HOST DEFENCE MECHANISMS

Immunoglobulin and non-immunoglobulin components of human milk inhibit *Clostridium difficile* toxin A-receptor binding

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Summary. *Clostridium difficile* is isolated from the intestinal tracts of > 50% of healthy infants. The mechanism by which intestinal colonisation of infants by toxigenic *C. difficile* is generally asymptomatic is unknown but may reflect the presence in human milk of neutralising activity against *C. difficile* toxin A. On this basis, the ability of human milk to inhibit the binding of toxin A to a purified hamster brush border membrane receptor was determined. Ten milk samples from healthy volunteers in various stages of lactation inhibited the binding of toxin A to the receptor by an average of 90%. Heating and dialysis did not significantly alter the inhibitory activity of any of the milk samples. Human milk protected adult hamsters against a lethal challenge with toxin A but had no effect on the cytotoxic activity of the toxin. SDS-PAGE and ligand blot analyses showed that there were at least four distinct factors in human milk that specifically bound toxin A. Thiophilic adsorption chromatography was used to separate immunoglobulin from non-immunoglobulin components of human milk. IgA was the only immunoglobulin detected in human milk and > 90% of this immunoglobulin was recovered after purification by thiophilic adsorption. Both the unbound non-immunoglobulin and bound immunoglobulin fractions of human milk inhibited the binding of toxin A to the purified receptor. These results suggest that human milk may be important in protecting infants against *C. difficile*-associated intestinal disease.

Introduction

Clostridium difficile-induced antibiotic-associated intestinal disease is toxin mediated. Pathogenic strains of *C. difficile* produce two protein exotoxins: toxin A, an enterotoxin, and toxin B, a cytotoxin. These two toxins are antigenically, physically and biologically distinct.^{1,2} Toxin A, a 308-kDa protein, is believed to cause both the diarrhoea and the destruction of the colonic mucosa which is characteristic of intestinal disease caused by *C. difficile*.^{2,3} Toxin B appears to have no effect on undisturbed colonic mucosa.^{3,4}

During the last few years investigators have noted that enteric disease does not develop in all individuals who harbour toxigenic *C. difficile* and cytotoxin in their intestinal tracts. In adults, intestinal colonisation with *C. difficile* frequently leads to disease ranging in severity from a mild, self-limiting diarrhoea to a life-threatening fulminant pseudomembranous colitis

(PMC).^{5,6} On the other hand, *C. difficile* has been isolated from the faeces of > 50% of healthy neonates and infants under 2 years of age.^{7–9} Although there are some reports of *C. difficile* disease in infants, this is frequently associated with some debilitating condition.^{10,11} Carrier rates for *C. difficile* fall sharply after 1 year of age, although in the second year they are still significantly higher than the average 2% carrier rate in healthy adults.^{8,9} Concentrations of *C. difficile* and toxins A and B in the faeces of healthy infants are frequently similar to concentrations found in the intestinal tracts of adults with clinical manifestations of *C. difficile*-associated intestinal disease.^{7–9,12}

It has been suggested that asymptomatic *C. difficile* colonisation of infants may reflect the presence of neutralising activity in human milk against *C. difficile* toxin A.^{9,11} Human milk may protect against *C. difficile* colitis by inhibiting the binding of toxin A to its intestinal epithelial receptor. The binding of toxin A to specific intestinal brush border receptors is undoubtedly one of the initial events in toxin A-mediated

colitis. Numerous studies have shown that human milk protects the neonatal gastrointestinal tract against various diseases caused by infectious agents such as *Salmonella* and *Shigella* spp., *Vibrio cholerae*, *Escherichia coli*, poliovirus and rotavirus.¹³⁻¹⁸ The protective capacity of milk is based on various immunological and non-immunological factors.¹⁹ Immunological factors in human milk which may protect against enteric pathogens include immunoglobulins (predominately IgA) and other factors such as lysozyme, lactoferrin, the lactoperoxidase system, and cellular components (macrophages and lymphocytes). Antibodies against toxin A in human milk may protect against *C. difficile*-associated intestinal disease by inhibiting or modifying the binding of toxin A to its intestinal receptor, thereby preventing its toxic activity. Protection by human milk can also be ascribed to non-immunological components such as glycolipids, glycoproteins and free oligosaccharides.²⁰⁻²⁴ These components may function as structural analogues of cell membrane toxin A receptors and competitively inhibit toxin A binding to its intestinal target receptor.

Therefore, these investigations were designed to characterise the ability of whole human milk, and the immunoglobulin and non-immunoglobulin components of human milk, to inhibit toxin A binding to an intestinal brush border membrane receptor.

Materials and methods

Purification and radiolabelling of C. difficile toxin A

C. difficile toxin A was purified from Brain Heart Infusion Broth (BBL Microbiology Systems, Cockeysville, MD, USA) filtrates of dialysis-bag cultures of a highly toxigenic strain of *C. difficile* (VPI strain 10463; Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA) by previously described techniques.^{3, 25}

Purified toxin A was radiolabelled enzymically by the Enzymobead lactoperoxidase-glucose oxidase (BioRad Laboratories, Richmond, CA, USA) method.²⁶ The average specific activity for radiolabelled toxin A was 4×10^8 cpm/ μ g. Radiolabelled toxin A retained > 90% of its biological activity as demonstrated by cytotoxicity in HeLa tissue culture cells, mouse lethality (after intraperitoneal inoculation), hamster lethality (after intraperitoneal inoculation) and induction of hamster ileocaecitis (after intracaecal inoculation).²⁶

Purification of the hamster brush border membrane receptor for toxin A

Brush border membranes (BBMs) were prepared from the small intestines of 7-day-old hamsters according to methods described previously.²⁶ The degree

of purification of the BBMs was evaluated morphologically by examination under phase-contrast microscopy and biochemically by comparing the specific activities of sucrase, lactase and alkaline phosphatase with those activities in the initial homogenate.²⁶ A toxin A receptor was purified from the isolated infant hamsters BBMs by a combination of Triton X-114 extraction and affinity chromatography.²⁷

Binding of toxin A to the brush border membrane receptor

Specific binding of toxin A to the BBM receptor was monitored by measuring the amount of [¹²⁵I]toxin A bound to the receptor absorbed to plastic wells of microtitration plates (Immunolon II; Dynatech Laboratories, Inc., Alexandria, VA, USA).²⁷

Human milk

Human milk was obtained from healthy volunteers in various stages of lactation, who were not taking antibiotics or other medication at the time of donation. The milk was centrifuged (10000 g for 15 min at 4°C) and the clear middle layer was separated from the upper and lower layers, which contained fat and cells, respectively. The middle layer was passed through a membrane filter (pore size, 0.45 μ m) to remove residual cells and fat. Filtrates were dialysed against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.05 M NaCl (Tris-HCl buffer) and stored at -20°C. In some experiments, the milk from 10 individuals was pooled and then centrifuged, membrane filtered and dialysed as described above.

Inhibition by human milk of toxin A binding was determined by diluting milk in Tris-HCl buffer. The diluted milk was then mixed with an equal volume of [¹²⁵I]toxin A (500 ng/ml) and incubated for 2 h at room temperature. The human milk-toxin A mixture was then assayed for toxin A binding activity in the microtitration well binding assay.

Characterisation of the inhibitory activity against toxin A binding

Heat stability. For tests of heat stability, dialysed fractions of human milk were diluted 1 in 4 in Tris-HCl buffer and heated for 5 min at 100°C. After heating, the samples were examined for inhibitory activity against toxin A binding as described above.

Ultrafiltration. For estimation of the size of the inhibitory factors, a 1 in 4 dilution of pooled human milk in Tris-HCl buffer was subjected to positive pressure ultrafiltration in a concentrator (Amicon Corporation, Danvers, MA, USA) with a 100-kDa cut-off membrane. The volumes of the retentate (> 100 kDa) and filtrate (< 100 kDa) were adjusted to the volume of the initial milk sample with Tris-HCl buffer.

Gel filtration. Ascending chromatography, per-

formed in a column (2.0 × 20 cm) of Sepharose CL-6B (Pharmacia Fine Chemicals, Inc., Piscataway, NJ, USA), was used to characterise the interaction between toxin A and human milk. [¹²⁵I]toxin A (15 µg/ml) was mixed with an equal volume of pooled human milk or Tris-HCl buffer and incubated for 2 h at room temperature; 1 ml of the mixture was applied to the column and eluted with Tris-HCl buffer at room temperature. Each 1.0-ml fraction was monitored for γ emissions. The *K_{av}* values of standard proteins (Pharmacia Fine Chemicals) was related to that of [¹²⁵I]toxin A.

Native polyacrylamide gel electrophoresis. The interaction of [¹²⁵I]toxin A with human milk was also characterised by polyacrylamide gel electrophoresis (PAGE) with a 7.5% w/v lower gel and an upper stacking gel.²⁸ [¹²⁵I]toxin A (178 µg/ml) was mixed with an equal volume of pooled human milk or Tris-HCl buffer and incubated for 2 h at 4°C; 50 µl of the mixture was added to the top of the polyacrylamide gel and electrophoresis was performed at 4°C with a constant current of 30 mA/gel until the tracking dye was c. 1 cm from the end of the gel. Gels were dried and exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY, USA) at -70°C for 4–6 h with an intensifying screen.

Neutralisation of toxin A cytotoxicity. Pooled human milk was examined for its ability to neutralise the cytotoxic activity of toxin A in a HeLa cell tissue culture assay. Toxin A was diluted in Tris-HCl buffer to a concentration four-fold greater than the highest dilution that caused complete rounding of 100% of the HeLa cells in a microtitration well (TCD₁₀₀). The TCD₁₀₀ for toxin A was 150 ng of protein. The diluted toxin A was mixed with an equal volume of pooled human milk or Tris-HCl buffer. After incubation at room temperature for 2 h, 50 µl of each mixture was added to a microtitration well containing a monolayer of HeLa cells and 200 µl of cell culture medium. The HeLa cells were prepared as described previously.²⁵ At 24 and 48 h after exposure to toxin, each well was examined for evidence of cytotoxicity (increased refractility, change in morphology, or partial loss of adherence).

Neutralisation of hamster lethality. Adult golden Syrian hamsters (Sasco, Omaha, NE, USA) were housed individually in a conventional animal room and fed Purina Laboratory Rodent Chow 5001 (Ralston Purina, Richmond, IN, USA) and water *ad libitum*. (These investigations were approved by the Texas Tech University Health Sciences Center Animal Care and Use Committee.)

Human milk was tested for protection of adult hamsters against intragastric challenge with toxin A as follows. A lethal dose of toxin A (100 µg/ml) was mixed with an equal volume of undiluted pooled human milk or 50 mM phosphate buffered saline (PBS), pH 7.2, and incubated at room temperature for 2 h before intragastric inoculation (1 ml/100 g body weight) of adult hamsters. Hamsters were not per-

mitted to feed for 12 h before challenge with toxin A. Stomach acidity was neutralised with 1 M carbonate buffer (1 ml/100 g of body weight) administered orogastrically immediately before toxin A challenge, and drinking water was withdrawn 4 h before NaHCO₃ treatment. After treatment, hamsters were observed every 8 h for evidence of diarrhoea or moribund condition. Moribund animals were killed immediately.

Thiophilic chromatography removal of immunoglobulins in human milk

A thiophilic adsorbent column (divinyl sulphone-activated agarose) was used to separate the non-immunoglobulin from the immunoglobulin fractions of human milk according to the manufacturer's instructions (T-Gel Purification Kit; Pierce, Rockford, IL, USA). Briefly, 1 ml of pooled human milk was added to 87 mg of K₂SO₄. The sample was loaded on to a 3-ml column of T-Gel adsorbent and washed with T-Gel binding buffer (0.5 M K₂SO₄, 50 mM Na₂PO₄ and sodium azide 0.05% w/v, pH 8.0) at room temperature. The unbound eluate was recirculated three times through the column to further deplete it of residual immunoglobulin. The specifically bound material was eluted with T-Gel elution buffer (50 mM Na₂PO₄ and sodium azide 0.05% w/v, pH 8.0). Fractions (2 ml) of the unbound (immunoglobulin-depleted human milk) and bound (isolated immunoglobulins from human milk) eluates were monitored for absorbance at 280 nm. The unbound and bound fractions were pooled, dialysed against Tris-HCl buffer and concentrated to the starting volume by ultrafiltration with a 10-kDa cut-off membrane filter.

Quantitation of human immunoglobulin

Quantitation of human immunoglobulins in the pooled milk and in the bound and unbound fractions from the thiophilic adsorption gel was performed by single radial immunodiffusion (RID) in plates prepared by the Binding Site (San Diego, CA, USA) which were specific for IgA, IgM and IgG.²⁹ Known concentrations of human immunoglobulins were used to produce linear calibration curves. The immunoglobulin concentrations in the milk samples were determined from the calibration curves.

SDS-PAGE of milk samples

Samples to be analysed by SDS-PAGE were diluted with an equal volume of 0.25 M Tris buffer, pH 6.8, containing SDS 4% w/v and 2-mercaptoethanol 10% v/v. The samples were heated at 100°C for 5 min and centrifuged at 13000 *g* for 1 min. The supernates were then subjected to SDS-PAGE with a 7.5% w/v lower gel and an upper stacking gel 1.5 mm thick, essentially as described by Laemmli.³⁰ After electrophoresis, the gels were fixed and stained with Coomassie Brilliant Blue as described previously.³¹

Fig. 1. Effect of human milk on [125 I]toxin A binding to the BBM receptor: untreated milk (■); milk heated at 100°C for 5 min before addition of toxin A (▨); milk dialysed against Tris-HCl buffer before addition of toxin A (▤); milk added to a microtitration well containing BBM receptor, incubated and washed before addition of toxin A to the well (□). Each bar represents the mean of three experiments. SDs were < 10% of the mean.

Fig. 2. Gel filtration analysis of [125 I]toxin A-receptor interaction. [125 I]toxin A was mixed with pooled human milk (○—○) or Tris-HCl buffer (●—●) and incubated for 2 h at room temperature, and separated on a Sepharose CL-6B column eluted with Tris-HCl buffer. Arrows indicate the approximate migration of mol. wt (kDa) markers. V_o , void volume (≥ 1000 kDa).

Mol. wts were estimated by comparison to known mol. wt markers (BioRad Laboratories).

Autoradiography of toxin A-milk interactions

The SDS-PAGE-separated preparations of human milk were transferred electrophoretically to nitrocel-

lulose membranes (Hoefer Scientific Instruments, San Francisco, CA, USA; 0.45 μ m pore size) essentially by the procedure of Towbin.³² After transfer, the membranes were rinsed in PBS, blocked with bovine serum albumin (BSA) 3% w/v and newborn calf serum 1% v/v and overlaid with [125 I]toxin A.²⁷ The membranes were then washed and dried at room temperature.

Autoradiography was performed by exposing the nitrocellulose membranes to X-OMAT AR film at -70°C for 18–24 h with an intensifying screen.

Protein determination

Protein content was determined by the method of Lowry *et al.*³³ with BSA as the standard.

Results

Inhibition of toxin A binding by milk

The cell-free aqueous phases of 10 human milk samples inhibited the binding of toxin A to the purified BBM receptor by an average of 90 % (range: 80–96 %) (fig. 1). In the absence of milk, 1 ng of purified BBM receptor bound *c.* 12 pg of radiolabelled toxin A. Heating (100°C for 5 min) and dialysis (8–10-kDa cut-off) did not significantly alter the inhibitory activity in any of the milk samples. Pre-incubation of the BBM receptor with human milk had minimal effect on toxin A binding as long as the milk was removed by washing

before addition of [^{125}I]toxin A, indicating that inhibition of toxin A binding was due to the interaction of milk with toxin A and not the BBM receptor.

Characterisation of the inhibitory activity

The cell-free aqueous phase preparations from the 10 milk samples were pooled for further characterisation. Ultrafiltration through a 100-kDa cut-off membrane filter demonstrated that the aqueous material with a mol. wt > 100 kDa completely inhibited toxin A binding whereas material with a mol. wt < 100 kDa had no effect on binding of toxin A.

The interaction of [^{125}I]toxin A with human milk was investigated by gel filtration and native PAGE to characterise the mechanism of toxin A binding inhibition. Fig. 2 shows that [^{125}I]toxin A by itself has a mol. wt of *c.* 300 kDa as determined by gel filtration. This agrees closely with the published mol. wt of 308 kDa for toxin A.³⁴ However, when [^{125}I]toxin A was mixed with pooled human milk, incubated for 2 h at room temperature and separated on a Sepharose CL-6B column, the mol. wt of [^{125}I]toxin A increased to *c.* 600 kDa. These data suggest that the factors present in human milk which inhibit toxin A binding are complexing with the toxin rather than breaking the toxin into inactive subunits. However, it is also possible that human milk is causing toxin A to complex with itself under these conditions.

The complexing of toxin A with factors present in human milk was further substantiated by native PAGE analyses. [^{125}I]toxin A by itself migrated *c.* 1 cm into an acrylamide 7.5 % w/v gel during electrophoresis (fig. 3, lane A). However, [^{125}I]toxin A remained close to the top of the gel when mixed with pooled human milk and incubated for 2 h at room temperature before electrophoresis in an acrylamide 7.5 % w/v gel (fig. 3, lane B).

To better characterise the factors in human milk complexing with toxin A, the binding of [^{125}I]toxin A to transblots of electrophoretically separated components of human milk was investigated. In these experiments, pooled human milk was separated by SDS-PAGE under reducing conditions. One gel was stained with Coomassie Brilliant Blue whereas the separated proteins on the other gel were transferred to nitrocellulose and probed with [^{125}I]toxin A before visualisation by autoradiography. Fig. 4 (lane B) shows that there were at least four distinct bands from human milk that specifically bound [^{125}I]toxin A. These bands corresponded to mol. wts of *c.* > 200 , 112, 71 and 56 kDa. None of these toxin A binding bands could be associated with dominant protein bands on the corresponding gel (fig. 4, lane A) suggesting that carbohydrate moieties play a dominant role in binding toxin A.

Inhibition of toxin A biological activity by human milk

The effect of pooled human milk on the biological activity of toxin A in the adult hamster and in tissue

Fig. 3. Autoradiograph of native PAGE analysis of toxin A interaction with human milk. [^{125}I]toxin A was mixed with an equal volume of Tris-HCl buffer (lane A), pooled human milk (B), non-immunoglobulin fraction of human milk (C) or immunoglobulin fraction of human milk (D) and incubated for 2 h at 4°C before PAGE.

Fig. 4. SDS-PAGE and ligand blot analysis of human milk. Pooled human milk (lanes A and B), non-immunoglobulin fraction of human milk (C and D) and immunoglobulin fraction of human milk (E and F) were separated by SDS-PAGE under reducing conditions. One gel was stained for protein (Lanes A, C and E); the other gel was transferred to nitrocellulose and probed with [125 I]toxin A before visualisation by autoradiography (B, D and F). Arrows indicate the migration of mol. wt markers.

culture was also tested. All 10 hamsters that received a lethal dose of toxin A ($50 \mu\text{g}/100 \text{ g}$ body weight) died within 48 h of intragastric challenge; 60% of these hamsters had evidence of diarrhoea at the time of death while the remaining 40% had no diarrhoea. However, all the dead animals had evidence of ileocaecitis (i.e., caeca that were enlarged, fluid filled and haemorrhagic). Only one (7%) of 15 hamsters died when the same final dose of toxin A was mixed with human milk and incubated for 2 h at room temperature before intragastric challenge. Interestingly, 67% of the hamsters that received the human milk-toxin A mixture developed diarrhoea within 24 h of intragastric challenge. However, in all instances the diarrhoea disappeared within 72 h after intragastric challenge.

The pooled human milk did not protect HeLa tissue culture cells against the cytotoxic activity of toxin A. Mixing a toxin A solution (150 ng/ml) with an equal volume of tissue culture medium or human milk and incubating for 2 h at room temperature before inoculation of the monolayers resulted in actinomorphic changes in 100% of the HeLa cells within 24 h.

Thiophilic adsorption of immunoglobulins from human milk

Human milk was divided into immunoglobulin and non-immunoglobulin fractions by thiophilic adsorption chromatography. The bound immunoglobulin

and unbound non-immunoglobulin fractions were pooled and concentrated to the original starting volume. IgA was the only immunoglobulin present in the human milk and was present at a concentration of 985 mg/L . This concentration is in close agreement with the amount of sIgA reported to be present in human milk at 1–6 months of lactation;³⁵ > 90% of this immunoglobulin was recovered in the bound fractions of the thiophilic adsorption column whereas the unbound fractions did not contain detectable immunoglobulin. Both the unbound non-immunoglobulin T-Gel fraction of human milk and the T-Gel bound immunoglobulin fraction of human milk inhibited the binding of [125 I]toxin A to the BBM receptor (fig. 5). The inhibitory activity in the non-immunoglobulin fraction was heat stable (100°C for 5 min) but heating caused *c.* 25% loss of inhibitory activity in the immunoglobulin fraction.

Native PAGE analysis of toxin A interaction with the bound and unbound fractions from the thiophilic adsorption column is shown in fig. 3. As was demonstrated previously with whole milk (fig. 4, lane B) the unbound (lane C) and bound (lane D) fractions complexed with toxin A, as was evident through the apparent increase in mol. wt of [125 I]toxin A.

The immunoglobulin and non-immunoglobulin fractions of human milk were also characterised by SDS-PAGE and ligand blotting with [125 I]toxin A. Ligand blot analysis demonstrated that the > 200- and 56-kDa components that bound toxin A in human

Fig. 5. Inhibition of toxin A binding to the purified BBM receptor by whole human milk and milk fractionated by thiophilic absorption; whole milk and both fractions were examined before (■) and after (▨) heating at 100°C for 5 min. Each bar represents the mean of three experiments. SDs were < 10% of the mean.

milk appeared in the non-immunoglobulin fraction whereas the immunoglobulin fraction contained the 112-, 71- and 56-kDa components (fig. 4).

Discussion

Several studies have shown that human colostrum passively protects the neonatal gastrointestinal surface against enteric pathogens during the period before active development of intestinal immunity.^{13, 14, 36, 37} It has been reported that breast-fed infants are less likely to be colonised with *C. difficile* than formula-fed infants.^{7, 8, 11} In this study, human milk inhibited the binding of toxin A to a receptor purified from infant hamster brush border membranes. Binding of secreted enterotoxins to specific cell-membrane receptors are obligatory pathogenic events in diarrhoeal diseases due to enterotoxigenic bacteria. The toxin A receptor from infant hamsters was used in these investigations because of the difficulty in obtaining healthy intestinal tissue from human infants. Furthermore, the infant hamster has been used to elucidate the mechanism(s) of asymptomatic *C. difficile* intestinal colonisation³⁸ and it has been shown that infant hamsters have an age-dependent susceptibility to asymptomatic enteric *C. difficile* colonisation similar to the restricted age distribution of human neonatal asymptomatic colonisation. Administration of *C. difficile* to non-antibiotic-treated infant hamsters resulted in multipli-

cation of the organism in the intestinal tracts of 4–13-day-old animals. These infant hamsters did not display evidence of toxicity despite toxin titres similar to the titres found in the intestinal tracts of adult hamsters with *C. difficile*-associated disease.

The purified toxin A receptor used in these investigations is a galactose- and N-acetylglucosamine-containing glycoconjugate that appears to be identical to the toxin A receptor reported on adult hamster and rabbit small intestine BBMs.^{26, 39, 40} This receptor involves the trisaccharide sequence Gal α 1-3Gal β 1-4GlcNAc on the reducing end of glycoconjugates.^{39, 41} Both infant and adult BBMs have this receptor and differences in the binding kinetics of toxins A or B, or both, to this receptor do not account for the observed differences in the animals' susceptibility to *C. difficile*-associated intestinal disease.²⁶ Several lines of evidence support the likelihood that the receptor used in the binding studies of this investigation is the physiologically functional receptor for toxin A in hamsters: (1) cell lines that produce large amounts of this trisaccharide receptor are more sensitive to toxin A-induced cytotoxicity than are cell lines that lack detectable receptor activity;⁴² (2) hamsters can be protected from *C. difficile*-associated intestinal disease by immunisation with a recombinant peptide that contains the portion of toxin A that binds to the trisaccharide;⁴³ (3) monoclonal antibody that blocks specific binding of toxin A to the trisaccharide also neutralises its biological activity;⁴⁴ and (4) the lectin

produced by *Bandeirea simplicifolia* that binds the trisaccharide blocks the binding of toxin A to cells in culture and protects them from the cytotoxic activity of toxin A.⁴⁵

The inhibitory activity present in human milk against toxin A binding was stable at 100°C for 5 min and restricted to compounds > 100 kDa. The significance of this inhibitory activity *in vivo* was demonstrated in experiments in which human milk protected adult hamsters against a lethal intragastric challenge with toxin A. Other investigators have also demonstrated the neutralisation of toxin A *in vivo* by human milk or milk products. Kim *et al.*⁴⁶ demonstrated that approximately one-third of the human colostrals samples examined reduced toxin A-induced fluid accumulation in suckling mice. Lysterly *et al.*⁴⁷ showed that hamsters treated prophylactically with orally administered hyperimmune bovine IgG concentrate prepared from colostrum at parturition were protected against *C. difficile* disease. Kim *et al.*²⁵ demonstrated that hamsters immunised against toxins A and B are protected against clindamycin-induced *C. difficile*-associated ileocaecitis and that this protection is transferred to infant hamsters through maternal milk.

Even though human milk inhibited toxin A binding to the BBM receptor and protected hamsters against a lethal intragastric challenge with toxin A, it did not prevent the cytotoxic activity of toxin A against HeLa tissue culture cells. However, other investigators have reported neutralisation of toxin A cytotoxic activity by human milk. For example, Wada *et al.*⁴⁸ reported that c. 25% of colostrals samples from Japanese women neutralised the cytotoxic activity of crude *C. difficile* toxins. However, no attempt was made by these investigators to examine specific neutralising activity against individual toxins A and B. Kim *et al.*⁴⁶ reported that approximately one-third of colostrals samples tested neutralised the cytopathic effect of *C. difficile* toxins A and B. These investigators concluded that IgA played a major role in neutralisation of toxin A cytotoxicity because immune precipitation of the human milk samples with anti-human IgA abolished the neutralising activity.

Human milk contains various specific antibodies and non-immunoglobulin factors that could contribute toward neutralisation of toxin A binding. Therefore, thiophilic adsorption chromatography was used to separate the immunoglobulin from the non-immunoglobulin components of human milk. The thiophilic gel is a β -mercaptoethanol derivative of divinyl sulphone-activated agarose which has been used for both the selective removal and purification of all classes of immunoglobulins from serum, ascites fluid, hybridoma cell culture media and human milk.^{49,50} In these investigations, both the immunoglobulin and non-immunoglobulin fractions of human milk inhibited toxin A binding to the purified BBM receptor. Radial immunodiffusion indicated that IgA was the only immunoglobulin detectable in human milk and was therefore, responsible for the inhibition of binding

of toxin A in the immunoglobulin component of human milk. Other investigators have also demonstrated inhibition of toxin A binding by IgA. Kelly *et al.*⁵¹ showed that colonic aspirates containing IgA antitoxin inhibit the binding of toxin A to rabbit ileal brush border membranes whereas such inhibition was not seen in the presence of colonic aspirates that did not contain IgA antitoxin.

The finding that the inhibitory activity against toxin A binding in the non-immunoglobulin fraction was heat stable and caused an apparent increase in the mol. wt of radiolabelled toxin A suggests that the inhibitory component may be a glycoconjugate toxin A receptor analogue. Recent studies have shown an array of oligosaccharides and glycoconjugates in human milk that act as receptor analogues that can inhibit the binding of certain enteric bacterial pathogens and their toxins.^{20,52-55} For example, receptor-like components in the non-immunoglobulin components of human milk have been shown to inhibit the action of *E. coli* and *V. cholerae* enterotoxins *in vivo* and *in vitro*.^{52,53,55-58} Toxin A binds to the carbohydrate antigens designated I, X and Y which are present on the intestinal epithelium of infants and adults.⁵⁹⁻⁶² These antigens are conformationally very similar to the trisaccharide sequence Gal α 1-3Gal β 1-4GlcNAc present on hamster BBMs.⁵⁹ The I, X and Y antigens have been proposed as receptors for toxin A in the human intestinal tract. Human milk is known to contain > 100 fucosylated oligosaccharides, including the X antigen.^{21,23,24,63} Further work must be performed to determine if the X antigen in breast milk is inhibiting toxin A binding in these investigations. It is interesting to note that the X antigen also occurs on the secretory component of human antibodies.⁶⁴ The secretory component has a mol. wt of 79 kDa which is the approximate mol. wt of one of the bands in the immunoglobulin fraction of human milk which bound to toxin A in the ligand blotting analyses. Therefore, the neutralisation of toxin A binding in the immunoglobulin fraction of human milk may be the result of non-specific interaction of sIgA with toxin A.

These data indicate that human milk may protect against *C. difficile*-induced intestinal disease in infants by inhibiting the binding of toxin A to its intestinal receptor. The specific neutralisation of toxin A binding by human milk would allow for the continued presence of *C. difficile* as well as toxin B in the intestine of asymptomatic infants. Both immunoglobulin and non-immunoglobulin components may be responsible for neutralisation of toxin A binding. Infant formula based on cow's milk also inhibits the binding of toxin A to the intestinal brush border membrane (unpublished observations). This could be the mechanism by which formula-fed infants are asymptotically colonised with toxigenic *C. difficile*. In the future, detailed structural determination of the components in human and cow's milk that inhibit toxin A binding will be required. Once these protective factors are understood it may be possible to enhance not only the resistance of

newborn infants to *C. difficile* toxins but other age groups as well.

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