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Bacterial invasion of HT29-MTX-E12 monolayers: Effects of human breast milk

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Abstract

Aim: The supramucosal gel, crucial for gut barrier function, might be compromised in necrotizing enterocolitis (NEC). Breast milk is associated with a reduced incidence of NEC. We compared the effects of human breast milk (BM) versus a neonatal formula, Nutriprem 1 (FF), on adherence, internalisation, and penetration of NEC-associated *Escherichia coli* through monolayers of mucus producing intestinal cells, HT29-MTX-E12 (E12).

Methods: E12 cells were grown to confluence on membranes permeable to bacteria. *E. coli,* reference strain and isolated from a NEC-affected intestine, were cultured in LB broth, labelled with fluorescein and biotinylated. Bacteria were suspended in tissue culture medium (TC) or mixtures of TC with BM or FF and applied to the E12 cultures. Bacterial numbers were assessed by fluorescence. DyLight 650-labelled neutravidin, which cannot cross cell membrane, evaluated extracellular bacteria. Fluorescence of basolateral medium was measured to quantify translocation. Bacterial concentrations were compared using the Mann Whitney U test.

Results: After 1h exposure, E12 cultures adhered or internalised more NEC-derived bacteria than standard strain *E. coli* and more suspended in FF than BM ($P<0.001$). A greater proportion of NEC-derived bacteria internalised when suspended in TC or BM. In FF, the NEC-derived strain internalised least. More translocation occurred in BM incubations compared to FF in the first 1–4 h: NEC-*E. coli* less than the reference strain. After 24 h translocated bacterial populations were equal.

Conclusion: In this pilot study, breast milk was associated with relatively less adhesion and internalisation of NEC-associated *E. coli* to mucus covered E12s compared to formula milk.

The pathogenesis of necrotizing enterocolitis (NEC) is multifactorial; microbes, immature gut mucosa, diminished innate defence, and the presence and type of feeds contribute variously to disease onset [1]. The contribution of microbes to NEC has been recently reviewed by Morowitz et al. [2].

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E. coli is one of the most frequently cultured species in the faeces of neonates with NEC [3]. A predominance of faecal Enterobacteriaceae prior to NEC onset has been observed in preterm infants [4]. Panigrahi et al. assessed adherence of *E. coli* and *Klebsiella* spp (both Enterobacteriaceae) isolated from the stools of infants with NEC to Caco-2 cells; the most adherent of these also induced NEC type changes in rabbit ileal loop models [5]. Both fresh and donor breast milk feeds are associated with a reduced risk of NEC compared to infant formula [6], but it is not clear why this occurs.

The supra-mucosal gel (SMG) coats the intestinal epithelial cell surface and is the first layer met by organisms in the gut [7]. The SMG and glycocalyx are crucial for a functioning barrier and the relationship between host and developing microbiota [8,9]. Mucins, a family of very large glycoconjugates, control the properties of luminal SMG (largely MUC2, MUC5B, MUC5AC) [10]; MUC1, MUC3, MUC4, MUC16 also participate in the glycocalyx that closely lines the cell surface. Mucins are tissue specific and their expression changes during foetal development [11]. Sub-normal total mucin turnover in Hirschsprung disease (HD) [12,13] and down-regulation of trefoil peptide expression in enterocytes affected by NEC [14] suggest a role for SMG and glycocalyx dysfunction in the pathogenesis of enterocolitis in infants. We used a mucin gel-producing cell line, HT29-MTX-E12 (E12), to study the effects of human breast milk on *Escherichia coli* isolated from an infant with confirmed NEC. E12s have been used extensively to study the activity and penetration of mucosal pathogens such as *Campylobacter jejuni* and *Helicobacter pylori* through mucous gel layers [15–17].

The aim of these experiments was to evaluate the effect of different feed types on bacterial invasion. We hypothesized that breast milk limits the progression of bacteria through the intestinal supra-mucosal gel, glycocalyx and underlying epithelium in the premature gut.

1. Methods

Ethical approval was obtained from the Southmead Research Ethics Committee (ref: 09/H0102/60). The experimental design involved quantification of extracellular and intracellular bacteria that penetrated through the cell layer, following timed exposure of cultures to bacteria suspended in medium or 1:1 mixtures of medium and milk or medium and formula.

1.1. Cell culture

The sub-clone of HT29-MTX used in this study, was differentiated to a goblet-type cell from HT29 using methotrexate as a selection agent [18]. It produces an apical mucous gel formed mainly of MUC2 and MUC5AC. The cells, passage number 50–52, were a generous gift from Dr Marguerite Clyne, University College Dublin. E12 cultures were grown in Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose and L-glutamine (GIBCO), 10% foetal bovine serum (Invitrogen), and 1% non-essential amino acids (Sigma-Aldrich) [17]. Medium was replenished every two to three days. Antibiotic-antimycotic (gentamicin/amphotericin, Invitrogen) was omitted from the medium more than 48 h prior to each experiment. For experiments, E12 cells were seeded into 24 well plates and polycarbonate membrane supports (Greiner Thin-Cert Tissue Culture Inserts; pore size 3.0 μm, Greiner Bio-One). 600 μl of medium was applied basolaterally in the well and 200 μl apically in the inserts. E12 cultures were used on days 13–15, based on serial assessments of transepithelial resistance (TEER), measured with Millicell electrodes (Millicell-ERS, Millipore, Billerica, MA, USA) and microscopic appearance.

1.2. Bacteria

1.2.1. Preparation of bacterial strains

E. coli isolated from the diseased ileum at laparotomy of a 32 week gestation neonate with histologically confirmed NEC was compared to a reference strain from the Bristol Oral Microbiology Laboratory stocks (MG1655). Inoculants were prepared from freshly streaked Fastidious Anaerobic Agar (FA) plates (prepared in house). Colonies were cultured overnight in 15 ml Luria–Bertani (LB) broth (Difco) at 37 °C with vigorous shaking, and harvested by centrifugation at 5000 *g* for 5 min at 10 °C. Pellets were resuspended in sterile PBS (GIBCO pH 7.2, Invitrogen), washed twice and diluted to the desired $OD_{600}=0.8$ using a photometer (Chroma Model 260, Sherwood Scientific, Cambridge, UK).

1.2.2. Labelling of bacteria

To distinguish between adherent bacteria and those which had internalised, a method of labelling was employed which takes advantage of the fact that neutravidin does not cross the cell membrane [19]. Bacteria, 10^9 cfu/ml, were labelled with fluorescein by incubation under constant shaking at 4 °C with 0.4 μg/ml 5-(6)-carboxyfluorescein-succinylester in PBS (Thermo Scientific Inc, UK). This mixture was then diluted 1:1 with 0.3 mg/ml sulfo-NHS-LC-biotin in PBS (Thermo Scientific Inc, UK) and incubated at the same temperature for a further 30 min. After three further washes with PBS the bacteria were suspended in the culture medium to achieve a concentration of approximately 8.4×10^8 cfu/ml.

1.2.3. Human milk and formula feed

Pasteurized human breast milk was obtained from the Queen Charlotte's and Chelsea Hospital Milk Bank. The donor breast milk was used in accordance with Milk Bank protocols and donor consent for its use for research purposes had been obtained. Pasteurized donor breast milk, like freshly expressed human milk, is associated with a lower risk of NEC compared to formula feed [6]. A single 200 ml sample from one mother was defrosted, gently mixed and