The protective effect of breast feeding in relation to sudden infant death syndrome (SIDS): I. The effect of human milk and infant formula preparations on binding of toxigenic *Staphylococcus aureus* to epithelial cells

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Abstract

Epidemiological studies indicate that breast-fed infants are at a decreased risk of sudden infant death syndrome (SIDS) compared to formula-fed infants. Increasing evidence suggests that infectious agents might be involved in some of these deaths, in particular bacteria which colonise mucosal surfaces and produce superantigenic toxins. One species implicated in recent studies of SIDS infants is *Staphylococcus aureus*. We tested the hypothesis that in comparison to infant formula, human milk might be a better inhibitor of binding of *S. aureus* to epithelial cells. In this study, two protocols were used for the binding assays which were assessed by flow cytometry: the in vitro method in which bacteria were treated with milk or formula, washed and added to epithelial cells; and a method more closely reflecting the competitive interactions in vivo in which cells, bacteria, and milk or infant formula were added at the same time. With the in vivo method, breast milk caused enhancement of bacterial binding to cells whilst infant formula caused inhibition; however, for the in vitro method, both human milk and infant formula caused consistent enhancement of binding. Flow cytometry and light microscopy studies indicated that the enhancement was due to the formation of bacterial aggregates. Human milk and infant formula preparations were also compared for components (antibodies or oligosaccharides) that could inhibit binding of *S. aureus* using the in vitro method. Human milk contained both IgA and IgG. Neither human milk nor infant formula contained oligosaccharides reactive with the *Ulex europaeus* lectin but both contained components that bound monoclonal antibodies to Lewis a and Lewis b antigens which can act as receptors for *S. aureus*. With both methods, synthetic Lewis a and Lewis b inhibited *S. aureus* binding in a dose-dependent manner. With human milk, however, only the component which showed a significant correlation with inhibition of binding was the IgA specific for the staphylococcal surface component that binds Lewis a. Both human milk and infant formula contain components which could potentially inhibit bacterial binding but only breast milk contains the IgA specific for the bacterial adhesin that binds Lewis a. Studies using the in vivo method suggest that protection associated with breast feeding in relation to

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SIDS could be due mainly to the formation of bacterial aggregates. The studies have implications for further research into constituents of infant formula. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Sudden infant death syndrome; Breast feeding; Staphylococcus aureus; Lewis antigen

1. Introduction

Epidemiological studies of sudden infant death syndrome (SIDS) demonstrated a protective effect for breast feeding compared with infant formula feeding [1]. Many of the risk factors for respiratory infections are also associated with SIDS. Infants exclusively breast-fed have a significantly lower risk of SIDS than infants who are bottle-fed. Breast feeding reduces susceptibility to respiratory tract infections [2] and oligosaccharide components in human milk have been demonstrated to inhibit binding of the bacterial respiratory pathogens Streptococcus pneumoniae and Haemophilus influenzae to human pharyngeal or buccal epithelial cells [3].

Prior to death, many SIDS victims had mild symptoms of respiratory tract infections. No definite association between SIDS and specific viruses or invasive bacteria has been reported [4,5]; however, several groups have proposed that bacterial toxins play a role in precipitating the series of events leading to SIDS [5-9]. Compared to healthy infants in the same age range, toxigenic bacteria such as Staphylococcus aureus and Streptococcus species are more likely to be identified among SIDS infants [10]. In a longitudinal survey of healthy infants in the age range at greatest risk of SIDS, 56% were colonised by S. aureus, but these bacteria were identified in 86% of 39 local SIDS infants examined during the same period [11].

Toxic shock syndrome toxin (TSST-1) and staphylococcal enterotoxin C (SEC) are superantigens that induce strong inflammatory responses. The toxins have been identified in body fluids or tissues of SIDS infants by immunohistochemical methods [12,13]. By enzyme-linked immunosorbent assay (ELISA) and flow cytometry methods, pyrogenic staphylococcal toxins have been identified in over half of the specimens from SIDS infants from three different countries examined in a recent study [14].

The Lewis\textsuperscript{a} antigen is expressed by 80-90% of infants during the 2-4-month age range in which most SIDS deaths occur. Lewis\textsuperscript{a} is a receptor for S. aureus [15] and an adhesin has been isolated from these bacteria by affinity purification with synthetic Lewis\textsuperscript{a} [16]. Epidemiological studies have found isolation of S. aureus from infants parallels expression of the antigen [11,17,18]. Work by other groups has suggested that oligosaccharides and glycoconjugates present in human milk can act as analogues of epithelial cell receptors that bind bacterial adhesins, thereby reducing the ability of bacteria to colonise epithelial surfaces [3,19].

The first objective of this study was to compare the ability of human milk and infant formula for their effect on binding of S. aureus to epithelial cells. The second objective was to assess the effect of individual human milk components and the synthetic Lewis\textsuperscript{a} and Lewis\textsuperscript{b} antigens on bacterial binding. Two different methods were used in this study. The first was an in vivo method in which bacteria, cells and the milk or formula were added together and binding allowed to occur. The second was an in vitro or more experimental approach in which bacteria were initially incubated with the milk or formula, washed and the treated bacteria added to the cells. Previous studies have used the in vitro approach but the in vivo approach was assessed to try to simulate more closely competitive interactions that occur on mucosal surfaces.

2. Materials and methods

2.1. Collection of milk specimens and preparation of infant formula

Milk was collected from mothers participating in a longitudinal survey of infants’ nasopharyngeal flora. Ethical permission for the study was obtained. Informed written consent was given by the mothers at recruitment into the study by the research nurse
and health visitors at the 6-week post-natal medical examination. Information on socioeconomic background and medical history was obtained by the research nurse who helped the mother fill in a standardised questionnaire at the first interview [11].

Milk specimens were collected from women in various stages of lactation by manual expression. The specimens were transported within 2–4 h to the laboratory and stored at −20°C until examined. Whole and defatted pools of human milk containing aliquots of 42 specimens were prepared. To prepare the defatted pool, an aliquot of the whole pool was centrifuged twice at 2000 × g for 15 min at 4°C. The clear middle layer was separated from the upper fatty layer and from cells at the bottom of the tube. Both pools were stored in aliquots at −20°C.

Five commercially available infant formula powders were prepared with sterile water according to the manufacturers’ instructions. All dilutions of milk and infant formula were made in phosphate-buffered saline (PBS).

2.2. Bacteria

*S. aureus* NCTC 10655 which produces TSST-1 was grown on nutrient agar plates for 24 h at 37°C.

2.3. Human cells

Kato III, an epithelial cell line derived from gastric carcinoma (CB769) (European Collection of Animal Cell Cultures), was used in this study. Cells were cultured in 75-cm² tissue culture flasks (Greiner) at 37°C in 5% CO₂ in growth medium (RPMI 1640, Gibco) containing 20% foetal calf serum (FCS) (Gibco), 1% (w/v) L-glutamine (Gibco), penicillin (100 IU ml⁻¹) and streptomycin (200 µg ml⁻¹) (Gibco). Kato III cells were chosen for use in this model system to minimise differences in expression of H and Lewis antigens noted for individual donors in association with diet, blood group or smoking [20].

For binding assays, cells were detached from the flask by gentle scraping using a cell scraper (Greiner). The cells were washed twice in PBS by centrifugation at 300 × g for 10 min, resuspended in PBS and counted using a Neubauer haemocytometer by preparing a 1 in 10 dilution of the cells in 0.5% (w/v) trypan blue (Northumbria Biological, UK). The cell number was adjusted to 5 × 10⁵ cells ml⁻¹ with PBS.

2.4. Bacterial binding assays

Bacteria were washed in PBS by centrifugation at 2000 × g for 20 min. The bacterial pellet was labelled with fluorescein isothiocyanate (FITC) (Sigma) as described previously [15]. For the protocol used in previous studies (in vitro method) [15], bacteria (200 µl) were incubated for 1 h at 37°C in an orbital incubator (100 rpm) with breast milk, infant formula or the synthetic Lewis antigen, washed twice and added to an equal volume of Kato III cells at a ratio of 400 bacteria per cell. After incubation as above, the cells were washed twice by centrifugation at 300 × g for 10 min with PBS and fixed with 1% (v/v) paraformaldehyde. The results were analysed with an EPICS XL flow cytometer (Coulter) as described previously [15]. The results are expressed as binding index (BI) of each sample calculated by multiplying the percentage of fluorescent cells by the mean fluorescence channel value.

For the in vivo approach, breast milk, infant formula preparations, synthetic Lewis a or Lewis b antigens (Dextra) were mixed with cells and bacteria and the experiment carried out as above.

2.5. Purification of total IgA and glycoconjugates containing Lewis a or Lewis b from milk

Cyanogen bromide-activated Sepharose 4B (1 g) (Pharmacia, Uppsala, Sweden, code no. 17-0430-01) was swollen in 10 ml of 1 mM HCl in a universal container, placed on a rotary mixer for 15 min and centrifuged at 50 × g for 10 min. The supernatant was discarded and 20 ml of coupling buffer (0.1 M NaHCO₃ and 0.5 M NaCl, pH 9) was added to the beads. After mixing on a rotary mixer for 10 min at room temperature, the supernatant was discarded. This cycle was repeated twice.

Monoclonal anti-human IgA (300 µl) (Sigma 1-0636) diluted in 5 ml of coupling buffer, affinity-purified and concentrated monoclonal anti-Lewis a (5 ml) or anti-Lewis b (5 ml) from the Scottish Antibody Production Unit (SAPU) were added to the beads. The beads were incubated on a rotary mixer...
at 4°C and centrifuged at 50×g for 10 min. The beads were washed twice with net buffer (0.15 M NaCl, 0.04 M EDTA, 0.04 M Tris, 0.2 mM phenylmethylsulfonyl fluoride, pH 7) by centrifugation at 50×g. Ethanolamine (5 ml, pH 9) was added to the beads to block any remaining active groups and incubated on a rotary mixer for 2 h at room temperature. The beads were centrifuged at 50×g for 10 min and the supernatant discarded. They were washed by centrifugation for 10 min at 50×g with three cycles of buffers alternating acid or alkaline pH: acetate buffer (0.1 M sodium acetate and 1 M NaCl, pH 4) followed by a wash with bicarbonate buffer (0.1 M, pH 8). Aliquots of the pool of milk specimens (5 ml) were added to universal tubes containing Sepharose beads conjugated with anti-IgA, anti-Lewisα or anti-Lewisβ and incubated overnight at 4°C on a rotary mixer. After centrifugation at 50×g for 10 min, the supernatant was kept as unbound material. The beads were washed with two cycles of net buffer. The bound material was eluted from the beads with 1 M acetic acid (5 ml) on a rotary mixer for 20 min at room temperature. After centrifugation at 50×g for 10 min, the eluates were collected and the pH adjusted to 7 following dialysis overnight in PBS. The unbound milk was added to the beads several more times and the process repeated. The eluates were pooled, freeze-dried and the protein concentrations adjusted so the equivalent amounts of each preparation were used in the experiments.

2.6. Affinity purification of antibodies to the S. aureus adhesin that binds Lewisα

The purified adhesin [16] was diluted in coupling buffer (40 μg ml⁻¹) and added to the activated Sepharose beads as described above. After overnight incubation at 4°C the procedure was carried out as described in Section 2.5.

2.7. Protein estimation of the eluates

Protein content of the eluates was determined by the method of Bradford [21] with bovine serum albumin (Sigma) as the standard.

2.8. ELISA for detection of antibodies to the S. aureus adhesin

The wells of microtitre plates (M129B Dynatech, Billinghamurst, Sussex, UK) were coated overnight at 4°C with 100 μl of the affinity-purified adhesin of S. aureus NCTC 10655 that binds Lewisα [16] diluted in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃ and 3 mM NaN₃, pH 9.6) to a final concentration of 4 μg ml⁻¹. The plates were washed with washing buffer containing 0.1% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween-20 in 0.01 M PBS and blocked with blocking buffer 1% BSA (w/v) in PBS for 30 min at room temperature (RT). Defatted milk samples were diluted 1/8 in blocking buffer and 100 μl added to duplicate wells. The plates were incubated at RT for 2 h. The plates were washed, and 100 μl of horseradish peroxidase (HRP)-labelled mouse antibodies to human IgA (Sigma A-0295) or human IgG (Sigma A-6029) diluted 1 in 500 and 1 in 80 respectively in blocking buffer were added to the plates and incubated for 2 h at 37°C. The plates were washed and 50 μl of the substrate ortho-phenylenediamine (OPD) added to the wells. The substrate contained 40 mg in 100 ml of 0.1 M phosphate citrate buffer (0.1 M citric acid, 0.1 M Na₂HPO₄, pH 5), and was activated immediately before use by adding 40 μl 30% (v/v) H₂O₂. The substrate colour change was stopped after 20 min by adding 50 μl of 12.5% (v/v) H₂SO₄. The absorbance at 490 nm (OD₄₉₀) was measured in an ELISA plate reader (Dynatech) and the immunoglobulin concentrations of milk specimens (ng ml⁻¹) were obtained by comparing their absorbance with those obtained for dilutions of IgA and IgG standards (Sigma).

2.9. ELISA to detect Lewisα, Lewisβ and H antigens in human milk and formula

Ten individual milk samples and five infant formula preparations were assessed for the presence of Lewisα and Lewisβ. Plates were coated overnight at 4°C with breast milk or infant formula samples diluted 1 in 10, 1 in 100 and 1 in 1000 in coating buffer. Positive controls of saliva samples were also included on each plate. The plates were washed three
times with washing buffer and 50 μl of blocking buffer was added to each well for 30 min at RT. The plates were washed three times and 50 μl of anti-Lewis a or anti-Lewis b (Scottish National Blood Transfusion Service, SNBTS) both diluted 1 in 10 in blocking buffer was added to the plates for 2 h at RT. The plates were washed three times and 50 μl of HRP anti-mouse IgG (SAPU) diluted 1 in 100 in blocking buffer was added to the plates for 2 h at RT. The plates were then developed as described in Section 2.8. The presence of H antigen in milk was determined by the ELISA method of Rahat et al. [22] with the biotinylated Ulex europaeus lectin.

2.10. Statistical methods

For comparison of inhibition of binding of bacteria by the synthetic Lewis a or Lewis b antigens, the binding index (BI) of cells in each sample was compared with the control by the formula: % inhibition = 100 −[(BI of the sample/BI of the control)×100]. 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 control of cells with bacteria and PBS was given a value of 100% and all other values were expressed as a percentage of the control. Those results with a percentage value below 100 demonstrated inhibition of binding whilst those values greater than 100 demonstrated enhanced binding. The results for both methods were compared for each dilution by Student’s t-test for paired samples.

Levels of Lewis a, Lewis b and IgA specific for the staphylococcal adhesin were assessed in relation to inhibition of binding by Spearman’s correlation coefficient.

3. Results

3.1. Effect of whole and defatted human milk on binding of S. aureus to Kato III cells

Dilutions of human milk ranging from 1 in 10 to 1 in 10000 were tested for their effect on bacterial binding. No significant differences in binding were observed between the 1 in 10000 dilution and the control of bacteria, cells and PBS (data not shown).

<table>
<thead>
<tr>
<th>Dilution</th>
<th>%</th>
<th>Se</th>
<th>95% CI</th>
<th>P</th>
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<tr>
<td>A: in vivo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/10 W</td>
<td>127.6</td>
<td>31.4</td>
<td>47.0-208.3</td>
<td>0.418</td>
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<tr>
<td>1/100 W</td>
<td>176.7</td>
<td>21.3</td>
<td>121.9-231.5</td>
<td>0.016*</td>
</tr>
<tr>
<td>1/1000 W</td>
<td>124.3</td>
<td>15.1</td>
<td>85.5-163.1</td>
<td>0.168</td>
</tr>
<tr>
<td>1/10 D</td>
<td>172.9</td>
<td>36.4</td>
<td>79.2-266.6</td>
<td>0.102</td>
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<tr>
<td>1/100 D</td>
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<td>14.9</td>
<td>52.3-129.0</td>
<td>0.560</td>
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<tr>
<td>1/1000 D</td>
<td>67.4</td>
<td>13.1</td>
<td>33.8-101.1</td>
<td>0.055</td>
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<tr>
<td>B: in vitro</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/10 W</td>
<td>409.5</td>
<td>77.8</td>
<td>209.4-609.6</td>
<td>0.011*</td>
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<td>1/100 W</td>
<td>154.1</td>
<td>25.8</td>
<td>87.7-220.5</td>
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<tr>
<td>1/1000 W</td>
<td>108.4</td>
<td>26.4</td>
<td>40.4-176.4</td>
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<tr>
<td>1/10 D</td>
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<td>43.4</td>
<td>146.8-369.9</td>
<td>0.015*</td>
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<td>1/100 D</td>
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<td>41.5</td>
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<td>0.077</td>
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<tr>
<td>1/1000 D</td>
<td>110.7</td>
<td>22.6</td>
<td>52.7-168.7</td>
<td>0.656</td>
</tr>
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</table>

*Significant differences from control treated with PBS only.

In six experiments, whole and defatted human milk caused enhancement of bacterial binding with both methods (Table 1). Greater enhancement of binding was observed for the in vitro method compared with the in vivo method, and this enhancement effect was reduced with increasing dilutions of human milk. Significant enhancement of binding was observed for the in vivo method with whole milk at the 1 in 100 dilution (P = 0.016) and for the in vitro method with both whole and defatted milk at the 1 in 10 dilution (P = 0.011 and 0.015 respectively).

Paired sample t-tests were carried out to test for significant differences between the methods for the same dilution. For whole milk, the increase in binding was significantly higher with the in vivo method for the 1 in 100 dilution (P = 0.003) and the 1 in 1000 dilution (P = 0.004), but for defatted milk no significant differences between the two methods were observed for any dilution tested. Bacteria incubated with whole milk showed significantly higher binding than those incubated with defatted milk with the in vitro method at the 1 in 100 dilution (P = 0.011) and the 1 in 1000 dilution (P = 0.024) but none were observed for the in vitro method.
3.2. Effects of infant formula preparations on binding of *S. aureus* to Kato III cells

Three different infant formula preparations were investigated for their effect on binding of *S. aureus* to Kato III cells using both methods. There was no significant variation associated with formula type with either method; therefore, the results obtained for each method and for each dilution have been grouped together (Table 2). In contrast to whole and defatted human milk, the results obtained differed considerably. For the in vivo method, infant formula inhibited bacterial binding to the Kato III cells, and this inhibition of binding was reduced with increasing dilutions of the formula. In contrast, with the in vitro method, infant formula caused enhanced bacterial binding to the cells, and this enhancement was also reduced with increasing dilutions of the formula.

A significant reduction of binding was observed for the in vivo method at the 1 in 10 ($P=0.000$) and 1 in 100 ($P=0.000$) dilutions. A significant enhancement of binding was observed for the in vitro method with all three dilutions (for the 1 in 10, $P=0.004$, for the 1 in 100, $P=0.001$ and for the 1 in 1000, $P=0.000$). Significant differences between the in vivo and in vitro methods for the same dilution were found for the 1 in 10 dilution ($P=0.024$) and the 1 in 100 dilution ($P=0.005$).

3.3. Investigation of enhancement of bacterial binding

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

3.4. Detection of Lewis\textsuperscript{a}, Lewis\textsuperscript{b} and H in milk and formula preparations

None of the human milk samples nor the infant formula preparations tested contained material that bound \textit{Ulex} lectin indicating there were no components cross-reactive with H type 1 or H type 2 antigens. The human milk samples (Fig. 2, samples 1–10, diluted 1 in 100) contained components that bound to monoclonal antibodies to Lewis\textsuperscript{a} and Lewis\textsuperscript{b} antigens. The levels detected varied among donors depending on secretor status: women who were non-secretors (samples 2, 5, 6, 10) had very low readings for Lewis\textsuperscript{b}. In contrast to the human milk samples, very low levels of binding to monoclonal anti-Le\textsuperscript{a} and anti-Le\textsuperscript{b} were detected for the infant formulas (samples 11–15, diluted 1 in 100).

3.5. In vitro inhibition of binding by partially purified components of human milk

Fractions collected by affinity purification with anti-IgA, anti-Lewis\textsuperscript{a} or anti-Lewis\textsuperscript{b} inhibited binding of \textit{S. aureus} in experiments using the in vitro method; but there was no significant difference noted for the three preparations. In five experiments, the mean percentage inhibition observed for the three fractions compared with the controls treated with PBS was 38\% for IgA, 53.4\% for Lewis\textsuperscript{a} and 43.2\% for Lewis\textsuperscript{b}. There was no correlation between levels of Lewis\textsuperscript{a} or Lewis\textsuperscript{b} and inhibition of binding by the infant formula preparations. For eight individual milk samples, the only component for which there was a significant correlation with inhibition of binding was the level of IgA specific for the bacterial surface adhesin obtained by affinity purification with Lewis\textsuperscript{a} ($r = 0.8$, $F = 10.43$, $P = 0.025$).

3.6. Inhibition of binding of \textit{S. aureus} by synthetic Lewis\textsuperscript{a} and Lewis\textsuperscript{b} antigens

Dose-response experiments with synthetic Lewis\textsuperscript{a} and Lewis\textsuperscript{b} antigens were carried out using both methods. The dose response curves from three experiments are shown as the mean percentage inhibition of binding (Fig. 3a,b) and the error bars represent the S.E.M.

Different results were obtained with the two methods for inhibition of binding by the synthetic anti-
Higher levels of inhibition were obtained for Lewis\textsuperscript{b} with the in vivo method whilst higher levels of inhibition were obtained for Lewis\textsuperscript{a} with the in vitro method. Significant inhibition of binding was observed for Lewis\textsuperscript{a} between 8 and 10 \( \mu g \) \( ml^{-1} \) 47.3\% (95\% CI 15.5–79.1, \( P = 0.034 \)) and 56.2\% (95\% CI 29.7–82.7, \( P = 0.012 \)) respectively with a plateau of inhibition reached at 10 \( \mu g \) \( ml^{-1} \). For Lewis\textsuperscript{b}, consistent and significant inhibition of binding was observed between 4 and 6 \( \mu g \) \( ml^{-1} \) 42.0\% (95\% CI 7.1–76.8, \( P = 0.035 \)) and 54.7\% (95\% CI 21.0–88.4, \( P = 0.020 \)) with a plateau reached at 6 \( \mu g \) \( ml^{-1} \).

**4. Discussion**

Epidemiological studies found there was a reduced risk for SIDS in breast-fed infants which persisted during the first 6 months after controlling for confounding demographic, maternal and infant factors [1]. In recent studies in Britain, the evidence was not as clear. Compared with fully breast-fed babies, the crude odds ratio for SIDS in fully bottle-fed babies was 3.1 and for mixed breast- and bottle-fed infants 1.5. Ratios fell to 1.8 and 1.2 respectively after adjustment for maternal smoking, parental employment, pre-term gestation and sleeping position. The conclusion of the British study was that bottle feeding was not a significant independent risk factor [23]. These results have, however, been criticised on the basis of design and small numbers [24]. In our longitudinal study of mothers and their infants, smoking and formula feeding were significantly higher among lower socioeconomic groups [11].

Staphylococcal toxins have been identified in tissues of SIDS infants [12–14] and we have found a significantly higher isolation rate for *S. aureus* among SIDS infants 3 months of age or younger compared with healthy infants [11]. Our longitudinal epidemiological studies on nasopharyngeal flora of infants found no association between frequency of isolation of *S. aureus* with socioeconomic circumstances of the family, method of feeding or maternal smoking [11]. The objectives of the present study were to determine if human milk was better able to reduce the density of colonisation of epithelial cells by *S. aureus* compared with infant formula and to determine if oligosaccharides which act as receptors...
for *S. aureus* or antibodies to the adhesin that binds to Lewis*α* could inhibit bacterial binding.

4.1. Methodology

Kato III cells, an epithelial cell line, were chosen for use in the model system to minimise differences in expression of H and Lewis antigens noted for individual donors in other studies [20]. Most studies on inhibition of bacterial binding are carried out by the in vitro protocol in which the bacteria or the cells are treated with the inhibitory substance, the excess washed away and the binding assays carried out. This study found that there were significant differences in results obtained by the two protocols. The in vivo method is thought to reflect more closely the interactions between mucosal cells, bacteria and the human or formula milk. The in vitro method was more useful for identifying components involved in binding.

4.2. Effects of human milk and formula on bacterial binding

The effects of whole and defatted milk on bacterial binding were similar with both methods. Enhancement of binding rather than inhibition 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 in which there was more time for bacteria to aggregate before addition to the cells. For infant formula, significantly different results were obtained with the two methods. The in vivo method showed consistent inhibition of binding regardless of formula type whilst the in vitro method showed enhancement of binding due to bacterial aggregation. From the results obtained in this study, breast milk can be suggested to have a protective effect due to the aggregation. The factor(s) responsible for this aggregation effect was present in both whole and defatted milk. Bacterial aggregates are more readily engulfed by phagocytes than single bacteria which would result in greater bacterial clearance. If the in vivo method reflects conditions on the mucosal surface, formula preparations might reduce the numbers of bacteria attaching to the epithelium.

4.3. Components contributing to reduction of bacterial binding

The infant formula preparations did not contain IgA or IgG. Neither human milk nor infant formula preparations contained oligosaccharides that bound to the *Ulex* lectin (H antigens). Both human milk and formula preparations contained components that bound to the monoclonal antibodies specific for Lewis*α* and Lewis*β* although breast milk samples contained much higher levels.

There is increasing evidence that oligosaccharides and glycoconjugates present in human milk can reduce the ability of bacteria to colonise epithelial surfaces by acting as receptor analogues and binding to bacterial adhesins [19]. In this study, a dose-response effect was observed for inhibition of binding of *S. aureus* by the synthetic Lewis*α* and Lewis*β* antigens. Higher levels of the Lewis antigens were required to cause maximum inhibition of binding using the in vivo method compared to the in vitro method. With the in vivo method, the bacteria can bind either to the Lewis antigen expressed on the cell surface or to the Lewis antigen in solution; however, with the in vitro method, direct blocking of the bacterial adhesin occurs before addition to the cells.

For human milk, both IgA fractions and the fractions obtained by affinity purification with anti-Lewis antibodies were able to inhibit bacterial binding. The components obtained with the affinity methods were not pure. By ELISA, Lewis antigens could be detected in the material eluted from IgA, and IgA was detected in the material eluted from Lewis*α* and Lewis*β*. Although a dose-response effect was observed for inhibition with the synthetic Lewis antigens, there was no correlation between the levels of these antigens in individual human milk samples and their inhibitory effect. For individual samples of milk, the only significant correlation with inhibition of binding for an individual component was the level of IgA specific for the adhesin purified by affinity chromatography with Lewis*α*. Other workers have shown that free oligosaccharides purified from low-molecular-mass fractions of human milk can inhibit the ability of *S. pneumoniae* and *H. influenzae* to bind to human pharyngeal or buccal epithelial cells [3]. The Lewis antigens, including Lewis*α* (CD15), have been demonstrated to act as epithelial cell re-
ceptors for S. aureus and Bordetella pertussis [25] and synthetic Lewis$^a$ and Lewis$^b$ significantly inhibited binding of Clostridium perfringens to Kato III cells [26].

Antibodies to the adhesin of S. aureus in breast milk might reduce the density of nasopharyngeal colonisation; it does not, however, affect frequency of colonisation since there was no difference in isolation rate of S. aureus from breast-fed compared with bottle-fed babies [11].

4.4. Conclusions

In contrast to our original hypothesis, human milk did not reduce bacterial binding in experiments carried out by either the in vivo or in vitro method. This suggests that aggregation of bacteria might play a major role in the protection afforded by breast feeding. Infant formula was found to have inhibitory activity in the in vivo experiments but enhanced bacterial binding in the in vitro ones. These studies indicate that the method used for binding studies needs to be considered in planning experiments. The in vitro method is used most widely and is valuable for assessment of individual components for inhibitory activity. The in vivo method was developed to assess the complex interactions at mucosal surfaces. Density of colonisation is an important factor in development of bacterial diseases due to invasion or toxin production [27]. Our studies suggest that both human milk and infant formula have oligosaccharide components that might reduce binding of S. aureus to epithelial cells but IgA antibody against the S. aureus adhesin is present only in breast milk. The possibility of enhancing the inhibitory activity of infant formula preparations by addition of synthetic Lewis antigens is being explored.

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