Adherence of *Salmonella typhimurium* to Caco-2 Cells: Identification of a Glycoconjugate Receptor

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The mechanism by which *Salmonella* species adhere to the epithelium of the intestine is not well understood. To identify components on intestinal epithelial cells that may be involved in the initial adherence of *Salmonella typhimurium*, we correlated patterns of adherence to well-differentiated Caco-2_{BBc} cell monolayers with expression of brush border membrane components and lectin binding sites. This cloned cell line shows heterogeneous expression of sucrase-isomaltase and most lectin receptors. *S. typhimurium* adhered to a subpopulation of living or formaldehyde-fixed cells with a high multiplicity (up to 150 bacteria per cell). Bacterial binding to selected cells was not correlated with expression of the brush border hydrolases dipeptidyl-peptidase IV and sucrase-isomaltase or with binding of 10 of the 12 lectins tested. However, binding was correlated with the presence of binding sites for peanut agglutinin (PNA) [specific for Gal β (1-3)GalNAc] and soybean agglutinin (specific for terminal GalNAc). Preincubation of live and fixed Caco-2_{BBc} monolayers with PNA inhibited bacterial binding, while preincubation with soybean agglutinin did not. Electron microscopic analysis demonstrated that the initial adherence of *S. typhimurium* to Caco-2 cells in vitro involved peripheral components of the glycocalyx on apical microvilli. These results suggest that a Gal β (1-3)GalNAc epitope recognized by PNA and located in the glycocalyx is involved in the early recognition events between *S. typhimurium* and Caco-2 cells and that differences in glycosylation patterns among individual epithelial cells may be a determinant in cell-selective adherence of *S. typhimurium*.

Protein-carbohydrate interactions appear to play a critical role in the adherence of pathogens to epithelial surfaces (4, 12, 41, 42, 45). Colonization or invasion of gastrointestinal mucosal surfaces by enteric pathogens often involves microbial lectin-like adhesins that recognize specific glycoconjugates on intestinal epithelial cells (19, 45). In the mammalian intestine, carbohydrate structures on epithelial cell surfaces vary among species and strains, among different intestinal regions, among different cell types, and even among enterocytes on a single villus (9, 28). There is some evidence that the selectivity of microbial adhesins for specific epithelial surface carbohydrate structures may determine the patterns of susceptibility of certain species and individuals and the regional distribution of infection within the host gastrointestinal tract (4, 19, 26). The host epithelial cell molecules that serve as receptors, however, are largely unknown.

Salmonella species can adhere to and invade a wide variety of cell types, from kidney epithelial cells to macrophages (11). Genetic studies of *Salmonella typhimurium* have demonstrated that many genes may be involved in the complex process of adherence and cell invasion (10). In vivo, the first direct encounter of *Salmonella* spp. with host cells is the initial recognition of and adherence to the surface of the intestinal epithelium, and this event is a prerequisite for the subsequent steps in pathogenesis that lead to mucosal infection, systemic spread, and disease (5, 17). Fimbriae, which have been the subject of extensive genetic and functional studies (19, 33), are thought to play a role in initial adherence. *S. typhimurium* and *Salmonella typhi* rapidly adhere to M cells of the follicle-associated epi-

thelium (6, 21, 24) and subsequently also adhere to absorptive enterocytes (47). Whereas relatively little is known about the composition of M-cell membranes (14), the enterocyte brush border membrane has been analyzed in considerable detail (7, 16, 32, 44), and this should facilitate the identification of *Salmonella* receptors. Enterocyte apical surfaces bear a complex array of glycoconjugates that are potential receptors for microbial adhesins, including membrane glycolipids (23), intramembrane stalked glycoprotein enzymes (16, 27, 44), and the large mucin-like glycoprotein that forms the thick, membrane-anchored glycocalyx on the tips of microvilli (3, 20, 29). In vivo, all of these components are in place on differentiated absorptive cells when they emerge from the crypts (27, 34, 40), and adherence of *Salmonella* spp. has not been correlated with the presence of any single membrane component.

Certain human colon carcinoma cell lines, such as Caco-2, sequentially express and organize brush border membrane components in vitro in a way that mimics the differentiation of normal small-intestinal enterocytes (7, 34, 37). *S. typhimurium* adheres to and invades well-differentiated Caco-2 cell monolayers, providing a valuable in vitro model (11). Using such models, investigators have observed that a subpopulation of cells in epithelial monolayers are heavily infected with *S. typhimurium* while others are untouched (10), but the basis for this selectivity is not known. The initial interaction of *S. typhimurium* with an epithelial cell induces dramatic changes in the apical cell surface (13, 47), and it has been suggested that these changes could facilitate adherence and invasion of additional organisms on the same cell (13, 15). However, monolayers of cloned Caco-2 cells do not differentiate synchronously, and even "fully differentiated" monolayers show a mosaic pattern of enzyme expression (2, 7, 37). This raises the possibility that differences in membrane enzymes or glycoconjugates among individual Caco-2 cells could explain the preferential infection

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of certain cells by *Salmonella* spp. and could provide clues about the mechanism of *Salmonella* adherence in vivo.

The goal of this study was to take advantage of the cell-selective binding of *S. typhimurium* to Caco-2 cells, in conjunction with the variable pattern of expression of apical membrane enzymes and complex carbohydrates on these cells, to identify possible epitopes involved in the initial adherence of these bacteria. Our results indicate that a Gal β (1-3)GalNAc carbohydrate epitope recognized by peanut agglutinin (PNA) is involved in the initial binding of *S. typhimurium* to Caco-2 cells and that this epitope is located in the glycocalyx blanketing the microvilli.

MATERIALS AND METHODS

Antibodies, lectins, and bacteria. Mouse ascites fluids containing mouse monoclonal anti-sucrase-isomaltase and anti-dipeptidyl-peptidase IV (DPP-IV) antibodies were generous gifts from A. Quaroni (Cornell University, Ithaca, N.Y.) (40) and were used at a dilution of 1:100. Anti-*Salmonella* serum (Sal O Group B Factors 1, 4, 5, and 12) was purchased from Difco (Detroit, Mich.) and used at a dilution of 1:1,000. Goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) and anti-mouse IgG-tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Hyclone Labs (Logan, Utah), and streptavidin-TRITC was purchased from Molecular Probes, Inc. (Eugene, Oreg.); all of these secondary reagents were used at 2 μ g/ml. Biotinylated lectins were purchased from Vector Labs (Burlingame, Calif.) and used at 20 μ g/ml, except for *Limax flavus* agglutinin (LFA), which was purchased from EY Labs (San Mateo, Calif.) and used at 50 μ g/ml. *S. typhimurium* 14028 was a gift from J. Mekalanos (Harvard Medical School, Boston, Mass.).

Growth and differentiation of Caco-2_{BBe} cells. The Caco-2_{BBe} cloned cell line was a generous gift from M. Mooseker (Yale University, New Haven, Conn.). Cells were grown as described previously (37) in 75-cm² flasks at 37°C in 5% CO₂ in 25 mM glucose-Dulbecco's minimal essential medium containing 10% fetal bovine serum (Hyclone Labs), 100 IU of penicillin per ml, 100 μ g of streptomycin per ml, and 10 μ g of human transferrin (Sigma, St. Louis, Mo.) per ml. Approximately 4×10^5 cells in 1 ml of medium were seeded on each 12-mm-diameter glass coverslip (Bellco Glass Inc., Vineland, N.J.) in 24-well plates. Approximately 10^5 cells in 0.3 ml of medium were seeded into the apical compartment of Transwell-COL filter inserts (Costar, Cambridge, Mass.). Cells on filters were cultured in 24-well plates and given 1.0 ml of medium in the basal compartment. The medium of each culture was replaced every other day. Monolayers were used for experiments at 7, 14, or 21 days postconfluence. For bacterial adherence assays, the medium was replaced with antibiotic-free Dulbecco's minimal essential medium 3 h prior to the addition of organisms.

Growth and preparation of bacterial cultures for adherence assays. Bacterial suspensions were prepared according to the protocol of Lee and Falkow (25), which maximizes the invasiveness of *S. typhimurium* in vitro. An overnight culture was grown in Luria broth with shaking at 37°C from bacteria which were stored frozen at -80°C in 15% glycerol. A 1:50 dilution of the overnight culture was made, and the organisms were grown to late log phase (optical density of 0.6 at 600 nm; 6×10^8 bacteria per ml) without shaking in 10 ml of Luria broth in a 15-ml conical centrifuge tube. The organisms were centrifuged and washed twice with sterile saline. The bacteria were then resuspended at a concentration of 10^9 /ml in normal saline, and a sample of this suspension was further diluted in saline, plated on agar, and incubated overnight at 37°C to determine CFU per milliliter. The remaining bacteria were diluted with antibiotic-free medium to 10^8 /ml for adherence experiments. The number of CFU always ranged from 5×10^7 /ml to 1×10^8 /ml after dilution in medium.

***S. typhimurium* adherence and invasion of live and prefixed monolayers.** Monolayers were washed twice with Dulbecco's phosphate-buffered saline (PBS) with 1 mM CaCl₂ and 0.5 mM MgCl₂ (D-PBS). Some coverslips were fixed for 30 min at room temperature (RT) with 3% formaldehyde in PBS and washed three times with D-PBS, and the reactive aldehyde groups were quenched with 50 mM NH₄Cl for 10 min at RT. For bacterial adherence and invasion assays with live or prefixed monolayers, 1 ml of the bacterial suspension (10^8 /ml) was added to wells with coverslips, and 0.3 ml was added to the apical compartment of filter units. Monolayers were incubated with bacteria for 1 to 2 h at 37°C in 5% CO₂ and subsequently washed three times with D-PBS before processing. Although some attached *Salmonella* organisms may have detached during culture and washing, those that were resistant to washing were stabilized by fixation as described below. For inhibition assays, live or prefixed monolayers grown on coverslips were incubated with lectins (20 μ g/ml, 500 μ l per coverslip) diluted in D-PBS for 1 h at 37 or 23°C. All coverslips were then washed three times for 5 min each with D-PBS before the addition of bacteria.

The number of bacteria per square millimeter for each lectin treatment was determined from two to five random areas of each coverslip photographed at a magnification of $\times 25$. A minimum of 600 Caco-2 cells were included in the two

FIG. 1. Cell-selective pattern of binding of *S. typhimurium* to confluent monolayers of well-differentiated Caco-2_{BBe} cells. (A and B) One-micrometer section of Caco-2_{BBe} cells grown on a permeable support and incubated before fixation with *S. typhimurium*. Sections were labeled with anti-*Salmonella* antibody and viewed by immunofluorescence (A) and phase-contrast microscopy (B). *Salmonellae* adhered to a subpopulation of the cells, and cells of this subpopulation contained multiple bacteria. (C and D) Monolayers grown on glass coverslips and incubated either live (C) or after fixation (D) with *S. typhimurium*, labeled with anti-*Salmonella* antibody, and viewed by immunofluorescence. Live and fixed monolayers showed similar cell-selective patterns of *S. typhimurium* adherence. Bar, 25 μ m.

FIG. 2. Lack of correlation of *S. typhimurium* binding with expression of brush border hydrolases by Caco-2_{BBe} cells. Caco-2_{BBe} cells grown on glass coverslips were incubated with *S. typhimurium*, double labeled with antienzyme and anti-*Salmonella* antibodies, and viewed by immunofluorescence. (A) Expression of sucrase-isomaltase was heterogeneous, labeling 40 to 70% of the cells in the monolayers. (B) There was no correlation with expression of sucrase-isomaltase and adherence of bacteria. Bar, 50 μ m.

areas examined on each coverslip. These areas contained a minimum of 100 bacteria (under inhibiting conditions) and a maximum of 3,000 bacteria. The mean percent inhibitions $\pm$ standard deviations were calculated from two or more experiments, with coverslips pretreated with D-PBS as controls.

Detection of salmonellae by immunofluorescence of semithin sections of filter-grown cells. *Salmonella* binding and invasion on filter-grown cells were detected by high-resolution fluorescence microscopy of semithin plastic sections as previously described (14). Briefly, monolayers in filter units were fixed with 3% formaldehyde–0.1% glutaraldehyde after incubation with bacteria and subsequent washing. Filters were removed from the plastic inserts during dehydration in ethanol and embedded in Polybed 812/Araldite (Polysciences, Warrington, Pa.). Sections (0.6 μ m thick) were cut perpendicular to the monolayer surface and attached to glass slides, and the Epon resin was extracted. Free aldehydes were quenched for 10 min with 50 mM NH_4Cl in PBS, and nonspecific protein binding sites were blocked with 0.2% gelatin–PBS for 30 min at RT. The sections were incubated with rabbit anti-*Salmonella* serum for 1 h at RT, washed, and incubated with anti-rabbit IgG–FITC for 30 min at RT. Slides were mounted, examined with a Zeiss Axiophot microscope equipped for epifluorescence, and photographed with Kodak T-Max 400 film.

Detection of salmonellae and brush border components by double-label immunofluorescence of Caco-2 cells on coverslips. Live and prefixed Caco-2 cells grown on coverslips were exposed to *S. typhimurium* and washed as described above. The monolayers were then fixed with 3% formaldehyde in PBS for 30 min at RT, washed, quenched with 50 mM NH_4Cl , and blocked with 0.2% gelatin–PBS for 30 min at RT. Cells were labeled with various pairs of antibodies and biotinylated lectins (for example, rabbit anti-*Salmonella* serum plus mouse anti-sucrase-isomaltase monoclonal antibody, mouse anti-DPP-IV monoclonal antibody, or biotinylated lectin), diluted in gelatin–PBS, and incubated for 1 h at RT. After being washed, the monolayers were incubated with secondary reagents: (anti-rabbit IgG–FITC and either anti-mouse IgG–TRITC or streptavidin–TRITC) for 30 min at RT. Coverslips were mounted inverted onto glass slides and analyzed by epifluorescence as described above.

Light and electron microscopy of epithelial cells. Following incubation of live and prefixed monolayers with organisms, samples were fixed in 2.5% glutaraldehyde–2% formaldehyde in 0.1 M sodium cacodylate (pH 7.4) for 1 h at RT, washed three times with cacodylate buffer, and postfixed in 1% aqueous OsO_4 for 1 h. For visualization of the glycocalyx, Caco-2 cell monolayers or ileal tissue samples from anesthetized New Zealand White rabbits (Charles River, Wilmington, Mass.) were fixed with a freshly prepared 1:1 mixture of 1% OsO_4 containing 7.5 mg of $\text{K}_4\text{Fe}(\text{CN})_6$ per ml and 5% glutaraldehyde in cacodylate buffer for 90 min, and samples were changed to fresh fixative every 10 min. Tissues were stained en bloc with 0.5% uranyl acetate for at least 2 h, dehydrated, and embedded in Polybed 812/Araldite (Polysciences, Warrington, Pa.). Caco-2 cells were fixed and processed on coverslips and then removed from the coverslips and embedded as previously described (38). For high-resolution light microscopy of cell monolayers, semithin (1- μ m) sections of Polybed-embedded cells were prepared and stained with 1% toluidine blue. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100CX electron microscope.

Fluorescence microscopy of human mucosal tissue. Mucosal biopsies were obtained from the terminal ileum of a human subject as part of a separate study conducted by Alan Leichter of the Division of Gastroenterology and Nutrition at Children's Hospital, Boston, Mass., and approved by the Human Studies Committee of Children's Hospital under protocol no. 94-02-016. Biopsies were

TABLE 1. Lectins used in this study^a

Lectin	Specificity	Epitope(s)	Reference
Jacalin (<i>Artocarpus integrifolia</i>)	Terminal galactose	Gal β (1-3)GalNAc, Gal α (1-6)Gal	43
PNA (<i>Arachis hypogaea</i>)		Gal β (1-3)GalNAc	46
RCA I (<i>Ricinus communis</i> I)		Gal β (1-4)Glc	35
ECA (<i>Erythrina cristagalli</i>)		Gal β (1-4)GlcNAc	22
VVA (<i>Vicia villosa</i>)	Terminal GalNAc	GalNAc, NeuAc	48
DBA (<i>Dolichos biflorus</i>)		GalNAc α (1-3)Gal	39
SBA (<i>Glycine max</i>)		GalNAc α / β	39
WGA (<i>Triticum vulgaris</i>)	Sialic acid	Neu5Ac, (GlcNAc) ₂₋₅	1
LFA (<i>Limax flavus</i>)		Neu5Ac, Neu5Gc	30
sWGA (succinylated WGA)	GlcNAc	(GlcNAc) ₂₋₅	32
ConA (<i>Canavalia ensiformis</i>)	Mannose/glucose	Branched N-linked hexasaccharide	8
UEA I (<i>Ulex europaeus</i> I)	Fucose	Fuc α (1-2)Gal	36

^a GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid.

FIG. 3. Lack of correlation of *S. typhimurium* binding with expression of WGA binding sites. Coverslip-grown Caco-2 cells were incubated with *S. typhimurium* and double labeled with biotinylated WGA and anti-*Salmonella* antibodies. Expression of WGA binding sites (A) did not correlate with binding of *S. typhimurium* (B). Bar, 50 μ m.

fixed in 3% formaldehyde–0.1% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.2) and processed by the same protocol used for Caco-2 cells grown on filters. The Epon-Araldite embedding medium was extracted from 1- μ m sections prior to labeling with biotin-conjugated PNA.

RESULTS

Pattern of *S. typhimurium* adherence to differentiated Caco-2_{BBe} cells. We first examined whether Caco-2_{BBe} cells in confluent, differentiated monolayers are uniformly susceptible to *S. typhimurium* adherence. Caco-2 cells were cultured on collagen-coated filter inserts or on glass coverslips to 14 or 21 days postconfluence and then incubated with 10^8 *S. typhimurium* organisms per ml for 60 or 120 min. The cell monolayers were washed and fixed, and the bacteria were visualized by immunofluorescence of semithin sections of monolayers on filters (Fig. 1A and B) or of intact monolayers on coverslips (Fig. 1C). Adherent bacteria were present only on a subpopulation of cells that varied from 40 to 80% of the cells in individual monolayers. Cells in this subpopulation had up to 150 bacteria on the apical surfaces and in apical compartments, while neighboring cells had none. The pattern of cell-selective infection and the total numbers of bacteria on or in the host cells did not

differ dramatically at the two time points, but at 120 min, a larger proportion of the bacteria had been internalized.

It has been suggested that the cytoskeletal rearrangements and membrane alterations induced by initial attachment of *S. typhimurium* to an epithelial cell may promote adherence and invasion of the same cell by other salmonellae (13, 15). To test this hypothesis, differentiated Caco-2 cell monolayers were fixed with 3% formaldehyde to prevent epithelial cell responses. They were then washed and incubated with *S. typhimurium* as described for live monolayers. The adherence pattern for prefixed monolayers (Fig. 1D) was identical to that for live monolayers (Fig. 1C). These results suggest that the cell-selective pattern of binding of *S. typhimurium* to live Caco-2_{BBe} cells was not due entirely to bacterium-induced alterations in the apical surface but rather to inherent differences in apical surface components of individual epithelial cells.

Role of epithelial cell differentiation in *S. typhimurium* adherence. *S. typhimurium* adherence appeared to require some degree of apical membrane differentiation, since bacteria did not adhere to early (0 to 7 days postconfluence) monolayers (data not shown). The undifferentiated Caco-2 cells in these early monolayers were confluent and polarized but had not yet developed an organized brush border, did not have apical sucrase-isomaltase, and did not display the lectin binding sites typical of older Caco-2 cells with brush borders. It is known that the degree of differentiation of individual cells in Caco-2 cell monolayers 14 to 21 days postconfluence is not uniform, as demonstrated by morphological features and the expression of brush border hydrolases (7, 37). In order to determine whether the cell-selective bacterial binding pattern is correlated with expression of specific brush border enzymes, monolayers at 14 and 21 days postconfluence were incubated with *S. typhimurium*, fixed, and labeled with antibody to one of two brush border hydrolases which are considered to be differentiation markers for this cell type: DPPIV or sucrase-isomaltase (27, 34, 40). At both 14 and 21 days, DPPIV, an early differentiation marker, was expressed by all of the cells and therefore could not be correlated with adherence of bacteria (data not shown). Only 40 to 70% of cells expressed the late differentiation marker sucrase-isomaltase (Fig. 2A), but there was no correlation between expression of this enzyme and adherence of bacteria (Fig. 2B). These results suggest that *S. typhimurium* adherence to Caco-2_{BBe} cells is neither dependent on nor correlated with expression of these integral membrane glycoprotein enzymes.

TABLE 2. Lectin binding patterns and correlation with *Salmonella* binding

Lectin	% Positive cells in monolayer ^a	Staining intensity of positive cells	Correlation with <i>Salmonella</i> binding
Jacalin	98	Variable	No
PNA	80	Variable	Yes
RCA I	100	Uniform	No
ECA	100	Uniform	No
VVA	98	Variable	No
DBA	25	Variable	No
SBA	80	Variable	Yes
WGA	100	Uniform	No
LFA	60	Variable	No
sWGA	80	Variable	No
ConA	95	Variable	No
UEA I	80	Variable	No

^a Average percentages were calculated from four microscopic fields (magnification, $\times 63$) per coverslip from at least two experiments.

FIG. 4. Correlation of *S. typhimurium* binding with expression of PNA binding sites. (A and B) Prefixed Caco-2 cells on coverslips were incubated with *S. typhimurium* and double labeled with biotinylated PNA and anti-*Salmonella* antibodies. PNA, specific for Gal β (1-3)GalNAc, labeled 75 to 85% of prefixed cells in the monolayer (A), and the same subpopulation of cells harbored *S. typhimurium* (B). Bar, 50 μ m. (C and D) Live Caco-2 cells on coverslips were incubated with *S. typhimurium*, fixed, and labeled with biotinylated PNA and anti-*Salmonella* antibodies. A single large cell is shown at the center. The dark PNA-negative spots on the cell surface (arrows in panel C) correspond to sites of adherence or internalization of bacteria (arrows in panel D). Bar, 20 μ m.

Lack of correlation of *S. typhimurium* binding with expression of certain lectin binding sites on Caco-2_{BBe} cells. A panel of lectins with various carbohydrate specificities was applied to monolayers at 14 and 21 days postconfluence to determine which, if any, correlated with the binding pattern of *S. typhimurium*. Live and prefixed monolayers were incubated with bacteria, fixed, and labeled with the lectins listed in Table 1. Three of the lectins tested, including wheat germ agglutinin (WGA) (Fig. 3A), *Ricinus communis* I (RCA I), and *Erythrina cristagalli* (ECA), labeled all of the cells with a relatively uniform intensity (Table 2). Thus, the binding pattern of *S. typhimurium* did not correlate with the binding of any of these lectins (Table 2), including WGA (Fig. 3B). Other lectins, including Jacalin, *Vicia villosa* (VVA), *Dolichos biflorus* (DBA), LFA, succinylated WGA (sWGA), concanavalin A (ConA), and *Ulex europaeus* I (UEA I), exhibited heterogeneous binding patterns, labeling populations of cells ranging from 25 to 98% of the cells in the monolayers with various

degrees of intensity (Table 2). The binding patterns of these lectins did not correlate with *Salmonella* binding (data not shown).

Correlation of *S. typhimurium* with presence of PNA and SBA binding sites. The binding patterns of two lectins, soybean agglutinin (SBA) (specific for terminal α - and β -GalNAc) and PNA [specific for Gal β (1-3)GalNAc], correlated very well with the pattern of *Salmonella* adherence. Although PNA binding sites and bacterial adherence were closely correlated on both prefixed (Fig. 4A and B) and live (Fig. 4C and D) cells, a minority (1 to 5%) of PNA-positive cells failed to bind salmonellae. After incubation of live monolayers with bacteria for 30 min or more, infected cells showed PNA-negative spots on their apical surfaces (Fig. 4C) that roughly correlated with the size and shape of single bacteria (Fig. 4D). This was not observed with fixed monolayers. These spots are likely to represent internalization of apical surface domains and/or masking of lectin binding sites accompanying the adherence and inva-

FIG. 5. Correlation of *S. typhimurium* binding with Caco-2 cell apical membrane differentiation. (A) Islands of undifferentiated cells (U) (enclosed by arrowheads) were observed by phase-contrast microscopy within well-differentiated monolayers of coverslip-grown Caco-2 cells. Bar, 100 μ m. (B and C) Semithin toluidine blue-stained sections of Caco-2 cell monolayers showed that the well-differentiated areas (B) contained tall, polarized cells with brush borders. Cells in the undifferentiated islands (C) were flat and had no brush borders. Bar, 20 μ m. (D and E) Prefixed Caco-2 cells on coverslips were incubated with *S. typhimurium* and double labeled with biotinylated PNA and anti-*Salmonella* antibodies. Cells in the undifferentiated islands (U) (demarcated by arrowhead) did not possess binding sites for PNA (D), and bacteria failed to adhere to these areas (E). Bar, 25 μ m.

sion process. *Salmonella* invasion also resulted in localized losses of cell surface labeling by other lectins whose binding was not correlated with *Salmonella* binding (data not shown).

Correlation of *Salmonella* binding with brush border differentiation. In order to test the hypothesis that PNA and/or SBA binding sites are involved in *S. typhimurium* binding to Caco-2_{BBc} cells, we took advantage of the fact that differentiated Caco-2 monolayers grown on glass coverslips (14 to 21 days postconfluence) often contained several small islands of flat, undifferentiated cells (Fig. 5A). The cells in the islands did not express DPPIV or sucrase-isomaltase (not shown). Light microscopy of semithin sections showed that these cells were much flatter and less polarized than cells in well-differentiated areas of the monolayer (Fig. 5B and C), and they exhibited few microvilli and no visible glycocalyx by electron microscopy (not

shown). Cells in these islands did not possess any binding sites for the panel of lectins listed in Table 1, including SBA and PNA (Fig. 5D), suggesting that the apical membrane glycocalyx was not assembled. When live or prefixed monolayers (Fig. 5E) were exposed to *S. typhimurium*, the bacteria failed to bind to cells in the undifferentiated islands. Young, newly confluent monolayers (2 to 3 days postconfluence) were also analyzed for bacterial binding and PNA or SBA binding sites. Although low levels of lectin binding were observed on some cells, there was no significant bacterial binding after our washing and fixation procedure (not shown). We cannot rule out the possibility that some reversible adherence occurred.

Inhibition of *Salmonella* binding. To directly test whether PNA and SBA binding sites are involved in *S. typhimurium* adherence to Caco-2_{BBc} cells, live monolayers were preincubated with various lectins at 37°C, washed, and then challenged

TABLE 3. Inhibition of *Salmonella* binding with lectins

Lectin	Specificity	% Inhibition (mean $\pm$ SD) ^a with:	
		Live monolayers	Fixed monolayers
PNA	Gal β (1-3)galNAc	93.3 $\pm$ 3.1 (90.8 $\pm$ 3.2)	92.7 $\pm$ 0.6
SBA	GalNAc α / β	<5	ND ^b
Jacalin	Gal β (1-3)galNAc, Gal α (1-6)gal	70.0 $\pm$ 5.2	ND
ECA	Gal β (1-4)glcNAc	67.7 $\pm$ 11.0	ND
RCA I	Gal β (1-4)glc	37.7 $\pm$ 2.2	ND
WGA	Neu5Ac, (GlcNAc) ₂₋₅	65.7 $\pm$ 11.9 (34.7 $\pm$ 5.4)	59.5 $\pm$ 8.2
LFA	Neu5Ac, Neu5Gc	<5	ND
sWGA	(GlcNAc) ₂₋₅	<5	ND
UEA I	Fuc α (1-2)gal	<5	ND
VVA	GalNAc, NeuAc	<5	ND

^a Obtained from calculations of number of bacteria per square millimeter from at least two experiments. Data in parentheses are from experiments in which the lectin exposure was carried out at RT instead of 37°C.

^b ND, not determined.

with bacteria. Preincubation with PNA reduced binding of bacteria by 93% (Table 3). Surprisingly, SBA did not prevent adherence, showing 5% or less inhibition. Jacalin, ECA, and RCA I, which have specificities related to that of PNA (Table 1), inhibited binding by 70, 68, and 38%, respectively (Table 3).

Previous studies using neuraminidase had suggested that sialic acid may be involved in *S. typhimurium* binding to MDCK cells (11). In our system, WGA [specific for (GlcNAc)₂ and Neu5Ac] inhibited *Salmonella* binding (66%), but sWGA [specific for (GlcNAc)₂ only] and LFA (specific for Neu5Ac and Neu5Gc) did not (Table 3). Finally, UEA I and VVA did not inhibit binding (Table 3). Similar inhibition data were obtained either when monolayers were prefixed with formaldehyde prior to exposure to lectins (Table 3) or when live monolayers were exposed to lectins at 23°C to slow endocytosis. These results further implicate the PNA epitope, Gal β (1-3)GalNAc, in adherence of *S. typhimurium* to Caco-2_{BBE} cells and suggest that sialic acid residues may also play a role.

Interaction of *S. typhimurium* with the epithelial cell glycocalyx. To visualize the interaction of *S. typhimurium* with live and prefixed monolayers, electron microscopy was performed on well-differentiated monolayers of filter-grown cells. When bacteria were incubated with prefixed monolayers, multiple organisms adhered to a subpopulation of cells without ultrastructural alteration of apical cell surfaces and with a gap (up to approximately 100 nm) between the bacterial surface and the tips of the microvilli (Fig. 6A). When such monolayers were prepared for electron microscopy by a method which reveals the glycocalyx, adherent bacteria were seen in direct contact with the glycocalyx, which extended up to 75 nm from the tips of the microvilli (Fig. 6B). These observations suggest that components of the glycocalyx are involved in the initial bacterium-epithelial cell interaction.

On live monolayers, various stages of adherence and internalization were seen (Fig. 6C). Bacteria at microvillar tips appeared to interact with the glycocalyx. Internalization of bacteria was accompanied by microvillar disruption and internalization of the host cell membrane, as described by others (11, 24, 47). The electron microscopy images suggest that the local loss of lectin binding at sites of bacterial uptake, as shown in Fig. 4C, could have been due to internalization and sequestering of lectin binding sites and/or to local shedding of membrane and glycocalyx.

Comparison of the glycocalyxes of Caco-2_{BBE} cells and enterocytes in vivo. To assess the relevance of the interaction between *S. typhimurium* and Caco-2_{BBE} cells in vitro to *Salmonella* adherence in vivo, we compared the structure of the

glycocalyx on Caco-2_{BBE} cells grown on filters with that of enterocytes in rabbit small intestine. The microvillar tips of Caco-2 cells showed a thin coat of filaments extending 25 to 75 nm from the membrane (Fig. 7A). In contrast, the glycocalyx on normal enterocytes from rabbit small intestine consisted of a thick filamentous layer extending approximately 400 nm from the tips of the microvilli and forming a blanket over the entire apical cell surface (Fig. 7B), as previously observed for various species by others (20, 29).

To determine whether the apical glycocalyx on enterocytes in normal human ileum contains PNA binding sites, a section from an ileal biopsy was subjected to lectin labeling. PNA strongly labeled the apical borders of villus enterocytes, suggesting that cognate oligosaccharides were available in the glycocalyx (Fig. 8). Although a survey of many human subjects was beyond the scope of this study, this result shows that at least some individuals may display these binding sites.

DISCUSSION

This study has shown that in polarized, well-differentiated Caco-2 cell monolayers, a subpopulation of cells displays a Gal β (1-3)GalNAc carbohydrate epitope on the apical surface that is closely correlated with adherence of *S. typhimurium*. Our results suggest that one mechanism of initial binding of *S. typhimurium* to the apical surfaces of intestinal epithelial cells may involve the recognition of a membrane-associated glycoconjugate containing this epitope by a lectin-like microbial adhesin.

Although Caco-2 cells are considered to be among the best in vitro models for human villus epithelial cells currently available, it is important to note that they differ from normal small-intestinal enterocytes in several respects. This cell line was originally derived from a relatively well-differentiated colon carcinoma and shows features typical of the fetal colon, where villi are transiently present and small-intestinal enzymes are expressed in utero (34). Of the numerous samples and clones of Caco-2 cells available, we chose the Caco-2_{BBE} clone because these cells consistently assemble well-developed apical brush borders with microvilli and associated cytoskeletal structures analogous to those of normal enterocytes in vivo (37). This is important because differentiation of this highly specialized apical membrane domain is a prerequisite for proper targeting, retention, and organization of the integral membrane glycoproteins that provide an interface with the intestinal lumen (7, 16). Despite their well-formed brush borders,

FIG. 6. Interaction of *S. typhimurium* with Caco-2 cell surfaces. Filter-grown Caco-2_{BBe} cells, either live or prefixed, were incubated with *S. typhimurium* and prepared for electron microscopy. (A and B) On prefixed cells, multiple bacteria adhered to the tips of microvilli on certain cells. When prepared by a method that stains the glycocalyx (B), adherent bacteria were seen in direct contact with the glycocalyx but not with the microvillar membrane. (C) On live monolayers, bacteria interacted with the microvillar tips (1) but were also internalized (2) by a process including reorganization, vesiculation, and loss of microvilli. Bars, 1 μ m (A and C) and 400 nm (B).

however, “fully differentiated” Caco-2_{BBe} monolayers do not uniformly express all of the apical membrane components of villus enterocytes in vivo. For example, sucrose-isomaltase, which is generally considered a marker of terminal differentiation, is present on almost all normal villus enterocytes but is expressed by only a subpopulation of Caco-2_{BBe} cells scattered apparently at random through the monolayer (2, 7, 37). In the present study this mosaic pattern was exploited to show that neither sucrose-isomaltase nor an earlier-expressed brush border protease is likely to serve as a receptor for *S. typhimurium*.

We have shown in addition that although differentiated

Caco-2 cells display many lectin binding sites and have a “cell coat” visible by electron microscopy, these cells lack the carbohydrate-rich, 400- to 500-nm-thick glycocalyx that is anchored to microvillus tips of normal enterocytes (20, 29). Although the expression of glycoconjugates on enterocytes in vivo can vary (28), the glycosylation patterns of the apical membrane components on Caco-2 cells are extremely heterogeneous. In particular, the villus cells of normal human ileum (shown here) and mouse ileum (9, 14) can show nearly uniform binding of the lectin PNA, whereas Caco-2_{BBe} cell monolayers bind PNA in a mosaic pattern. Our data indicate that one “receptor” exploited by salmonellae on Caco-2 cells in vitro is

FIG. 7. Comparison of the glycolyxes on Caco-2_{BBE} cells and small-intestinal enterocytes. (A) The glycolyx on well-differentiated, filter-grown Caco-2_{BBE} cells appeared as filaments extending approximately 25 to 75 nm from the tips of the microvilli. (B) On normal rabbit enterocytes in vivo, the glycolyx consisted of a 400- to 500-nm-thick filamentous layer that uniformly blanketed the microvilli. Bar, 400 nm.

FIG. 8. Lectin binding sites on enterocytes of normal human terminal ileum. A 1- μ m section was incubated with PNA-biotin followed by streptavidin-FITC and was viewed by high-resolution fluorescence or phase-contrast microscopy. (A) PNA labeled apical borders of enterocytes with a strong, uniform intensity. Intracellular labeling corresponds in position to the Golgi complex. (B) Phase-contrast microscopy. Bar, 20 μ m.

the Gal β (1-3)GalNAc structure recognized by PNA. This suggests that brush border glycoproteins or glycolipids bearing this carbohydrate structure could serve as receptors for salmonellae on normal enterocytes, but studies in vivo are required to establish whether this is the case.

Other investigators had observed that treatment of MDCK cells with neuraminidase reduced binding and uptake of *S. typhimurium*, and they suggested that salmonellae may exploit sialic acid residues as receptors (12). In our Caco-2 cell system, however, cell-specific binding of *S. typhimurium* was not correlated with binding of WGA or other lectins that recognize sialic acid-containing glycoconjugates. Indeed, the lectin (PNA) that best correlated with and inhibited *S. typhimurium* adherence recognizes a Gal β (1-3)GalNAc structure that might be masked by the addition of a terminal sialic acid residue, as seen with *Pseudomonas aeruginosa* adherence to respiratory

epithelial cells (42). Since *S. typhimurium* synthesizes a neuraminidase (18), the organisms might enzymatically remove terminal sialic acid residues in order to expose underlying Gal β (1-3)GalNAc. It is therefore paradoxical that binding of *S. typhimurium* to Caco-2 cells was partially inhibited by WGA. It must be emphasized that *S. typhimurium* may use multiple adherence mechanisms, and we have not ruled out the possibility that some lectins may inhibit bacterial binding by steric hindrance or that oligosaccharides containing sialic acid and other carbohydrate structures may also serve as receptors on some cell types. Further work on *Salmonella* interactions with the normal mucosa is needed to resolve these issues.

Electron microscopic studies in vivo (24, 47) and in vitro (11), along with our own observations, have shown that *S. typhimurium* initially adheres to material on the tips of microvilli, presumably the glycocalyx, and subsequently induces a dramatic reorganization of the brush border membrane and cytoskeleton. The epithelial cell response to *Salmonella* binding might expose additional binding sites and render the apical cell surface more accessible to the bacteria. This could explain in part why multiple *Salmonella* organisms tend to infect certain target epithelial cells while leaving other cells untouched. However, we have demonstrated that a subpopulation of Caco-2 cells are selectively targeted by *S. typhimurium* even when the epithelial cells are aldehyde fixed and unable to alter their apical surfaces. Therefore, the epithelial cell response cannot completely account for the predilection of *S. typhimurium* for certain cells in the monolayer. Rather, our results suggest that differences in the glycosylation patterns and/or accessibilities of cell surface glycoconjugates may be sufficient to explain the selective adherence of *S. typhimurium* to certain Caco-2_{BBE} cells. It is now important to extend these observations to the normal intestine and to determine whether glycoconjugate heterogeneity or accessibility can explain the selective adherence of *Salmonella* species to subpopulations of enterocytes and other epithelial cells (6, 10, 21, 24).

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