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Occurrence of Necrotizing Enterocolitis May Be Dependent on Patterns of Bacterial Adherence and Intestinal Colonization: Studies in Caco-2 Tissue Culture and Weanling Rabbit Models[1]

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ABSTRACT

Necrotizing enterocolitis (NEC) is one of the leading causes of death in neonatal intensive care units. The underlying pathophysiology of NEC is poorly defined, although there is a suggestion that bacterial agents play an important role in the process. In this study, we evaluated bacterial isolates from 17 NEC cases and matched asymptomatic control infants. Isolates from NEC patients were no more likely than control isolates to be adherent to enterocytes, as assessed by a Caco-2 cell tissue culture model. Adherent *Escherichia coli* isolates, from both NEC cases and controls, were able to cause pathologic changes typical of NEC in a weanling rabbit ileal loop model. Adherence of *E. coli* strains to Caco-2 cells, and subsequent production of disease in weanling rabbits, could be blocked by coinfection with Gram-positive isolates from control children. In contrast, in three of four instances, adherent *E. coli* from NEC cases retained their adherence and caused illness in rabbits when coinfected with Gram-positive isolates from the homologous child. Our data suggest that patterns of intestinal adherence, as influenced by the underlying intestinal microbial ecology, play a role in the pathophysiology of NEC. (***Pediatr Res* 36: 115–121, 1994**)

Abbreviations
NEC, necrotizing enterocolitis
NICU, neonatal intensive care unit

NEC is a serious gastrointestinal disorder in newborns affecting predominantly premature or low-birth-weight infants. NEC is one of the leading causes of death in NICU, with an incidence of 2–3% in premature infants and a mortality of 10–55% (1–4). The etiology of NEC is unknown. Although there have been outbreaks of NEC associated with specific infectious agents (2, 5–10), the range of agents isolated is broad, and in studies of endemic disease it has not been possible to consistently link a single infectious agent with illness (11).

During the past decade, there has been increasing recognition that the environment or milieu in which a microorganism is placed can have a profound effect on its virulence (12). In experimental animals, for example, severe combined immunodeficient mice can be protected against lethal cryptosporidium infection by modification of intestinal microflora with a combination of avirulent bacteria (13). In humans, *Clostridium difficile* often colonizes the intestine without any apparent consequence (14, 15), with colitis occurring in settings in which there are perturbations of the intestinal flora due to administration of antibiotics or chemotherapeutic agents. There are also suggestions from rabbit studies that with some bacteria the simple process of intestinal adherence or colonization can lead to illness, without expression of specific toxins or other virulence factors (16). "Schaedler's Cocktail," a combination of harmless bacteria, is routinely used while raising specific pathogen-free rodents; when animals are raised germfree without such treatment, normal physiologic development of the gut does not occur, and they become susceptible to pathogens that would be normal flora for the same strain of rodent (13).

We hypothesized that similar processes were important in NEC—that normal flora bacterial isolates might be

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able to cause illness based on patterns of adherence to the intestinal mucosa and on the underlying microbial ecology of the neonatal gut. To test this hypothesis, we examined a collection of bacterial isolates from 17 NEC cases and matched controls. As a marker for adherence and colonizing ability, we evaluated adherence of each bacterial strain in a Caco-2 cell culture assay (17–20); we also looked at the effect of combinations of bacteria on colonization in this model. To assess the *in vivo* relevance of these observations, selected strains and strain combinations were inoculated into ileal loops in weanling rabbits, a model that has been used in prior NEC studies (21, 22).

Table 1. *Bacterial isolates in strain collection*

	Case isolates (n = 17)	Control isolates (n = 17)
Enterococcus species	8	12
Staphylococcus epidermidis	8	6
S. hemolyticus	0	1
Staphylococcus aureus	0	1
Escherichia coli	9	4
Klebsiella pneumoniae	6	6
Enterobacter aerogenes	4	1
K. oxytoca	4	1
Citrobacter freundii	0	2
Serratia marcescens	1	0
Pseudomonas aeruginosa	1	0
Proteus mirabilis	0	1

METHODS

Bacterial strains. Collection of bacterial isolates and clinical characteristics of cases have been previously reported (11). Briefly, stool samples were collected from 17 infants diagnosed with medical or surgical NEC and from matched control subjects. Children were hospitalized in the NICU at the University of Maryland Hospital between June 1990 and February 1992. Case patients were defined as those infants who fulfilled the modified Bell's criteria for staging of NEC (stage 1 to 3) (23). Matched controls were selected from infants who met the following criteria: *1*) no evidence of NEC; *2*) birth weight within 25% or 250 g of the birth weight of the case patient; *3*) presence in the NICU at the time of diagnosis of NEC in the matching case subject; *4*) not on antibiotics at time of enrollment; *5*) postnatal age within 1 wk of the matched case patient. Studies were reviewed and approved by the Human Volunteer Research Committee, University of Maryland at Baltimore.

A single stool sample was collected as soon as the definition of NEC was satisfied. Case patients were enrolled only if a stool was passed no later than 5 h after the first antibiotic dose. A single stool specimen was obtained in controls as soon as possible after enrollment. All representative colony types were picked and identified to the species level by using standard microbiological techniques, with confirmation by API20E or API-Staph Ident (Analytab Products, Plainview, NY). Isolates were frozen in Luria broth (L-broth) containing 25% glycerol and stored at −70°C. For each experiment, fresh bacteria regrown from frozen stock were used. A listing of all isolates in the collection is given in Table 1. In seven of eight instances in which infants subsequently had a positive blood culture, the organism isolated from blood was also present in stool; in five instances, *Staphylococcus epidermidis* was the blood isolate.

Isolates within the same species were further evaluated by plasmid profile analysis. As previously reported (11), these data suggested that each infant was colonized with a limited number of unique strains: isolates of the same species from different infants had different plasmid profiles, whereas almost all isolates of the same species from the same infant had the same plasmid profile. For the purposes of the current study, a single isolate of each species from each child was selected for evaluation. In the few instances in which more than one plasmid profile was present among isolates of the same species from the same infant, an isolate of the most common plasmid profile was selected. All *Escherichia coli* isolates were screened by DNA probes to confirm that they did not belong to previously identified pathogenic groups, including enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, or enteroaggregative *E. coli* (24). Only two isolates from control infants were positive for the gene encoding diffuse adherence.

In vitro adherence assay using Caco-2 cells. Ten-d-old Caco-2 cell monolayers grown on chamber slides (Nunc, Naperville, IL) were infected with 10^8 bacteria/mL in antibiotic-free medium at 37°C with gentle rocking for 1.5 h. The inoculum was then removed, and monolayers were washed, stained with Giemsa, and evaluated by light microscopy. Coinfection studies were conducted using a combination of Gram-negative and Gram-positive organisms. Adherence was graded on a scale of 0 to 4 (0: no adherent bacteria; 1: one to five bacteria; 2: six to 25 bacteria; 3: 25 to 50 bacteria; and 4: more than 50 bacteria per cell). Each experiment was repeated at least three times before assigning any adherence grade to a strain.

Ileal loop model in weanling rabbit. Ileal loops were prepared in weanling New Zealand White rabbits weighing between 392 and 430 g by following standard methods (21, 22, 25). Each bacterial isolate was inoculated in duplicate animals. Rabbits were killed after 16–18 h, gross changes in the loops were noted, fluid accumulation was measured, and tissue samples were fixed in formalin for histopathology. Only the center portion of the loop sufficiently away from the ligature sites was collected to avoid any local inflammatory changes due to physical trauma. All studies were approved by the Institutional Animal Care and Use Committee of the University of Maryland at Baltimore.

Representative adherent and nonadherent strains of *E. coli*, *Klebsiella*, *Staphylococcus*, and *Enterococcus* species from NEC patients and controls that had been already evaluated in the *in vitro* Caco-2 cell culture model were selected for study. A total of 5×10^7 organisms were used per loop (5 cm) for monocontamination exper-

iments. In coinfection (bicontamination) studies, Gram-negative and Gram-positive strains were inoculated into the ligated loop in PBS; both were inoculated at the same inoculum size. Control loops received sterile PBS.

RESULTS

In vitro adherence of bacteria to Caco-2 cells. Results of adherence assays performed on *E. coli*, *Klebsiella pneumoniae*, enterococci, and *S. epidermidis* are shown in Table 2. Strains of *E. coli* were most likely to have high grade (grade 4) adherence. Adherence of *Klebsiella* species was much less pronounced in our Caco-2 cell model. *Enterococcus* and *Staphylococcus* species showed moderate adherence (grade 1–3). All other strains isolated from case patients as well as from controls showed minimal (1+) or no adherence, with the exception of one strain of *Citrobacter freundii* that showed a 4+ adherence (data not shown). There was no clear association between the adherence grade of the colonizing strain and the occurrence or severity of NEC.

In vitro adherence after coinfection. Because there were no obvious differences in the adherence grade of isolates from case or control infants, we examined the adherence pattern of selected Gram-negative species in combination with Gram-positive bacteria isolated from the same infants. Studies were limited to strains from infants from whom both a Gram-negative and a Gram-positive species had been isolated.

As shown in Table 3, three of three highly adherent (grade 4) *E. coli* from control infants became nonadherent (grade 0) upon coinfection with a Gram-positive isolate from the homologous infant. The coinfecting Gram-positive strains retained adherence, albeit at levels comparable to or slightly below that seen when the Gram-positive strain was inoculated in pure culture. In two instances (for *E. coli* strains 6–1 and 32–1), *Enterococcus faecalis* was the Gram-positive partner; in the third, it was *Enterococcus faecium*.

In contrast, three of four highly adherent strains from NEC case patients remained adherent when combined with Gram-positive isolates from the homologous infant (Table 3). Of the three case *E. coli* strains that retained adherence, two had coinfections with enterococci and one with staphylococci; the *E. coli* strain from the fourth NEC case patient (which became nonadherent on coinfection) was coinfected with an enterococcal strain.

Coinfection studies were repeated by crossing the strains, *i.e.* combining an enterococcus from a control child with an adherent *E. coli* from an NEC case patient and enterococci from an NEC case patient with adherent *E. coli* from a control infant. When *E. faecium* strain 20–2 from a control patient was used in coinfection studies with *E. coli* strains 21–1, 25–1, 27–1, and 33–1 (from NEC case patients), all became nonadherent. In contrast, *E. fecalis* strain 21–2 (isolated from an NEC case patient) could not abrogate the adherence of *E. coli* strains 6–1, 20–1, and 32–1 isolated from control infants. Representative results are shown in Figure 1.

Experimental NEC in rabbit ileal loops. All control loops had a healthy gross and microscopic appearance, and there was minimal (<0.5 mL/loop) or no fluid accumulation. Fluid accumulation in loops infected with bacteria ranged from 1.6 to 2.4 mL/2.5 cm of loop.

Adherent strains of *E. coli* (isolated from NEC case patients and controls) produced grossly apparent damage to the loop, with purulent necrotic spots and hemorrhagic fluid accumulation (Table 4). Histopathology showed massive necrosis of luminal epithelium extending to the muscle layers, with edema, hemorrhage, and polymorphonuclear cell infiltration. Bacteria were seen that were adherent to necrosed epithelial cells. In keeping with reports of experimental NEC from other investigators, pneumatosis intestinalis was not observed in our model.

Table 2. *Adherence of NEC bacterial isolates to Caco-2 cells*

Bacterial isolate	Adherence grade				
	0+	1+	2+	3+	4+
Escherichia coli					
Case (9)	0	5	0	1	3
Control (4)	0	1	0	0	3
Klebsiella pneumoniae					
Case (6)	0	5	1	0	0
Control (6)	4	2	0	0	0
Enterococcus					
Case (8)	0	6	1	1	0
Control (12)	0	8	3	1	0
Staphylococcus epidermidis					
Case (8)	2	6	0	0	0
Control (6)	0	4	2	0	0

Table 3. *Adherence of Gram-negative bacteria to Caco-2 cells upon coinfection with Gram-positive bacteria isolated from the same infants*

Isolate	Patient	Adherence grade of Gram-negative isolate	
		Monocontamination	Coinfection
Escherichia coli			
1-1	Case	1+	1+
5-4	Case	1+	1+
9-5	Case	1+	1+
19-1	Case	1+	1+
21-1	Case	4+	4+
25-1	Case	4+	4+
27-1	Case	3+	3+
33-1	Case	4+	0+
6-1	Control	4+	0+
20-1	Control	4+	0+
32-1	Control	4+	0+
Klebsiella pneumoniae			
7-1	Case	2+	NA
9-11	Case	1+	1+
11-4	Case	1+	0+
23-1	Case	1+	1+
31-3	Case	2+	0+
4-2	Control	0+	0+
6-3	Control	0+	0+
12-2	Control	1+	0+
24-3	Control	0+	0+
30-2	Control	1+	1+

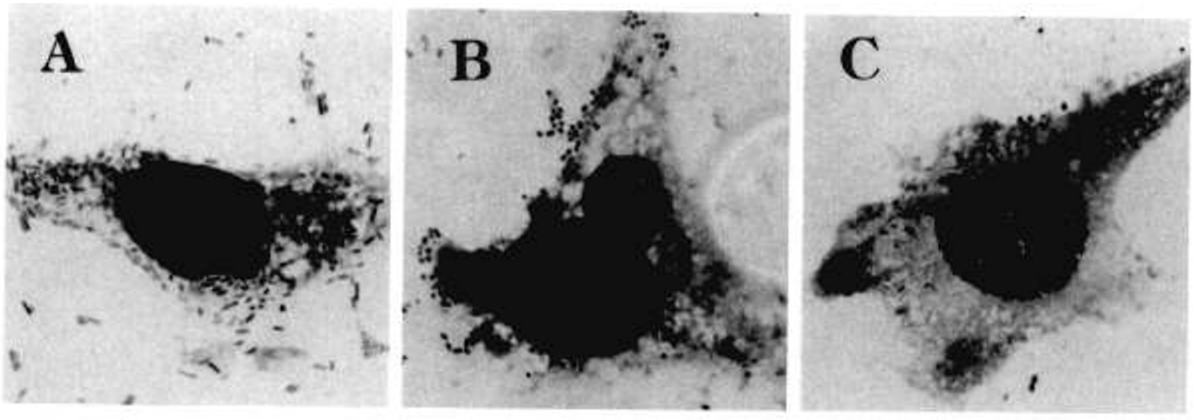

Figure 1. Bacterial adherence to Caco-2 cells. *A*, A typical 4+ adherence by *E. coli* strain 21–1; *B*, *E. faecium* strain 20–2 showing a 3+ adherence. Note the total abrogation of *E. coli* (21–1) adherence upon coinfection with *E. faecium* (20–2) (*C*). Adherence of the Gram-positive strain (enterococci) is maintained at a slightly lower degree.

Table 4. *Adherence and virulence of bacterial isolates upon mono- and coinfection in Caco-2 cell adherence assay and weanling rabbit ileal loop model**

Isolate	Adherence grade†		Ileal loop pathology	
	Monoinfection	Coinfection	Monoinfection	Coinfection
Escherichia coli (case)				
9-5‡	1+	1+	Healthy	Healthy
21-1	4+	4+	Necrosis	Necrosis
25-1	4+	4+	Necrosis	Necrosis
27-1‡	3+	3+	Necrosis	Necrosis
33-1	4+	0+	Necrosis	Healthy
E. coli (control)				
6-1	4+	0+	Necrosis	Healthy
14-1	1+	NA	Healthy	NA
20-1	4+	0+	Necrosis	Healthy
32-1	4+	0+	Necrosis	Healthy
Klebsiella pneumoniae (case)				
7-1	2+	NA	Healthy	NA
9-11‡	1+	1+	Necrosis	Necrosis
K. pneumoniae (control)				
26-3	0+	0+	Healthy	Healthy
Enterococci (case)				
21-2	3+	NA	Healthy	NA
25-2	2+	NA	Healthy	NA
Enterococci (control)				
20-2	3+	NA	Healthy	NA
26-2	2+	NA	Healthy	NA
32-2	2+	NA	Healthy	NA
Staphylococcus epidermidis (case)				
9-2	2+	NA	Healthy	NA
27-2	1+	NA	Healthy	NA
S. epidermidis (control)				
22-1	2+	NA	Healthy	NA

* NA, not applicable (Gram-positive organisms not isolated from these patients or monoinfection was done).
† Data from Table 3.
‡ *S. epidermidis* was present as coinfectant; other Gram-negative organisms had enterococci as co-infectant.

Strain invasion was confirmed by visualization of large numbers of bacteria in the lamina propria and submucosa of the infected loops (Fig. 2). All of these rabbits appeared sick, and one died between 12 and 16 h after inoculation due to rupture of the loop. Only the inoculated organisms were cultured from the infected loops at necropsy.

One *Klebsiella* strain caused pathologic changes comparable to those seen with adherent *E. coli*. The remaining two *Klebsiella* strains tested showed mild inflammatory reaction, with infiltration of neutrophils, congested vessels, and some blunted villi with hyperplastic epithelium. Villus necrosis was not produced, and lamina propria, submucosa, and the muscle layers were healthy in the loops infected by these *Klebsiella* strains.

Ileal loop coinfection with Gram-positive bacteria. Results of coinfection studies in rabbit loops correlated with results of adherence assays in the Caco-2 model (Table 4). In instances in which coinfection resulted in a loss of

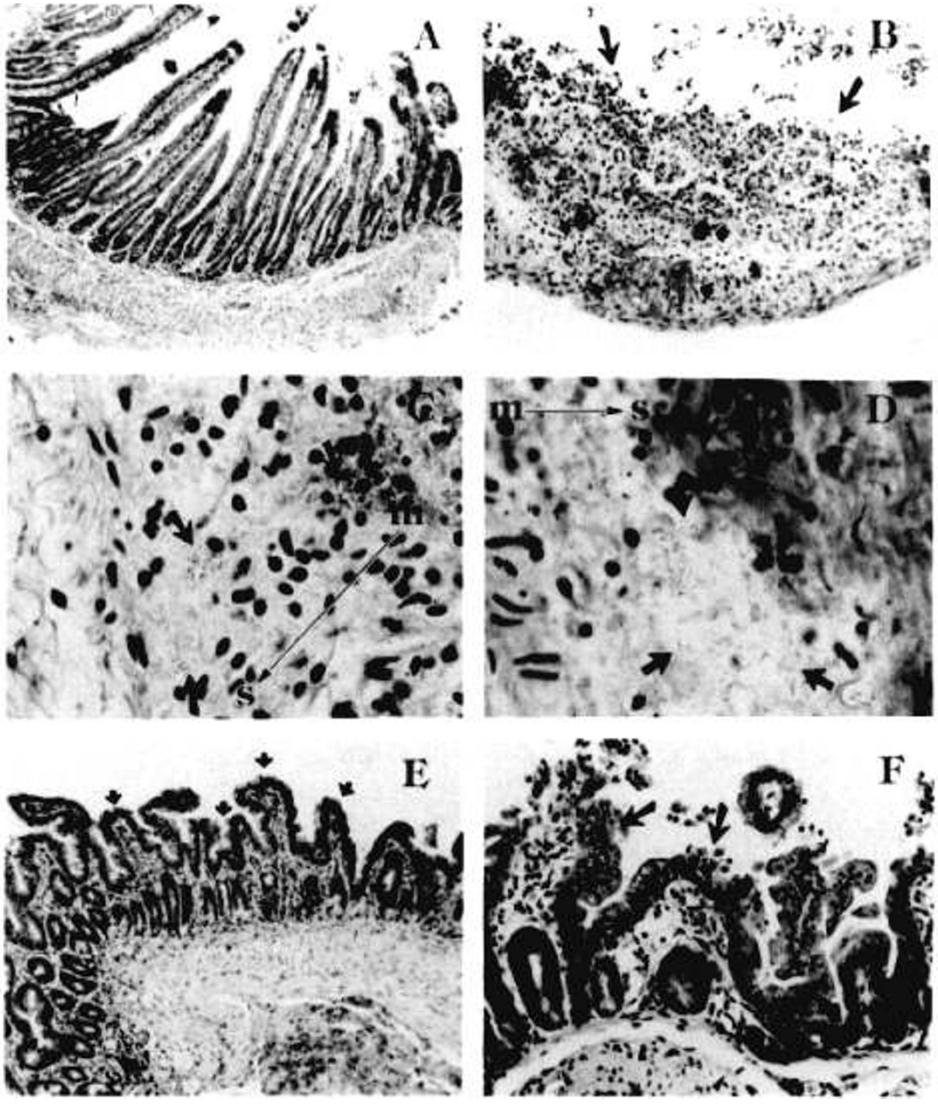

Figure 2. NEC in weanling rabbit ileal loops. *A*, Histopathology of a control rabbit ileal loop showing intact villi with simple columnar epithelium, intact deeper crypts, submucosa, and muscle layers. *B*, Complete severe transmural necrosis produced by adherent *E. coli* strain 21–1. There is complete loss of surface and glandular epithelium with collapse of mucosa (*arrows*). There is severe transmural acute inflammatory cell infiltration accompanied by areas of congestion (*arrowheads*) and hemorrhage. *C*, A higher magnification picture of *B* (adherent *E. coli* strain 21–1) showing clumps of bacteria (*arrows*) entering from the mucosal side (*m*) and extending toward the serosal side (*s*) (*long arrow*). *D*, The same section as in *C* showing a large number of bacteria (*surrounded by arrows*) in the submucosa close to the inner circular muscle layer. *E*, Ileal loop after coinfection with adherent Gram-negative and Gram-positive bacteria showing minimal damage and a regenerative response. Surface epithelium, deeper glands, and muscle layers are intact. Lamina propria contains low numbers of acute inflammatory cells. Some blunting of villi is observed (*arrows*). Villus and glandular epithelia are intact. *F*, The same section has focal areas of flattened villi lined by piled-up basophilic cuboidal cells (*arrows*), a sign of active regeneration. Lamina propria and submucosa are mildly edematous.

adherence, typical NEC-like pathology was not seen. There were signs of mild inflammation in a few focal areas showing flattened villi where the single-layered simple columnar epithelium was replaced by more basophilic, hyperplastic cuboidal epithelium, suggesting active regeneration after mild injury (Fig. 2). In contrast, strains that retained adherence in the Caco-2 coinfection assay produced typical NEC-like injury in the ileal loops in the presence of the Gram-positive partner.

DISCUSSION

This study was made possible by the availability of a unique collection of bacterial isolates from NEC case patients and matched controls. These strains were originally collected in an effort to identify bacterial strains or species that might be associated with NEC cases; as such, quantitative cultures were not obtained. However, the observation that in most instances all picks of a common species from a single patient had a common plasmid profile, and the intentional selection of the most common plasmid profile when multiple profiles were identified, suggests that the isolates studied represented the predominant strain of each species in each infant. In this study, seven of eight blood isolates were also present in the stool. It is possible that in some instances blood isolates reflect entry of intraluminal organisms through damaged mucosa, and may not be indicative of

the organism that triggered the underlying pathologic process.

Epidemics of NEC have been associated with single pathogens such as *E. coli*, *Enterobacter cloacae*, *K. pneumoniae*, *Salmonella* spp, *S. epidermidis*, *Clostridium butyricum*, *C. difficile*, corona virus, enterovirus, and rotavirus (2, 5–10). Although these outbreak reports have generated interest in the role of specific microorganisms in NEC, they may not be representative of "normal" endemic NEC in neonatal nurseries. In our studies here at the University of Maryland, we have found no evidence that a single agent is responsible for all NEC cases or even for isolated clusters of cases (11). We have observed no association between NEC and production of toxins such as the delta hemolysin of *S. epidermidis* (7, 8). We have also failed to find a correlation between disease and the ability of strains to ferment carbohydrate, as measured by β-galactosidase activity (21, 22, 26).

These observations led us to investigate other possible pathogenic mechanisms that might underlie NEC. Bacterial adherence to host cells is a well-studied phenomenon in almost all bacterial mucosal diseases (27–30). The process is frequently complex, and there is a wide range of host cell and bacterial responses that are evoked as a result of bacterial adherence that can culminate in tissue injury (31). This study represents the first effort to assess the possible role of bacterial adherence in the pathogenesis of NEC.

We screened all isolates from NEC case patients and controls for adherence using a Caco-2 cell assay (17). Caco-2 cells are derived from a moderately differentiated human colon adenocarcinoma that differentiates into enterocyte-like cells without any inducers during *in vitro* culture. The differentiation occurs at late confluence when the cells exhibit typical brush-border microvilli and tight junctions. At this polarized stage, the cells express typical small-intestinal enzymes, *e.g.* sucrase-isomaltase, lactase, aminopeptidase, and alkaline phosphatase (18, 19). We have previously described the use of these cells to study the pathogenesis and evaluate virulence of non-O1 *Vibrio cholerae* (20), and this model is now extensively used to study the pathogenesis of several other enteric bacterial species (31, 32). In this study, we were not able to show that isolates from NEC case patients were more likely to be adherent than isolates from control infants. However, when selected strains were evaluated in a weanling rabbit ileal loop model, highly adherent strains (from case patients as well as from controls) produced typical NEC-like injury. Pathologic changes were not seen after infection with Gram-positive organisms. Although they do not explain why NEC occurred in some infants but not in others, these data do suggest that, in the case of *E. coli*, a high level of adherence may be necessary but not sufficient for production of NEC-like injury.

Results became more interesting when we attempted coinfection with Gram-positive and Gram-negative isolates from the same infant. Control Gram-negative isolates that were adherent in pure culture lost their ability to adhere in the Caco-2 model when coinfected with Gram-positive isolates from the homologous infant. With one exception, adherence of Gram-negative isolates from NEC case patients was not affected by coinfection with a homologous Gram-positive isolate; however, when these strains were coinfected with an enterococcal strain isolated from a control child, adherence was lost. These results were mirrored in the weanling rabbit model, with loss of adherence correlating with failure to produce typical NEC-like changes. The mechanisms responsible for these changes in adherence seen in association with coinfection remain to be determined. Among other possibilities, Gram-positive organisms could be saturating the adherence-receptor sites on the epithelial cells for the Gram-negative bacteria, or expression of adherence and virulence factors by the Gram-negative bacteria may be affected by the presence of Gram-positive organisms. Inasmuch as we were concentrating on the effects of adherence and colonization, we did not introduce other variables (substrate, pH, ischemia, temperature, etc.) into our experiments. These factors will need to be taken into consideration in subsequent studies. It may also be noted that our results dealing with protection are most compelling with respect to *E. coli*. Although we have examined other Gram-negative strains in our model systems, there is a need for further investigation with larger numbers of other Gram-negative strains before we can generalize the phenomenon of protection.

NEC is undoubtedly the result of a complex interaction of factors, including gut maturity, prior ischemic injury, inflammatory mediators, and other unidentified factors (2, 33–37). We cannot yet explain the pathophysiologic mechanisms underlying our observations. However, our data suggest that bacterial adherence and patterns of bacterial colonization of the neonatal gut play a pivotal role in the development of NEC. In this context, factors that change or modify the innate pattern of microbial colonization should be given serious consideration in the management of preterm infants. To begin with, infants under aseptic NICU care have very limited exposure to microorganisms. Use of broad-spectrum antibiotics in the management of respiratory and other associated conditions in these group of infants could further damage the existing microflora. Endogenous antimicrobial peptides, such as defensins and cryptdins, have an increased antibacterial property on Gram-positive organisms compared with Gram-negative bacteria (38, 39). Elimination of or decrease in the population of such Gram-positive organisms may have a profound effect in circumstances in which the infant's intestine is colonized with only two to three species of bacteria. Although gaining insight into the multiplicity of these factors will require substantial additional research, our observations now provide a starting point for such investigations and for judicious therapeutic interventions in this devastating disease.

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