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Human Milk Mucin 1 and Mucin 4 Inhibit *Salmonella enterica* Serovar Typhimurium Invasion of Human Intestinal Epithelial Cells In Vitro[1–3]

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Abstract

Many human milk glycans inhibit pathogen binding to host receptors and their consumption by infants is associated with reduced risk of disease. *Salmonella* infection is more frequent among infants than among the general population, but the incidence is lower in breast-fed babies, suggesting that human milk could contain components that inhibit *Salmonella*. This study aimed to test whether human milk per se inhibits *Salmonella* invasion of human intestinal epithelial cells in vitro and, if so, to identify the milk components responsible for inhibition. *Salmonella enterica* serovar Typhimurium SL1344 (SL1344) invasion of FHs 74 Int and Caco-2 cells were the models of human intestinal epithelium infection. Internalization of fluorescein-5-isothiocyanate–labeled SL1344 into intestinal cells was measured by flow cytometry to quantify infection. Human milk and its fractions inhibited infection; the inhibitory activity localized to the high molecular weight glycans. Mucin 1 and mucin 4 were isolated to homogeneity. At 150 μg/L, a typical concentration in milk, human milk mucin 1 and mucin 4 inhibited SL1344 invasion of both target cell types. These mucins inhibited SL1344 invasion of epithelial cells in a dose-dependent manner. Thus, mucins may prove useful as a basis for developing novel oral prophylactic and therapeutic agents that inhibit infant diseases caused by *Salmonella* and related pathogens. J. Nutr. 142: 1504–1509, 2012.

Introduction

Human milk is widely accepted as containing the optimum nutrients for most infants, while it simultaneously conveys immunologic and other health benefits (1). Breast-fed infants have lower risk of enteric disease than those artificially fed. A number of human milk glycans have been identified that contribute to the protection of infants through inhibition of pathogen binding to host cell membranes (2). Many of the most common human enteropathogens are inhibited by human milk glycans, but only limited information is available regarding inhibition of *Salmonella* species.

Salmonella is among the most commonly recognized enteric pathogens, estimated to cause 1.4 million illnesses and 400 deaths each year in the United States (3). Infection by *Salmonella* is more frequent in children <1 y of age than among older age groups (3,4). The treatment for *Salmonella* infection is primarily antibiotic therapy, but resistant strains abound. Even when antibiotic therapy is effective against *Salmonella*, the treatment can also affect normal microbiota of the infant intestine, thereby inducing risk of secondary enteric disturbances (5). Thus, there is an urgent need to develop alternative safe and effective therapies against *Salmonella* infection in infants.

One promising approach is to search for molecules in human milk that specifically inhibit *Salmonella* invasion of human intestinal epithelial cells. The binding of an undefined clinical isolate *Salmonella typhimurium* to human HeLa epithelial cells was partially inhibited by a crude human milk glycan fraction; this inhibition was attributed to the free secretory component and lactoferrin of milk (6). We previously found that a mucin-associated molecule of human milk can bind to rotavirus and inhibit viral replication (7). Recently, a bovine milk molecule whose size by PAGE is similar to that of mucin 1 was reported to inhibit binding of enteric bacteria to Caco-2 cells (8). *S. typhimurium* specifically binds mucins of the intestinal mucosa (9). Human milk mucins competitively inhibit some types of enteropathogens binding to target receptor glycans on host cells (7,10,11). Thus, human milk glycans, and especially the milk mucins, are interesting candidates as potential inhibitors of *Salmonella* infection.

Human mucins are high-molecular weight glycoproteins whose typical size ranges from 200 to 2000 kDa and are primarily found in the extracellular glycocalyx region of extracellular matrix and in human milk. Their diverse functions

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include regulating cell signaling and transcription and modulating the binding of bacteria to the intestinal mucosa epithelium, including binding by both mutualists and pathogens (12). The glycans expressed on mucins include many common moieties that can act as cell surface targets for pathogens.

In the studies described herein, the specific hypothesis is that human milk mucins could inhibit *Salmonella* invasion of human intestinal epithelia. Two cell lines, FHs 74 Int (derived from normal human fetal intestine) and Caco-2 (derived from human colon adenocarcinoma), were used as models of the immature human intestinal epithelial cell. *S. typhimurium* is the most common of the *Salmonella* serovars that cause human infections (13). Therefore, *Salmonella enterica* serovar Typhimurium SL1344 (SL1344)[6] invasion of FHs 74 Int and Caco-2 cell lines were developed as models of human salmonellosis. These models were used to determine whether human milk mucins affect SL1344 invasion of FHs 74 Int and Caco-2 cells in vitro, thereby indicating the potential utility of human milk mucins to protect infants from *Salmonella* infection.

Materials and Methods

Human milk. Use of human milk was approved by the Institutional Review Boards of Massachusetts General Hospital. Human milk was collected with a breast pump from 40 healthy donors and stored at −20°C. This pooled milk from donors at different stages of lactation was tested as representative of human milk.

Bacterial strains and culture. The invasive wild-type SL1344 (14) was obtained from the American Type Culture Collection (ATCC) and grown to the stationary phase in Luria-Bertoni medium at 37°C with constant shaking.

Cell lines and culture. The normal small intestine epithelial cell line FHs 74 Int was obtained from ATCC and was cultured in Hybri-Care medium (ATCC) 10% FBS (Atlanta Biologicals) in the presence of 30 μg/L epidermal growth factor (Invitrogen) at 37°C in 5% CO_2. The human epithelial colorectal adenocarcinoma cell line Caco-2 was obtained from ATCC and cultured in DMEM (Gibco-BRL)/10% FBS at 37°C in 5% CO_2.

Isolation, purification, and analysis of mucins. Pooled frozen human milk (40 donors) was thawed and centrifuged at 1000 × *g* for 40 min at 18°C to obtain cream and skim milk. The cream was washed 3 times with PBS to remove soluble protein. The cream was then resuspended in PBS and subjected to 3 cycles of freeze-thaw followed by sonication for 20 min to disrupt the milk fat globule. Milk fat globule membrane (MFGM) was collected as the pellet after centrifugation at 100,000 × *g* for 1 h. Whole milk, skim milk, and MFGM were freeze-dried and stored at −20°C for testing their biologic activity.

To isolate the human milk mucins, MFGM was dissolved in PBS with 8 mol/L urea and applied to a Sepharose CL-2B (GE Healthcare) column. Fractions containing mucin 1 and mucin 4 were collected and further purified over a Sepharose CL-4B (GE Healthcare) column. The mucin 1 and mucin 4 fractions were pooled, dialyzed, freeze-dried, and stored at −20°C for assessment of their ability to inhibit *Salmonella*. The identity and purity of mucins were evaluated by SDS-PAGE. Purified mucin 1 and mucin 4 were analyzed on 4–12% gradient SDS-PAGE gels stained by silver and periodic acid-Schiff (PAS) reagent. The identity of mucin 1 and mucin 4 were confirmed by Western-blot analysis against the mucin 1 antibody (a gift from Dr. Sandra Gendler, Mayo Clinic) or mucin 4 antibody (a gift from Dr. Surinder Batra, University of Nebraska Medical Center).

[6] Abbreviations used: ATCC, American Type Culture Collection; FITC, fluorescein-5-isothiocyanate; IC_{50}, half maximal inhibitory concentration; MFGM, milk fat globule membrane; PAS, periodic acid-Schiff; SL1344, *Salmonella enterica* serovar Typhimurium SL1344.

Fluorescence confocal microscopy. The FHs 74 Int or Caco-2 cells were grown on polylysine-coated glass coverslips in 24-well cell culture plates to ~70% confluence. SL1344 was labeled with fluorescein-5-isothiocyanate (FITC) (Sigma) as previously described (8). Briefly, SL1344 (10^{12} CFU/L in PBS) were incubated with 100 μg of FITC for 30 min at room temperature and washed thrice with PBS. The epithelial cells were incubated with FITC-labeled SL1344 for 1 h at 37°C at a 100:1 bacteria/epithelial cell multiplicity of infection. The cells were washed with PBS to remove unattached SL1344 and then incubated in cell culture medium containing 100 mg/L of gentamicin (Sigma) for 1 h at 37°C. After gentamicin killing of extracellular bacteria, cells were washed by dipping the cover slip in PBS and the cells were fixed by dipping in 1% buffered formalin for 10 min at room temperature. Cells were rinsed twice and incubated with membrane-specific dye Vybrant DiI (Invitrogen) for 20 min according to the manufacturer's instructions. After rinsing with PBS, a drop of mounting media was applied to a microscope slide and the inverted cover slip was carefully laid on the slide. The mounting media included 4′,6-diamidino-2-phenylindole stain and was left to harden overnight. Imaging analysis was performed with a Leica TCS SP5 inverted confocal laser-scanning microscope (Plan-Apochromat 63× oil immersion lens, 1.4 numerical aperture).

Invasion assay. Intestinal epithelial cell lines, FHs 74 Int or Caco-2 cells were seeded onto 6-well plates at 10^6 cells/well. The cells were inoculated with suspensions of 1.0×10^8 CFU FITC-labeled SL1344, along with test samples or controls, and incubated for 1 h at 37°C, whereupon the intestinal epithelial cell monolayers were rinsed 5 times with fresh medium. The epithelial cells were then incubated for 1 h at 37°C in 5% CO_2 with cell culture medium containing 100 mg/L of gentamicin to kill extracellular bacteria. After incubation, extracellular SL1344 was removed by thorough washing and the cells were fixed with 1% paraformaldehyde in PBS and analyzed for invasion as fluorescence intensity of FITC-labeled SL1344 by flow cytometry in a C6 Flow Cytometer system (Accuri Cytometers). Cells were harvested and fluorescence intensity analyzed by flow cytometry as previously described (15). The mean fluorescence intensity was set to detect 10,000 cells/sample, which was then multiplied by the number of cells within each well to give total fluorescence per well. Invasion of SL1344 was:

$$\%\,\text{invasion} = \left[\left(I_{exp} - I_{neg}\right) / \left(I_{pos} - I_{neg}\right)\right] \times 100,$$

where I_{exp} = fluorescence intensity of well with epithelial cells, Salmonella, test sample; I_{neg} = fluorescence intensity of well with epithelial cells (background signal); and I_{pos} = fluorescence intensity of well with epithelial cells, Salmonella.

Inhibitor specificity. Inhibition could result from the test molecule interacting with the target epithelial cells, the test molecule interacting with the pathogen, or both. As a first step toward investigating these possible mechanisms, epithelial cells (FHs 74 Int or Caco-2) or the pathogen SL1344 were individually preincubated with the inhibitor for 1 h prior to the invasion assay. An increase in inhibition by the test substance in either condition would suggest that the inhibitor interacts with the target epithelial cell or with the pathogen, respectively.

Statistical analysis. Data are expressed as mean ± SD of at least 3 independent experiments. Relative inhibition by treatments was evaluated using 1-way ANOVA followed by Tukey's post hoc test. The dose dependency used logistic dose response of nonlinear curve fit analysis; the half maximal inhibitory concentration (IC_{50}) was calculated from each curve. The differences in IC_{50} between mucin 1 and mucin 4 were compared by Student's *t* test for paired samples. All statistical analyses were conducted using the Originpro (OriginLab) and MATLAB (MathWorks). Differences were considered significant at $P < 0.05$.

Results

Preparation and analysis of mucin. Two mucins, mucin 1 and mucin 4, were isolated from human MFGM, purified by

size exclusion chromatography, and identified as mucin 1 and mucin 4 by SDS-PAGE and Western blot (**Supplemental Fig. 1**). Because these 2 mucins are glycoproteins, they stained with PAS reagent for carbohydrates and silver stain for proteins. PAGE with silver and PAS stains indicated that mucin 1 and mucin 4 were purified to >90%. The identities of mucin 1 and mucin 4 were confirmed by Western blot against authentic mucin 1 and mucin 4 antibodies.

Mucin inhibits Salmonella invasion of intestinal epithelial cells. Confocal fluorescence microscopy indicates that FHs 74 Int and Caco-2 intestinal epithelial cells were invaded by FITC labeled SL1344 in vitro (**Supplemental Fig. 2**). FITC-labeled SL1344 invasion into epithelial intestinal cells was measured by flow cytometry to quantify inhibition of invasion by milk fractions (**Supplemental Fig. 3**). Human milk fractions inhibited SL1344 invasion of epithelial cells; the purified mucins displayed the highest specific activity of inhibition by several orders of magnitude. BSA, the negative control, did not inhibit invasion. Mucin 1 and mucin 4 were each found in human milk at concentrations of ~150 μg/L, based on gravimetry of lyophilized purified fractions (data not shown). At these concentrations of 150 μg/L, mucin 1 and mucin 4 significantly inhibit SL1344 invasion of epithelial cells, whereas BSA, whole milk, skim milk, and MFGM at this concentration did not exhibit inhibitory activity (data not shown). Even at 1000 times this concentration (150 mg/L), inhibition was less than that of the mucins at 150 μg/L (**Table 1**); mucin 1 and mucin 4 showed much stronger inhibition of SL1344 invasion of FHs 74 Int cells than whole milk, skim milk, MFGM, or BSA ($P < 0.05$). Mucin 1 inhibited more strongly than mucin 4 ($P < 0.05$). Likewise, similar results were obtained in Caco-2 cells. Human milk mucins were more effective than the other human milk fractions to reduce invasion. Mucins and other human milk fractions did not reduce viability of either SL1344 or the epithelial FHs 74 Int and Caco-2 cells (data not shown) under the conditions of the assays for 3 h, the maximum incubation duration used in these studies. Mucins and other human milk fractions did not change the distribution of SL1344 in culture, indicating that decreased invasion was not due to agglutination. Altogether, these data indicate that human milk mucin 1 and mucin 4 account for essentially all of the inhibition of SL1344 invasion of epithelial cells by human milk.

Mucins inhibit Salmonella invasion in a dose-dependent manner. Both mucin 1 and mucin 4 inhibited *Salmonella* invasion at doses similar to those found in human milk. To determine the dose dependency of mucin inhibition, serial dilutions of purified mucin 1 and mucin 4 were tested for their relative ability to inhibit *Salmonella* infection of FHs 74 Int or Caco-2 cells. Mucin 1 inhibited SL1344 invasion of FHs 74 Int (**Fig. 1***A*) and Caco-2 (Fig. 1*B*) cells in a dose-dependent manner (1–1000 μg/L); likewise, mucin 4 inhibition was also dose dependent in FHs 74 Int (Fig. 1*C*) and Caco-2 (Fig. 1*D*) cells. The inhibition strongly fit a logistic dose-response curve (adjusted $R^2 \geq 0.98$). The IC_{50} of mucin 1 was 7 ± 1 and 8 ± 1 μg/L for FHs 74 Int and Caco-2 cells, respectively. The corresponding IC_{50} for mucin 4 was 33 ± 2 and 39 ± 2 μg/L for FHs 74 Int and Caco-2 cells, respectively. Thus, in FHs 74 Int and Caco-2 cell models, the 2 mucins inhibit *Salmonella*, but mucin 1 inhibits this major serovar of *Salmonella* more strongly than mucin 4 for each comparison within a cell line ($P < 0.001$).

Mucin interaction with epithelial cells and SL134. As a first step toward defining the mechanisms by which mucins inhibit the invasion of SL1344, either the epithelial cells or the *Salmonella* were separately preincubated with mucin 1 or mucin 4 for 1 h prior to performing the 1-h invasion assay (**Table 2**). The results were compared with those from the simultaneous incubation of mucins, epithelial cells, and SL1344 for 1 h only. Preincubation of either FHs 74 Int cells (host cell) or SL1344 (bacterial pathogen) with mucin 1 for 1 h followed by the simultaneous incubation of all together for 1 h reduced invasion relative to only the 1-h simultaneous incubation of all reagents ($P < 0.05$). Pretreatment also reduced invasion in the Caco-2 cell model. Preincubation with mucin 4 likewise further reduced infection. Thus, inhibition by mucin 1 and mucin 4 may be due to interaction with both the target epithelial cells and SL1344 bacteria.

TABLE 1 Mucin 1 and mucin 4 inhibit SL1344 invasion of epithelial cells in vitro[1,2]

	SL1344 invasion, %	
Inhibitor	of FHs 74 Int cells	of Caco-2 cells
BSA	96 ± 6[a]	96 ± 7[a]
Whole milk	33 ± 7[b]	36 ± 5[b]
Skim milk	35 ± 8[b]	42 ± 8[b]
MFGM	28 ± 4[b]	33 ± 4[b]
Mucin 4	20 ± 3[c]	25 ± 3[c]
Mucin 1	16 ± 2[d]	19 ± 2[d]

[1] Values are mean ± SD, $n = 3$. Means in a column without a common letter differ, $P < 0.05$. MFGM, milk fat globule membrane SL1344, *Salmonella enterica* serovar Typhimurium SL1344.

[2] BSA, whole milk, skim milk, and MFGM were tested at 150 mg/L. Mucin 1 and mucin 4 were tested at 150 μg/L.

Discussion

Mucin 1 and mucin 4 are transmembrane glycoproteins with extensive *N*- and *O*- linked glycosylation (16,17), containing highly sialylated glycan moieties (18,19). Human milk mucin exhibits biological activity, including blocking transmission of human immunodeficiency virus from dendritic to T cells (11,20) and binding Norwalk virus (16). The biological activities of mucins observed herein could reside in either specific carbohydrate moieties or peptide motifs. For example, sialic acid-containing carbohydrate moieties can act as key receptors for the adherence of major strains of *S. typhimurium*. Cell surface sialic acid is required for the adherence of *S. typhimurium* to Caco-2 epithelial cells in vitro (21). The mucin sialic acid is essential for bovine mucin 1 inhibition of pathogen binding to Caco-2 cells (8). Both mucin 1 and mucin 4 contain sialic acid. The ability of human milk mucins to inhibit SL1344 binding to epithelial cells is consistent with their carbohydrate moieties acting as competitive inhibitors of SL1344 binding to receptors on the epithelium. If this were so, the differences in inhibition observed between mucin 1 and mucin 4 could be due to differential glycosylation. On the other hand, the differences in inhibition that were observed could be due to differences in the protein backbone. For example, mucin 1 contains a sperm protein enterokinase and agrin (SEA) domain. Mucin 4 lacks the SEA domain, which may function in protein binding to carbohydrate moieties (22). If the SEA domain of mucin 1 participates in bacterial binding, this could account for the stronger inhibition of SL1344 by mucin 1 relative to mucin 4.

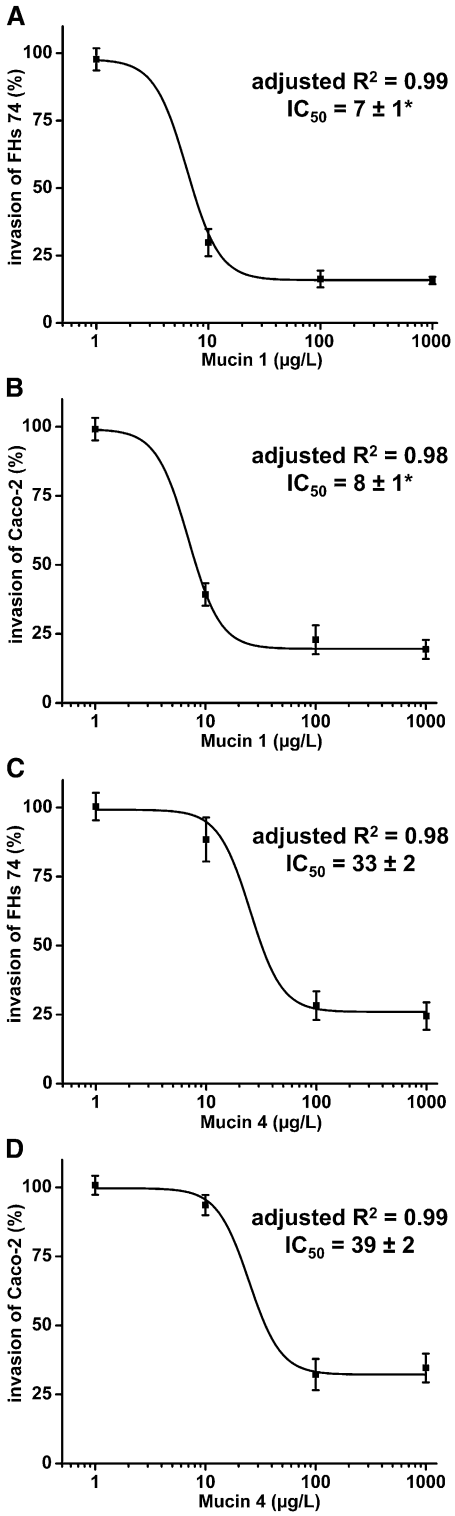

FIGURE 1 Mucin 1 inhibits SL1344 invasion of FHs 74 Int (*A*) or Caco-2 (*B*) human intestinal epithelial cell lines and mucin 4 inhibits SL1344 invasion of FHs 74 Int (*C*) or Caco-2 (*D*) cells. Values are mean ± SD, $n = 3$. *Different from corresponding mucin 4, $P < 0.001$.

The mechanisms whereby *Salmonella* invade epithelial cells include 2 type III secretion systems: *Salmonella* pathogenicity island 1 allows *Salmonella* to invade the intestinal epithelium, whereas *Salmonella* pathogenicity island 2 promotes its survival within cells (23). There is a dearth of evidence supporting the specific interaction of human milk mucins with these systems. However, heavily glycosylated mucins inhibit binding to other receptors of epithelial cell binding. For example, mucin 1 and mucin 4 interact with galectin-3 (24,25), a ligand found in epithelial cells, fibroblasts, dendritic cells, and inflammatory cells. Galectin-3 binds to LPS in intact bacteria and enhances bacterial interaction with host epithelial cells and macrophages (26). Thus, human milk mucins, by binding galectin 3, may inhibit LPS-mediated *Salmonella* binding to epithelial cells. This is supported by the increased inhibition of SL1344 invasion of epithelial cells by preincubation of the epithelial cells with the mucin. Various pili are expressed by *Salmonella* strains and binding of the pili to host target molecules is an essential step in pathogenesis. Many pili target specific glycans of the epithelial cell surface (27). Mucins have been demonstrated to bind to some *Salmonella* strains via mucin mannose interacting with pili adhesins (9). Other strains bind through interactions with other glycan motifs, but the binding specificity of most remains undefined. Thus, human milk mucins may inhibit *Salmonella* pathogenesis through the inhibition of as-yet-undefined binding by pili adhesins to epithelial cell surface glycans. This is consistent with the increased inhibition after preincubating the *Salmonella* with the human milk mucins. Altogether, human milk mucins may inhibit *Salmonella* invasion by binding to both the pathogens and epithelial cells but through different moieties of this large, highly glycosylated, complex molecule.

In this study, the target cells chosen to develop models of *Salmonella* invasion of human intestinal epithelial cells were undifferentiated Caco-2 and FHs 74 Int cells. Caco-2 cells, originally derived from colonic carcinoma, are classically chosen for in vitro models of *Salmonella* invasion assays (28). FHs 74 Int cells are derived from normal fetal small intestine and have epithelial features more similar to normal intact immature mucosa, including morphology that is epithelial like, reduction of doubling time by epidermal growth factor, and stimulation of growth by amniotic fluid and human milk that is tyrosine kinase dependent (29). FHs 74 Int cells are often used to model the immature infant intestinal epithelium (30). Infants are at higher risk for *Salmonella* infection than adults. Thus, the FHs 74 Int cell is a relevant model for studying inhibition of *Salmonella* invasion by human milk and yielded results that were consistent with those provided by the more widely used Caco-2 cell model.

Human milk contains many bioactive substances that function in protecting breast-fed infants. Only some human milk components have analogs in bovine milk and among them is mucin. Bovine milk mucin 1 inhibits binding of enteric bacteria to Caco-2 cells in vitro *(8)*. The model used to test bovine milk mucin was a strain of *Salmonella* different from the SL1344 used herein and binding was to Caco-2 cells. That notwithstanding, the magnitude of inhibition by the bovine and human MFGM was roughly comparable. The mucin of bovine milk, which was assumed to be mucin 1, also displayed a specific activity of inhibition that was comparable with that of the human milk mucin 1. However, there is no mention of mucin 4 in bovine milk. Due to the known differences between bovine and human milk glycosylation for many other proteins, it seems likely that qualitative differences exist between the mucin 1 of human and bovine milk as well as potential qualitative differences in the types of mucins in each type of milk. For example, the molecular weight of bovine mucin 1 is <200 kDa by SDS-PAGE, and human milk mucin 1 is >250 kDa, consistent with greater glycosylation. In general, human milk components are more heavily glycosylated than analogous components of bovine milk (31). Moreover, the glycans of human milk are also qualitatively different in that human milk glycans tend to be more heavily fucosylated than bovine milk glycans (32). Many biologically active human milk

TABLE 2 Mucin 1 and mucin 4 interact with epithelial cells and SL1344[1–3]

Experimental group	Mucin 1		Mucin 4	
	FHs 74 Int	Caco-2	FHs 74 Int	Caco-2
	Invasion, %			
BSA	96 ± 6[a]	97 ± 7[a]	100 ± 3[a]	99 ± 5[a]
SL1344, epithelial cell mixed simultaneously	17 ± 2[b]	23 ± 5[b]	29 ± 7[b]	34 ± 7[b]
SL1344 pretreated with mucin	8 ± 4[c]	14 ± 4[c]	19 ± 7[c]	25 ± 6[c]
Epithelial cells pretreated with mucin	10 ± 3[c]	16 ± 3[c]	22 ± 5[c]	24 ± 6[c]

[1] Values are mean ± SD, $n = 3$. Means in a column without a common letter differ, $P < 0.05$. SL1344, *Salmonella enterica* serovar Typhimurium SL1344.
[2] Mucin 1, mucin 4, and BSA were tested at 150 μg/L.
[3] 10^6 epithelial cells were inoculated with 1.0×10^8 FITC-labeled SL1344.

glycans inhibit specific human pathogens in vitro and in vivo and reduce risk of enteric diseases in infants (2,33–35). The data herein indicate that human milk mucin strongly inhibits *Salmonella* invasion of human epithelial cells, providing a basis for future comparisons of the structural basis of this inhibition by bovine milk mucin and each of the human milk mucins.

In summary, mucin 1 and mucin 4 were isolated, purified, and identified from human milk. To our knowledge, this is the first purified mucin 4 isolated from human milk. These mucins inhibit SL1344 invasion of human intestinal epithelium cells (FHs 74 Int and CaCo-2 cells) at the concentrations in milk and account for essentially all of this inhibitory activity of human milk. Mucin 1 is a stronger inhibitor than mucin 4. Therefore, human milk mucins are promising as potential prophylactic and therapeutic agents that inhibit infant diseases caused by *Salmonella* and perhaps other pathogens.

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