Quantitative changes in faecal microflora preceding necrotising enterocolitis in premature neonates

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Abstract

Quantitative studies of faecal bacterial flora were carried out during the week preceding the clinical onset of 12 episodes of neonatal necrotising enterocolitis. There were considerable quantitative changes in the faecal flora preceding the clinical onset of both definite and possible episodes of necrotising enterocolitis. There was a decline in the numbers of some species from up to 72 hours before the clinical onset of the disease. Enterobacteriaceae were isolated from samples collected during the 48 hours preceding the clinical onset of all four definite episodes of necrotising enterocolitis. These were 'new' isolates in two episodes, and considerably increased numbers in another.

The changes that we found are probably the result of changes in intraluminal conditions that precede the clinical onset of necrotising enterocolitis.

The aetiology of neonatal necrotising enterocolitis remains unknown; its incidence rises with increasing prematurity and may reach over 13% in very low birthweight infants.¹ There may be temporal and geographical clustering of cases, suggesting that a transmissible agent may play a part.² Previous studies of faecal flora associated with neonatal necrotising enterocolitis have concentrated on the speciation of the microbial flora present at the onset of clinical signs of disease. *Clostridium* spp.,³⁻⁴ enterobacteriaceae,⁵⁻⁷ and—most recently—coagulase negative staphylococci⁸ have been associated with the disease. Studies of faecal α_1 -antitrypsin⁹ and breath hydrogen concentration,¹⁰ however, together with histological evidence (W Ballance, B Dahir, N Sheuher, R Kliegman. Proceedings of a Symposium 'Neonatal necrotising enterocolitis: current concepts and controversies'. Columbus, USA, 1989) suggest that the pathological process leading to necrotising enterocolitis may precede the onset of clinical signs of disease by hours or even days. The bacterial species present at the clinical onset therefore may not be representative of those present at the onset of the pathological process.

We describe quantitative changes in faecal microflora in the weeks preceding the onset of 12 episodes of neonatal necrotising enterocolitis.

Materials and methods

We studied the faecal flora of premature neonates with episodes of illness associated with specific signs of gastrointestinal dysfunction

including abdominal distension, blood in the stools, gastric retention, abdominal wall erythema, and abnormal abdominal radiographs. Radiological abnormalities were evidence of ileus, thickening of the bowel wall, or 'rigid' loops of bowel. Episodes were divided into those associated with intramural or intrahepatic gas (definite) and those without such gas (possible).

SAMPLE COLLECTION AND STORAGE

Sample of faeces (when available) were collected by nursing staff each day from all neonates of less than 32 weeks' gestational age who had been admitted to the Peter Congdon Regional Neonatal Unit with respiratory distress syndrome between February and September 1989. Approximately 0.2 g of faeces was inoculated into a phial containing 1.8 ml of prereduced glycerol citrate broth¹¹ in an anaerobic cabinet (Wise Anaerobic Work Station, Don Whitley, Shipley). The weight of the sample was determined by subtracting the known weight of the phial from the weight after the addition of the sample. Phials containing faecal samples were stored at -70°C. Dry weight as a percentage of wet weight was determined after drying a weighed sample in a hot air oven for 24 hours.

DETERMINATION OF QUANTITATIVE BACTERIOLOGY

Quantitative bacteriology was carried out within eight weeks of storage of the samples at -70°C. Samples were thawed at room temperature and 100 μ l of the contents of the phial were serially diluted tenfold in brain heart infusion broth (Oxoid CM 225). From each dilution 100 μ l was inoculated onto the following solid media and incubated under the following conditions: blood agar base (Oxoid CM 55) containing 5% horse blood incubated at 37°C in air; heated blood agar at 37°C in 5% carbon dioxide; MacConkey agar without salt (Oxoid CM 7b) at 37°C in air; Sabouraud's agar (Oxoid CM 41) with chloramphenicol 50 mg/l at 30°C in air; VCAT agar (GC agar base (Oxoid CM 367) containing 10% lysed horse blood, GC supplement (Oxoid SR 105), vancomycin 5 mg/l, colistin 6 mg/l, amphotericin B 1 mg/l, and trimethoprim 5 mg/l) at 43°C under microaerophilic conditions; and Slanetz-Bartley agar (Oxoid CM 377) at 37°C in 5% carbon dioxide. The following solid media were inoculated and incubated at 37°C under anaerobic conditions (Wise Anaerobic Work Station): Columbia blood agar base (Oxoid CM 331) with 5% horse blood; Rogosa's agar (Oxoid CM 627);

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Wilkins-Chalgren agar (Oxoid CM 619) with 5% horse blood, sodium pyruvate 1 g/l, and nalidixic acid 10 mg/l; Veillonella agar (Difco Ltd); Columbia blood agar with kanamycin 100 mg/l and vancomycin 7.5 mg/l; and fructose agar (*Clostridium difficile*) (Oxoid CM 601) with 7% horse blood, cycloserine 500 mg/l, and cefoxitin 16 mg/l. Media incubated under anaerobic conditions were supplemented with hemin, menadione, and sodium bicarbonate. In addition, for the isolation of clostridia, 100 µl of the contents of the phial were added to 100 µl of 95% ethanol.¹² This mixture was incubated for 30 minutes at 37°C before inoculation on to egg yolk agar,¹² prepared with blood agar base (Oxoid CM 55), which was then incubated at 37°C under anaerobic conditions. All agar plates were examined for growth for up to one week after inoculation. Colonies were counted using an automated colony counter. Isolates were identified using standard laboratory methods.¹²

'New' isolates were defined as those strains isolated in the 48 hours preceding the clinical onset of necrotising enterocolitis but not from the samples collected in the five days before that time.

Results

A total of 752 stool samples were obtained from 90 neonates during the eight month period of the study. Seven definite episodes of necrotising enterocolitis and 11 possible episodes were diagnosed. Faecal samples were obtained during the week preceding the clinical onset of four (four babies) of the seven definite episodes and eight (seven babies) of the 11 possible episodes. The neonates who developed possible necrotising enterocolitis and from whom samples were available had a median gestational age of 27 weeks (range 26–30) and median birth weight of 995 g (range 660–1160). Neonates who developed definite necrotising enterocolitis and from whom samples were available had a median gestational age of 28 weeks (range 27–30) and median birth weight of 725 g (range 620–1440). Antibiotics were given intravenously to the babies in the week before three of the eight episodes of possible and one of the four episodes of definite necrotising enterocolitis.

All the episodes of possible and definite necrotising enterocolitis followed the introduction of nasogastric feeding (by intervals ranging from three to 16 days). In the intensive care unit there is a trend towards an increase in numbers of strains present in the faeces with time after the introduction of nasogastric feeds. A sudden decline in numbers of facultative species such as *Streptococcus faecalis* or *Staphylococcus* spp is unusual, unless it is associated with antibiotic treatment. The quantitative changes in flora before three definite episodes of necrotising enterocolitis (in three neonates) are shown in figs 1–3. Samples were available only from the 48 hours before the onset of the fourth definite episode of necrotising enterocolitis; a strain of *Klebsiella pneumoniae* was isolated at a concentration in excess of 10^{11} colony forming units (cfu)/g dry weight in the 48 hours before this fourth episode. Bacterial strains from the genus enterobacteriaceae were isolated from samples collected in the 48 hour periods before the onset of all four definite episodes of necrotising enterocolitis.

The species involved were *Escherichia coli*, *K pneumoniae* (two episodes) and *Enterobacter cloacae*. Two of these strains were 'new' isolates (figs 1 and 3) and in a third episode (fig 2), a strain of *E cloacae* increased considerably in concentration before the onset of necrotising enterocolitis. There

Figure 1 Changes in facultative and anaerobic species of faecal flora during the week before the clinical onset of neonatal necrotising enterocolitis.

Figure 2 Changes in numbers of *E cloacae*, *S faecalis*, coagulase negative staphylococci, and *Veillonella* sp during the week before the clinical onset of neonatal necrotising enterocolitis.

Figure 3 Changes in numbers of *E. coli*, *S. faecalis*, coagulase negative staphylococci types I and II, and *Candida* sp during the week before the clinical onset of neonatal necrotising enterocolitis.

was a quantitative decline in the concentration of other bacterial species including both facultative and anaerobic species from up to 72 hours before all three definite episodes of necrotising enterocolitis.

In the week before the onset of one of the definite episodes of necrotising enterocolitis (fig 1) four species of clostridia were isolated; this was an unusual pattern of colonisation. Although *Clostridium* spp were isolated from 19 of 121 samples (nine neonates) collected from 29 neonates admitted to the unit between six weeks before, and six weeks after, the onset of this episode of necrotising enterocolitis, in only three samples from one neonate were two species of clostridia isolated. There were no samples with three or more species.

Four of the eight possible episodes of necrotising enterocolitis were not preceded by quantitative changes (rise or fall of two or more microbial species by twice $\log_{10}$ cfu/g dry weight) or by the detection of 'new' isolates. 'New' isolates associated with the other four possible episodes of necrotising enterocolitis were *Serratia marcescens* and *S. faecalis* (episode 1); *S. faecalis*, *Clostridium perfringens*, and *Veillonella* spp (episode 2); *S. faecalis* (episode 3); and *Veillonella* spp, *Propionibacterium* spp, *Candida* spp, and *E. cloacae* (episode 4).

Discussion

The intramural (or intrahepatic) gas that is characteristic of necrotising enterocolitis is probably a result of bacterial fermentation of intraluminal substrates,¹³ and bacterial fermentation of carbohydrates in the small intestine before the clinical onset of the disease.¹⁰ Clark *et al* reported that mucosal lesions could be induced in the rabbit ileum by the intraluminal injection of acidified casein, and suggested that the reduction in pH resulting from bacterial fermentation of excess substrate within the small bowel of neonates, particularly by enterobacteriaceae that ferment lactose rapidly,^{14 15} may lead to similar conditions to those that induce lesions in the rabbit model of necrotising enterocolitis.

The concentration of breath hydrogen has been positively correlated with the numbers of enterobacteriaceae in the faeces of neonates.¹⁶ Previous studies have suggested a link between enterobacteriaceae and necrotising enterocolitis,^{5-7 17-20} but no one species has consistently been linked

with it. In this study, enterobacteriaceae were isolated from samples collected during the 48 hours before the clinical onset of all four definite episodes of necrotising enterocolitis. These were 'new' isolates in two episodes and a considerable increase in numbers in another episode. By contrast with episodes of possible necrotising enterocolitis, new isolates of other genera were not found in the 48 hours before the clinical onset of the definite episodes. Enterobacteriaceae were isolated from samples preceding two of the eight possible episodes of necrotising enterocolitis, and were isolated in association with 'new' isolates from other genera in those episodes. The decline in numbers of some bacterial species in the faecal flora probably resulted from alterations in substrate availability and pH after bacterial fermentation in the small intestine.

Bacterial fermentation in the small intestine may contribute directly to the pathogenesis of necrotising enterocolitis,^{14 15} or it may result from changes in intraluminal conditions arising as a result of the disease. The changes that we have described probably result from changes in intraluminal conditions that precede the clinical onset of necrotising enterocolitis.

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