INFECTION AND IMMUNITY, Sept. 1987, p. 2268–2273
0019-9567/87/092268-06$02.00/0

Vol. 55, No. 9

Delta-Like Toxin Produced by Coagulase-Negative Staphylococci Is Associated with Neonatal Necrotizing Enterocolitis

DAVID W. SCHEIFELE,[1]* GORDEAN L. BJORNSON,[1] ROGER A. DYER,[1] AND JAMES E. DIMMICK[2]

Division of Infectious Diseases, Department of Pediatrics,[1] and Department of Pathology,[2] British Columbia's Children's Hospital, Vancouver, British Columbia, Canada

Received 29 December 1986/Accepted 27 April 1987

Necrotizing enterocolitis (NEC) is a serious neonatal disorder of uncertain cause, although fecal bacteria have been implicated in some outbreaks. We examined coagulase-negative staphylococci (CONS) as possible etiologic agents. In our unit, CONS colonized the bowels of most infants studied, including 46% of 70 NEC cases (mean concentration, $10^{9.1}$ CFU/g of stool). Over 90% of tested isolates produced a hemolysin resembling delta toxin of *Staphylococcus aureus*. Toxin purified from a NEC-associated isolate of *Staphylococcus epidermidis* resembled reference delta toxin from *S. aureus* in size, biologic properties, and antigenicity. This delta-like toxin was enteropathic, causing mucosal necrosis and hemorrhage in injected loops of the bowels of infant rats. Adjacent, nonexposed bowel remained normal, as did loops injected with lecithin-neutralized toxin. Using a new enzyme-linked immunosorbent assay (ELISA), we detected delta-like toxin in the stools of 11 of 35 infants colonized with CONS positive for delta-like toxin (Tox^+). Positive tests were strongly associated with NEC. Of 18 cases with Tox^+ CONS, 10 were positive (56%), whereas only 1 of 17 control infants so colonized was positive (6%, $P = 0.002$). In NEC patients, the mean fecal toxin concentration was 1,012 ng/g. Toxicity to fibroblasts was demonstrable in filtrates of each of six ELISA-positive samples tested but was absent in all five ELISA-negative samples tested. We conclude that delta-like toxin is elaborated in the bowels of some infants with Tox^+ CONS, and its association with NEC suggests that such CONS are enteropathic. In our unit, this mechanism was apparent in 23% of 44 recent cases of endemic NEC.

Neonatal necrotizing enterocolitis (NEC) is a serious bowel disorder that principally affects infants in intensive-care settings (17, 18). It occurs in endemic and epidemic patterns, with immature infants (birth weight of <1,500 g) affected most often. In our special-care unit, the endemic attack rate for NEC among immature infants has averaged 15% during the past several years, a rate typical of many large nurseries (17, 32).

For neonatologists, NEC is a frustrating enigma. Its cause is uncertain, precluding development of rational preventive and therapeutic measures. Recent reviewers (17, 18) envisioned a multifactorial etiology, with an interplay of vascular insults, gut immaturity, and infectious agents. A role for infection is strongly suggested in outbreaks of NEC (24) but it has been difficult to discern a common mechanism of injury. A few clusters have been associated with high-grade enteropathogens (e.g., salmonella, *Clostridium difficile*) but most have involved seemingly ordinary gut bacteria (e.g., *Klebsiella* and *Enterobacter* spp.) (24).

In our nursery, the species most often found in stools of infants with NEC are coagulase-negative staphylococci (CONS). In other nurseries, such organisms have also been associated with NEC (1, 29), as well as with a milder enterocolitis syndrome (11). A possible mechanism by which CONS might injure the bowel is by elaborating delta-like toxin. Delta toxin is produced in vitro by most isolates of *Staphylococcus aureus* and has been extensively studied (8, 9, 12, 19). It is distinguished from other staphylococcal hemolysins by its heat stability, neutralization by lecithin (14) and normal serum (7, 30), and the pattern of its activity on erythrocytes of various species. Delta toxin can damage a variety of cell types as a result of its detergentlike action on cell membranes (9). It was reported to cause dose-dependent damage to the bowels of guinea pigs (23), leading to speculation that delta toxin is the mediator of staphylococcal membranous enterocolitis (15). Delta-like toxin activity has also been reported in most tested clinical isolates of CONS (10, 16), but these did not include fecal isolates. In this report, we present evidence in support of CONS positive for delta-like toxin (Tox^+) as a cause of neonatal NEC.

MATERIALS AND METHODS

Cultures of stools at NEC onset. We tried to obtain stools from all new cases of NEC occurring in our intensive-care nursery between January 1983 and August 1986. Only patients with compatible clinical signs and pneumatosis intestinalis on abdominal radiographs were considered to have definite NEC (3).

Stools were also obtained from control infants who were at increased risk of NEC because of immaturity (birth weights of <1,500 g) but who experienced no feeding problems while in the nursery. Stool samples were collected during the first 2 weeks of oral feeding, when NEC most often occurs (17). Control infants were recruited randomly during the first 3 months (25 infants) and final 4 months (24 infants) of the study.

Stools were refrigerated immediately after collection and were processed within 24 h in most instances. Initial experiments showed no significant alteration in counts of CONS or coliforms with storage for up to 72 h. Samples were mixed with nutrient glycerol broth in a 1:2 weight-to-volume ratio and vortexed vigorously with sterile glass beads to obtain a uniform suspension. Serial 10-fold dilutions were made in 0.9% saline, and from each of the dilutions from 10^{-2} to 10^{-7}, 100-μl samples were spread evenly over two culture plates, one containing MacConkey agar and the other containing blood agar (Columbia agar base with 5% sheep blood). After January 1986, Columbia CNA agar (Difco

* Corresponding author.

Laboratories, Detroit, Mich.) with 5% sheep blood was added to select for staphylococci. Plates were incubated at 37°C for 24 to 48 h. Each type of colony on each medium was enumerated by, and the number of CFU in the initial sample was calculated from the averaged counts of plates containing 30 to 300 colonies. Each type of colony was subcultured for identification. Staphylococcal species were identified by using API Staph-Ident strips (Analytab Products, Inc., Plainview, N.Y.), and gram-negative species were identified by using API 20E strips (Analytab).

Testing staphylococcal isolates for cytotoxin production. CONS isolates which had been stored at −70°C in skim milk were subcultured in enriched broth (2). After incubation at 37°C for 18 h in a shaking water bath, bacteria were removed from the broth by centrifugation and the supernatant was heated at 100°C for 2 min to inactivate any non-delta hemolysins and then tested for ability to hemolyse washed human type O erythrocytes (25) and, in some instances, rabbit and sheep erythrocytes. Hemolysin-containing broths were retested after incubation for 40 min with 1% lecithin solution or 10% calf or human serum. These solutions alone had no hemolytic activity.

CONS culture supernatants, heated as described above and filter sterilized, were tested for cytotoxicity to human foreskin fibroblasts (10). Cell monolayers were washed free of medium containing fetal bovine serum before challenge. Culture supernatant was diluted serially in minimal essential medium containing penicillin, streptomycin, bacitracin, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and L-glutamine, and each dilution through 1:20 was tested for the ability to kill 100% of the monolayer after incubation at 37°C in 5% CO_2 for 24 h. Fresh broth and broth supernatants from nonhemolytic isolates caused no cytopathic effects.

Identification of the cytotoxin in *Staphylococcus epidermidis*. The cytotoxin detected in our CONS isolates was compared with delta toxin of *S. aureus*. Toxin from a representative NEC-associated isolate of *S. epidermidis* (T7b) (API biotype 3000) was purified to homogeneity by using a combination of the methods of Heatley (12) and Kreger et al. (19), with additional modifications. Bacteria were grown for 18 h at 37°C in enriched broth (2) with enhanced aeration (200 rpm) in an incubator shaker (New Brunswick Scientific, Edison, N.J.). Bacteria were pelleted by centrifugation at 14,000 × *g* for 40 min and then discarded. The supernatant was concentrated approximately 10-fold by using tangential flow filtration (Minitan; Millipore Corp.) across a filter (cutoff, 30,000 molecular weight). Hemolytic activity was measured by endpoint dilution (1 hemolytic unit [HU_{50}] is defined as the amount of activity required to lyse 50% of the blood cell suspension) (25). Hemolytic activity concentrated in the retentate, which was then acidified to pH 5.3 and combined with chloroform to precipitate toxin (12). The precipitate was suspended and further purified by phase shifts in chloroform-methanol, followed by acetone extraction (12). The product was then passed over an hydroxylapatite column (Bio-Rad Laboratories, Richmond, Calif.), a modification of the method of Kreger et al. (19). The elution fractions containing the highest hemolytic activity were pressure dialyzed in an Amicon cell (Amicon Corp., Lexington, Mass.) to concentrate activity and to remove excess salts, by using a filter (cutoff, 500 molecular weight) and intermittent addition of water to the cell.

The purity of the preparation was assessed by polyacrylamide gel electrophoresis by using a 15% gel in a vertical slab apparatus (Bio-Rad). Comparison was made with purified delta toxin prepared from *S. aureus* (25) (kindly donated by W. V. Shaw). Gels were stained with silver (Bio-Rad) for optimal resolution. Toxin preparations were also compared by immunodiffusion (28) by using antiserum prepared in rabbits (see below).

Toxin-induced enteropathy. To determine whether delta-like toxin prepared from *S. epidermidis* was capable of damaging the bowel, we injected toxin into loops of bowels of Wistar rats 2 to 5 days old. Under general anesthesia, the abdomen was opened aseptically by a midline incision and a 2-cm segment of midbowel was identified and tied at each end. A test solution (50 to 100 μl) was injected into this loop, the injection being made medial to a tie so that the puncture site could be isolated between it and an additional ligature, which was tightened as the needle was withdrawn. The bowel was returned to the peritoneal cavity, and the abdominal wall was closed in two layers. Four hours later, animals were sacrificed and the injected loop was identified and removed. The specimen was opened lengthwise and placed immediately in Bouin solution for fixation. An adjacent segment of bowel was removed and fixed as an internal control. Animals that appeared ill were sacrificed before 4 h in order to avoid agonal histopathologic changes (D. W. Scheifele, G. L. Bjornson, and J. E. Dimmick, Can. J. Vet. Res., in press). Specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Coded specimens were submitted to a pathologist (J.E.D.) for interpretation.

Test solutions consisted of 0.9% saline, brain heart infusion broth, crude supernatant from 18-h cultures of *S. epidermidis* (three isolates), partially and fully purified toxin from strain T7b, toxin preparations mixed with 0.2% lecithin, and lecithin solution alone.

Testing stools for delta-like toxin. An enzyme-linked immunosorbent assay (ELISA) was developed to detect delta-like toxin. Antisera were prepared in three rabbits by the method of Heatley (13), except that doses 2 to 6 were decreased by approximately 50%. Purified delta-like toxin from *S. epidermidis* was given with Freund adjuvant, with each dose divided among several injection sites. Animals were sacrificed after 6 weeks. Since serum lipoproteins interact nonimmunologically with delta toxin (30), rabbit sera were depleted of lipoproteins by using colloidal silicic acid (Cab-O-Sil; Eastman Kodak Co., Rochester, N.Y.) (26). Silicic acid was added to serum to a final concentration of 5% (wt/vol). The mixture was tumbled overnight at 4°C and then centrifuged at 10,000 *g* for 4 min. The supernatant was shown to be virtually free of lipoproteins, although up to 60% of antibody content was also lost. Normal sera treated in this manner no longer neutralized delta toxin, and treated immune sera formed only a single precipitate with delta toxin in immunodiffusion gels.

To detect delta-like toxin in stools, we took advantage of the propensity of this toxin to adhere strongly to nonpolar (plastic) surfaces (22). Emulsified stool was diluted 1:50 in coating buffer and clarified by centrifugation at 10,000 × *g* for 4 min at 4°C. Each sample was subsequently processed in triplicate. Clear supernatant was serially diluted in coating buffer, and 0.1-ml samples were added to wells of a microtiter plate (Immulon 1; Dynatech Laboratories, Inc., Alexandria, Va.). Plates were incubated overnight at 4°C to allow delta-like toxin to bind to the plastic. Control preparations included dilutions of stool known to be negative and stool dilutions spiked with delta-like toxin to final concentrations of 1 to 50 ng/ml. Plates were washed five times with washing buffer before the addition of delta antiserum, normal rabbit

serum, and sample buffer without serum. Delta antiserum was found to be optimally active at a 1% concentration. Both this and normal rabbit serum had been previously treated to remove lipoproteins. After 1 h of incubation at 37°C, serum was removed with four rinses of washing buffer. Next, alkaline phosphatase-conjugated goat anti-rabbit antiserum (A-7539; Sigma Chemical Co., St. Louis, Mo.) was added according to the recommendations of the manufacturer, and the plates were incubated at 37°C for 1 h. Finally, plates were again rinsed four times with washing buffer and phosphatase substrate buffer (104; Sigma) was added. The reaction color was read after an appropriate interval by using an ELISA reader (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.). A positive sample was considered one with an average immune serum/control serum reaction ratio of ≥2.0. Only reactions with an optical density of ≥0.1 were used for calculations. The amount of delta-like toxin present in positive samples was estimated from a graphic plot of results of simultaneously tested positive control samples, and an average value was calculated from each of several routinely performed dilutions. With the necessary dilutions, minimum sensitivity of the assay was 50 ng of toxin per g of wet stool.

The ELISA was applied to stools from patients with definite NEC as defined earlier (3) and from control infants (birth weight of <1,500 g) who experienced no feeding difficulties. Control infants in this instance were tested during the final 4 months of the study. Their fecal flora were determined as described above, except that cultures were not limited to the initial 2 weeks of feeding.

Testing for cytotoxicity was done on six ELISA-positive stool samples by using filter-sterilized supernatant (5). Supernatant in minimal essential medium (diluted 1:20 or greater) was added in duplicate to wells containing confluent monolayers of human foreskin fibroblasts, previously washed free of serum-containing medium. Plates were incubated for 24 h at 37°C in 5% CO_2, and the monolayers were examined for damage. A positive test was defined as one showing lysis of ≥50% of the monolayer. Supernatants from five ELISA-negative samples were tested in similar fashion.

RESULTS

Cultures of stools at NEC onset. Stools were obtained at onset of 70 cases, representing approximately 75% of cases that occurred during the study period. The most frequent isolates were CONS, present in 32 infants (46%), and various coliforms, present in 32 infants (46%). In 20 infants (29%), only CONS were identified. Other species, e.g., streptococci (15) and *S. aureus* (2), were encountered infrequently.

The CONS species identified were: *S. epidermidis* (22 isolates, 3 biotypes), *Staphylococcus hominis* (7 isolates, 2 biotypes), untypable (3 isolates), and single isolates of three other species. The geometric mean concentration of CONS isolates in stools was $10^{9.1}$ CFU/g (wet weight). CONS concentrations in stools were similar whether patients had them alone or in combination with other species.

The coliform species identified were *Escherichia coli* (10 isolates, 9 biotypes), *Klebsiella pneumoniae* (6 isolates, 5 biotypes), *Klebsiella oxytoca* (5 isolates, 3 biotypes), *Pseudomonas* sp. (3 isolates, 1 biotype), and others (7 isolates, 7 biotypes). The geometric mean concentration of coliforms in stools was $10^{9.45}$ CFU/g (wet weight).

CONS were detected in stools from 43 (88%) of 49 control infants; the incidence rates were similar during both survey periods. In 28 infants (57%), only CONS were identified in stools. The principal species were *S. epidermidis* (25 isolates), *S. hominis* (9 isolates), and *Staphylococcus saprophyticus* (4 isolates). Seven isolates were not fully speciated or were untypable. The mean concentration of CONS in stools of this group was $10^{8.9}$ CFU/g (wet weight).

TABLE 1. Hemolytic and cytotoxic activities in boiled culture supernatants of CONS associated with NEC

Staphylococcus species	No. of isolates tested	No. (%)	
		Hemolytic	Cytotoxic
S. epidermidis	19	19 (100)	19 (100)
S. hominis	7	6 (86)	6 (86)
S. cohnii	1	1	1
S. capitis	1	0	0
S. haemolyticus	1	1	0

Cytotoxicity of CONS isolates. Hemolytic activity was detected in heated broth supernatants of 27 (93%) of 29 NEC-associated CONS isolates tested (Table 1) and in 26 (90%) of 29 isolates from control infants. In each instance, the hemolysin was neutralized by lecithin. Hemolysin was also neutralized by 10% calf and human serum, but this was not done on all isolates.

Cytotoxicity to human fibroblasts was detected in broth supernatants from 26 (96%) of 27 NEC-associated hemolytic strains. The response of fibroblasts to estimated toxin concentrations of ≥10 HU_{50}/ml consisted of rapid lysis of virtually 100% of the monolayer. With one exception, culture supernatants of hemolytic strains caused lysis of 100% of the monolayer at dilutions of 1:3 or greater, with a mean 100% lysis endpoint dilution of 1:5.

Identification of the CONS cytotoxin. The purification process yielded a solution which contained a single band on silver-stained polyacrylamide gels, the migration of which was identical to the reference preparation of delta toxin from *S. aureus*. The potency of our preparation was 600 HU_{50}/ml or 2.5 $HU_{50}/\mu g$ of protein. Purified toxin had the same biological properties (hemolysis pattern, heat stability, and lecithin and serum neutralization) as the heated crude preparation and reference delta toxin. When our preparation and the reference delta toxin were reacted in an immunodiffusion assay with lipoprotein-depleted antiserum raised against our preparation in rabbits, both formed a precipitate, with a line of identity joining them. No spurs were evident.

Enterotoxicity of *S. epidermidis* delta-like toxin. Fifty animals were used, and they tolerated the test procedure well. At sacrifice, test loops injected with ≥10 HU_{50} of purified delta-like toxin per ml showed lysis and detachment of apical epithelial cells. Concentrations of ≥20 HU_{50}/ml caused severe necrosis of villi with hemorrhage into the bowel loop lumen (Fig. 1). Grossly, these loops were black and friable. Minimal fluid accumulation occurred in toxin-damaged loops. Histopathologic changes in bowel were similar after exposure to crude, semipurified, and pure toxin preparations and were prevented in each instance by the addition of lecithin. Bowel adjacent to test loops or loops injected with saline, sterile culture broth, or lecithin solution were consistently normal.

Detection of delta-like toxin in stools of infants. Stool samples from 44 definite NEC cases were tested by ELISA for delta-like toxin (Table 2). Among 18 babies colonized with toxigenic CONS, the ELISA was positive in 10 initial samples (56%). The concentration of toxin estimated in these samples ranged from 50 to 3,000 ng/g, with an average of 1,012 ng/g. Follow-up samples were available from three

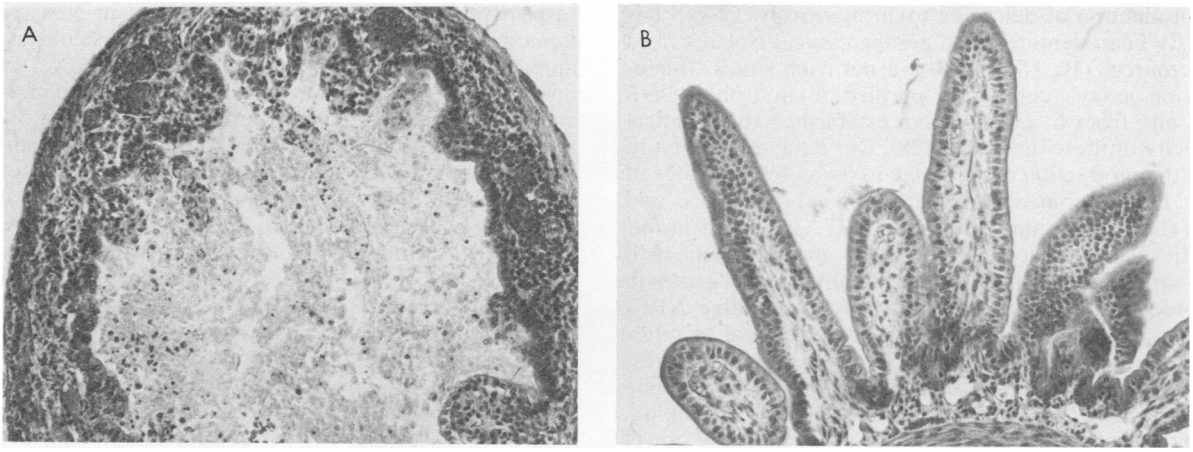

FIG. 1. Extensive mucosal necrosis is evident 4 h after injection of purified delta-like toxin from *S. epidermidis* into a ligated loop of bowel of a 2-day-old Wistar rat (A), although adjacent bowel not exposed to toxin (B) remains normal. Note total destruction of villi and submucosal hemorrhage in the test loop, which imparted a black discoloration to the gross specimen. Approximately 20 HU_{50} of toxin were injected into this loop. Original magnification, ×100.

patients who were initially positive. Each became negative for toxin and CONS during treatment with vancomycin, which is part of the standard therapy for NEC in our unit. Vancomycin therapy appeared also to influence the sensitivity of the test in patients colonized with toxigenic CONS. Of 16 samples obtained within 4 days of treatment onset, 9 (56%) were positive compared with only 1 (17%) of 6 samples obtained later.

Delta-like toxin was seldom found in NEC patients without toxigenic CONS (Table 2). None of 20 samples from the first 4 days of illness and none of the initial samples from this cohort of 26 infants was positive ($P < 0.001$, Fisher's exact test, one-sided). Of 42 samples obtained serially from this group, 2 (5%) were falsely positive. The two corresponding patients had earlier and later stool samples which were negative, and supernatants from broth cultures of the bacteria present in the positive samples were nonreactive in the ELISA. The amount of activity was 50 ng/g in each instance.

Delta-like toxin was also found uncommonly in stools from premature infants without feeding problems (Table 2). No toxin was found in stools from 15 infants who either lacked CONS (13 samples) or had toxin-negative CONS (21 samples), and only one test was positive from 17 infants with Tox^+ CONS in stools.

Overall, the ELISA was positive in only 2 of 86 samples from 41 patients without detectable Tox^+ CONS, a specificity (recognition of true negatives) of 98%. Among those with Tox^+ CONS, the detection of free toxin differed between normals (1 of 17 positive, 6%) and those with NEC (10 of 18 positive, 56%) ($P = 0.002$, Fisher's exact test, one-sided). Among five Tox^+ CONS-colonized infants with suspected NEC (radiographs nonconfirmatory), three had toxin detected in stools (60%).

Each of six ELISA-positive stool samples tested for cytotoxicity to fibroblasts produced a positive test at a dilution of 1:20. The ELISA-negative samples tested were not toxic to fibroblasts.

TABLE 2. Detection rate of delta-like toxin in stools by ELISA

Clinical condition (no. of patients)	Stools with Tox^+ CONS		Stools without Tox^+ CONS		*P* value, Fisher's exact test
	No.	No. ELISA positive (%)	No.	No. ELISA positive	
NEC (initial sample) (44)	18	10 (56)[a]	26	0	<0.001
Feeding well (32)	17	1 (6)[a]	15	0	NS[b]
All (76)	35	11 (31)	41	0	<0.001

[a] $P = 0.002$.
[b] NS, Not significant.

DISCUSSION

Our observations indicate that CONS were frequently present in stools of infants in our intensive-care nursery. Among cases of NEC, CONS were the aerobes most often detected in stools and were often present alone. CONS were also prominent in stools of premature infants without bowel symptoms during the first 2 weeks after the initiation of oral feedings, the time when NEC most often develops (17). In both groups, the density of colonization with CONS was great, averaging 10^9 CFU/g of stool. In surveys of healthy term infants during the first weeks of life, CONS were reported in stools infrequently (6), with colonization density seldom exceeding 10^6 CFU/g (31).

The overgrowth of CONS noted in our patients is consistent with observations from other nurseries (4, 20, 29) where the fecal flora of neonates in intensive care was seen to develop abnormally, with initial colonizers growing to great numbers for lack of interspecies competition. Early colonizers often included species not normally prominent in stools of healthy infants, reflecting the greater risk to premature babies of acquiring organisms from the hospital environment. The source of CONS in our patients was not studied, but many were multiply resistant to antibiotics, suggesting nosocomial origin.

Lawrence et al. (20) were the first to suggest that overgrowth of CONS in the bowels of neonates might cause NEC. They fed *S. epidermidis* isolated from stools of a baby with NEC to germfree infant rats and observed the development of bloody diarrhea after several days. These animals had necrosis of the mucosa of the distal bowel. A mechanism of injury was not determined. We have found that their isolate (kindly provided by G. Lawrence) plus most of our nursery CONS isolates produce a hemolysin or cytotoxin with unusual properties, consistent with delta toxin of *S. aureus* (9). Hemolytic activity was present in most isolates from NEC cases, as well as from control infants.

The production of delta-like toxin in vitro by CONS has previously been demonstrated among clinical isolates from various sources (10, 16, 21, 28) but not from stools. Immunodiffusion assays comparing purified toxin from CONS isolates and from *S. aureus* have established the identical antigenicity of these molecules (28). Our data are the first to confirm the production of delta-like toxin by fecal isolates of CONS. The combined properties of heat resistance and lecithin (14) and serum neutralization (7, 30) found in our hemolytic or cytotoxic CONS isolates are consistent with delta toxin and are unlike any other staphylococcal exotoxin (9). Moreover, toxin purified from a representative NEC-associated isolate of *S. epidermidis* was identical to reference delta toxin from *S. aureus* in size, biologic properties, and antigenicity. Since the toxins might differ in more subtle ways, we refer to the CONS product as a delta-like toxin.

The potential for CONS delta-like toxin to cause bowel injury is clearly indicated by the animal inoculation experiments. Introduction of delta-like toxin into ligated loops of the midbowels of infant rats caused cell damage in a dose-dependent fashion, comparable with the effects of delta toxin of *S. aureus* on the bowels of guinea pigs (23). A concentration of delta-like toxin sufficient to lyse 100% of a fibroblast monolayer also damaged the bowel. Such concentrations were produced by most toxigenic isolates after overnight culture in broth. With a toxin dose of 10 HU_{50}/ml, lysis of apical mucosal cells occurred in test loops, and with doses of $\geq$20 HU_{50}/ml, necrosis of whole villi was evident, with intralumenal accumulation of blood and cell debris (Fig. 1). These histopathologic changes resemble those found in NEC (27). The addition of lecithin, an inhibitor of delta-like toxin, abolished the enterotoxicity of CONS culture broths. This unusual property suggests that delta-like toxin was the mediator of injury, but we could not rule out the existence of another heat-resistant, lecithin-inhibited molecule by using antiserum to neutralize delta-like toxin because our antisera are nonneutralizing. No such molecule has been described in staphylococci (9) however.

Using our newly developed ELISA for delta-like toxin, we were able to confirm that this toxin is released by CONS in the stools of some colonized infants. To our knowledge, this is the first evidence of elaboration of delta-like toxin in vivo. Our assay is specific, for positive tests were obtained in only 2 of 86 samples that contained nontoxigenic CONS or other species. The ELISA does not determine the functional status of delta-like toxin, but filtrates from each of six positive stools tested on fibroblast monolayers caused cytolysis typical of delta toxin, suggesting that the toxin detected was intact and functional.

Among infants who were colonized by Tox^+ CONS, only 11 of 35 tested had delta-like toxin that was detectable in stools (Table 2). The basis for this variability in toxin expression remains to be explored. It has been demonstrated previously that toxin production in vitro varies considerably between species and among isolates of a given species, with *S. epidermidis* being the most active (10). We attempted to titrate hemolytic activity in culture broths of our isolates but found that the results were too variable for meaningful analysis. Redox potential within the lower bowel and residual dietary lecithin might also influence toxin expression and activity.

Importantly, among infants colonized by Tox^+ CONS, toxin was more often detected in stools of infants who had NEC (56%) or suspected NEC (60%) than in those feeding normally (6%; $P = 0.002$). Toxin concentrations were higher in NEC patients than in controls (means, 1,012 and 50 ng/g, respectively) and fell sharply to negative in NEC patients treated intravenously with vancomycin. These observations implicate Tox^+ CONS in the pathogenesis of bowel damage, but our evidence that delta-like toxin is the mediator is not complete, for lack of a delta-neutralizing antiserum. With the six ELISA-positive, cytotoxic stool samples from NEC patients mentioned above, we were unable to specifically neutralize the cytotoxin present by using antiserum (not available) or lecithin (toxic to fibroblasts). Thus, it remains possible that some other substance produced by Tox^+ strains caused tissue damage. An alternative possibility is that delta-like toxin is more readily expressed in the milieu of an already damaged gut, but this is unlikely because toxin was readily detected at the onset of NEC.

The full spectrum of bowel injury attributable to Tox^+ CONS has not been determined in this study. We examined only NEC cases and normal control infants and excluded those with other bowel symptoms. It is conceivable that such isolates contribute to the CONS-associated enterocolitis syndrome of Gruskay et al. (11) and to the feeding intolerance that plagues many immature infants. In the NEC patients we tested, delta-like toxin was detected at onset in 23% (10 of 44). The contribution of Tox^+ CONS to NEC might have been underestimated by our assay. If every Tox^+ CONS present in NEC patients caused injury, this mechanism could account for as much as 41% of cases in our study. In either event, the apparent contribution of this mechanism to endemic NEC in our unit is considerable. A similar situation might exist in other units where bowel colonization with CONS is prominent among babies at risk.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council of Canada and the B.C. Medical Services Foundation.

We gratefully acknowledge the help of Elizabeth Hancock with the ELISA, Marty Puterman with statistical analyses, and Shannon M. da Silva with manuscript preparation.

LITERATURE CITED

1. **Anderson, C. L., M. F. Collin, J. P. O'Keefe, M. Challapalli, T. F. Myers, C. C. Caldwell, and G. S. Ahmed.** 1984. A widespread epidemic of mild necrotizing enterocolitis of unknown cause. Am. J. Dis. Child. **138**:979–983.
2. **Arvidson, S., T. Holme, and T. Wadstrom.** 1971. Influence of cultivation conditions on the production of extracellular proteins by *Straphylococcus aureus*. Acta Pathol. Microbiol. Scand. Sect. B **79**:399–405.
3. **Bell, M. J., J. L. Ternberg, R. D. Feigin, J. P. Keating, R. Marshall, L. Barton, and T. Brotherton.** 1978. Neonatal necrotizing enterocolitis—therapeutic decisions based upon clinical staging. Ann. Surg. **187**:1–7.
4. **Blakey, J. L., L. Lubitz, G. L. Barnes, R. F. Bishop, N. T. Campbell, and G. L. Gillam.** 1982. Development of gut colonization in pre-term neonates. J. Med. Microbiol. **15**:519–529.
5. **Chang, T.-W., M. Lauermann, and J. G. Bartlett.** 1979. Cytotoxicity assay in antibiotic-associated colitis. J. Infect. Dis. **140**: 765–770.
6. **Cooperstock, M. S., and A. J. Zedd.** 1983. Intestinal flora of infants, p. 79–99. *In* D. Hentges (ed.), Human intestinal microflora in health and disease. Academic Press, Inc., New York.
7. **Donahue, J. A.** 1969. Antistaphylococcal hemolysins and delta hemolysin inhibitor in adult human serum. Can. J. Microbiol. **15**:957–959.
8. **Fitton, S. E., A. Dell, and W. V. Shaw.** 1980. The amino acid sequence of the delta hemolysin of *Staphylococcus aureus*. FEBS Lett. **115**:209–212.
9. **Freer, J. H., and J. P. Arbuthnott.** 1983. Toxins of *Staphylococcus aureus*. Pharmacol. Ther. **19**:55–106.

10. **Gemmell, C. G., and M. Thelestam.** 1981. Toxinogenicity of clinical isolates of coagulase-negative staphylococci toward various animal cells. Acta Pathol. Microbiol. Scand. Sect. B **89**:417–421.
11. **Gruskay, J. A., S. Abbasi, E. Anday, S. Baumgart, and J. Gerdes.** 1986. *Staphylococcus epidermidis*-associated enterocolitis. J. Pediatr. **109**:520–524.
12. **Heatley, N. G.** 1971. A new method for the preparation and some properties of staphylococcal delta-haemolysin. J. Gen. Microbiol. **69**:269–278.
13. **Heatley, N. G.** 1977. Production of antibody to staphylococcal delta-haemolysin in the rabbit. Br. J. Exp. Pathol. **58**:400–411.
14. **Kapral, F. A.** 1972. Inhibition of *Staphylococcus aureus* delta hemolysin by phospholipids. Proc. Soc. Exp. Biol. Med. Proc. **141**:519–521.
15. **Kapral, F. A.** 1985. *Staphylococcus aureus* delta toxin as an enterotoxin. CIBA Found. Symp. **112**:215–229.
16. **Kleck, J. L., and J. A. Donahue.** 1968. Production of thermostable hemolysin by cultures of *Staphylococcus epidermidis*. J. Infect. Dis. **118**:317–323.
17. **Kliegman, R. M., and A. A. Fanaroff.** 1984. Necrotizing enterocolitis. N. Engl. J. Med. **310**:1093–1103.
18. **Kosloske, A. M.** 1984. Pathogenesis and prevention of necrotizing enterocolitis: a hypothesis based on personal observation and a review of the literature. Pediatrics **74**:1086–1092.
19. **Kreger, A. S., K.-S. Kim, F. Zaboretzky, and A. W. Bernheimer.** 1971. Purification and properties of staphylococcal delta hemolysin. Infect. Immun. **3**:449–465.
20. **Lawrence, G., J. Bates, and A. Gaul.** 1982. Pathogenesisis of neonatal necrotizing enterocolitis. Lancet **i**:137–139.
21. **Mahajan, V. M., T. N. Reddy, and L. P. Agarwal.** 1982. Toxigenic strains of *Staphylococcus epidermidis* and their experimental corneal pathogenicity in rabbits. Int. Ophthalmol. **5**:155–161.
22. **Nolte, F. S., and F. A. Kapral.** 1981. Hydrophobic interaction chromatography of *Staphylococcus aureus* delta-toxin. Infect. Immun. **31**:1094–1098.
23. **O'Brien, A. D., and F. A. Kapral.** 1976. Increased cyclic adenosine 3′,5′-monophosphate content in guinea pig ileum after exposure to *S. aureus* delta-toxin. Infect. Immun. **13**: 152–162.
24. **Rotbart, H., and M. Levin.** 1983. How contagious is necrotizing enterocolitis? Pediatr. Infect. Dis. **2**:406–413.
25. **Smith, G., and W. Shaw.** 1981. Comparison of three methods for purification of the delta haemolysin of *Staphylococcus aureus*. J. Gen. Microbiol. **124**:365–374.
26. **Stephan, V. W., and L. Roka.** 1968. Adsorption von lipoproteinden. Z. Klin. Chem. Klin. Biochem. **6**:186–190.
27. **Swanson, V. L., and B. H. Landing.** 1980. Pathology, p. 129–141. *In* E. G. Brown and A. Y. Sweet (ed.), Neonatal necrotizing enterocolitis. Grune & Stratton, Inc., New York.
28. **Turner, W. H., and D. J. Pickard.** 1979. Immunological relationship between delta-hemolysins of *Staphylococcus aureus* and coagulase-negative strains of staphylococci. Infect. Immun. **23**:910–911.
29. **Westra-Meijer, C. M., J. E. Degener, G. Dzoljec-Danilovic, M. Michel, and J. W. Mettau.** 1983. Quantitative study of the aerobic and anaerobic faecal flora in neonatal necrotizing enterocolitis. Arch. Dis. Child. **58**:523–528.
30. **Whitelaw, D. D., and T. H. Birkbeck.** 1978. Inhibition of staphylococcal delta-haemolysin by human serum lipoproteins. FEMS Microbiol. Lett. **3**:335–338.
31. **Yoshioka, H., K. Iseki, and K. Fujita.** 1983. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics **72**:317–321.
32. **Yu, V. Y., R. Joseph, B. Bajuk, A. Orgill, and J. Astbury.** 1984. Necrotizing enterocolitis in very low birthweight infants: a four year experience. Aust. Paediatr. J. **20**:29–33.