Lactoferrin protects neonatal rats from gut-related systemic infection

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1Department of Pediatrics, University of Arizona, Tucson, Arizona 85724; 2Department of Pediatrics, University of California, Davis, California 95616; 3Agennix Incorporated, Houston, Texas 77046; 4Ross Products Division, Abbott Laboratories, Columbus, Ohio 43215; and 5Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

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Ede, Lynn, Ronaldo B. Hipolito, Freda F. Y. Hwang, Denis R. Headon, Robert A. Shalwitz, and Michael P. Sherman. Lactoferrin protects neonatal rats from gut-related systemic infection. Am J Physiol Gastrointest Liver Physiol 281: G1140–G1150, 2001.—Lactoferrin is a milk protein that reportedly protects infants from gut-related, systemic infection. Proof for this concept is limited and was addressed during in vivo and in vitro studies. Neonatal rats pretreated orally with recombinant human lactoferrin (rh-LF) had less bacteremia and lower disease severity scores (P < 0.001) after intestinal infection with Escherichia coli. Control animals had 1,000-fold more colony-forming units of E. coli per milliliter of blood than treated animals (P < 0.001). Liver cultures from control animals had a twofold increase in bacterial counts compared with cultures from rh-LF-treated pups (P < 0.02). Oral therapy with rh-LF + FeSO4 did not alter the protective effect. In vitro studies confirmed that rh-LF interacted with the infecting bacterium and rat macrophages. An in vitro assay showed that rh-LF did not kill E. coli, but a combination of rh-LF + lysozyme was microbicidal. In vitro studies showed that rat macrophages released escalating amounts of nitric oxide and tumor necrosis factor-α when stimulated with increasing concentrations of rh-LF. The in vitro studies suggest that rh-LF may act with other “natural peptide antibiotics” or may prime macrophages to kill E. coli in vivo.

bacteremia; Escherichia coli; disease severity score; human lysozyme; macrophage-related priming or activation; recombinant human lactoferrin

THE HUMAN FETUS RECEIVES nutrients, growth factors, and immunoglobulins via active or passive placental transport (1, 23). The fetal intestine is also nourished and prepared for birth by swallowed amniotic fluid translocation. A logical choice for a milk protein that...
could be added to formula was lactoferrin (LF). LF is the most abundant whey protein in human colostrum and milk (38). LF is also enriched in the milk of mothers who deliver prematurely (54). Recently, an increasing number of antimicrobial and anti-inflammatory attributes have been credited to LF (2). Human LF can now be produced in commercial quantities via recombinant technology (71). The three-dimensional structure of this recombinant human LF (rh-LF) has been determined by X-ray crystallography and does not differ from that of the native protein (62).

The choice of LF as a therapeutic protein was not based solely on the preceding criteria. A prior study indicated that LF may reduce bacterial translocation from the gut (63). Adult mice develop bacterial translocation from the intestine when fed bovine milk, but this event can be suppressed by adding bovine LF to the diet (63). No such studies are reported in neonatal animals, and here we describe such a model. Further support for this concept comes from recent studies that showed that oral administration of LF improves survival in neonatal piglets that are later challenged with intravenous endotoxin (37). Piglets that were fed bovine LF at 0, 8, and 20 h after birth had a 74% survival rate after they were given endotoxin intravenously at 23 h of age. Meanwhile, control piglets given oral albumin rather than bovine LF at the same time intervals had a 17% survival rate after induced endotoxemia. Piglet (25, 37) and human (30) studies suggest that the neonatal intestine is permissive for the passage of LF. Through its antimicrobial and anti-inflammatory characteristics (2), LF transported from the gut into the systemic circulation may alleviate the adverse effects seen when gram-negative bacteria translocate from the intestine lumen into the bloodstream. Bacterial invasion of the intestine may occur more readily in neonates via transcytosis and/or paracellular routes (6, 22). Here we report that feeding oral rh-LF to neonatal rats protects them from subsequent systemic infection induced by instilling massive amounts of Escherichia coli into the intestine. Using in vitro systems, we also describe that rh-LF acted (along with lysozyme) to kill the infecting bacterial strain and primed (or activated) the primary rat host defense cell against bacteria, the macrophage. We undertook an examination of the interaction of rh-LF with rat macrophages because these phagocytes have been proposed as a key defense that prevents bacterial translocation (12, 32).

### MATERIALS AND METHODS

**Animals.** Specific pathogen-free Harlan Sprague-Dawley rats were used in these studies. Dams that had previously delivered at least one litter were shipped with their pups to the vivarium on the day after birthing. Dams and their litters were housed in an isolation room in isolator cages. Rat chow and water were sterilized. At all times, the pups were kept with their mother for suckling. Litter size was limited to eight pups to insure maximum nutrition. The Animal Use Committees at the Baylor College of Medicine (where the animal studies began) and the University of California, Davis approved the protocol and procedures used in this study.

**Bacteria.** *E. coli* strain Ec5 (O18:K1:H7) was provided by Dr. Kwang Sik Kim (Johns Hopkins Univ. School of Medicine, Baltimore, MD). This strain was used previously to induce meningitis in neonatal rats after oral infection of the intestine (34). Three liters of *E. coli* Ec5 were grown to stationary phase in flasks of trypticase soy broth (Difco Laboratories, Detroit, MI) by shaking for 16 h in a water bath at 37°C. The bacteria were recovered by centrifugation and washed twice by suspension in sterile saline followed by centrifugation. The final pellet was suspended in 10 ml of sterile saline. For each experiment, the final bacterial slurry was quantified by serial pour plate techniques and had an average concentration of 1 × 10¹¹ colony-forming units (CFU)/ml. Purity of the bacterial slurry was confirmed by inoculation onto 5% sheep blood agar followed by Gram stain and biochemical analyses of the colonies.

**Lactoferrin.** The human LF gene was expressed in *Aspergillus niger* var. *awamori* (27, 71). During the fermentation of *Aspergillus*, LF is secreted. The rh-LF is then purified and subjected to freeze drying to yield a powder. The freeze-dried rh-LF was mixed in sterile 0.45% normal saline (NS) and filtered through a 0.22-μm filter before its oral administration to the rat pups. For each experiment, this suspension of rh-LF was prepared at a concentration of 10 mg/ml. Repeated measurements (spectrophotometric analyses at A₄₆₅) of this suspension revealed that rh-LF was 8–11% saturated with iron. After production, each lot of freeze-dried rh-LF was analyzed for purity by performing high-performance liquid chromatography and NH₂-terminal sequencing of the protein. Additionally, each lot of the freeze-dried rh-LF, after its resuspension in 0.45% sterile NS, was subjected to electrophoresis on SDS-polyacrylamide gradient gels in the presence of molecular weight standards. Coomassie Brilliant Blue R-stained gels always had a single protein band identified at ~87 kDa (glycosylated rh-LF), consistent with a pure preparation of rh-LF.

**Experimental protocol.** The procedures used to treat and infect the neonatal rat pups with *E. coli* are summarized in Table 1. The control groups received either 0.45% sterile NS (0.4 ml/pup) or 0.45% sterile NS + FeSO₄ (fer-in-sol) at a

<table>
<thead>
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<th>Table 1. Schedule of treatment and infection</th>
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<td><strong>DOL</strong></td>
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<td>Control group 1</td>
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<td>Treatment group 1</td>
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<td>PM</td>
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<td>Control group</td>
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<td>Treatment group</td>
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DOL, day of life; 0.5NS, 0.45% sterile normal saline (dose = 0.4 ml/pup); rh-LF, recombinant human lactoferrin resuspended in 0.5NS (rh-LF dose = 350 mg·kg⁻¹·dose⁻¹ in 0.4 ml/pup); FeSO₄, ferrous sulfate (fer-in-sol) given orally with rh-LF at a dose of 5 mg·kg⁻¹·day⁻¹.
dose of 5 mg·kg⁻¹·day⁻¹ by gastric gavage. The treatment groups received either rh-LF (350 mg·kg⁻¹·day⁻¹) or rh-LF + FeSO₄ instilled into the stomach (0.4 ml/pup). By random assignment, three litters were studied in the 0.45% NS group and compared with three litters given rh-LF, and two litters each were used to ascertain the effects of 0.45% sterile NS + FeSO₄ compared with rh-LF + FeSO₄. The dose of rh-LF given to the neonatal rats represented a simulated intake of human colostrum by human preterm infants shortly after birth and the content of LF in colostrum. The calculation was performed as follows. The volume of oral intake by human preterm infants shortly after birth is in the range of 80 ml/kg body wt, and the LF concentration in human colostrum is ~5 mg/ml (42) [1-kg infant (80 × 5) = 400 mg·kg⁻¹·day⁻¹]. On the basis of weight changes during the 2 days of treatment, the adjusted average dose of rh-LF given to the rat pups was ~350 mg·kg⁻¹·day⁻¹. This intake is within 2 standard deviations of the LF taken by 1-mo-old human infants (7). Within the first week of life, the intake of LF by human infants should be higher than that reported by Butte and colleagues (7) in 1-mo-old infants. Saline was used instead of albumin because of the assumption that an irrelevant protein might still act as a nutrient for the bacteria. Either 0.45% NS or rh-LF was instilled through a sterile 2-Fr silicone elastomer catheter that had been advanced to the level of the stomach. E. coli was slowly instilled into the stomach with a sterile 3-Fr polyurethane catheter as the delivery system. On the basis of average daily weight, ~3 × 10¹² CFU of E. coli were given by orogastric gavage for each kilogram of body weight. A physical measurement was made in each pup to ascertain the distance from the anterior mandible to the epigastrium so that catheters used for gavage would be properly placed.

A scoring system that defined the magnitude of illness after intestinal infection was formulated (Table 2). The scoring system was designed to produce uniformity among observers scoring the animals, but the investigators were not blinded to the treatment given to individual litters. Animals were examined twice daily at 8 AM and 6 PM to determine whether pups were dying or had died. If a pup was scored as dying, the animal was removed and subjected to euthanasia with pentobarbital sodium. As described below, blood and liver bacterial cultures were obtained after euthanasia. Animals that survived until the sixth day of life were assigned an illness severity score, weighed, and then underwent euthanasia.

Blood and liver cultures were then obtained in the following manner. After euthanasia, the skin over the sternum, xiphoid area, and midline of the abdomen was cauterized. A minimum of 0.2 ml of blood was obtained for culture from a sterile cardiocentesis. The blood (0.1 ml) was diluted serially in sterile saline, placed onto 5% sheep blood agar plates, and dispersed with a spreader. Thereafter the abdomen was aseptically entered. The liver was isolated and aseptically removed, and the right lobe was divided equally with a sterile knife blade. The cut surface of the liver was immediately placed on a blood agar plate. The extruded liquid was spread over the surface of the plate. After 24 h of incubation at 37°C, the colonies were counted. For liver cultures, the maximum number of CFU that could be reliably counted was 300 per plate. Plates having >300 CFU were assigned a value of 300.

In vitro studies of E. coli-related killing by rh-LF. An in vitro system was developed to ascertain the killing of E. coli strain Ec5 by LF. The bacterium was suspended in Hanks’ balanced salt solution that had final concentrations of 2 mM dextrose and 0.1% peptone no. 3 broth (Difco). The growth medium was equilibrated to pH 7.0 before and during the assay by incubating and mixing the suspension in 6.5% CO₂. Except for the oxygen tension and the absence of bile salts, the suspension and conditions of incubation were intended to simulate the pH and nutrient composition of the intestinal lumen. An initial inoculum of ~1 × 10⁵ CFU/ml of E. coli was incubated with rh-LF (10 µg/ml), lysozyme from human milk (10 ng/ml, Sigma, St. Louis, MO), or both proteins. Portions of the suspensions were removed after 1 or 2 h, serially diluted in saline, and spread onto 5% sheep blood agar plates. After 48 h of incubation at 37°C, the colonies were counted. These studies were performed on three separate occasions with duplicate plates.

In vitro studies of rat macrophage activation by rh-LF. A primary rat macrophage cell line (CRL-21192, American Tissue Type Collection, Manassas, VA) was cultured and used to study the activation of macrophages as we reported previously (14). After stimulation of the macrophages with rh-LF, lipopolysaccharide (LPS from E. coli O128:B12; Sigma), or both agents, the production of nitric oxide (NO) and the secretion of tumor necrosis factor-α (TNF-α) were measured in culture supernates. Macrophages were cultured in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) with 5 × 10⁶ cells/well in 1 ml of MEM (Gibco-BRL, Grand Island, NY) with 2% fetal calf serum (Atlanta Biologicals, Norcross, GA) and antibiotics (Irvine Scientific, Santa Ana, CA). This medium was used for all studies described below. The cells were incubated using the following conditions for 24 h: 1) media alone, 2) LPS alone at doses of 1, 10, 100, and 1,000 ng/ml, 3) rh-LF alone at doses of 10, 50, 100, 250, and 500 µg/ml, and 4) 500 µg/ml rh-LF + LPS at concentrations of 1, 10, 100, and 1,000 ng/ml.

Nitrite determination. NO production was measured as its metabolite nitrite (NO₂⁻) with the Griess reaction (14). Briefly, the color reagent consisted of 5 mM sulfanilic acid, 5 mM N-(1-naphthyl)ethylenediamine, and glacial acetic acid combined in a ratio of 1:1:3. The assay was performed in 96-well plates by adding 0.5 ml of color reagent to 0.25 ml of standard or sample. The reaction was activated by adding 0.25 ml of 350 mM ammonium chloride buffer (pH 9.6). With an ELISA plate reader, the resulting absorbance was measured at 550 nm after a 10-min incubation in dim light at 23°C. Nitrate standards were prepared in MEM at concentrations ranging from 0 to 100 µM.

TNF-α assay. An ELISA was used to quantify murine TNF-α in tissue culture supernates. The ELISA was performed according to the manufacturer’s specifications (Quan-

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Table 2. Illness scoring scale

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<th>Condition</th>
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<tr>
<td>No signs of life or rigor mortis</td>
<td>Dead 4</td>
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<tr>
<td>Cyanosis and/or poor perfusion, labored breathing, marked lethargy, no righting response, shaking, no gastric milk, poor skin turgor (dehydration) (3 or more required)</td>
<td>Dying 3</td>
</tr>
<tr>
<td>Pallor or gray color, abnormal breathing, reduced activity, decreased suckling and gastric milk, diminished skin turgor (2 or more required)</td>
<td>Moderately ill 2</td>
</tr>
<tr>
<td>Pale, but perfusion acceptable, less activity, rapid breathing pattern, gastric milk present (1 or more required)</td>
<td>Mildly ill 1</td>
</tr>
<tr>
<td>Normal breathing, color, activity, and suckling; copious milk in stomach</td>
<td>Normal 0</td>
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tikine M Mouse TNF-α Assay, R&D Systems, Minneapolis, MN). Briefly, this is a sandwich enzyme immunoassay technique. An affinity-purified polyclonal antibody specific for murine TNF-α was precoated onto a microplate. Standards, controls, and samples were instilled into 96-well plates. Rat TNF-α present in culture supernates binds to the immobilized antibody. After any unbound substances were washed away, an enzyme-linked polyclonal antibody for murine TNF-α was added to the wells. After a wash to remove any unbound antibody enzyme reagent, substrate solution was added to the wells. The intensity of the color measured was proportional to the amount of murine TNF-α bound in the initial step. Sample values were calculated from concurrently analyzed standards.

Assessment of nuclear factor-κB activation. Macrophages (1 × 10^7/10 ml MEM) were stimulated for a period of 2 h with rh-LF (500 μg/ml), LPS (100 ng/ml), lysozyme (10 μg/ml), or different combinations of these agents. A nuclear extract was prepared from the stimulated macrophages, and a mobility shift assay was performed to determine nuclear factor-κB activation as previously reported (14). The NF-κB specific consensus sequence, GGRRGAC TTT CCC, was prepared in our oligonucleotide facility and labeled with [32P]ATP with the use of T4 polynucleotide kinase. Binding reactions incubated 5 μg of nuclear extract with 2.5 μl of binding buffer (100 mM Tris, pH 7.5, 400 mM NaCl, 10 mM EDTA, 40% glycerol, 10 mM β-mercaptoethanol) and 2 μl of poly dI:dC in the presence and absence of competitor for 5 min followed by addition of probe (∼30,000 cpm) for 20 min (final volume was 25 μl). The products were resolved on a 1× Tris-boric acid-EDTA polyacrylamide gel, dried, and analyzed by radiography.

Northern analysis for expression of early growth response gene-1. After 2 h of stimulation with rh-LF, LPS, or both agents, total cellular RNA from 1 × 10^7 macrophages cultured in 10 ml of MEM was isolated by the guanidinium thiocyanate-phenol-chloroform method, and Northern analysis was performed as previously described (28). A rat cDNA probe for early growth response gene-1 (Egr-1) was used for these studies. Hybridization was performed with the addition of 10^6 dpm/ml of [32P]-labeled cDNA for 16 h at 42 °C. After hybridization, the membranes were washed with 1× SSPE (0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) + 0.1% SDS for 3 min at 23°C, 0.5× SSPE + 0.1% SDS at 42°C for 30 min, and finally 0.1× SSPE + 0.1% SDS for 30 min at 50°C. Membranes were then exposed to Kodak XOMAT-AR film for an average time of 6 h at ~70°C. The extent of hybridization was assessed by laser densitometry of the corresponding autoradiograms. A mouse β-actin probe was used to standardize for loading and transfer of the RNA (Invitrogen, Carlsbad, CA).

Statistical analyses. The Levene test was used to delineate whether values were normally distributed. After this assessment, either the Mann-Whitney test or the two-tailed t-test was applied (21). Data are presented as means ± SE, and a P value of <0.05 was considered significant.

RESULTS

Control studies consisted of one litter each (n = 8 pups/litter) in which the pups received saline, rh-LF, or sham therapy (gavage tube placement only) during days 3 and 4 of life but no intestinal infection was induced thereafter. All pups in each group remained well at 2 wk of age. After euthanasia, blood obtained from these animals proved to be sterile, whereas 5 of 24 liver touch cultures had <10 CFU of mixed bacterial flora.

In studies involving infected animals, the average body weight/pup at the beginning of the study was 9.4 g in the 0.45% sterile NS group, 9.5 g in the 0.45% sterile NS + FeSO₄ group, 9.6 g in the rh-LF group, and 9.3 g in the rh-LF + FeSO₄ group. Just before euthanasia on day 6 of life, the average weights in the surviving infected pups were 14.8 g in the 0.45% NS group, 14.5 g in the 0.45% sterile NS + FeSO₄ group, 14.6 g in the rh-LF group, and 14.3 g in the rh-LF + FeSO₄ group. Differences in either the initial weight or the weight at euthanasia among the four groups were not statistically significant. Our experiments showed that pre-treating rats with either rh-LF or rh-LF + FeSO₄ before massive intestinal infection with E. coli substantially reduced the severity of illness and death rate (Fig. 1). A weighted clinical illness score was formulated that assigned values ranging from a score of 0 for well animals to a score of 4 for pups that died. The cumulative illness score per group was significantly higher in the NaCl group (score = 52) than in the rh-LF pretreatment group (score = 15) [z score = −4.069, P < 0.001]. A similar comparison of cumulative illness score between the NS + FeSO₄ group (score = 33) and the rh-LF + FeSO₄ group (score = 7) also showed a significant difference [z score = −3.604, P < 0.01]. All animals that had E. coli infection of the intestine subsequently had the same bacterium isolated from the blood and liver. E. coli was easily distinguished as brownish, convex, and mucoid colonies on 5% sheep blood agar inoculated with either whole blood or the effluent from liver slices. Gram stains and biochemical analyses of the isolated colonies confirmed that the bacterium was E. coli. The numbers of E. coli cultured from blood and liver were significantly lower in the animals that were pretreated with either rh-LF or rh-LF + FeSO₄ compared with either NaCl-treated or NaCl + FeSO₄-treated pups, respectively (Fig. 2).

![Fig. 1. Clinical illness scores of neonatal rats infected with intestinal Escherichia coli in treatment groups as indicated. No. of animals is presented on the abscissa, and clinical status or degree of illness is displayed on the ordinate.](http://ajpgi.physiology.org/)
All animals in both control groups had positive blood cultures, whereas 2 of 22 pups in the rh-LF-treated group and 1 of 16 pups in the rh-LF FeSO₄ group had negative blood cultures. The average number of E. coli in the blood of dying control animals was 9.5 × 10⁸ ± 1.2 × 10⁸ CFU/ml.

To test whether rh-LF could kill E. coli in vitro, a bacterial suspension that contained rh-LF, lysozyme, or both “natural antibiotic” proteins was incubated with ~10⁴ CFU/ml of E. coli strain Ec5. The assay tried to recreate the pH, P CO₂, ionic strength, and nutrient conditions in the small intestinal lumen. rh-LF did not kill E. coli over 2 h (Fig. 3). Conversely, human milk lysozyme did slightly reduce the numbers of E. coli in the suspension, whereas a combination of both natural antibiotic proteins lowered the presence of E. coli to <10 CFU/ml (Fig. 3). A microscopic examination of the bacterial suspension after 2 h showed that the combination of rh-LF and lysozyme did not aggregate the E. coli, thereby causing a reduction in the colony counts, but rather caused bacterial cell wall disruption.

Rat milk lysozyme and rh-LF could have acted together in vivo to reduce the numbers of E. coli in the intestinal lumen of treated pups, but because substantial amounts of bacteria were still present in their liver and blood, we considered alternative mechanisms of action for rh-LF. We speculated that rh-LF might activate macrophages residing in the intestinal wall or liver. This action would enhance the ability of intestinal wall and hepatic macrophages to kill bacteria that invaded from the intestinal lumen. The stimulatory effect of rh-LF was tested with rat macrophages in culture. Escalating amounts of rh-LF were shown to induce heightened production of NO (Fig. 4A) and secretion of TNF-α (Fig. 4C) by rat macrophages. By itself, LPS stimulated macrophages to generate far greater amounts of NO and TNF-α than those measured after treatment with rh-LF (Fig. 4, B and D). When LPS and rh-LF were mixed together before they were added to macrophage cultures, there was no significant increase or decrease in the content of NO and TNF-α in supernates (Fig. 4, B and D).

Although LF is reportedly a LPS-binding protein (15, 73), its inability to inhibit LPS-mediated stimulation of macrophages was confirmed when the activation of NF-κB and the expression of Egr-1 were evaluated. LPS activated NF-κB in rat macrophages to a larger extent than did rh-LF, but coinubation of LPS with rh-LF did not diminish the response to LPS (Fig. 5A). The expression of Egr-1 by rat macrophages showed less difference when LPS or rh-LF alone was the stimulus for macrophages (Fig. 5B). The induction of Egr-1

Fig. 3. In vitro killing of E. coli by recombinant human lactoferrin (rh-LF), human milk lysozyme (Lys), or a combination of both agents. Nos. of bacteria as CFU are depicted on the ordinate. Points are the means of 3 separate experiments. Error bars are too narrow for display. Treatment of E. coli with rh-LF (10 μg/ml) and Lys (10 ng/ml) significantly reduced the bacterial counts compared with 0 h and the other 2 conditions at 2 h (P < 0.001).
in cultured macrophages was not increased or reduced significantly when these phagocytes were stimulated with a mixture of LPS and rh-LF (Fig. 5B).

**DISCUSSION**

Host defense mechanisms directed at preventing bacterial invasion of the neonatal intestine differ significantly from those in older infants, children, and adults (12, 41). Translocation of bacteria from the intestine to the systemic circulation is hindered by many defense mechanisms. These defenses include the mucus layer overlying the epithelia, “natural peptide antibiotics” and IgA (sIgA) secreted into the intestinal lumen, the presence of host defense cells in the subepithelial region, the lamina propria, and muscular layers of the intestinal wall, and organized immune tis-

**Fig. 4.** Production of nitric oxide and secretion of tumor necrosis factor-α (TNF-α) by rat macrophages treated with rh-LF and/or lipopolysaccharide (LPS). Production of nitric oxide (measured as nitrite, NO₂⁻) and the secretion of TNF-α (immunoassay) were measured in tissue culture supernates. A: NO₂⁻ generation in the presence of increasing amounts of rh-LF. B: amount of NO₂⁻ produced when macrophages were stimulated with LPS in the presence and absence of rh-LF. C: secretion of TNF-α in the presence of increasing amounts of rh-LF. D: amount of TNF-α produced when macrophages were stimulated with LPS in the presence and absence of rh-LF. The ordinate indicates NO₂⁻ and TNF-α released into supernates. Shaded areas in A and B show the basal production of NO₂⁻ by nonstimulated macrophages. There is no basal release of TNF-α by nonstimulated macrophages. Points represent means ± SE for 3–5 separate experiments performed in triplicate.

**Fig. 5.** Mobility shift assay showing activation of nuclear factor-κB (NF-κB) (A) and Northern analysis showing the expression of early growth response gene-1 (Egr-1) (B) by rat macrophages treated with LPS, rh-LF, human milk Lys, or a combination of agents. Loading and transfer of the RNA were normalized to a murine β-actin probe.
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sues (e.g., Peyer’s patches) (3, 12). This list is not meant to be exhaustive but is presented as a link to the immaturity of host defenses in the neonatal intestine. The intestinal mucus layer (49, 55), the secretion of lysozyme and/or defensins by salivary glands (64, 75) and Paneth cells (8, 10, 40, 56), the production of sIgA by B lymphocytes (11, 24), the integrity of gut epithelium (11, 50, 57), and the immune cells present in the intestine (31, 36, 39, 67, 69) are each undergoing maturation in neonates. It is presumed that each may be a factor that facilitates bacteria translocation from the intestinal lumen of newborn infants.

Despite the immaturity of antibacterial host defenses in the neonatal intestine, the infant has a special system that assists in reducing the risk of bacterial translocation. That host defense system is milk from the mammary gland. Mammalian milk has many antimicrobial peptides/proteins akin to those eventually produced by the mature intestine (22, 23, 65). The leukocytes (e.g., macrophages) that are present in milk also have a critical role in preventing bacterial translocation (52). The host defense protein with the highest concentration in human milk is LF (colostrum: 5–7 mg/ml, mature milk: 1–2 mg/ml; Refs. 38, 42). Alternatively, LF has been reported to be undetectable in rat milk (42), hence the selection of the neonatal rat as an animal model for this study. We theorized that rh-LF given orally to neonatal rats would reduce systemic bacterial infection induced by inoculating massive numbers of an invasive E. coli into the intestine. This hypothesis was supported by a recent study showing that bovine LF given orally to neonatal piglets had a protective effect during subsequent endotoxemia (37). Here we extend that observation and show that orally administered rh-LF protects neonatal rats from systemic bacterial infection, illness, and death following massive intestinal infection with E. coli (Figs. 1 and 2). The survival rate in the neonatal rats pretreated with either rh-LF or rh-LF + FeSO₄ followed by massive gut infection with E. coli compares favorably with the outcome of piglets given oral bovine LF before induced endotoxemia.

The present study differs, however, from the classic investigation of Bullen et al. (5), which suggested that the iron-binding properties of LF prevent E. coli infections in infants. Those studies examined mainly the effects of LF on gut colonization by E. coli rather than examining overwhelming intestinal infection that causes significant bacteremia and death. The rh-LF used in these studies was 8–11% saturated with iron. This percent saturation of LF with iron is within the range reported for human milk (19, 29). The current study could be criticized for not including a control group in which the infant rats were pretreated with LF that was fully saturated with iron followed by infection of the gut with E. coli. We did not perform studies using LF that was fully saturated with iron for several reasons. First, such an experiment would not be physiological in the following ways. When the mammary gland and the mother are healthy, human milk has LF that has very low iron binding (<10% bound iron). Moreover, an infant receiving full feedings from human milk would have no source of ferrous ion in the diet to fully saturate the LF present in the intestine. In our studies using rat macrophages exposed to either rh-LF (8–11% iron saturated) or fully iron-saturated human LF showed that the holo-LF induced the macrophages to release significantly higher concentrations of NO (data not shown). The association between NO production in the neonatal intestine and injury to enterocytes during necrotizing enterocolitis (18) discouraged us from conducting an experiment using oral administration of fully iron-saturated human LF. Furthermore, ferric chloride is classified as an astringent, and ferric iron formulations could themselves have adverse effects on the intestinal mucosa. We concluded that adding a ferric iron preparation to the diet was not consistent with the nutritional care of human neonates. Rather than those studies, control studies were performed that used FeSO₄ in therapeutic amounts. The intent was to simulate rh-LF and iron therapy as might take place during clinical trials of rh-LF in human newborn infants. Infant rats pretreated with FeSO₄ alone had bacteriologic and clinical findings similar to those seen in the 0.45% saline control group, whereas rh-LF + FeSO₄ pretreatment had findings akin to the results when rh-LF alone was given orally before gut-related bacterial infection. The studies using FeSO₄ do not allow the conclusion that rh-LF restricts bacterial growth in the intestinal lumen via the sequestration of iron because the flux of ferrous ion to ferric ion and vice versa in the intestine of the neonatal rat is unknown. Thus the putative binding of iron by LF in the neonatal gut as a mechanism for bacteriostasis remains an unanswered question. Since the study by Bullen et al. (5) was reported, a substantial amount of new information has been published regarding the mechanisms whereby LF enhances host defense. These alternative explanations may be operative as to why rh-LF was protective in our neonatal rat model.

In this new model of gut-related E. coli infection of the newborn, it would have been ideal to delineate the mechanisms for actions of rh-LF in vivo. Although in vivo approaches are being undertaken, the investigations entail applying old methods to this animal model and the development of new techniques to study actions believed to be mediated by LF. Models that ascertain the clearance (killing) of bacteria within the intestinal lumen of neonates have not been described compared with similar studies of bacterial clearance performed in the lung (60). Although the lung is sterile after birth and remains relatively so thereafter, bacterial colonization of the neonatal gut takes place rapidly and therefore makes studies similar to pulmonary clearance of bacteria problematic. For this reason, the numbers of E. coli in the intestine of the infected pups were not quantified. An approach currently being pursued is the use of a rifampin-resistant isogenic mutant of E. coli strain Ec5. This spontaneous mutant should allow quantitative measurements of E. coli strain Ec5 in the neonatal intestine. The use of rifampin-contain-
ing media should restrict the growth of most “contaminating” bacteria present in the gut lumen and the intestinal wall.

Because methods to study the in vivo actions of rh-LF have not been fully developed, it was imperative to know that rh-LF interacts with the infecting bacterium, *E. coli* strain Ec5, and with rat immune cells. The macrophage was selected to study rat immune cells because macrophages are the primary phagocytes that usually encounter invading bacteria. The interactions between rh-LF and *E. coli* strain Ec5 are discussed here first. The possibility that rh-LF participated in the killing of *E. coli* within the intestinal lumen and was responsible for preventing some degree of bacterial translocation cannot be excluded on the basis of the studies performed. rh-LF may have some degree of membrane damage (73), the bacteria may have been either less invasive or more susceptible to killing by phagocytes once translocation from the intestine occurred.

Additionally, rh-LF may have acted in concert with lysozyme or other antimicrobial factors present in milk (22, 23, 65). Mammalian milk of all species reportedly contains lysozyme (53), and rat milk is no exception. Lysozyme is present in rat milk (2.4 units/mg protein), but the units used to quantify its concentration make it difficult to correlate the lysozyme content of rat milk with other species (72). Furthermore, although lysozyme is absent in the intestine of the rat fetus, Paneth cells of 8- to 12-day-old rats are strongly positive for lysozyme (35). Given the presence of lysozyme in rat milk and Paneth cells of 8-day-old rats, we had presumed that lysozyme would be present, to some extent, in the intestinal lumen of our suckling rats (3–6 days old). We stress, however, that no studies have analyzed the intestinal fluid obtained from suckling rats for its concentration of lysozyme. Nevertheless, it is reasonable to assume that some lysozyme was present and that the killing of *E. coli* by a mechanism that combines the actions of rh-LF and lysozyme is probable. The extent of the killing of *E. coli* by this mechanism within the lumen of the neonatal animals was not answered in the current study, but it may be in future studies.

Other “antibiotic peptides” (e.g., defensins) may also have been secreted by the salivary and other glands, Paneth cells, epithelia, and macrophages in milk (8, 10, 40, 56, 64, 75), and they may have acted along with rh-LF to kill *E. coli* within the intestinal lumen. Again the concentrations of other antibiotic peptides in the intestinal fluid of newborn rats has not been tested. This information is badly needed to ascertain the role of peptide/protein antibiotics in host defense mechanisms against bacteria during intestinal development. Our in vitro findings regarding the ability of rh-LF and lysozyme to kill *E. coli* in vitro are consistent with previous studies that showed that bovine LF and lysozyme act synergistically to kill this bacterium (16, 73). Future studies must determine whether rh-LF can induce the production and stimulate the secretion of peptide/protein antibiotics by epithelia and glands of the neonatal alimentary tract. Investigations must also focus on the development of peptide/protein antibiotics in the gut and define during ontogeny whether “natural antibiotics” produced by the intestine have synergy among themselves or act with antimicrobial effectors in milk to kill pathogenic bacteria. The concept of synergy among natural peptide/protein antibiotics and/or joint actions with therapeutic antibiotics is emerging as a strategy to prevent or treat bacterial infection (61, 74). The large numbers of *E. coli* introduced into the intestine, and the high percentage of rat pups treated with rh-LF that still had bacteremia and hepatic colonization, make it doubtful that bacterial killing in the intestinal lumen via the combined actions of LF, lysozyme, and other natural peptide antibiotics was the sole mechanism for the actions of rh-LF.

For the aforementioned reason, we also considered other explanations for the improved clinical status of the rat pups pretreated with rh-LF and then infected via the gut with *E. coli*. There has been recent interest in the role that intramural intestinal macrophages play as a barrier against bacterial invasion (12, 32). Furthermore, once bacteria traverse epithelial barriers of the intestine and enter the portal circulation, Kupffer cells in the liver provide the largest macrophage pool in the body to ingest bacteria and clear them from the bloodstream (26). Because isolation of macrophages from the intestine or liver often injures their membrane structure and receptors, we chose to examine the effects of rh-LF on macrophages by using a rat macrophage cell line. These studies would establish that rh-LF could interact with cells of the rat immune system. Upregulation of NF-κB, the production of N2-centered reactive species (e.g., ·NO), the secretion of cytokines (e.g., TNF-α), and expression of the macrophage differentiation gene Egr-1 have been considered to be indicators of macrophage priming or activation after exposure to proinflammatory agents such as LPS (13, 45, 66). Escalating concentrations of rh-LF stimulated macrophages to progressively increase the amount of ·NO and TNF-α that they produced (Fig. 4). Although it is difficult to establish a relative potency for each agent, exposure of macrophages to rh-LF produced ·NO and TNF-α at a far lower rate than did LPS stimulation. For instance, exposure of macrophages to 500 μg/ml of rh-LF would likely be a very high local concentration in vivo, whereas 100 ng/ml of LPS might be considered a modest stimulus at sites of infection. At the highest concentration of 500 μg/ml, rh-LF activated NF-κB and induced expression of Egr-1 to a lesser extent than did 100 ng/ml of LPS (Fig. 5). LF given orally would have its local concentration diluted as it passes through the intestine, crosses intestinal epithelial barriers, and enters the portal and/or systemic circulations. Thus macrophage-related exposure to rh-LF in the intestinal wall or liver was probably quite low. Considering that passage of proteins beyond the intestinal epithelia may be more permissive in neonates, the local concentration of rh-LF in tissues was probably closer to a priming
dose rather than an activating dose. Priming of macrophages is a concept whereby they are made ready for a second insult but are not yet activated and secreting biofactors associated with inflammation (43). In other words, the macrophages are armed but are not firing. We propose that tissue macrophages in rat pups given rh-LF orally were rendered more capable of killing bacterial invaders from the gut, and thus these animals had lower bacterial counts in their liver and blood after infection. Studies using in situ hybridization to characterize activation markers in tissue macrophages will be required to confirm that this hypothesis is correct. Such studies could concurrently examine the transfer and binding of radiolabeled LF to macrophages in the intestinal wall and liver. Pretreatment with rh-LF may have also desensitized macrophages so that they produced lower concentrations of reactive O$_2^{-}$ and N$_2$-centered intermediates and proinflammatory cytokines in the aftermath of systemic infection caused by massive infection of the gut with *E. coli*. Future studies could address this assumption by measuring the relative concentrations of NO metabolites, peroxida
tion products, and cytokines in the blood of neonatal rats that are pretreated or not with rh-LF before intestinal infection with *E. coli*.

The actions of rh-LF cannot be thought of as having effects solely on bacteria in the intestinal lumen or tissue macrophages. Evidence exists that proteolytic enzymes secreted by bacteria or leukocytes might degrade LF and release lactoferricin (4). Lactoferricin and lactoferricin-like peptides have antibacterial activity that is orders of magnitude higher than that of LF (46, 73). These peptides may have been generated in our neonatal rats, and the concept can be explored in this neonatal rat model. Evidence also exists that LF and lactoferricin have effects on other immune cells such as tissue lymphocytes (59) and Langerhans cells (9), and these effects may also be important before, during, and after bacterial infection. Intestinal epithelia undergo apoptosis when infected with enteroinvasive bacteria (33). Perhaps one of the most intriguing effects of LF is the in vitro observation that LF may facilitate apoptosis when bacteria are replicating inside intestinal epithelia (68). We theorize that LF may induce cellular apoptosis during intracellular bacterial replication and limit the infection by establishing an isolated “bag of bacteria” that can then be ingested and digested by other cells (e.g., adjacent epithelia, macrophages). Alternatively, if LF does not initiate apoptosis, necrosis of epithelia may occur and viable intracellular bacteria would be disseminated into the environment. This hypothesis can be achieved in the neonatal rat model described here. Finally, the recent literature emphasizes the anti-inflammatory (2, 37) and antioxidant (20, 70) properties of LF. Intestinal epithelia invaded by bacteria play a vital role in the proinflammatory response to infection (13), but whether LF mediates an anti-inflammatory response in this scenario has not been examined. The neonatal model described here may be able to achieve the answer. Any or all of the beneficial effects of LF described above might help explain the reduced bacteremia and improved clinical state that was seen in neonatal rats that received prophylactic rh-LF before gut-related infection with *E. coli*.

rh-LF is presently available for use in clinical trials involving human infants. As more proteins secreted in human milk become available through recombinant technology, it is possible that infants who cannot receive their mother’s milk will benefit from the inclusion of these proteins in a cow milk-based formula. With this advance in nutritional supplementation and augmentation of host defenses in the neonatal intestine (and other organs), prematurely born human infants may experience improved survival and reduced morbidity.

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