The protective effect of breast feeding in relation to sudden infant death syndrome (SIDS): II. The effect of human milk and infant formula preparations on binding of Clostridium perfringens to epithelial cells

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Abstract

Breast feeding is known to protect an infant against gastrointestinal pathogens and epidemiological studies indicate that compared to breast fed infants, formula fed infants are at a greater risk of dying from sudden infant death syndrome (SIDS). Many SIDS infants have symptoms of gastrointestinal infections prior to death and one gastrointestinal pathogen associated with SIDS is Clostridium perfringens. Studies have found that a significantly higher number of formula fed SIDS infants have C. perfringens and its enterotoxin in their faeces compared to breast fed infants. The aim of the study was to compare the effects of human milk and infant formula on binding of C. perfringens to epithelial cells.

Two protocols were used to assess the effect of human milk and infant formula to inhibit binding of C. perfringens to epithelial cells. Binding was assessed by flow cytometry. For the in vivo protocol which more closely represents interactions on the mucosal surface, breast milk enhanced bacterial binding but infant formula caused inhibition of binding; however for the in vitro method, both human milk and infant formula resulted in consistent enhancement of binding. Flow cytometry studies indicated that enhancement of binding was due to the formation of bacterial aggregates. Lewis a and Lewis b antigens, found in both breast milk and infant formula, inhibited C. perfringens binding in a dose dependent manner.

The Lewis a and Lewis b antigens in human milk and infant formula can inhibit C. perfringens binding to epithelial cells. While infant formula reduced binding of C. perfringens to epithelial cells in the experiments carried out with the in vivo protocol, the protective effects of breast feeding in relation to colonisation with C. perfringens are more likely to be due to formation of bacterial aggregates. These findings have implications for improving infant formula preparations. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: SIDS; Breast feeding; Clostridium perfringens; Lewis antigen; Infant formula

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1. Introduction

Breast feeding is associated with protection against enteric infections [1], and compared to formula fed infants, breast fed infants have a lower risk of dying from sudden infant death syndrome (SIDS) [2]. Many SIDS infants have symptoms of gastrointestinal infections prior to death, and compared to healthy infants in the same age range, toxigenic strains of *Clostridium perfringens* are more likely to be identified in SIDS infants [3]. A number of studies have found significantly higher levels of *C. perfringens* and their toxins in SIDS cases compared to non-SIDS cases [4,5], and one study found *C. perfringens* in faeces from 45.4% of SIDS infants compared to 19.6% of healthy babies [5]. This study also found that formula fed SIDS infants had a significantly higher incidence of *C. perfringens* and its enterotoxin in their faeces than breast fed infants [5]. *C. perfringens* enterotoxin A has been identified in body fluids of SIDS infants [6] and because of its superantigenic nature, has been suggested to have a significant role in some SIDS deaths [3,7].

Adherence to the intestinal mucosa allows bacteria to resist expulsion by peristaltic clearing mechanisms and is the first stage in development of a gastrointestinal infection. Human milk is unique in its content of free oligosaccharides, glycoproteins and glycolipids. There is evidence that free oligosaccharides and glycoconjugates can act as soluble receptor analogues of intestinal cell surface carbohydrates. These interfere with either bacterial binding, thereby preventing colonisation, or with binding of toxins to their receptors [8,9]. The Lewis antigens are oligosaccharide components of both breast milk and infant formula [10].

Previous work by our group investigated the effect of human milk, infant formula and synthetic Lewis antigens on binding of the respiratory pathogen *Staphylococcus aureus* expressing an adhesin that binds the Lewis antigens [10]. Two different protocols were used in this study. The first was the in vitro or conventional experimental approach in which bacteria were initially incubated with the test component (for example milk or oligosaccharide), washed and then added to the cells. The second was an in vivo method in which bacteria, cells and the test component were added together and binding allowed to occur. This was developed to try to simulate more closely the complex interactions between microorganisms, components of body fluids and epithelial cells on the mucosal surface of the infant. The previous study of a toxigenic strain of *S. aureus* found the method used significantly influenced the results obtained with human milk compared with infant formula. The objective of the current study was to determine if similar effects were obtained with a gastrointestinal organism implicated in SIDS.

2. Materials and methods

2.1. Collection of milk specimens and preparation of infant formula

Milk specimens and infant formula preparations were the same as those used in the accompanying paper [10].

2.2. Human cells

Kato III, a gastric carcinoma cell line (CB769) (European Collection of Animal Cell Cultures), was used in this study. The methods for growth and preparation of the cells are the same as those described in the accompanying paper [10].

2.3. General protocol for binding assays

*C. perfringens* NCTC 2661 (kindly provided by Mr R. Brown of this department) were grown on blood agar plates for 24 h at 37°C under anaerobic conditions (Oxoid Gas Generating Kit) and washed in PBS by centrifugation at 2000 \( \times \) g for 20 min. The bacterial pellet was labelled with fluorescein isothiocyanate (FITC) (Sigma) as described previously [11]. Bacteria (200 µl) were tested at a ratio of 400 bacteria per cell. The in vivo and in vitro protocols described in the accompanying paper were used and the results were analysed with an EPICS XL flow cytometer (Coulter) as described previously [10]. The results were expressed as the binding index (BI) of each sample calculated by multiplying the percentage of fluorescent cells by the mean fluorescence channel value.
2.4. Statistical methods

For comparison of inhibition of binding of bacteria by the synthetic Lewis\textsuperscript{a} or Lewis\textsuperscript{b} antigens, the binding index (BI) of cells in each sample was compared with the control by the formula: \( \% \text{ inhibition} = 100 - \left[ \frac{(\text{BI of the sample/BI of the control}) \times 100}{} \right] \). The results were assessed by Student’s \( t \)-test. For the effect of whole and defatted breast milk or infant formula on bacterial binding to epithelial cells, the positive controls of cells with bacteria and PBS were given a value of 100% and all other values were expressed as a percentage of the control. The results between the in vivo and in vitro methods were compared by Student’s \( t \)-test for paired samples.

3. Results

3.1. Effect of whole and defatted human milk on binding of C. perfringens to Kato III cells

Whole and defatted milk were tested at dilutions of 1 in 10, 1 in 100, 1 in 1000, and 1 in 10 000. The results for the 1 in 10 000 dilution were not significantly different from the controls and the data for these are not shown.

In six experiments, for both methods with whole or defatted human milk, there was a general pattern of enhancement of bacterial binding (Table 1a and b), but the enhancement was not significant compared with the untreated controls. Greater levels of binding were observed with the in vitro method, and this was reduced with increasing dilutions of human milk. A small but significant reduction in binding

Table 1

<table>
<thead>
<tr>
<th>Dilution</th>
<th>%</th>
<th>Se</th>
<th>95% CI</th>
<th>( P )</th>
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</thead>
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<tr>
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<td>117.0</td>
<td>29.1</td>
<td>42.1–191.9</td>
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<td>1/100 W</td>
<td>171.5</td>
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<td>83.2–259.8</td>
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<td>1/1000 W</td>
<td>130.7</td>
<td>12.3</td>
<td>99.0–162.4</td>
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<tr>
<td>1/10 D</td>
<td>160.3</td>
<td>26.0</td>
<td>93.5–227.1</td>
<td>0.068</td>
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<td>1/100 D</td>
<td>135.3</td>
<td>15.8</td>
<td>94.6–176.1</td>
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<td>1/1000 D</td>
<td>88.7</td>
<td>3.4</td>
<td>79.9–97.5</td>
<td>0.022*</td>
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Table 1b

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<th>Se</th>
<th>95% CI</th>
<th>( P )</th>
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<td>232.1</td>
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<td>74.2</td>
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<td>31.2</td>
<td>67.5–228.1</td>
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<td>1/1000 D</td>
<td>120.3</td>
<td>30.9</td>
<td>40.9–199.7</td>
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</table>

*Significant differences from controls treated with PBS only.

Fig. 1. Aggregation of C. perfringens by pooled whole human milk detected by flow cytometry.
was observed for the defatted milk at higher dilutions.

Paired sample t-tests were carried out to test for significant differences between the two methods for the same dilution. With the in vitro method, significantly greater binding was observed for whole milk at the 1 in 10 dilution ($P = 0.000$) and the 1 in 1000 dilution ($P = 0.000$) and for defatted milk at each of the three dilutions: 1 in 10 dilution $P = 0.001$; 1 in 100 dilution $P = 0.000$; 1 in 1000 dilution $P = 0.001$. Paired sample t-tests were also carried out to test for significant differences between whole and defatted milk. Enhancement of binding was significantly higher for whole milk than defatted milk with the in vivo method at the 1 in 1000 dilution ($P = 0.045$). With the in vitro method, enhancement of binding with whole milk was significantly greater than that for defatted milk at the 1 in 10 dilution ($P = 0.026$).

### 3.2. Effects of three different infant formula preparations on binding of C. perfringens to Kato III cells

Three different infant formula preparations were investigated for their effect on binding of C. perfringens to Kato III cells using the two methods. When the results from the three different formulas were analysed, it was found that within the methods, there was no significant variation associated with formula type; therefore, the results obtained for each method and for each dilution have been grouped together (Table 2a and b). In contrast to whole and defatted human milk, the results obtained for the in vivo and in vitro methods differed. With the in vivo method, infant formula reduced bacterial binding to Kato III cells at the first two dilutions and this effect was reduced with increasing dilution of the formula. In contrast, with the in vitro method, infant formula caused enhanced bacterial binding to the cells, and this enhancement was reduced with increasing dilu-

### Table 2

Effect of infant formula preparations on binding of C. perfringens to Kato III cells using (a) in vivo and (b) in vitro methods for the mean of 13 and 12 experiments respectively expressed as a percentage of results for control bacteria treated with PBS (100%)%

(a) In vivo

<table>
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<th>Dilution</th>
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<th>Se</th>
<th>95% CI</th>
<th>$P$</th>
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<tr>
<td>1 in 10</td>
<td>85.7</td>
<td>9.5</td>
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<td>1 in 100</td>
<td>86.6</td>
<td>5.1</td>
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<td>1 in 1000</td>
<td>106.3</td>
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<td>101.9–110.8</td>
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(b) In vitro

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<th>Se</th>
<th>95% CI</th>
<th>$P$</th>
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</thead>
<tbody>
<tr>
<td>1 in 10</td>
<td>559.0</td>
<td>132.2</td>
<td>268.0–849.9</td>
<td>0.005a</td>
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<td>1 in 100</td>
<td>456.3</td>
<td>90.2</td>
<td>257.7–654.8</td>
<td>0.002a</td>
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<tr>
<td>1 in 1000</td>
<td>241.7</td>
<td>40.2</td>
<td>153.3–330.1</td>
<td>0.005a</td>
</tr>
</tbody>
</table>

*Significant differences from controls treated with PBS only.

![Inhibition of binding of C. perfringens by the synthetic Lewis* (diamond) and Lewisb (rectangle) antigens (0–80 μg ml⁻¹): a: in vivo method; b: in vitro method.](image)
tion of the formula. A significant reduction in binding was observed for the in vivo method for the 1 in 100 dilution and a small but significant increase in binding for the 1 in 1000 dilution. Significant enhancement of binding was observed for the in vitro method for each of the three dilutions.

Paired sample t-tests were carried out to test for significant differences between the two methods for the same dilutions of infant formula. Significant differences between the two methods for the same dilution were found for *C. perfringens* for all three dilutions: 1 in 10 (*P* = 0.020); 1 in 100 (*P* = 0.000); 1 in 1000 (*P* = 0.000).

### 3.3. Investigation of enhancement of bacterial binding

To investigate the enhancement of binding effect, flow cytometry and microscopy studies were carried out and the results from the former are shown in Fig. 1. The enhancement of binding observed following preincubation of the bacteria with either of human milk or formula was found to be due to the formation of bacterial aggregates.

### 3.4. Inhibition of bacterial binding by synthetic Lewis\(^a\) and Lewis\(^b\) antigens

Dose response experiments with synthetic Lewis\(^a\) and Lewis\(^b\) antigens were carried out with both methods. Results of three experiments are shown as the mean percentage inhibition of binding in Fig. 2a and b and the error bars represent the standard error of the mean (S.E.M.).

For *C. perfringens*, similar results for inhibition of binding by Lewis\(^a\) and Lewis\(^b\) were obtained for both methods. For the in vivo method, significant inhibition of binding was observed for Lewis\(^a\) from 2 μg ml\(^{-1}\) 18.8% (95% CI 10.2–27.4) (*P* = 0.011) with a plateau of inhibition at 20 μg ml\(^{-1}\) 52.2% (95% CI 36.7–67.6) (*P* = 0.005). For Lewis\(^b\), significant inhibition of binding was also observed from 2 μg ml\(^{-1}\) 15.3% (95% CI 13.8–16.8) (*P* = 0.001) with a plateau at 20 μg ml\(^{-1}\) 51.9% (95% CI 32.4–71.4) (*P* = 0.008). For the in vitro method, significant inhibition of binding was observed for Lewis\(^a\) from 4 μg ml\(^{-1}\) 40.0% (95% CI 29.0–51.0) (*P* = 0.004) with a plateau at 10 μg ml\(^{-1}\) 47.2% (95% CI 5.8–88.5) (*P* = 0.039). For Lewis\(^b\), significant inhibition of binding was observed from 2 μg ml\(^{-1}\) 24.1% (95% CI 8.0–40.2) (*P* = 0.023) with a plateau at 5 μg ml\(^{-1}\) 55.5% (95% CI 35.8–75.2) (*P* = 0.007).

### 4. Discussion

For *C. perfringens*, both methods gave similar results for whole and defatted human milk. Enhancement of binding rather than inhibition of binding was observed and flow cytometry studies suggest that enhanced binding occurs due to the formation of bacterial aggregates. Greater enhancement of binding was observed using the in vitro method because bacterial aggregates are formed when they are incubated with milk or formula. Significantly different results were obtained with infant formula. The in vivo method showed inhibition of binding at the lower dilutions regardless of formula type. The in vitro method showed enhancement of binding. The results with *C. perfringens* are similar to those observed with *S. aureus* [10].

Oligosaccharides and glycoconjugates present in human milk have been found to inhibit binding of certain enteric pathogens and their toxins by acting as receptor analogues [9]. Preincubation of epithelial cells with fucose containing glycopeptides from lactoferrin was found to inhibit adherence of *Shigella flexneri* to intestinal cells [12]. This suggests fucosyl containing components are binding to a receptor on the cell and blocking adherence. Fucosyl containing oligosaccharides protected against the heat stable enterotoxin of *Escherichia coli* [13]. Breast feeding has also been found to be protective against salmonella. Human milk antibodies to *Clostridium difficile* have been found to inhibit the binding of toxin A to its intestinal receptor. Bacteria producing soluble exotoxins have been found in the gastrointestinal tract of some SIDS infants and a small number of deaths have been due to infant botulism [14–18].

In this study a dose response effect was observed for inhibition of binding of *C. perfringens* by the synthetic Lewis\(^a\) and Lewis\(^b\) antigens. These antigens are components of breast milk and infant formula. Higher levels of the Lewis antigens are required to cause maximum inhibition of binding using the in vivo protocol in which the bacteria can bind to either the Lewis antigen on the cell surface or to antigens in
the solution. In the in vitro protocol, direct blocking of the bacterial adhesin occurs before addition to the cells.

Density of colonisation is an important factor in development of bacterial disease due to invasion or toxin production [19]. Our studies suggest that the Lewis antigens in both human milk and infant formula might reduce binding of C. perfringens to epithelial cells. The results obtained for synthetic Lewis antigens with the two methods follow the same trends as those observed for S. aureus [10].

Significantly higher levels of C. perfringens and their toxins have been identified in SIDS cases compared to non-SIDS cases [4,5], and formula fed infants had a significantly higher incidence of C. perfringens and its enterotoxin in their faeces than breast fed infants [5]. Compared with healthy infants, enterotoxigenic strains of S. aureus and their toxins have also been isolated significantly more often from the gastrointestinal tracts of SIDS infants. Formula fed SIDS infants had a higher incidence of S. aureus and its enterotoxin than breast fed babies, but the increase was not significant [5].

In conclusion, although the free oligosaccharides Lewisa and Lewisb were shown to inhibit binding of both C. perfringens and S. aureus, the major protective effect of whole human milk is likely to be due to aggregation of the bacteria by antibodies and glycoconjugates. This could result in enhanced clearance by phagocytic cells or other innate mechanisms such as ciliary action or peristalsis. The presence of Lewisa and Lewisb in infant formula [10] and their ability to reduce bacterial binding in the in vivo protocol was not predicted. The current studies indicate these antigens are naturally present in all five commercial preparations of infant formula milk tested. The effect of increasing the concentrations of the Lewis antigens in these compounds is an area for further exploration in relation to improving the composition of infant formula.

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References


