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Role of micro-organisms in necrotizing enterocolitis

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The frequency with which necrotizing enterocolitis occurs in outbreaks makes it likely that the illness can have an infective origin. Immunological and non-immunological defences of the gastrointestinal are impaired in early life. Consequently the gut of the preterm infant is predisposed to bacterial overgrowth. A wide range of pathogenic bacteria and viruses have been isolated from infants with necrotizing enterocolitis or detected histologically. The presence of bacterial metabolites in the breath, intestinal bullae (hydrogen) and urine (D-lactate) during the course of the illness is further confirmatory evidence. The presence of bacteria or bacterial products (such as exo- and endotoxin) in the circulation will lead to ischaemia of the intestine and other organs either directly or via mediators such as cytokines or platelet activating factor. Future studies in necrotizing enterocolitis should be directed to understanding and modulating inflammatory mediators in necrotizing enterocolitis and preventing the disease with breast milk and nutritional supplements (glutamine, short chain fatty acids), chemoprophylaxis, and antibodies.

Witnessing an outbreak of necrotizing enterocolitis first-hand convinced one of us that this illness must have an infectious aetiology. Despite strong indirect epidemiological evidence for such an aetiology, the search for a specific microbial cause has been frustrating.

Direct evidence for a microbial aetiology for necrotizing enterocolitis

Many species of micro-organisms have been isolated from infants with necrotizing enterocolitis; some of these are listed in Table 1. Specific species have been associated with some outbreaks, for example *Salmonella* sp., enterotoxigenic *Escherichia coli*, *Klebsiella*, *Enterobacter cloacae*, *Pseudomonas*, and viruses such as rotavirus, and coronavirus.

The demonstration that *Clostridium welchii* type F (later reclassified as *Clostridium perfringens* type C)

was responsible for a related disease, 'pig-bel', and the subsequent prevention of this disease by immunization with β -toxoid, helped to stimulate the search for Clostridia in necrotizing enterocolitis. Darmbrand, a necrotizing enterocolitis occurring in adults in post-Second World War Germany was found to have the same aetiology. Gram-negative

Table 1. Micro-organisms associated with necrotizing enterocolitis

| | |
|---------------|--|
| Coliforms | <i>Escherichia coli</i> <i>Klebsiella</i> sp. <i>Enterobacter cloacae</i> <i>Pseudomonas</i> sp. <i>Salmonella</i> sp. |
| Staphylococci | <i>Staphylococcus epidermidis</i> <i>Staphylococcus aureus</i> |
| Anaerobes | <i>Clostridium butyricum</i> <i>Clostridium perfringens</i> <i>Clostridium difficile</i> |
| Viruses | rotavirus enterovirus coronavirus Coxsackie B |

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Table 2. Factors contributing to immaturity of gastro-intestinal host defences in preterm infants

| |
|--|
| Decreased intraepithelial lymphocytes |
| Decreased IgA |
| Malnutrition |
| 'Stress' |
| Reduced gastric acid production |
| Pancreatic insufficiency |
| Bile salts insufficiency |
| Impaired mucus production |
| Immature gastro-intestinal motility |
| Glutamine deficiency |
| Decreased concentrations of short chain fatty acids in the colonic lumen |

rods have been demonstrated in the bowel wall of resected gut from infants with necrotizing enterocolitis and *Cl. perfringens* has been cultured from peritoneal fluid [1, 2]. At the Royal Free Hospital, London, UK, *Clostridium butyricum* was isolated in 9 of 10 babies examined after prolonged incubation of cultures and examination of samples by gas-liquid chromatography [3], giving rise to a second 'Royal Free Disease'. Nevertheless, Clostridial spp. have been isolated subsequently only from a minority of infants with necrotizing enterocolitis.

Bacterial colonization, mucosal integrity, host resistance, and necrotizing enterocolitis

Bacterial overgrowth

The intestinal flora of low-birth-weight infants differs from that of healthy, full term infants. The establishment of bowel flora in new-born infants receiving intensive care is a protracted process, with a limited number of strains being present initially [4]. Overgrowth of the intestinal tract with *Klebsiella* species has been suggested as a risk factor for necrotizing enterocolitis [5]. There are many factors present in preterm infants which might predispose to bacterial overgrowth of the small intestine, some of which are listed in Table 2.

Gastric acid production

Gastric acid production has been described as markedly decreased in the first month of life [6].

Although preterm babies can produce an acid intra-gastric pH, overgrowth of the stomach with bacteria in such babies is a frequent occurrence (B. Patel and S. P. Devane, unpublished observations) and may predispose to the development of an abnormal intestinal flora.

Immature gastro-intestinal motility

The cleansing effect of normal gastro-intestinal motility is a further defensive mechanism against colonization of the small intestine by bacteria and the damaging effects of the by-products of fermentation of malabsorbed nutrients by bacteria; antro-duodenal motility is immature in preterm infants [7].

Pancreatic insufficiency

In an analogous situation to pig-bel in which pancreatic insufficiency leads to decreased intraluminal destruction of β -toxin, pancreatic insufficiency has also been described in low-birth-weight babies who developed necrotizing enterocolitis [8]. Pancreatic insufficiency was measured indirectly by faecal chymotrypsin activities.

Impaired mucus production

Intestinal mucus provides non-specific protection to the intestine, by binding bacteria and bacterial toxins. This protective mechanism is poorly developed in the new-born mouse [9] due to defective mucus production; the same may also apply to human infants.

Glutamine deficiency

Adequate intake of glutamine is important for the integrity of the small bowel mucosa and immune function [10]. Glutamine deficiency could occur in preterm babies on parenteral nutrition, or receiving artificial diets.

Decreased concentrations of short chain fatty acids in the colonic lumen

Decreased concentrations of short chain fatty acids in the colonic lumen could also decrease integrity

of the colonic mucosa [11]. The delayed establishment of a normal flora in preterm infants receiving intensive care [4] could lead to deficiency of short chain fatty acids in the colonic lumen. Interaction of this and other factors impairing the barrier function of the gastrointestinal mucosa could explain the frequent isolation of enteric micro-organisms from blood cultures of infants with necrotizing enterocolitis. The balance is a fine one, because the by-products of fermentation of malabsorbed nutrients by bacteria can damage the intestinal mucosa.

Gastrointestinal immune defences

Both cellular and immunological defence mechanisms are immature in infancy. Intraepithelial B lymphocytes are decreased [12]. Secretory IgA production is virtually non-existent in new-borns [13]. T lymphocytes are decreased in the intestinal mucosa of new-born animals [14]. They are important in monitoring the integrity of the mucosa and may destroy cells colonized with an infecting agent.

Indirect evidence for a microbial aetiology for necrotizing enterocolitis

Epidemiology of necrotizing enterocolitis

The strongest indirect evidence for a microbial aetiology arises from the epidemiology of the disease [15]. During a survey lasting one year in the UK, 6 of 54 neonatal centres reported outbreaks. Within the six units, 24 of the 56 cases were diagnosed within 3–11 days of each other. The clinical features of this group were indistinguishable from sporadic cases.

Blood cultures

One in three affected neonates have been reported to have a positive blood culture. The organisms isolated are unlikely to be causal and probably translocated from the bowel lumen through a damaged mucosa. Nevertheless, such organisms

must contribute to the parlous state of these patients and add to the endotoxaemia to which they are exposed.

Protective effect of human milk

Breast milk has been shown to contain specific cellular and antibody activity against a variety of bacterial, viral and food-related antigens. Furthermore, it protects against necrotizing enterocolitis [16].

Protective effect of oral human IgG+IgA

Oral administration of pooled IgG and IgA has successfully prevented necrotizing enterocolitis, possibly by passively protecting against bacterial overgrowth of the intestinal tract [17]. IgA prevents attachment of antigens (bacterial, viral and dietary) to the small intestinal mucosa. IgG alone may be effective [18].

Protective effect of oral antibiotics

Oral vancomycin has been administered to low-birth-weight infants identified as having a high risk of developing necrotizing enterocolitis prior to the introduction of oral feeds. Necrotizing enterocolitis developed in 1 of 84 infants, compared with 17 of 120 low risk infants not given vancomycin. While the trial was not double-blind or randomized, this study [19] suggests that vancomycin could have a prophylactic role.

Raised breath hydrogen

Breath hydrogen concentrations have been studied prospectively in 122 preterm infants as a potential aid to earlier diagnosis [20]. Maximum elevation of breath hydrogen occurred in infants 8–20 hours prior to the onset of necrotizing enterocolitis. The presence of elevated breath hydrogen concentrations indicates that bacteria are metabolizing luminal substrates.

D-lactic acidosis

D-lactate, a product of bacterial metabolism, is found in the urine of infants with necrotizing enterocolitis [21].

Exposure of Thomsen-cryptantigen on RBC

Nine of 26 infants with necrotizing enterocolitis had exposure of the Thomsen-cryptantigen (T-antigen) with the titre of the antigen correlating with severity. The T-antigen is exposed by cleavage of N-acetylneuramic acid from the red cell surface; neuraminidase is produced by *Cl. perfringens*, *Cl. butyricum* and *Bacteriodes fragilis*. This finding is indirect evidence for the presence of circulating bacterial neuraminidase, and neuraminidase-producing Clostridia were isolated from two patients.

Animal models

Necrotizing enterocolitis can be induced in neonatal rats colonized with *Klebsiella* sp. and subjected to hypoxia or cold stress which reduce mesenteric blood flow [22]. Breast milk has been shown to be protective. Necrotizing enterocolitis has been induced in infant rats by injecting platelet activating factor (PAF) into the splanchnic circulation [23]. Platelet activating factor is a phospholipid secreted in response to injection of endotoxin (lipopolysaccharide component of gram negative bacterial cell wall). Platelet activating factor may induce intestinal necrosis by release of the vasoconstrictor leukotriene C₄ [24]. When mesenteric blood flow is re-established the stage is set for reperfusion injury which can be prevented by free-radical scavengers such as superoxide dismutase, catalase or the xanthine oxidase inhibitor, allopurinol [25].

Histological changes in necrotizing enterocolitis

Various authors have cited the presence of inflammatory changes in necrotizing enterocolitis as evidence for an infective aetiology while others have emphasized that the appearances support an ischaemic aetiology. In a systematic review of 84 patients from Cleveland, Ohio in which histological changes were correlated with clinical findings, ischaemic changes (tissue necrosis with loss of cellular detail but preservation of overall structure) were confirmed in 75 patients of whom 5 had mesenteric artery thromboses [25]. Acute (neutrophils) or chronic (lymphocytes, plasma cells and histiocytes) inflammation was seen in 50 and

Table 3. Bacterial toxins and necrotizing enterocolitis

| |
|---|
| <i>Clostridium difficile</i> toxin A |
| <i>Clostridium perfringens</i> type C β toxin |
| <i>Escherichia coli</i> heat labile toxin |
| Coagulase - ve staphylococci delta toxin |

evidence of bacterial overgrowth was also present in 55. Mixed colonies of cocci and bacilli and occasional fungi were demonstrated in the lumen and bowel wall specimens. The authors felt that the degree of overgrowth was greater than seen in other types of ischaemic bowel injury such as volvulus, intussusception, vasculitis and mesenteric artery thrombosis or embolism. Pneumatosis was present in 40 patients and the authors stress that the bullae contains hydrogen, a by-product of bacterial metabolism. Nevertheless, any hypothesis implicating infection in the aetiology of necrotizing enterocolitis must take into account the ischaemic changes highlighted in this study.

Possible pathophysiological relationships between bacteria and necrotizing enterocolitis

Bacterial toxins and necrotizing enterocolitis

Bacterial toxins are capable of inducing a lesion resembling necrotizing enterocolitis. Bacterial toxins previously implicated in necrotizing enterocolitis are listed in Table 3. Toxin A, produced by *Clostridium difficile* induced necrosis of intestinal mucosa *in vivo* and *in vitro* [27] the *in vitro* data indicating that a direct effect on the mucosa was responsible, rather than an effect on mucosal blood supply. The species has been implicated in necrotizing enterocolitis but is ubiquitous in healthy neonates [28]. *Clostridium perfringens* has been isolated from necrotizing enterocolitis and β -toxin is involved in the analogous conditions of pig-bel. *E. coli* producing heat-labile enterotoxin were associated with two clusters of necrotizing enterocolitis [29].

Scheifele's group has suggested that *Staphylococcus epidermidis*, which commonly colonize low-birth-weight infants including those with necrotizing enterocolitis produces a cell-damaging toxin resembling the delta toxin of *Staphylococcus*

aureus. The cytotoxin can induce severe mucosal necrosis in rat intestinal loops [30]. They suggest that hypertoxigenic strains may be responsible for necrotizing enterocolitis.

Impact of intestinal infections on intestinal mucosal blood supply

The effects of intestinal infections on mucosal blood supply have not been studied in detail, yet if such effects occur they could explain the link between mucosal infection and ischaemia. The anatomy of the microcirculation of intestinal villi of infant mice has been studied using a histochemical technique that specifically stained erythrocytes [31]. Over 8–14 days there was little variation between the same region of the gut with the exception of the distal ileum which was markedly less well perfused with erythrocytes in 8 day old mice than older mice. In the upper intestine, the capillary beds were more complex than in the middle and lower regions. If the anatomy of the villous microcirculation is duplicated in human villi, this may contribute to the vulnerability of the distal small intestine to ischaemia. Age-matched infant mice infected with rotavirus developed marked ischaemia and atrophy of the villi between 18 and 48 hours post infection [32] with preservation of the crypt microcirculation. Between 72 and 96 hours post infection, the capillaries of the small intestinal mucosa became engorged, with recovery of villous height. During this phase, the mucosa could have been subjected to a reperfusion injury. A second phase of villous atrophy followed between 120 and 144 hours which was less marked than the initial episode. The villous microcirculation recovered by 168 hours, and the diarrhoea ceased. These findings could explain how rotavirus infection could initiate necrotizing enterocolitis.

Perturbations of the enteric microcirculation have also been described in clinical or experimental cholera, salmonellosis, shigellosis, and acute diarrhoea. These data all provide the link between abnormal colonization and ischaemia of the intestinal mucosa.

Role of endotoxaemia

Endotoxin is a component of the cell wall of Gram negative bacteria which consists of lipopolysaccharide. Some systemic effects of endotoxin are

Table 4. Some systemic effects of endotoxin

| |
|--|
| Disseminated intravascular coagulation |
| Thermal instability |
| Vascular permeability |
| Metabolic acidosis |
| Hypotension |
| Left ventricular failure |
| Bowel necrosis |

listed in Table 4. Although only providing circumstantial evidence, there are clearly similarities between the signs of necrotizing enterocolitis and the effects of endotoxaemia. Low-birth-weight babies have been shown to experience endotoxaemia when fed [33]. Endotoxaemia has variable effects on intestinal blood flow but can induce ischaemia and increased permeability of the intestinal mucosa which could predispose to absorption of more endotoxin or entry of bacteria from the lumen of the gut.

The systemic effects of endotoxin are mediated by the release of proteins called cytokines from monocytes and probably endothelial cells [34]. In low concentrations, cytokines co-ordinate essential functions such as immune responses, but higher concentrations may have adverse effects. If endotoxaemia is implicated in the pathogenesis of necrotizing enterocolitis then it is likely to be due to the effects of excessive production of cytokines, which in turn trigger the production of substances such as platelet activating factor [35].

Severe shigellosis is an example of a disease of the distal bowel in which endotoxaemia is associated with mucosal and on occasion, full thickness necrosis. It may be complicated by disseminated intravascular coagulation, haemolytic uraemic syndrome and occasionally gangrene and perforation of the large bowel. Endotoxaemia induced fibrin degradation products, and deposition of fibrin in glomeruli and in rectal microvasculature have been reported in patients with uncomplicated shigellosis. Infusion of either the cytokine tumour necrosis factor or endotoxin in rabbits produced similar pathology including disseminated intravascular coagulation with thrombocytopenia, damage to glomeruli with leukocyte infiltration, segmental ischaemia, haemorrhage, and necrosis in the liver, bowel, adrenals, pancreas, lung and other tissues. Microscopically there were fibrin deposits, polymorphonuclear infiltration and arterial thromboses. Similarities between the pathology of shigellosis and endotoxin or TNF infusions prompted

measurement of cytokine concentrations in sera and stool extracts from children with shigellosis [36].

Serum interleukin 6 (IL-6) and tumour necrosis factor concentrations were significantly elevated during the acute phase of *S. dysenteriae* 1 infection in children with a complicated course compared to convalescence, and IL-6 concentrations correlated with the presence of complications such as haemolytic uraemic syndrome, microangiopathic haemolytic anaemia, leukemoid reactions, thrombocytopenia or thrombocytosis and severe colitis associated with persistent diarrhoea. IL-6 has been shown to be a better indicator of disease severity in other septic states although tumour necrosis factor release is essential for the initiation or amplification of IL-6 release. IL-6 and IL-1 are thought likely to be important mediators of the pathological effects of tumour necrosis factor [34] including endothelial cell damage caused by increasing the adhesiveness of neutrophils and endothelial cells endothelial rearrangement, production of a procoagulant factor by endothelial cells, reduced expression of thrombomodulin, increased production of interleukin 1, which can in turn activate leukocytes to initiate coagulation and stimulation of endothelial cells, polymorphs, and macrophages to produce platelet activating factor [35]. Future work should be directed to the investigation of the roles of endotoxin, cytokines and their mediators in the pathogenesis of necrotizing enterocolitis. In common with shigellosis, serum tumour necrosis factor concentrations were elevated in necrotizing enterocolitis compared with age-matched control infants [23]. Tumour necrosis factor levels did not correlate with severity or outcome. Larger, longitudinal studies are required to investigate the role of inflammatory mediators in necrotizing enterocolitis.

Micro-organisms and management of necrotizing enterocolitis

Chemoprophylaxis

(See above: Protective effect of oral antibiotics.) A report from Leeds, UK suggested that the use of vancomycin and aztreonam to treat episodes of sepsis prevented necrotizing enterocolitis compared with vancomycin and gentamicin; infants

treated with vancomycin and aztreonam had lower viable counts of enterobacteriaceae in the stools [37].

Antibiotics

Broad spectrum antibiotics with anaerobic cover are part of the treatment regimen for established necrotizing enterocolitis.

Barrier nursing

The documentation of outbreaks of necrotizing enterocolitis suggests that affected infants should be nursed with particular attention to the prevention of cross-infection [15].

Breast milk

Lucas *et al.* [38] estimated that necrotizing enterocolitis was 6–10 times as common in artificially fed infants (20 times more common in infants over 30 weeks gestation) and that 500 infants are developing necrotizing enterocolitis in the UK every year as a result of artificial feeding. They attribute the protective effect to the IgA present in breast milk.

IgA, IgG

(See above: Protective effect of oral human IgG+IgA.)

Glutamine and short chain fatty acids supplementation

Supplementation of parenteral and enteral feeds with glutamine and short chain fatty acids could enhance mucosal barrier function of the small and large bowel, respectively [10, 11].

Protection against endotoxaemia

If endotoxaemia does play a role in the pathogenesis or complications of necrotizing enterocolitis then affected infants may benefit from therapeutic attempts to neutralize endotoxins or their mediators. Potential approaches are summarized in Table 5.

Table 5. Protection in endotoxaemia

| | |
|--|--|
| Anti-endotoxin antibodies | Human polyclonal anti- <i>E. coli</i> J5 Human monoclonal (HA-1A) anti-lipid A Murine monoclonal (E5) anti-lipid A |
| Anti-cytokine antibodies | Monoclonal anti-tumour necrosis factor antibody Monoclonal anti IL-6 antibody |
| Receptor antagonists | Soluble tumour necrosis factor receptor IL-6 receptor antagonist (ra) |
| Platelet activating factor antagonist Pentoxifylline (xanthine blocks tumour necrosis factor synthesis) | |

Table 6. Classification of necrotizing enterocolitis. (Modified from R. M. Kliegman and A. A. Fanaroff [40] *N Engl J Med* 1984; 310: 1093–1103.)

| | |
|-------------------------------|--|
| Necrotizing enterocolitis | Endemic Epidemic Post-exchange transfusion Post-cardiac catheterization |
| Pneumatosis coli | |
| Pseudomembranous colitis | |
| Primary bowel pathology | |
| Spontaneous bowel perforation | |
| Intestinal obstruction | |
| Appendicitis | |
| Primary mucosal injury | |
| Hypertonic feeds | |
| Gastroenteritis | |
| Milk allergy | |

While these potential interventions await clinical trials, there is experimental evidence that platelet activating factor antagonist prevents necrotizing enterocolitis. Endotoxin and tumour necrosis factor act synergistically to induce shock, neutropaenia, focal bowel necrosis in young rats; these effects are inhibited by platelet activating factor receptor antagonists [39].

Conclusion and classification

Conventionally, a review such as this should conclude with a complex diagram of 'vicious cycles' summarizing our uncertainty about precise causes. The basic cause of necrotizing enterocolitis remains a mystery, not the least because of occurrence of the disease in infants who 'break the rules' on necrotizing enterocolitis by being full-term or older, previously well, unfed or exclusively breast

fed. Perhaps a note of greater clarity would be sounded by reproducing a classification of necrotizing enterocolitis (Table 6) [40]. Any studies on necrotizing enterocolitis should clearly differentiate between these subgroups, which are likely to have different mechanisms with a final common pathway.

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