOH washes followed by three washes in xylene. Slides were mounted with Permount mounting medium (Sur-gipath, Richmond, IL) and allowed to dry overnight. All sections were examined by light microscopy (×400).

mRNA detection by RT-PCR. Total RNA was extracted using acid guanidinium isothiocyanate-phenol-chloroform extraction as previously described (7). RNA integrity was confirmed by gel electrophoresis. The concentration of mRNA was determined spectrophotometrically at 260 and 280 nm; no mRNA sample was used with a ratio of 260 to 280 nm of <1.7. Total RNA (2.5 μg) was reverse transcribed in a 20-μl reaction containing 1 μl oligo(dT) primer, 10 μl diethyl pyrocarbonate water, 4 μl 5× first-strand buffer, 2 μl dithiothreitol, 1 μl dNTPs, and 1 μl of RT Superscript. All reagents were purchased from GIBCO Canada. cDNA (5 μg) was then amplified via PCR. All oligonucleotide primers were designed such that the products were only obtained from cDNA and not genomic DNA. Oligonucleotide primers were obtained from GIBCO Canada (Table 1).

PCR was performed in a 50-μl reaction containing 0.4 μM of each cytokine primer or 0.2 μM glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 10 μM dNTPs, 10 μM

Table 1. Oligonucleotide primer sequences

| IL-4 | 5’-TCCATGCGACCCAGATTTTGTACC | 3’-GCAAAGATTTTCCCCCTGAGATGC |
| IL-6 | 5’-GCCAGTGCTCATCAGGAGATGTAG | 3’-TTGGGATATCAGGTTTCTGGTCTG |
| IL-10 | 5’-TAAGGGTGTTCTGGGTTGGTGGC | 3’-AGGGGAAAAATCGATGACACGC |
| GAPDH | 5’-CGGAGTCAAGCGTTTGGTTCGAT | 3’-AGCCTTCCTTACGGTGTTAGAC |

IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydroge-

nase.
MgCl₂, 5 µl 1× PCR buffer, 34.8 µl sterile water, and 5 units of Taq polymerase. The temperature profile of the amplification consisted of 1-min denaturation at 95°C, 1-min annealing, and an extension at 72°C for 1 min for 35 cycles. Ten microliters of the reaction mixture were electrophoresed in a 1.5% agarose gel (GIBCO Canada) and stained with ethidium bromide to visualize the amplification products. Primers for GAPDH, IL-4, IL-6, and IL-10 yielded products of 306, 299, 668, and 174 bp, respectively. PCR products were sequenced to verify that the desired product was amplified.

**Statistical analysis.** Data are presented as means ± SE for the number of animals (n) per experiment. Statistical significance (SigmaStat software) was assessed by ANOVA and a Dunnett’s test or a Mann-Whitney test. P < 0.05 was considered significant.

**RESULTS**

**Histological analysis of mucosal injury.** Sections of colon excised from control animals revealed a normal histological appearance, with a maximum microscopic damage score of 0.8 ± 0.1 for suckling rats and 1.0 ± 0.2 for weaned rats. After treatment with LPS, there was a significant increase in the extent of damage for both groups of rats compared with control animals (Fig. 1). Furthermore, 4 h after LPS treatment, 10-day-old rat pups displayed a significantly higher damage score of 8.1 ± 0.4 compared with 25-day-old rats, with an average damage score of 5.7 ± 0.6 (Fig. 1).

**LPS-induced changes in MPO activity.** MPO activity in whole colon tissue samples from 10- and 25-day-old rats treated with LPS was significantly higher than that observed in saline-treated animals (data not shown). MPO activity in colon tissue of 10- and 25-day-old animals treated with ANS before LPS was observed to be significantly lower than that of rats treated with LPS alone (Fig. 2, A and B). Compared with weaned rats, preweaned animals had significantly higher MPO activity in whole colon tissue samples 2, 3, and 4 h after LPS treatment (Fig. 2, A and B).

**LPS-induced cytokine production in the neonatal rat.** IL-4 levels in the colon were not found to be significantly different between 10-day-old animals treated with saline or LPS (Fig. 3A). Weaned animals were observed to have an increase in IL-4 levels at 2 h after LPS over saline-treated animals (Fig. 3B). Additionally, the difference in IL-4 at 2 h after LPS in weaned rats was also significant from levels seen in 10-day-old animals at 2 h after LPS.

**Fig. 2.** Effect of LPS (3 mg/kg ip) and anti-neutrophil serum (ANS; 10 µl ip) on myeloperoxidase activity in colonic tissue of 10 (A)- and 25 (B)-day-old rats examined 1–4 h after treatment. Data are presented as means ± SE (n = 6). A: *significant increase in LPS-treated rats over ANS- and LPS-treated rats; **significant increase in LPS-treated rats over respective control rats at 0 h; B: *significant increase in LPS-treated rats over ANS- and LPS-treated rats. #significant increase in LPS-treated animals over control rats at 0 h; $significant increase in LPS-treated rats at 3 h after injection compared with 1, 2, and 4 h after LPS injection. Statistical significance was determined by ANOVA and a Dunnett’s test (P < 0.05).

**LPS-induced cytokine production in the neonatal rat.** IL-4 levels in the colon were not found to be significantly different between 10-day-old animals treated with saline or LPS (Fig. 3A). Weaned animals were observed to have an increase in IL-4 levels at 2 h after LPS over saline-treated animals (Fig. 3B). Additionally, the difference in IL-4 at 2 h after LPS in weaned rats was also significant from levels seen in 10-day-old animals at 2 h after LPS.

**Fig. 1.** Histological damage score of colonic mucosal sections stained with hematoxylin and eosin. Ten- and 25-day postpartum rat pups were treated with lipopolysaccharide (LPS; 3 mg/kg ip), and colon specimens were harvested at 1–4 h after treatment. Data are presented as means ± SE (n = 6). *Significant increase in 10-day-old over 25-day-old rats after LPS treatment as determined by ANOVA and a Dunnett’s test (P < 0.05).
duce significantly higher levels of IL-6 in the colon 3 and 4 h after LPS treatment.

In suckling rats treated with LPS, the level of colonic IL-10 was significantly increased over the level observed in saline-treated control animals 3 and 4 h after injection (Fig. 5A). In 25-day-old rats, there were no observed differences in IL-10 levels for animals treated with saline or LPS (Fig. 5B). Compared with the 25-day-old animals treated with LPS, 10-day-old rats had significantly higher levels of IL-10 observed at 1, 2, 3, and 4 h after LPS.

Cytokine localization in the colonic mucosa after LPS treatment. To determine staining specificity, whole colon sections were treated with normal serum rather than primary antibody. A low level of background staining was evident for the muscle layer only. Peroxidase staining was completely absent from the epithelial cell layer and the submucosa. Positive staining for IL-6 appeared 1 h after treatment with LPS and was observed at all time periods up to and including 4 h after LPS in the 10-day-old animals (Fig. 6C). Staining was observed predominantly in the epithelial cell layer and was also seen in the submucosal layer. LPS-treated 25-day-old rats displayed positive staining for IL-6 at 1 h after LPS, and this continued until 3 h after treatment (Fig. 6D). The peroxidase staining was observed consistently in the epithelial cell layer, with some staining seen in the submucosa. Positive staining for IL-10 was observed in 10-day-old animals up to and including 4 h after LPS treatment (data not shown). Staining for IL-10 was observed predominantly in epithelial cells at all times after LPS administration, with some staining in the submucosal layer. For 25-day-old rats treated with LPS, staining was also evident 1–4 h after administration. Positive staining for IL-10 was again found in the epithelial cell layer for all times after LPS treatment. Staining was also evident.
Cytokine mRNA detection in the colonic mucosa after LPS treatment. In neonatal rats, IL-4 mRNA was detected after both saline and LPS treatment (Fig. 7A and C). In contrast, IL-4 mRNA was not detected after saline or LPS treatment in 25-day-old animals (Fig. 7B and D). IL-6 mRNA was not detected in saline-treated neonatal rats at any time after treatment (Fig. 7A). However, the detection of IL-6 mRNA in LPS-treated neonatal 10-day-old animals was evident 2 h after treatment and persisted until 4 h after LPS (Fig. 7C). This was not the case in weaned animals, where IL-6 expression was not found at any time after saline or LPS administration (Fig. 7B, D). In saline-treated 10- and 25-day-old rats, IL-10 mRNA was not evident at any time after treatment (Fig. 7A and B). However, the mRNA for this cytokine was evident in neonatal animals 2 h after LPS and continued through to 4 h after LPS treatment (Fig. 7C). In 25-day-old rats, IL-10 mRNA expression was not evident at any time after LPS treatment (Fig. 7D).

Cytokine mRNA detection after ANS and LPS treatment. In 10-day-old animals treated with ANS and LPS, IL-4, IL-6, and IL-10 mRNA expression was similar to that seen in LPS-treated animals (Fig. 7E). However, in 25-day-old rats, cytokine expression after treatment with ANS was not similar to rats treated with LPS alone. IL-4 expression was evident after ANS treatment and persisted until at least 3 h after ANS-LPS treatment (Fig. 7F). Positive detection of IL-6 and IL-10 was also evident in 25-day-old rats treated with ANS-LPS.

DISCUSSION

The administration of bacterial LPS to the suckling rat results in colonic damage, and, compared with more mature animals, the colon displays an increased susceptibility to LPS challenge (3). This observation is confirmed by the present findings that preweaned rats have a higher index of histological damage in the colonic mucosa and an increased degree of neutrophil infiltration in the large bowel compared with weaned rats after LPS administration. The reasons for this enhanced susceptibility to colonic injury in the suckling rat are currently unknown. Recent evidence has suggested that immunological immaturity or a dysfunctional inflammatory response in the large bowel of neonates may contribute to a decreased capacity to maintain mucosal integrity and barrier function during an inflammatory response (15). The regulation of immune reactivity at mucosal surfaces is a complex phenomenon, involving the participation of multiple cell types and protein mediators (38). Cytokines play a dominant role in the regulation of gut immune responses (11). Because of their potent proinflammatory and immunoregulatory activities, a local defect in cytokine generation or function could be relevant to the high susceptibility of the neonatal colon to LPS challenge.

In the present study, a small increase in the level of the immunoregulatory cytokine IL-4 was observed in weaned but not preweaned rat colon after LPS challenge. It is questionable to conclude that this increase is a definitive example of differences between the two groups of rats, because a difference was only observed at 2 h and was not large. However, in children with inflammatory bowel disease, it has been shown that the number of IL-4-secreting T cells is significantly reduced from that in normal children (21). Furthermore, a study by Tang and Kemp (35) reported levels of IL-4 to be reduced in neonates and children under 10 yr of age compared with adults, showing an age-dependent increase in IL-4 production. These findings would imply that IL-4 production in neonates seems to be lower than that of mature or full-term animals, and the present study would lend some support to those observations.
In the present study, LPS-induced colonic production of the proinflammatory cytokine IL-6 was significantly increased in suckling animals compared with weaned littermates. Similarly, elevated levels of IL-6 in premature infants with either sepsis or inflammatory bowel disease have been reported previously (14, 16). Harris et al. (16) also demonstrated that, in human infants with both sepsis and necrotizing enterocolitis, IL-6 levels were 5- to 10-fold higher than in children with sepsis alone or control groups. This would appear to implicate the colonic mucosa in the neonate as a source of IL-6. IL-6 has been found by many investigators to be involved in inflammatory bowel disease, both in adults and neonates (8, 14, 16, 25, 29). IL-6 has also been previously found to be directly associated with causing colonic tissue damage. In adult rats, an endoscopic injection of IL-6 in the colonic mucosa can result in crypt distortion and goblet cell depletion, two characteristic tissue indexes of colonic inflammation (25). From these data, it is apparent that IL-6 is produced in the large bowel of neonates in levels higher than those produced by more mature animals. Furthermore, in consequence of being localized to the large bowel, IL-6 may contribute to the enhanced susceptibility to injury observed in suckling animals.

Levels of the immunoregulatory cytokine IL-10 were found to be significantly higher in prewean rats than those observed in weaned rats after LPS treatment. IL-10 is generally considered beneficial because it can reduce inflammation by inhibiting the production of proinflammatory cytokines and other proinflammatory mediators (13). Several studies have also reported that IL-10 can inhibit the production of cytokine, chemokine, and prostaglandin synthesis by LPS-stimulated neutrophils (5, 26, 28). Because inflammatory damage was evident 1 h after LPS and increased thereafter, it is possible that the late increase in colonic IL-10 observed in the suckling rat is produced to decrease the inflammatory response. This explanation would also appear to support the observation that an increase in colonic IL-10 was not seen in the weaned rats with less inflammatory damage. Additionally, Keel et al. (22) have reported that IL-10 was found to significantly counteract neutrophil apoptosis, an effect that appears to be regulated through alterations in signal transduction pathways such as tyrosine phosphorylation. IL-10 has also been shown to have effects on circulating neutrophil content. Administration of recombinant IL-10 in healthy volunteers has been demonstrated to cause a transient rise in circulating neutrophils and
monocytes (18). Therefore, an increased production of IL-10 in the colon of neonates may contribute to a higher degree of neutrophil infiltration, a result observed in the present study.

The localization of proinflammatory and immunoregulatory cytokines to the inflamed neonatal colon is not currently well characterized. This study demonstrates that, in suckling and weaned rats, IL-6 and IL-10 are localized to the colonic mucosa after LPS challenge, predominantly to the colonic epithelial cells. Intestinal epithelial cells have been shown to produce a variety of cytokines, including IL-1, IL-6, IL-8, tumor necrosis factor-α, and transforming growth factor-β (10, 20, 37). Furthermore, colonic epithelial cells from patients with inflammatory bowel diseases produce IL-6 protein and IL-6 mRNA (19, 23). The colonic epithelial cells may therefore play a role in local colonic inflammation and may possibly contribute to the differences in cytokine production observed between pre- and postweaned LPS-treated rats. Furthermore, these observations may point to an important role of intestinal epithelial cells as an essential component of colonic mucosal defense.

Previous studies have demonstrated that neutrophil infiltration in the large bowel after LPS treatment is significantly higher in neonates compared with adult animals (3). Neutrophils have also been implicated in mediating the greater degree of damage seen in experimentally induced colonic inflammation in the suckling rat. Furthermore, in the present investigation, RT-PCR demonstrated that neutrophils may have an inhibitory effect on cytokine production in the weaned rat. LPS-treated 25-day-old rats did not display any positive cytokine detection, whereas rats pretreated with ANS did show positive detection for all cytokines. These data would suggest that neutrophils in the weaned rat may influence cytokine expression in the colonic tissue. These results also suggest that the neutrophil may exert different roles in weaned vs. preweaned rats, because the absence of neutrophils in preweaned animals did not produce the same response in cytokine mRNA expression. It is not understood whether the difference is in function or in maturity of these immune cells. The functional capacity of neonatal neutrophils has been investigated previously (17, 33, 39). Neutrophil adherence and chemotaxis appear to be decreased in neonates while phagocytosis and microbial killing are intact (1). Additionally, studies in neonatal rats with experimentally induced sepsis have indicated that transfusion of adult human neutrophils can decrease mortality in neonates with serious infection (31). Thus, in addition to the functional impairment of neonatal neutrophils that has been established previously, it may be proposed that the function or immaturity of neonatal neutrophils is important in the expression of cytokines in the large bowel after LPS challenge in rats.

In conclusion, we have shown the suckling rat to display different levels of colonic IL-4, IL-6, and IL-10 compared with weaned littermates after LPS challenge. Furthermore, the difference in production and mRNA detection of cytokines seems to be, in some part, dependent on the presence of neutrophils or possibly the function or maturity of neonatal neutrophils.

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REFERENCES


