HOST RESPONSE TO INFECTION

Binding of Clostridium difficile toxin A to human milk secretory component

S. D. DALLAS and R. D. ROLFE

Department of Microbiology and Immunology, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

Toxigenic Clostridium difficile is isolated from a majority of healthy human infants. The exact mechanism of asymptomatic colonisation is unclear; however, previous studies in this laboratory have shown that components of both the immunoglobulin and non-immunoglobulin fractions of human milk bind to toxin A and prevent its interaction with hamster intestinal brush border membranes (BBMs). Secretory IgA (sIgA) is the primary immunoglobulin found in human milk. As sIgA resists digestion in the infant stomach and passes at high levels into the colon, its ability to bind toxin A was the subject of this investigation. Purified sIgA in concentrations at and below those found in human milk inhibited the binding of toxin A to purified BBM receptors. Heating sIgA to 100°C for 5 min did not affect its inhibitory activity. IgM, IgG and serum IgA did not appreciably inhibit the binding of toxin A to BBM receptors. SDS-PAGE separated sIgA into three major bands: secretory component, heavy chains and light chains. Autoradiography with radiolabelled toxin A revealed that toxin A bound to the secretory component (SC) of sIgA. When the three purified subunits of sIgA were coated on to microtitration wells, SC bound significantly more toxin A than the heavy or light chains of sIgA. Purified SC also inhibited toxin binding to receptors in a dose-dependent fashion similar to sIgA. The heavy and light chains of sIgA did not inhibit toxin A receptor binding. Removing carbohydrates from sIgA and SC by enzymic digestion showed that toxin A binds much less to deglycosylated SC than to glycosylated SC. These data suggest that SC in human milk binds to toxin A and may function as a receptor analogue, protecting human infants against C. difficile-associated disease.

Introduction

Clostridium difficile is recognised as a frequent cause of antibioticassociated diarrhoea and as the major cause of antibiotic-associated pseudomembranous colitis (PMC) in man [1, 2]. Concomitant with the widespread use of antibiotics, C. difficile has emerged as a serious life-threatening pathogen in the last 20 years. Virulent C. difficile strains produce two protein exotoxins with glucosyltransferase enzymic activity. Toxin A, a single polypeptide enterotoxin of 308 kDa, is the primary virulence factor. The target of toxin A is the cellular protein Rho A, a GTP-binding protein and regulator of actin filament formation [3, 4]. The effects of toxin A on the intestinal tract include disruption of intracellular tight junctions, fluid influx, diarrhoea, inflammation and neutrophil recruitment [1, 5]. Toxin B, a single polypeptide of 269 kDa, does not affect undisrupted colonic mucosa [6]. The hamster intestinal brush border membrane receptor for toxin A has been characterised as a cell surface glycoconjugate containing the non-reducing terminal sequence Galβ1-3Galβ1-4GlcNAc [7-11]. This trisaccharide is not present on human cells. However, the human carbohydrate Lewis X blood group antigen, which is found on human intestinal epithelium, is conformationally similar to the hamster receptor (Fig. 1). There is evidence that this molecule, which contains the core structure Galβ1-4GlcNAc, is the toxin A receptor in man [12].

Three factors are key to C. difficile pathogenesis: disruption of the normal flora, acquisition of the organism from an environmental source, and, most interestingly, age-related susceptibility to disease. Surprisingly, toxigenic C. difficile has been isolated from a majority of healthy human infants [13]. C.
Difficultie toxin levels in asymptomatic infants are frequently similar to those that cause disease in adults [14]. Carriage of C. difficile is age-dependent with rates dropping to 30% by 2 years of age, but still higher than the adult carriage rate of 3-4% [15]. Formula-fed infants have higher carriage rates than breast-fed infants [16]. Several theories have been proposed to explain why infants may carry high levels of C. difficile and its toxins and remain asymptomatic. These theories include immaturity of the inflammatory response, masking of receptors, immature receptors and lack of receptors [17]. Hamsters have an age-dependent susceptibility to C. difficile disease analogous to that of man [18]. Previous research in this laboratory with a hamster model showed no difference in the ability of infant and adult purified brush border membranes to bind toxin A in vitro [9], thus discounting the theory that lack of susceptibility to disease can be explained by lack of receptors. It has also been proposed that protective factors in milk may block toxin A activity [19]. It is well documented that milk blocks the pathogenic effects of Vibrio cholerae toxin and Escherichia coli enterotoxins, without preventing colonisation of the intestinal tract by these organisms [20-22]. In a similar fashion, factors in human milk may block the toxin activity of C. difficile while allowing the organism to persist in the infant colon. One protective mechanism could be an antigen-antibody interaction, as in toxin-neutralising specific slgA. Another possible mechanism may be toxin A-carbohydrate receptor analogue interactions in the infant colon. Previous studies in this laboratory have shown that both the immunoglobulin and non-immunoglobulin fractions of human milk contain substances that interact with toxin A and prevent its binding to purified receptors [23]. The glycoprotein secretory component contains the same Lewis X trisaccharide structure found on human intestinal epithelium [24-26]. Secretory component is composed of 20% carbohydrate by mol wt; it is found at high levels in human milk in its free form and is bound to IgA to form dimeric secretory IgA [27-29]. Large quantities of free SC and slgA survive transit through the infant intestinal intact and are excreted in the faeces [30-33]. Toxin A-secretory component interactions in the colon might prevent the toxin from binding to an intestinal cell receptor, thus explaining asymptomatic colonisation.

This study was designed to characterise the ability of human milk secretory IgA (slgA) and free secretory component to interact with toxin A in vitro and the ability of these molecules to prevent toxin A-receptor interactions. Results from this study may help explain the mystery of asymptomatic colonisation of human infants with this otherwise serious pathogen and may lead to alternative approaches to treatment.

**Materials and methods**

**Immunoglobulins**

Secretory IgA (pooled from human colostrum) was purchased from Sigma. Serum IgA, IgG and IgM were purchased from Pierce Chemical Company, Rockford, IL, USA.

**Purification of C. difficile toxin A**

C. difficile toxin A was purified from brain heart infusion broth filtrates of dialysis bag cultures of a highly toxigenic strain (VPI strain 10463; Virginia Polytechnic Institute and State University, Blacksburg, VA, USA) by gel filtration, ion-exchange chromatography and affinity chromatography with bovine thyroglobulin [34, 35].

**Purification of hamster brush border membranes**

Brush border membranes (BBMs) were purified from the small intestines of 7-day-old hamsters according to well established methods [9, 10].

**Radiolabelling of C. difficile toxin A**

Purified toxin A was radio-iodinated by the Iodo-bead N-chlorobenzenesulphonamide method (Pierce Chemical Company). Unbound 125I was separated from radiolabelled toxin A by gel filtration with Sephadex G-25 (Pharmacia Biotech, Piscataway, NJ, USA) in a 1 X 10-cm column. Protein content of pooled radiolabelled toxin was determined by the Lowry method [36]. Average specific activity was 5.0 X 10^7 cpm/μg of toxin A. Incorporation of 125I averaged 70% as determined by trichloroacetic acid precipitation. Toxin A biological activity after radiolabelling was verified by cytotoxicity assay with human foreskin fibroblast cells (Barrels Cytotoxicity Assay for Clostridium difficile Toxins, Deerfield IL, USA) and compared with unlabelled toxin A controls. Purity and integrity of the toxin were also evaluated by SDS-PAGE essentially by the method of Laemmli [37]. The radiolabelled toxin appeared as a single band of c. 300 kDa.

**Radiolabelled toxin A receptor binding**

125I labelled toxin A receptor binding was evaluated by coating hamster purified BBMs to Immulun 2 microtitration wells (Dynatech Laboratories, Chantilly, VA, USA) at 1 μg/ml, 150 μl/well, blocking with bovine serum albumin 3% in phosphate-buffered saline with...
Purification of secretory component, heavy chains and light chains

slgA (5 mg) was separated by SDS-PAGE in a 3-mm acrylamide 10% preparative gel. A vertical segment was stained with Coomassie Blue, rapidly de-stained and used as a template to excise SC, HC and LC bands. Gel strips were electro-eluted in Tris-glycine tank buffer with reverse current for 18 h in a Hoefer model 600 apparatus essentially by the method of Abramovitz [39]. Completeness of elution was evaluated by staining gel strips with Coomassie Blue after electro-elution. Eluted proteins were then dialysed against PBS for 48 h and concentrated with Centriprep 10 000 MW cut-off concentrators (Amicon, Beverly, MA, USA). Protein content of eluted proteins was determined by the method of Lowry et al. [36]. Purity of eluted proteins was evaluated by SDS-PAGE and Coomassie Blue staining.

Binding of toxin A to SC, HC and LC coated to microtitration plates

Experiments were first conducted to quantify the amount of purified SC, HC and LC which were coated to microtitration plates. The three proteins were radiolabelled as described above for toxin A. Specific activities were calculated, the radiolabelled proteins were coated to microtitration wells, and the wells were washed. The radioactive counts/min of each well were determined and converted into ng of protein coated/well. Radiolabelled toxin A was then added to the wells and, based on specific activity of toxin A and subtracting cpm of coated SC, HC or LC, toxin A binding (ng) to each immunoglobulin component was calculated.

Neutralisation of toxin A cytotoxicity

slgA and SC were examined for their ability to neutralise the cytotoxicity of toxin A in human fibroblast cells (Bartels Cytotoxicity Assay for Clostridium difficile Toxins). Toxin A was diluted in PBS buffer to a concentration two-fold greater than the highest dilution that caused complete rounding of 100% of the human fibroblast cells in a microtitration well (TCD100) [23]. The TCD100 of toxin A was 50 ng. The diluted toxin A was mixed with an equal volume of serial dilutions of slgA. After incubation at room temperature for 3 h, 50 µl of each mixture were added to microtitration wells containing human fibroblast cells and 200 µl of cell culture medium. The wells were examined for cytotoxicity at 24 and 48 h after exposure to toxin A.

Competitive inhibition of toxin A binding to slgA

Unlabelled toxin A was used in a competitive inhibition assay to examine the specificity of binding of 125I-labelled toxin A to slgA. Immulon 2 microtitration wells were coated with slgA at 10 µg/ml, 150 µl/well and blocked with BSA-PBST. Serial two-fold dilutions of unlabelled toxin A were pre-mixed with a constant amount of labelled toxin A and immediately added to the coated microtitration wells and incubated for 3 h at 4°C. After incubation, the wells were washed four times with PBST and counted in a gamma counter. The results were corrected for the binding of toxin A to microtitration wells in the absence of slgA.

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Hamster BBMs were coated to Immulon 2 microtitration wells at 1 µg/ml in 15 mM carbonate-35 mM bicarbonate coating buffer, pH 9.6, 150 µl/well, overnight at 4°C. The wells were washed with PBST and blocked with BSA-PBST for 2 h. During this incubation, serial 10-fold dilutions of immunoglobulins were incubated with radiolabelled toxin A at 1 µg/ml in PBS at 25°C. After incubation, wells were washed with PBST and 150 µl of the immunoglobulin-toxin A mixtures were added to the microtitration wells coated with hamster BBMs and incubated for 3 h at 4°C. After incubation, wells were washed four times with PBST and counted in a gamma counter. Results were corrected for the binding of toxin A to the microtitration wells in the absence of BBMs. Heat stability of binding inhibition was characterised by heating immunoglobulins to 100°C for 5 min, cooling and adding radiolabelled toxin A and performing the assay as described above. Purified secretory component (SC), heavy chains (HC) and light chains (LC) of slgA were serially diluted 10-fold and mixed with radiolabelled toxin A as described above to determine toxin A receptor binding inhibition by immunoglobulin components.

 Autoradiography of toxin A–secretory IgA interactions

Purified secretory component and immunoglobulins (10 µg of each) were separated by SDS-PAGE with acrylamide 10% lower gels. Proteins were transferred to nitrocellulose membranes by the method of Towbin et al. [38] and blocked overnight at 4°C in 50 mM Tris-HCl buffer, pH 7.4, with BSA 3% and fetal bovine serum 1%. Toxin A was added at 1 µg/ml in BSA 3%, fetal bovine serum 1% in 50 mM Tris·HCl, 50 mM NaCl buffer, pH 7.4, and incubated for 3 h at 4°C, and excess toxin was removed by five washes of 30 min each at 4°C with Tris·HCl buffer. Membranes were air dried overnight. Autoradiography was performed at −70°C with X-OMAT film (Kodak, Rochester, NY, USA) and an intensifying screen.
Deglycosylation of proteins

slgA, purified secretory component, heavy chains and light chains (10 μg of each) were vacuum dried, resuspended in H2O and sequentially treated with O-Glycosidase, NAnase II and PNGase F following the manufacturer’s protocol to remove Asn and Thr-ser linked carbohydrates (Glyco, Novato, CA, USA); 10 μg of bovine thyroglobulin (a glycoprotein containing the trisaccharide sequence known to bind toxin A) were used as a binding and deglycosylation control. After deglycosylation, the proteins were separated by SDS-PAGE and stained with Coomassie Blue. Deglycosylated proteins were compared to glycosylated controls by observing migration shifts. Mol. wts of deglycosylated proteins were calculated and compared to the known percentage carbohydrate content of the proteins to assess completeness of carbohydrate removal.

Verification of deglycosylation

Glycosylated and deglycosylated bovine thyroglobulin, slgA and slgA components (10 μg of each) were separated by SDS-PAGE and transferred to nitrocellulose membranes. A digoxigenin-alkaline phosphatase-based enzyme immunoassay was used to evaluate the completeness of deglycosylation of SC, HC and LC of slgA, following the manufacturer’s instructions (Glycan Detection Kit, Boehringer Mannheim Biochemica, Indianapolis, IN, USA).

Autoradiography of toxin A–deglycosylated secretory IgA component interactions

Glycosylated and deglycosylated bovine thyroglobulin, secretory IgA, and SC, HC and LC (10 μg of each) were separated by SDS-PAGE, transferred to nitrocellulose and blocked with BSA 3% and fetal bovine serum 1%. Radiolabelled toxin A was added to the membrane at 1 μg/ml in BSA 3%, fetal bovine serum 1%, 50 mM Tris-HCl, 50 mM NaCl buffer, pH 7.4. After incubation for 3 h at 4°C, the excess toxin was removed by washing with Tris-HCl buffer. Autoradiography was performed as described above.

Results

Inhibition of toxin A receptor binding to intestinal BBMs by immunoglobulins

Purified immunoglobulins in serial 10-fold dilutions were individually pre-incubated with radiolabelled toxin A and then added to microtitration wells coated with hamster intestinal BBMs (Fig. 2). slgA at 500 μg/ml, which is approximately one-half the level of slgA found in human milk, inhibited toxin A binding by 74%, compared with toxin A binding control (p < 0.0001). slgA showed significantly greater toxin A binding inhibition than serum IgA, IgG or IgM, (p < 0.0001); serum IgA, IgG and IgM at 500 μg/ml inhibited toxin A binding by 21, 30 and 31%, respectively. All four immunoglobulins exhibited a dose-dependent reduction in binding inhibition as immunoglobulin concentration decreased.

In the absence of immunoglobulins, c. 7 ng of radiolabelled toxin A bound to each well when 150 ng of BBMs were coated. Heating all four immunoglobulins to 100°C for 5 min did not significantly alter the binding inhibition. Pre-incubation of BBMs with immunoglobulins in serial 10-fold dilutions followed by washing and addition of radiolabelled toxin A did not inhibit toxin A binding to BBMs, indicating that the immunoglobulins interacted with toxin A and not with the BBM receptors.

Inhibition of toxin A receptor binding to intestinal BBMs by secretory component purified from secretory IgA

The protein components of slgA were separated by SDS-PAGE and purified by electro-elution, dialysis and centrifugal filter concentration. Serial 10-fold dilutions of SC, HC and LC were pre-incubated with radiolabelled toxin A and added to microtitration wells coated with hamster intestinal BBMs. Secretory component at 200 μg/ml (approximate milk concentration) significantly inhibited toxin A receptor binding by 79%, compared with toxin A binding control (p < 0.0001) (Fig. 3). Serial 10-fold dilutions showed a reduction in binding inhibition in a dose-dependent fashion similar to that of slgA. The HCs and LCs of slgA did not appreciably inhibit toxin A binding at any concentration tested.
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Inhibition of toxin A binding to intestinal BBMs by free SC. Serial 10-fold dilutions of purified SC were mixed with radiolabelled toxin A at 1 μg/ml, incubated for 2 h and added to microtitration wells coated with hamster BBMs. Inhibition of toxin A binding was measured by comparing cpm of SC/toxin A wells to phosphate-buffered saline/toxin A control wells.

Inhibition of toxin A cytotoxicity by sIgA and SC

The amount of toxin A that caused 100% rounding of human foreskin fibroblast cells in a commercial cytotoxicity kit was determined empirically to be 50 ng (200 ng/ml). Serial dilutions of sIgA and SC were incubated with toxin A in tissue culture medium for 3 h at room temperature and then added to microtitration wells. Cells were observed for characteristic cell rounding. Neither sIgA nor SC at any dilution tested inhibited the cytotoxicity of toxin A.

SDS-PAGE characterisation of toxin A binding to immunoglobulins and sIgA components

Purified SC, sIgA, IgA, IgG and IgM (10 μg of each) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. A duplicate gel was stained with Coomassie Brilliant Blue. The membrane was then probed with radiolabelled toxin A and autoradiography was performed. The autoradiograph revealed that toxin A bound to the purified SC and the SC of sIgA (Fig. 4). The identity of the SC band in both SC and sIgA lanes was verified by transferring the proteins from a gel to nitrocellulose and probing with a mouse monoclonal anti-human secretory component antibody followed by an alkaline phosphatase conjugated goat anti-mouse antibody. Toxin A did not appreciably bind to the heavy or light chains of IgA, IgG or IgM.
Quantification of toxin A binding to SC

To quantify the relative amounts of toxin A binding to SC, HC and LC, the purified proteins were coated to microtitration wells. The amounts of SC, HC and LC that bound to microtitration wells when added at a concentration of 1 μg/ml were 9.48, 14.41 and 45.34 ng, respectively. Radiolabelled toxin A was added to the wells and, after washing, individual wells were counted in a gamma counter. The cpm in each well were converted to ng of toxin A binding/100 ng of coated protein. The averages of three separate experiments were as follows: SC bound significantly more toxin A than the heavy or light chains of sIgA, compared with HC and LC (p<0.005). SC bound 10.72 (SD = 3.10) ng of toxin A; HC bound 1.18 (SD = 0.64) and LC bound 0.05 (SD = 0.07) ng of toxin A.

Competitive binding of non-labelled and radiolabelled toxin A to sIgA

Serial two-fold dilutions of unlabelled toxin A were added to a constant amount of 125I-radiolabelled toxin A in PBS. The toxin mixtures were then added to sIgA-coated wells, incubated for 3 h and washed; cpm of non-labelled/radiolabelled toxin A mixture wells were compared with radiolabelled toxin A controls, and the percentage inhibition was calculated. The percentage inhibition of radiolabelled toxin A binding increased in a dose-dependent fashion as the level of competing unlabelled toxin was raised (Fig. 5). At 1000 μg of unlabelled toxin A/ml, the binding of radiolabelled toxin A was completely inhibited.

Binding of toxin A to glycosylated and deglycosylated sIgA components

To determine if toxin A was binding to the carbohydrate chains of secretory component, enzymic deglycosylation was performed with a commercial kit that cleaves sugar chains at the asparagine link. sIgA and purified SC, HC and LC were lyophilised, denatured by SDS-PAGE and deglycosylated following the manufacturer's instructions. Completeness of deglycosylation was determined by migration shifts of deglycosylated proteins as compared to untreated proteins when visualised by SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 6). The mol. wt of secretory component shifted from 78 to 59.57 kDa, a difference of 23.9%, indicating complete removal of carbohydrate, which has been previously reported at 20–25%. The heavy chains of sIgA shifted from 60.8 to 56.75 kDa, a difference of 6.7%. The mol. wt of the light chains did not shift, as they contain no carbohydrate. Completeness of deglycosylation was also determined with a commercial carbohydrate detection kit. The deglycosylated proteins showed no detectable carbohydrate by this method. Additional gels containing 10 μg of each glycosylated and deglycosylated protein were prepared, transferred to nitrocellulose, probed with radiolabelled toxin A and developed by autoradiography. The deglycosylated SC showed a
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Fig. 6. Glycosylated and deglycosylated proteins were separated by SDS-PAGE and stained with Coomassie Blue. Ten μg of each protein were enzymically deglycosylated and run in duplicate with a glycosylated control. MW, mol. wt markers; BTG, bovine thyroglobulin (glycoprotein containing the trisaccharide Galα1-3Galβ1-4GlcNAc, a toxin A-binding and deglycosylation control); sIgA, secretory IgA; SC, secretory component; HC, secretory IgA heavy chains; LC, secretory IgA light chains; L/D, deglycosylated proteins.

marked reduction in toxin A binding as compared with glycosylated SC (Fig. 7). The glycosylated and deglycosylated HC and LC showed no appreciable toxin A binding.

Discussion

C. difficile was originally described by Hall and O'Toole [1] as a component of the normal intestinal flora of human infants. In recent years toxigenic strains of C. difficile have been isolated from > 50% of healthy human infants [13]. The fact that C. difficile colonisation and toxin production may occur in man with or without disease symptoms has complicated the study of this important pathogen. The mechanism of age-dependent asymptomatic colonisation by C. difficile remains unclear, but numerous studies have shown that human milk protects infants from the effects of several other pathogenic enteric bacteria, parasites and viruses [20–22, 33, 40]. An obvious difference between human adults and infants is that the diet of infants is exclusively or primarily milk. Experiments in this laboratory have shown that human milk pre-incubated with toxin A protects hamsters from a lethal intragastric dose of toxin A and that components in both the immunoglobulin and non-immunoglobulin fractions of milk interacted with toxin A and prevented its binding to intestinal BBM receptors [23]. Also, hamsters exhibit an age-dependent window of susceptibility to asymptomatic colonisation by toxigenic C. difficile analogous to that of human infants [10] and the intestinal epithelium of infant hamsters expresses a functional toxin A receptor at levels higher than those in adult hamsters [10], suggesting that asymptomatic colonisation cannot be attributed to absence of receptors. Furthermore, differences in the binding kinetics of toxin A to adult and infant hamster
intestinal cells do not account for age-dependent differences in susceptibility to *C. difficile* toxin A. These facts suggest that the infant hamster [41] is suitable for studying toxin A–receptor interactions and the effects of human milk fractions on these interactions. Purification of a specific receptor from the intestines of hamsters has greatly aided these studies [10]. The carbohydrate Lewis blood group X antigen has been proposed as the likely human intestinal receptor [12]. This trisaccharide structure is similar to the hamster receptor with the exception of fucose substituted for the terminal galactose [17]. It is interesting to note that the Lewis X antigen is also expressed on human neutrophils and on SC [27]. SC is a glycosylated protein of the immunoglobulin superfamily [25], synthesised by mucosal epithelial cells and attached to IgA dimers through disulphide bonds [26], forming secretory IgA. slgA is the primary immunoglobulin of mucosal secretions. SC is secreted in human milk in its free form and bound to slgA. Free SC is found in mature milk at c. 200 μg/ml [42]. slgA is secreted in mature milk at c. 1000 μg/ml [32, 33]. It is believed that the attachment of SC to slgA explains this immunoglobulin’s unique resistance to acid and protease [26]. It is generally assumed that the abundant carbohydrates, lipids and proteins comprising milk are all bioavailable to the infant, yet surprisingly, up to 80% of an infant’s daily intake of slgA can be recovered intact from faeces [30, 31, 33, 43]. Free SC is also relatively resistant to digestion and also passes in large quantities into the infant colon. slgA purified from human milk inhibited the binding of toxin A to intestinal receptors. Other immunoglobulins showed lesser degrees of inhibition. It is possible that the small amounts of inhibition shown by serum IgA, IgG and IgM are caused by interactions of toxin A with the glycosylated immunoglobulin HCs. The increased binding inhibition by slgA and the fact that the SC attached to slgA carries the Lewis X carbohydrate antigen lead us to initiate inhibition studies with free SC. SC was purified from slgA by SDS-PAGE and electro-elution. Free SC inhibited the binding of toxin A in a fashion similar to slgA. The heat stability of binding inhibition by slgA and SC is evidence that the toxin binds to a glycoconjugate. These studies also showed that toxin A bound to the secretory component of slgA separated by SDS-PAGE and to purified SC coated to microtitreation wells. Enzymic deglycosylation of SC led to a significant
reduction in toxin A binding, further indicating that toxin A interacts with the carbohydrate portion of this molecule. Although SC and slgA inhibited toxin A binding to hamster intestinal receptors, neither protected human fibroblast cells from the cytopathic effects of toxin A. The inability of SC and slgA to neutralise toxin A cytotoxicity may possibly be caused by entry of toxin A into tissue culture cells by non-specific uptake [14]. Interestingly, other investigators have reported that toxin A neutralisation could be attributed to the slgA fraction of colostrum, as immune precipitation of the samples with anti-human IgA removed the neutralising activity [19]. There are numerous published examples of the protective effects of milk components against enteric pathogens. Studies have shown that components in human milk inhibited the actions of *E. coli* and *V. cholerae* enterotoxins without preventing colonisation of the intestinal tracts [22]. Both free SC and lactoferrin inhibited the adhesion of enterotoxigenic *E. coli* [21].

The in-vitro interactions of milk components with toxin A are of note; however, as milk has undergone digestion by the time it reaches the colon, any protective factors in milk against toxin A must survive this process and reach the colon intact. Previous studies in this laboratory have shown that the intestinal contents of infant hamsters receiving maternal milk block the binding of toxin A to purified receptors [17], whereas the intestinal contents of adult hamsters fed a standard solid food diet showed minimal effect on toxin A binding. These results suggest that this inhibitory activity could possibly be attributed to factors in maternal milk passing intact into the infant hamster intestine. Human milk contains many possible toxin A carbohydrate receptor analogues including soluble I, X and Y antigens, other oligosaccharides and glycoproteins [20, 32, 44]. The vast majority of potential toxin A carbohydrate receptor analogues are absorbed or degraded by the time they reach the colon [44, 45]; two exceptions are slgA and SC. Several considerations support slgA and free SC as possible receptor analogues: they both carry the Lewis X carbohydrate antigen, they are uniquely resistant to digestion, the amount of these substances in milk (gram quantities) received per day [30] by the breast-feeding infant and, lastly, the fact that they are detectable in infant stools intact. The binding of toxin A to secretory component in *vivo* could prevent binding of toxin A to intestinal receptors. This interaction in the infant colon could at least partly explain asymptomatic colonisation. Other investigators have shown that human milk has immunostimulatory effects, with breast-fed infants producing their own intestinal slgA sooner than formula-fed infants [32]. This increased slgA production could further protect infants from *C. difficile* toxin A.

Interestingly, cow and formula milks also show inhibition of toxin A receptor binding, but to a lesser degree than human milk [46]. The inhibitory sub-

stances also appear to be carbohydrates, as boiling did not affect binding inhibition. Fresh cow’s milk, which contains SC, shows the greatest degree of inhibition, followed by cow-based formula. Soy-based formulas show lower degrees of binding inhibition. It is possible that asymptomatic colonisation of formula-fed infants could also be explained by carbohydrate receptor analogue-toxin A interactions.

Other mechanisms to explain asymptomatic colonisation by toxigenic *C. difficile* have been suggested. These include maternal neutralising antibodies in human milk [19], lack of an inflammatory response to toxins caused by the general immaturity of the infant immune system and toxin A interactions in the colon with unabsorbed or undigested soluble receptor analogues other than SC and slgA, such as fucosylated oligosaccharides or the glycoprotein lactoferrin.

This study provides evidence that toxin A and SC interact in *vivo* and that this interaction prevents the binding of toxin A to purified intestinal receptors. Large-scale purification of free SC and slgA from human milk by affinity chromatography [42] is in progress for future studies to determine the protective effects of these glycoproteins in hamsters oro-gastrically challenged with toxin A. Detection of toxin A–secretory component or toxin A–slgA complexes in infant colon contents, or both, would support a role for these receptor analogues in explaining asymptomatic colonisation by toxigenic *C. difficile*.

The irony of *C. difficile*-associated disease is that it is both induced and cured by antibiotics. *Saccharomyces boulardii*, a non-pathogenic yeast, has been used as a successful non-antibiotic treatment for *C. difficile*-associated diarrhoea [47]. Interestingly, it has been shown that treatment of weaning rats with *S. boulardii* leads to increased release of slgA and SC into the intestinal lumen [48]. This is notable considering the relative immaturity of the immune system in the 14–22-day-old rats examined in this study. It is tempting to postulate that a possible mechanism of *S. boulardii*’s effectiveness is the production of slgA and SC toxin A receptor analogues. Knowledge of toxin A receptor analogues may lead to non-antibiotic approaches to enhance the resistance or improve treatments of adults infected with this important intestinal pathogen.

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