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Gut-Derived Bone Infection in the Neonatal Rat

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ABSTRACT

The risk of osteomyelitis is increased in the premature and critically ill neonate. Although potential sites of bacterial entry are present in many of these infants, the source of infection frequently cannot be established. This study was performed to assess the possible role of bacterial translocation from the intestine in the origin of bone infection using models of breast-fed and formula-fed rat pups. Newborn Sprague-Dawley rats suckled either *ad libitum* by the dam ($n = 30$), or were fed a rat milk-simulated formula ($n = 30$). After 3 d, the animals were killed, and the left femur, heart blood, mesenteric lymph nodes, liver, spleen, and terminal ileum were excised. Organs were analyzed for bacteria by standard microbiologic procedures. Bacterial translocation occurred in 23% of breast-fed rats; the bone was not infected in any of these animals. After feeding of formula diet, bacterial counts of the ileum were markedly elevated ($p < 0.001$), and the composition of the gut flora was disrupted. Bacterial translocation was noted in all formula-fed rats. Bone cultures were positive in 23 of 30 (77%) rats after formula-feeding ($p < 0.001$ *versus* breast-feeding). Organisms translocated to the bone included *Enterococci, Proteus, Enterobacter,* and *Escherichia coli*. Bacterial species cultured from the bone correlated with the individual colonization pattern of other extraintestinal organs and with the composition of the ileal flora. Members of the gut flora can escape the intestine and colonize the bone in formula-fed rats. The gut should be considered as a potential source for osteomyelitis in the neonate. **(*Pediatr Res* 50: 767–771, 2001)**

Abbreviation

CFU, colony forming units

Acute osteomyelitis in the neonate is a leading cause of destruction of the physis resulting in high rates of permanent handicaps (1–3). Because the major route of infection is via the bloodstream, opportunities for organisms to gain access to the circulation may favor the development of osteomyelitis. Premature and sick neonates represent the high-risk group, and in this population potential sites of bacterial entrance are frequently present. Many of these infants undergo vascular manipulation including insertion of central catheters for total parenteral nutrition or invasive monitoring, which have long been linked to the development of skeletal infection. In most cases, however, the origin of bacterial entry remains unknown (4). Therefore, alternative routes of infection should be considered.

Among the organisms causing neonatal osteomyelitis, *Staphylococcus aureus* is most common, followed by group B streptococci and Gram-negative bacteria (1, 5–7). All of these organisms have the ability to colonize the neonatal gut. They may represent normal luminal residents, or members of an abnormal microflora, as frequently seen in hospitalized neonates (8). It is now widely accepted that organisms can escape from the gastrointestinal tract and invade extraintestinal organs, a process termed as bacterial translocation (9, 10). In normal adult individuals, translocation of bacteria from the gastrointestinal tract is limited by a normal permeability of the intestinal mucosa, a well-established intestinal microflora, and a normal functioning of the immune system (11). Each of these factors is potentially altered in neonatal animals (12–15) and infants (16, 17), especially when premature and critically ill, and by feeding of a formula diet (18). Consequently, it has been argued that bacterial translocation is facilitated early in life (19). In support of this concept, translocation of parts of the intestinal flora occurs spontaneously and decreases with age in conventionally reared neonatal rodents (20, 21), with the intensity of translocation apparently being higher in rats (which are relatively immature at birth) than in the more mature rabbit pups. The age-dependant fall in spontaneous bacterial translocation is associated with a reduction of the intestinal permeability to small molecular weight particles (22), with the maturation of the gut-associated lymphoid tissue (23), and with the establishment of a normal gut flora (20). Whereas the spontaneous escape of bacteria from the intestine has no documented adverse effects in normal breast-fed animals, more pronounced translocation, as observed after artificial feeding (24) or induced by oral inoculation of various strains of *Escherichia coli* (25–27) and group B streptococcus (28), can

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result in septicemia (24–28), pneumonia (28), meningitis (26, 28), and death (26, 28).

The present experiments were performed to assess the possible role of bacterial translocation from the intestine in the etiology of bone infection in the premature neonate. Newborn rats were chosen as subjects for two reasons. First, these animals are relatively immature and therefore serve as a model for the preterm neonate (29), in whom the risk of neonatal osteomyelitis is highest. Second, we knew from previous experiments that rat pups are prone to bacterial translocation, especially when fed a formula diet (24).

METHODS

Animals. Time-gestation pregnant Sprague-Dawley rats were purchased from the Himberg Breeding Laboratories (Vienna, Austria). The animals had free access to water and regular chow and were kept on a 12-h light-dark cycle. The rats were allowed to deliver spontaneously. Only witnessed births were used for these experiments. The studies have been approved by the institutional review board.

Experimental groups. Sixty newborn male and female rat pups from nine litters were randomly assigned to one of two groups: in the first group, rats were fed adequate amounts of formula per gavage calculated to provide a daily energy intake of 500 kcal/kg as described previously in more detail (24, 30). In the second group, rats suckled *ad libitum* by the dam and were sham intubated every 6 h. An additional group of 17 rat pups were studied immediately after delivery and served as newborn controls. All organs cultured from this control group remained sterile. Formula-fed pups were housed in individual Styrofoam cups floated in a water bath maintained at 37°C. The rat milk formula (Milupa Nutrition Co., Austria) was composed according to published data on rat milk composition (31). Each 100 mL contained 175 kcal, 9.6 g of protein, 2.9 g of carbohydrate, and 12.9 g of fat with minerals and vitamins approximating that present in rat milk.

Testing for bacterial translocation. After 3 d the animals were killed by cervical dislocation. Using sterile techniques, the left femur was removed under lens magnification. The femur was used for microbiologic investigation because rapidly growing ends of the large long bones, especially those of the lower extremity, are major sites of osteomyelitis in the neonate (7, 32). The femur was cut longitudinally, and the bone marrow was removed and homogenized with 9 volumes of brain heart infusion using sterile ground glass stoppers. Then, the chest and abdominal cavities were opened. Blood was obtained by cardiac puncture under direct visualization, and 100 μL was incubated for 24 h at 37°C in 5 mL of brain heart infusion. The mesenteric lymph node complex, liver, and spleen were removed, and all organs were weighed and homogenized with 9 volumes of brain heart infusion. To determine bacterial concentrations, homogenates of the bone marrow, liver, and spleen were serially diluted from 10^1 to 10^6 in sterile saline. Finally, the distal ileum was excised, weighed, and homogenized. Homogenates were serially diluted from 10^1 to 10^{10}. Portions (0.1 mL from the various dilutions and 0.1 mL from the remaining organ homogenates) were plated on blood agar to culture for aerobic and facultative anaerobic Gram-positive bacteria, on Endoagar to culture for aerobic and facultative anaerobic Gram-negative bacilli, and on MRS agar to culture for lactobacilli (Oxoid Company, Hampshire, UK). The homogenized mesenteric lymph nodes and the remaining homogenates of the bone marrow from the femur, liver, and spleen were incubated aerobically in 5 mL of brain heart infusion at 37°C and then subcultured on the three different agar plates. Hypothetically, as few as one viable bacterium in the homogenates of the organs can be detected using these culturing procedures. All agar plates were incubated at 37°C under aerobic conditions. After 24 h the cultures were analyzed by a microbiologist who was unaware of the study design. The Gram-negative bacteria were identified with the API 20 E system (Analytab Products, Plainview, NY, U.S.A.) and the Gram-positive cocci by standard microbiologic procedures. Quantitative culture results were determined by the number of CFU per gram of tissue calculated from the dilutions of organ homogenates and positive tissue cultures.

Statistics. Continuous data were compared using the unpaired *t* test. Discontinuous data were evaluated by χ^2 analysis; p values < 0.05 were considered statistically significant.

RESULTS

Bacterial colonization of the ileum. As shown in Table 1, formula-feeding resulted in an increase in the ileal concentration of all bacterial species, and the total bacterial counts were substantially higher than those in breast-fed animals. Furthermore, the ilea of formula-fed pups were colonized with a higher variety of species, and also included *Proteus, Enterobacter,* and *Acinetobacter*, organisms that were not found in breast-fed animals.

Bacterial translocation. Table 2 compares the frequency of bacterial translocation to extraintestinal organs. After 3 d of breast-feeding, bacteria were cultured from the mesenteric lymph nodes in 20%, and from the liver and spleen in 10% of rats. All cultures from the blood or from the bone remained sterile in breast-fed rats. Formula-feeding dramatically increased bacterial translocation to all organs investigated. Blood cultures were positive in 63%, and in 77% of formula-fed pups enteric bacteria had translocated to the bone.

Table 1. *Bacterial colonization of the ileum*

Species	Bacterial concentration ($\log_{10}$ CFU/g tissue)*		
	Breast-feeding (n = 30)	Formula-feeding (n = 30)	p value
Escherichia coli	6.17 ± 0.22 (30)	7.06 ± 0.18 (26)	0.004
Proteus	—	6.61 ± 0.27 (20)	—
Enterobacter	—	8.90 ± 0.11 (10)	—
Acinetobacter	—	6.51 ± 0.59 (7)	—
Lactobacillus	5.67 ± 0.22 (29)	7.14 ± 0.21 (22)	< 0.001
Enterococcus	4.19 ± 0.17 (25)	7.09 ± 0.33 (22)	< 0.001
Staphylococcus epidermidis	4.38 ± 0.30 (8)	5.74 ± 0.79 (6)	0.10
Staphylococcus aureus	3.94 ± 0.05 (3)	5.31 ± 0.37 (2)	0.018
Total	6.55 ± 0.16 (30)	8.08 ± 0.18 (30)	< 0.001

* Concentration data are expressed as mean ± SEM and are based on positive cultures only. Values in parentheses indicate incidences.

Table 2. *Incidence of bacterial translocation*

Group	Weight* (g)	MLN	Liver	Blood	Spleen	Bone	Rats positive
Breast-feeding	8.4 ± 0.3	6 (20)	3 (10)	—	3 (10)	—	7 (23)
Formula-feeding	8.1 ± 0.3	30 (100)	26 (87)	19 (63)	14 (47)	23 (77)	30 (100)
p value	NS	< 0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001

Abbreviation used: MLN, mesenteric lymph nodes.

* Data are mean ± SEM. Values in parentheses indicate percentages.

The incidences and concentrations of viable species translocated to the various organs are summarized in Table 3. In breast-fed pups, translocated organisms were identified as *E. coli*, *Enterococcus*, *Lactobacilli*, and *Staphylococcus epidermidis*. In addition to these organisms, formula-feeding was associated with translocation of *Proteus* and *Enterobacter*, species that were not present in organs of breast-fed pups.

Bacteria translocated to the bone. In normal suckling rats all cultures from the bone remained sterile. Among formula-fed animals, 23 of 30 (77%) had positive bone cultures. Different translocated bacterial species included *Enterococci*, *Proteus*, *Enterobacter,* and *E. coli*; the mean concentrations of these species varied between 2.8 and 5.4 log CFU/g tissue (Table 3). Bacterial species cultured from the bone were correlated with the individual colonization pattern of other extraintestinal organs and with the composition of the bacterial flora in the ileum.

DISCUSSION

Various animal models of osteomyelitis have been developed to study the pathophysiology of this disease and to compare the efficacy of different antibiotic regimens (33–36). However, no reports exist that address the role of the gut as a possible source of neonatal bone infection. Our current findings confirm and expand the results of a previous study, showing that formula-feeding dramatically increases bacterial translocation in formula-fed rat pups (24). Using the same model, we now demonstrate that bacteria may escape the intestine and colonize the bone.

For the onset of hematogenous osteomyelitis bone lodgment of circulating bacteria is crucial. Experimental data indicate that among the factors that determine the ability of a microbe to colonize bone tissue and eventually induce osteomyelitis, the concentration and specific virulence factors of the offending organism have importance. For example, Emslie and Nade (33) injected *S. aureus* i.v. in chickens and observed that the incidence of osteomyelitis increased in a dose-dependent fashion. In their model, the 50% inhibitory dose for osteomyelitis was estimated as 5.5×10^5 viable organisms per kg of body weight. Others inoculated *S. aureus* UAMS-1 directly into the bone marrow of adult rabbits, and required 2×10^4 CFU to produce histologic and radiographic features of osteomyelitis (37). After inoculation of less virulent strains, bone lodgment of bacteria was rare or absent, despite the fact that a 100-fold dose of bacteria was used. During the current experiments we did not examine the bone histologically, and thus our findings do not prove that osteomyelitis was actually present. However, the presence of an average of 1.8×10^4 CFU of viable gut bacteria in the bone of our formula-fed rats clearly demonstrates that, under certain circumstances, members of the intestinal microflora can colonize bone tissue and thus become a potential source of osteomyelitis.

Several factors might explain the high incidence of positive bone cultures in our formula-fed rats. Formula-feeding dramatically enhances the concentration of enteric organisms and disturbs the composition of the gut flora. In addition, formula diet lacks the antiinfective properties normally present in breast milk (38, 39) and may thus further compromise the already limited host defense mechanisms present in the rats during the neonatal period. This may have resulted in the high load of systemically spreading organisms, which *per se* can increase the risk of bacterial lodgment to the bone. Various factors are

Table 3. *Species and concentrations of translocated bacteria*

			Bacterial concentration ($\log_{10}$ CFU/g tissue)*			
Group	Species	MLN	Liver	Blood	Spleen	Bone
Breast-feeding	*E. coli*	(2)	4.38 ± 0.61 (2)	—	2.76 ± 0.64 (2)	—
	Enterococcus	(2)	3.31 (1)	—	—	—
	Lactobacilli	—	3.11 (1)	—	—	—
	S. epidermidis	(3)	4.31 (1)	—	3.62 (1)	—
	Total	(6)	4.31 ± 0.55 (3)	—	3.06 ± 0.46 (3)	—
Formula-feeding	*E. coli*	(15)	4.16 ± 0.39 (17)	(12)	3.55 ± 1.07 (2)	3.56 ± 0.38 (9)
	Proteus	(14)	5.07 ± 0.49 (11)	(4)	3.22 ± 0.23 (9)	2.81 ± 0.21 (11)
	Enterobacter	(9)	4.69 ± 0.38 (10)	(7)	4.73 ± 0.31 (9)	5.48 ± 0.37 (9)
	Enterococcus	(21)	4.11 ± 0.48 (13)	(4)	3.60 ± 0.36 (11)	3.31 ± 0.31 (17)
	Lactobacillus	(5)	4.41 ± 0.49 (7)	—	3.15 ± 0.15 (2)	—
	S. epidermidis	(4)	3.45 ± 0.65 (5)	(1)	3.85 ± 0.51 (3)	—
	Total	(30)	4.80 ± 0.31 (26)	(19)	4.25 ± 0.25 (19)	4.26 ± 0.31 (23)

Abbreviation used: MLN, mesenteric lymph nodes.

* Concentration data are expressed as mean ± SEM and are based on positive cultures only. Frequency of positive cultures for various species are given in parentheses.

known to favor the deposition of bacteria in neonatal bone tissue, including the sluggish circulation near the growth plate (40), specific virulence factors of the offending organisms (41), a particular tropism to the growth-plate cartilage matrix (42) of some species, and a deficiency of local host defense mechanisms (43). It is not surprising that in the presence of a deficient immune system and a high load of circulating bacteria, the clearance of organisms from the bone is incomplete, allowing the bacteria to multiply. Bacterial translocation to systemic organs (liver and spleen) was also observed in breast-fed animals; however, in none of them the bone was colonized. This may be explained by a lower intensity of translocation in combination with a better functioning of the immune defense system in the breast-fed rats.

Despite the growing evidence derived from experimental animals, as alluded to in the "Introduction," there exist no systematic studies documenting that bacterial translocation occurs commonly and causes clinically significant disease in human neonates. Some limited and circumstantial data, however, support the existence and pathogenetic impact of translocation in the pediatric population. For example, Albers *et al.* (44) cultured bacteria from the peripheral blood in 21 of 131 normal 3-d-old infants, and in three of them blood cultures remained positive for the same organisms for 4 to 6 d. The authors argued that this was a benign phase that some infants undergo during the process of acquiring normal bacterial flora. Goldmann (8) found that infection caused by Gram-negative bacilli usually occurs in neonates already colonized with these organisms in the pharynx or intestine. Further, Lambert-Zechovsky *et al.* (45) described a 2150-g male infant born at 33 wk of gestation who demonstrated systemic bacteremia and meningitis caused by *Enterobacter cloacae*. Using molecular analysis, the authors provided genetic evidence that the strains isolated sequentially from cultures of stool, blood, and cerebrospinal fluid were identical. Pierro *et al.* (46) studied neonates and infants receiving long-term parenteral nutrition. The authors found that six of 94 patients experienced 15 episodes of septicemia caused by bacteria of enteric origin; in all six patients, the causative organisms were also present in the throat or rectum. We are aware of two studies on bacterial translocation in infants in which cultures from the mesenteric lymph nodes or the liver were performed in an effort to better trace the path of bacterial entrance. Cicalese *et al.* (47) studied 50 children undergoing small bowel transplantation. Blood, stool, liver biopsies, and peritoneal fluid were collected when infection was clinically suspected. Bacterial translocation (defined as the simultaneous presence of microorganisms in blood or liver biopsy and feces) was present in 44% of patients. Finally, among 28 infants with short bowel syndrome and sepsis, 19 harbored the causative organisms in the fecal flora, and in 12 of them the pathogens were present in the mesenteric lymph nodes (48).

Although our findings in rats cannot be directly applied to the situation in infants, several observations are consistent with the hypothesis that skeletal infection of the neonate may be caused by translocation of bacteria from the gut. Unlike in the older child, organisms frequently present in the neonatal gastrointestinal tract, like *E. coli*, *Klebsiella*, *Enterobacter*, and *Enterococci*, are pathogens for osteomyelitis in this age group (1, 6). It is conceivable that these organisms could pass through the immature intestinal barrier and cause manifest infection in the neonatal host with an unusual susceptibility to many organisms that may be considered normal flora. Further, *S. aureus* and group B streptococcus, the most frequently encountered causative species of neonatal skeletal infection, may also colonize the intestine (8), particularly in hospitalized neonates, in whom the risk of osteomyelitis is increased. The ability of these bacteria to translocate from the gut into the systemic circulation has been documented in experimental animals (28, 49), and may be operative when asymptomatic bacteremia develops in human neonates (44). It is interesting to note that in the present study *S. aureus* was not detected in the bone, despite the fact that some rat pups harbored this organism in the small intestine. Possible explanations for the failure of *S. aureus* to translocate include the relatively low intestinal concentration and the small number of rats colonized with this species.

In conclusion, we have shown that intestinal bacteria escape the gut and can colonize bone tissue in formula-fed rat pups. It is tempting to speculate that neonatal osteomyelitis is mediated by bacterial translocation in a subgroup of patients, especially in those who are premature and sick. To substantiate this hypothesis, clinical studies are required that are designed to identify the causative organisms of osteomyelitis in the patient's own gut flora. The absence of bacteria in bone tissue of suckling rats supports the importance of breast-feeding.

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