Delta Toxin Activity in Coagulase-Negative Staphylococci from the Bowels of Neonates

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Coagulase-negative staphylococci are prominent in stools of neonates in some intensive care units and have been associated with necrotizing enterocolitis. A plausible mediator of bowel damage is delta-like toxin, which is produced in vitro by most coagulase-negative staphylococci, but factors influencing the expression of toxin in the bowel are unknown. We examined 105 coagulase-negative staphylococcus isolates from stools of neonates by using an enzyme-linked immunosorbent assay and detected delta toxin production by 92 isolates (88%). The amount present in 18-h broth cultures varied over 100-fold, from 933 to 125,000 ng/ml. All broths positive by enzyme-linked immunosorbent assay except one caused hemolysis of human erythrocytes. The threshold concentration for consistent cytotoxicity to fibroblasts was ≥24,000 ng/ml. Only 56% of Staphylococcus epidermidis isolates were capable of producing this much toxin, and these were more often obtained from premature infants in intensive care than from healthy full-term infants ($P = 0.003$) and were more often resistant to multiple antibiotics ($P < 0.001$). Cultures grown anaerobically seldom caused hemolysis (4 positive of 29 tested; $P < 0.001$) because potency of the toxin was decreased (at least ninefold for S. epidermidis isolates).

We conclude that only a portion of the fecal coagulase-negative staphylococci tested produced enough delta toxin in vitro to be cytotoxic, that such isolates have accumulated in our intensive care nursery, and that development of toxin-mediated bowel injury may also require a favorable redox potential within the host bowel.

Coagulase-negative staphylococci frequently colonize neonates in intensive care units and are emerging as important opportunistic pathogens in this setting (6, 12, 22, 23). We recently reported (26) evidence indicating that coagulase-negative staphylococci might be capable of causing necrotizing enterocolitis (NEC) in neonates.

Neonatal NEC is a serious bowel disorder that principally affects premature infants in intensive care settings. Its cause is uncertain and may be multifactorial, involving an interplay of gut immaturity, vascular stresses, and infectious agents (18). A role for the latter is strongly suggested when cases occur in epidemics (25), but it has been difficult to discern a common mechanism of injury. A few clusters were associated with high-grade enteropathogens, but most have involved seemingly ordinary gut bacteria. Lawrence et al. (20) postulated that it is the tendency for infants in intensive care to suffer overgrowth of initial bowel flora that predisposes them to NEC. Should these initial colonizers produce toxins capable of damaging the bowel, their spontaneous overgrowth in the bowel would predispose to development of disease. This interesting theory is supported by our recent observations with coagulase-negative staphylococci.

In our unit, coagulase-negative staphylococci colonize the bowels of most infants during the initial weeks of feeding and are often present without other aerobes. The density of colonization with coagulase-negative staphylococci during this time averaged $10^5$ CFU/g of stool, far in excess of concentrations reported in healthy full-term infants (30). We found that most fecal coagulase-negative staphylococcus isolates from our unit produced a delta-like toxin in vitro (26). Originally described in Staphylococcus aureus, delta toxin (9, 24) is an exoprotein with detergentlike action on membranes of many cell types (14), resulting in rapid cell lysis. Kapral (16) presented evidence for enteropathogenic effects of delta toxin and speculated that the toxin might be the mediator of membranous enterocolitis caused by S. aureus. A comparable toxin was described by Kleck and Donahue (17) and Gemmel and Thelestam (11) in clinical isolates of coagulase-negative staphylococci. This was identical to the S. aureus toxin by immunodiffusion (28). We showed (26) that delta-like toxin from Staphylococcus epidermidis caused severe necrosis when injected into loops of bowel of infant rats, confirming its potential enterotoxicity. Using a newly developed enzyme-linked immunosorbent assay (ELISA), we demonstrated (26) the presence of delta-like toxin in stools of 23% of recent cases of NEC (representing 56% of coagulase negative staphylococcus-colonized cases) but rarely detected toxin in infants without bowel dysfunction, even in those colonized with toxigenic coagulase-negative staphylococci ($P = 0.002$).

Taken together, these observations suggest that overgrowth in the bowel of toxigenic coagulase-negative staphylococci places infants at risk of developing NEC should toxin be elaborated in sufficient amounts. Factors influencing the expression of delta toxin in the bowel remain to be delineated, but this would appear to be an important issue, as only a minority of coagulase-negative staphylococcus-colonized infants in our unit develop NEC.

We undertook this study to learn more about the phenomenon of delta toxin production by fecal isolates of coagulase-negative staphylococci in vitro, using our ELISA to make quantitative observations. We were particularly interested in identifying factors that might have a major influence on toxin production in vivo.

MATERIALS AND METHODS

Coagulase-negative staphylococci were obtained from stools of (i) randomly chosen, healthy neonates in a maternity hospital; (ii) premature infants with definite necrotizing enterocolitis (7); and (iii) other infants in the same intensive care nursery; (ii) premature infants with definite necrotizing enterocolitis (7); and (iii) other infants in the same intensive care nursery; (iv) and (v) from healthy full-term infants (6, 12, 22, 23).
care unit matched with the latter for age and weight but without bowel dysfunction. Isolates able to grow on Columbia CNA agar (Difco Laboratories, Detroit, Mich.) with 5% sheep blood were identified by using the API Staph-Ident system (1, 8) (Analytab Products, Plainview, N.Y.), supplemented by Gram stains and tests for catalase and novobiocin susceptibility (1) and growth on glycerol-supplemented purple agar (Difco) (4) and bile esculin agar (Difco). Coagulase-negative staphylococci were stored at −70°C in skim milk and were subcultured on brain heart infusion agar (Difco) prior to being tested for delta toxin production. For aerobic cultures, 10⁶ organisms was added to 12 ml of enriched broth (3) in a 250-ml flat-bottom flask and incubated in a rotary shaker (200 rpm) at 37°C for 18 h. For anaerobic cultures, 8 ml of broth contained in a 10-ml capped plastic tube (Falcon; Becton Dickinson Labware, Oxnard, Calif.) was used instead, with incubation continued for 80 h at 37°C in an anaerobic jar (GasPak; BBL Microbiology Systems, Cockeysville, Md.). The optical density of completed cultures was measured at a wavelength of 620 nm, and the concentration of organisms was estimated from a previously constructed correlation curve. Bacteria were removed by centrifugation, and the supernatant was heated at 100°C for 2 min to inactivate any non-delta hemolysins (9).

Delta toxin was detected and quantitated by using an ELISA assay as previously described (26), except Immulon 2 plates (Dynatech Laboratories, Inc., Alexandria, Va.) were used. Hemolytic activity was assessed as described by Smith and Shaw (27), by using washed human type O erythrocytes. A single lot of blood was used from a donor who maintained a low-fat diet for the preceding 3 days. Hemolytic activity was considered present if samples caused ≥10% hemolysis at a 1:4 dilution during a 30-min incubation at 37°C. Cytotoxicity of culture broths was assessed on monolayers of human foreskin fibroblasts as described previously (26).

Antibiotic susceptibility of isolates was tested by the Kirby-Bauer disk diffusion method (5) by using Mueller-Hinton agar medium (Difco).

The statistical tests used to compare proportions were the chi-square test, with the Yates correction for continuity, and Fisher’s exact test, one sided.

RESULTS

Fecal isolates from 105 infants were identified as coagulase-negative staphylococci. Under aerobic growth conditions, in vitro production of delta-like toxin was detected in 92 isolates (88%) by ELISA. Of 73 isolates of *S. epidermidis*, 67 produced toxin (92%), as did 23 of 24 isolates of *Staphylococcus hominis* (96%). Among eight remaining isolates, which belonged to three other species, toxin production was detected in three (38%).

The amount of delta toxin produced under aerobic conditions varied greatly among isolates, even though all reached estimated climax populations of 2 × 10⁹ to 5 × 10⁹ CFU/ml. With *S. epidermidis*, toxin concentrations ranged from 933 to 100,388 ng/ml (mean, 31,830 ng/ml) and with *S. hominis*, the concentrations ranged from 1,600 to 125,000 ng/ml (mean, 23,458 ng/ml).

All ELISA-positive culture broths except one caused hemolysis of human erythrocytes. The observed threshold for detectable hemolysis (≥10% of the test suspension) was ≥933 ng of toxin per ml for *S. epidermidis* and ≤1,600 ng/ml for *S. hominis*. The non-hemolytic isolate was a *S. hominis* strain that produced 5,000 ng of toxin per ml by ELISA. In an immunodiffusion assay using lipoprotein-depleted rabbit anti-delta serum (26), culture broth from this isolate formed a precipitate which aligned with that of purified delta toxin, suggesting the presence of an inactive toxin. A single ELISA-negative sample caused hemolysis, likely as a result of incomplete heat-inactivation of the nondelta hemolysin of *Staphylococcus hemolyticus*.

A total of 24 culture broths of *S. epidermidis* were tested for toxicity to fibroblasts. Cytotoxicity was observed only with delta toxin concentrations exceeding 16,000 ng/ml, with a consistent effect (≥50% lysis of the monolayer with each sample) requiring ≥24,000 ng/ml. A similar threshold was observed with toxin produced by *S. hominis*.

*S. epidermidis* isolates capable of producing delta toxin in cytotoxic amounts (≥24,000 ng/ml) were more often recovered from patients in the intensive care nursery (ICN) (36 of 54 isolates; 67%) than from patients in the normal nursery (5 of 19 isolates; 26%) (*P* = 0.003). No difference was noted in the frequency of cytotoxic strains from babies with NEC and their matched controls in ICN for either *S. epidermidis* or *S. hominis*. There were too few *S. hominis* isolates from full-term babies to permit comparison. It is noteworthy that almost all coagulase-negative staphylococci isolates (14 of 15; 93%) producing concentrations of delta toxin in excess of 50,000 ng/ml came from ICN (*P* = 0.04).

Antibiotic susceptibility testing was limited to *S. epidermidis* isolates, including all those available from full-term infants and representative samples of ICN isolates with high-level (≥24,000 ng/ml) and low-level delta toxin production in vitro. As summarized in Table 1, high-level delta toxin producers from ICN infants were more often resistant to erythromycin, gentamicin, and oxacillin (20 of 25 isolates; 80%) than were isolates from term infants (1 of 19 isolates; 5%) (*P*² = 21.4; *P* < 0.001). Regardless of the source, high-level delta toxin producers were more likely than low-level producers to be resistant to erythromycin (*P* = 0.004), gentamicin (*P* < 0.001), or oxacillin (*P* < 0.001).

Coagulase-negative staphylococcus isolates were unable to grow to the same density under anaerobic conditions and produced less delta toxin. The mean toxin concentration in 90 broths in which it was detectable was 2,553 ng/ml (range, 105 to 10,625 ng/ml). However, when toxin concentration was related to a standardized cell mass (10⁶ CFU), no difference existed between aerobic and anaerobic cultures. The toxin produced under anaerobic conditions was less active; only 4 supernatants of 29 tested caused hemolysis.

### Table 1. Relationship between antimicrobial susceptibility and capacity to produce delta toxin in vitro among fecal isolates of *S. epidermidis* from neonates

<table>
<thead>
<tr>
<th>Isolate source and delta toxin production</th>
<th>No. of isolates</th>
<th>Erythromycin</th>
<th>Gentamicin</th>
<th>Oxacillin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Full-term infants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High¹</td>
<td>5</td>
<td>5 (100)</td>
<td>1 (20)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Low</td>
<td>14</td>
<td>5 (36)</td>
<td>0</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>10 (53)</td>
<td>1 (5%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td><strong>ICN infants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High¹</td>
<td>25</td>
<td>23 (92)</td>
<td>24 (96)</td>
<td>23 (92)</td>
</tr>
<tr>
<td>Low</td>
<td>15</td>
<td>13 (87)</td>
<td>11 (73)</td>
<td>7 (47)</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>36 (90)</td>
<td>35 (88)</td>
<td>30 (75)</td>
</tr>
</tbody>
</table>

¹ High, ≥24,000 ng/ml.  
² *P* = 0.004.  
³ *P* < 0.001.  
⁴ *P* < 0.001.
despite toxin concentrations ranging from 2,550 to 10,625 ng/ml ($r^2 = 40.6; P < 0.001$). Such concentrations invariably caused hemolysis when toxin was produced under aerobic conditions. A threshold for consistent hemolytic activity was not evident within the range of toxin concentrations observed under anaerobic conditions. For S. epidermidis, the anaerobic product was at least ninefold less potent as a hemolysin.

**DISCUSSION**

Our study differs from previous characterizations of delta toxin activity in staphylococci because we did not rely on hemolysis assays, preferring instead an ELISA method, the accuracy of which we documented previously (26). We found that the hemolysis assay for delta toxin was unreliable. Regular inclusion of purified delta toxin as a positive control revealed substantial variation in susceptibility of donor type O erythrocytes (data not shown), perhaps resulting from variations in the dietary fat intake of donors (29). Occasional blood samples were refractory to toxin, and most contained a subpopulation of refractory cells. Such variations were not controlled in previous studies of delta hemolysin in coagulase-negative staphylococci (11, 17, 19, 21). Nevertheless, when we used a single batch of susceptible erythrocytes to test all of our isolates for delta-like hemolysin, activity was detectable ($\pm 10\%$ hemolysis) in all ELISA-positive samples, even with toxin concentrations as low as 933 ng/ml.

We demonstrated that most fecal coagulase-negative staphylococci tested had the capacity to produce delta toxin, but the amount produced under aerobic conditions in vitro differed over 100-fold among isolates. Comparable variation in toxin activity has been reported in coagulase-negative staphylococci from other sources (11, 17). A clinically significant amount of toxin might be that required to lyse fibroblast monolayers, because we observed previously that cytotoxic culture broths damaged intestinal epithelium in a rat ileal loop model but noncytotoxic broths did not (26). The observed toxin concentration above which all samples caused cytotoxicity ($\pm 50\%$ lysis of the monolayer) was 24,000 ng/ml. Such samples represented 47% of isolates in our series.

We found that S. epidermidis isolates capable of producing cytotoxic amounts of delta toxin were more often identified among isolates from premature infants requiring intensive care than among those from healthy full-term infants ($P = 0.003$). Other species were not present sufficiently often in both groups to permit comparison. Virtually all of the S. epidermidis and S. hominis isolates with the highest activities ($\geq 50,000$ ng/ml) came from ICN ($P = 0.04$). Isolates producing potentially cytotoxic amounts of delta toxin were more likely than noncytotoxic isolates to be resistant to one or more of oxacillin, gentamicin, and erythromycin. We were aware that multiply resistant coagulase-negative staphylococci had accumulated in our ICN, but the coexistence of the high-level toxinogenesis phenotype among them was unanticipated. The genetic basis for delta toxin activity in coagulase-negative staphylococci is unknown, but it is possible that high-level toxinogenesis is facilitated in strains containing R plasmids, as one expects to find in coagulase-negative staphylococci resistant to the above antibiotics (2). The existence of any direct association between high-level toxinogenesis and multiple antibiotic resistance will be important to determine, because if it exists, coagulase-negative staphylococcus isolates selected by antibiotic utilization in an ICN would be more enteropathic.

Our observation that coagulase-negative staphylococci produce an inactive hemolysin under anaerobic conditions is novel and would not have been made without the ELISA. Working only with a hemolysis assay, Kleck and Donahue (17) concluded that coagulase-negative staphylococci do not produce hemolysin under anaerobic growth conditions. In fact, molecules detected by the ELISA are produced equally well under aerobic and anaerobic conditions if one relates toxin concentration to the cell mass achieved. However, the anaerobically produced toxin molecule is much less active as a hemolysin ($P < 0.001$) and is unlikely to be cytotoxic. A structural basis for altered toxin activity remains to be defined. A possible explanation is that the anaerobic product fails to aggregate into the multimeric form of the toxin which is necessary for it to insert into and damage cell membranes (10).

Our study has identified two factors that might be important in determining which infants will develop bowel injury from toxigenic coagulase-negative staphylococci. Clearly, not all isolates have the same capacity to produce toxin in vitro. Only half of our toxigenic isolates produced toxin in amounts that are potentially cytotoxic and enteropathic. Toxinogenesis in vivo might also be influenced by diet, as it is clearly influenced in vitro by constituents of the growth medium (3). A potentially significant dietary factor is lecithin. This is commonly used in infant formulas as an emulsifier and is a potent neutralizer of delta toxin in vitro (15). We have detected a toxin neutralizing capacity in many liquid formulas (unpublished data).

The detrimental effect of anaerobic culture conditions on the potency of delta toxin suggests that the redox potential of the bowel lumen of the host may be the ultimate determinant of injury in patients colonized with toxigenic coagulase-negative staphylococci. Grutter et al. (13) reported a mean fecal $E_h$ in infants on days 0 to 1 of $+175$ mV, changing to $-113$ mV by day 2. In those fed only breast milk, $E_h$ remained positive ($+106 \pm 47$ mV). It is conceivable that babies who are heavily colonized with coagulase-negative staphylococci, either alone or with a limited diversity of companion species, might retain sufficiently aerobic conditions in their lower bowel to allow the formation of an active toxin. In six toxin-positive NEC cases previously reported (26), we found that the corresponding stool filtrates damaged fibroblasts in a manner consistent with delta toxin, suggesting that conditions do exist in the bowels of some infants to permit the formation of an active delta toxin. Subsequent maturation of the intestinal microflora with reduction of the redox potential may end the risk of delta toxin-mediated injury.

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**LITERATURE CITED**


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