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N-Glycosylated Proteins Are Involved in Efficient Internalization of *Klebsiella pneumoniae* by Cultured Human Epithelial Cells

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***Klebsiella pneumoniae* obtained from patients with urinary tract infections is able to invade cultured human epithelial cells. The internalization process is dependent upon both microfilaments and microtubules. To better understand the interaction of these invasive bacteria with the host cell receptor(s), bladder, lung, and ileocecal epithelial cells were infected with *K. pneumoniae* in the presence of various lectins possessing multiple glycan specificities. It was found that the *N*-acetylglucosamine (GlcNAc)-specific lectins concanavalin A, *Datura stramonium* agglutinin, and wheat germ agglutinin significantly inhibited the invasion of *K. pneumoniae* into these cells but did not interfere with the internalization of an invasive strain of *Salmonella typhimurium*. Conversely, internalization of *K. pneumoniae* but not *S. typhimurium* was also significantly inhibited when the bacteria were pretreated with GlcNAc or chitin hydrolysate, a GlcNAc polymer, prior to the gentamicin invasion assay. Other carbohydrates such as glucose, galactose, mannose, fucose, and *N*-acetylneuraminic acid had no inhibitory effects on *K. pneumoniae* uptake. Furthermore, internalization of *K. pneumoniae* but not *S. typhimurium* by HCT8 cells was also significantly inhibited when eukaryotic protein glycosylation was interrupted by tunicamycin or when host N-linked surface glycans were removed by pretreatment with *N*-glycosidase F. These studies suggest that a N-glycosylated protein receptor is involved in the internalization of *K. pneumoniae* by human epithelial cells in vitro. The results also indicate that internal GlcNAc residues might be a carbohydrate component of the receptor.**

Klebsiella pneumoniae, a widely recognized urinary tract pathogen, also causes opportunistic and nosocomial pneumonia with accompanying bacteremia and septicemia (9, 20, 22). Patients with complicated urinary tract infections (UTIs) are more prone to severe disease, such as renal damage and urosepsis (26). Treatment of *K. pneumoniae* infections has become increasingly difficult because of the predominance of multiple-antibiotic-resistant strains (2, 3, 15). Recently, Oelschlaeger and Tall described the ability of *K. pneumoniae* to invade cultured human epithelial cells (29). Therefore, invasion ability might be a new virulence factor for *Klebsiella*. The intracellular location of *K. pneumoniae* could be the cause of recurrent UTIs, as well as explaining antibiotic treatment failures. Evasion of host barriers as a result of transcytosis of epithelial cells may be another cause of urosepsis. However, no known clinical cases have been reported which document intracellular *Klebsiella* in urinary tract biopsy specimens. Therefore, a better understanding of the invasion mechanism is necessary for developing new therapies.

It is considered that internalization of invasive pathogenic bacteria is the result of interaction of bacterial ligands with eukaryotic cell surface receptors, many of which are glycosylated proteins (11, 14, 19, 21, 24, 28, 32, 43). It is still unclear whether the bacterial invasion proteins bind to the protein or carbohydrate moiety of the respective receptor or both. Taking into account that the outermost part of the epithelial cell is covered by the carbohydrate side chains of glycolipids and glycoproteins, it is likely that the receptor carbohydrate moiety is involved in binding of the bacterial ligands.

The present study was undertaken to determine if the invasion ability is common among several *Klebsiella* urinary tract isolates of different capsule types and to identify features of the eukaryotic cell which are essential for efficient internalization of *Klebsiella*. Using a variety of inhibitors which either blocked bacterial ligand-host receptor interaction or interfered with or modified the glycosylation of eukaryotic surface proteins, we investigated the involvement of the N-glycosylated protein(s) on the eukaryotic cell surface in the uptake of *K. pneumoniae* by cultured human epithelial cells. These new studies revealed that an N-glycosylated cell receptor(s) is needed for internalization of *K. pneumoniae* by human epithelial cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *K. pneumoniae* 3091 and IA565 and *Citrobacter freundii* 3009 are from the strain collection of the Department of Bacterial Immunology, Walter Reed Institute of Research, Washington, D.C. Strain 3091 was isolated at the Walter Reed Hospital from a patient with a UTI, and strain IA565 was originally obtained from the University of Iowa Hospitals and Clinics Special Microbiology Laboratory (29). The other *K. pneumoniae* strains, 375, 625, and 706, were clinical urinary tract isolates obtained from patients attending the Hygiene Institute of the University of Würzburg, Würzburg, Germany. They were isolated either from indwelling catheters or from renal puncture cultures (Table 1). *Salmonella typhimurium* C17 (23), *Escherichia coli* DH5α, and *E. coli* 536 (O6:K15:H31; isolated from a patient with an UTI) were from the strain collection of the Institut für Molekulare Infektionsbiologie, University of Würzburg. They were used in all invasion experiments as invasive and noninvasive controls, respectively. Strain DH5α/pRR5 expressing G pili was obtained from S. Saarela, Department of Pharmacy, University of Helsinki, Helsinki, Finland (35). *E. coli* TCFF15, harboring pCCF15 with the cloned sequence of the adhesin CF29K and pDSPH524 (accessory genes), was provided by A. Darfeuille-Michaud, Faculté de Pharmacie, Université d'Auvergne, Clermont-Ferrand, France (5). All strains were stored at −20°C in aqueous solution with 50% glycerol and 3 mM $MgSO_4$. The bacteria were cultivated at 37°C in

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TABLE 1. Invasion efficiencies of various *K. pneumoniae* isolates compared to *S. typhimurium* C17 and *E. coli* DH5α in HCT8 and T24 epithelial cells[a]

Strain	Capsule type[b]	Isolate source	% Invasion into[c]:	
			HCT8	T24
K. pneumoniae 3091	16	UTI	45.1 ± 30.1	7.1 ± 5.7
K. pneumoniae IA565	ND	UTI	11.9 ± 3.9	6.6 ± 3.7
K. pneumoniae 375	26	Renal puncture	15.8 ± 5.2	1.6 ± 1.1
K. pneumoniae 625	16	Indwelling catheter	10.1 ± 5.9	3.2 ± 1.7
K. pneumoniae 706	NC	Renal puncture	48.6 ± 26.5	14.6 ± 12.7
S. typhimurium C17			65.8 ± 51.2	34.5 ± 24.6
E. coli DH5α			0.5 ± 0.5	0.1 ± 0.0

[a] Human epithelial cells from the ileocecum (HCT8) and bladder (T24) were incubated with bacteria for 3 h before the addition of 100 μg of gentamicin per ml to kill extracellular bacteria.

[b] Abbreviations: NC, nonencapsulated; ND, not determined.

[c] The invasion efficiency is expressed as the mean percentage of the inoculum surviving gentamicin treatment ± standard deviation.

Luria-Bertani (LB) broth or on LB plates, supplemented if necessary with ampicillin (50 μg/ml) or tetracycline (30 μg/ml).

Cell lines, media, and culture conditions. The human epithelial cell line HCT8, from the ileocecum, was obtained from the Walter Reed Army Institute of Research (28, 29). T24, an epithelial cell line from the bladder, was purchased from the American Type Culture Collection (Rockville, Md.). The lung-alveolar cell line A549 was kindly provided by the Institute of Microbiology, University of Würzburg. HCT8 cells were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM pyruvate, and 10% fetal calf serum (FCS). T24 cells were grown in McCoy's 5A medium supplemented with nonessential amino acids and 10% FCS. The A549 cell line was cultivated in RPMI 1640 with 5% FCS. All cell lines were cultivated at 37°C in a 5% CO_2–95% air atmosphere at ca. 90% humidity and were split twice a week at a ratio of 1:5 to 1:10. All cell culture media and supplements were purchased from GIBCO-BRL (Gaithersburg, Md.).

Invasion assay. For invasion assays, the human epithelial cells were seeded into 24-well plates (Falcon) and incubated overnight at 37°C. The invasion assay was performed essentially as described previously (27) with minor modifications and is briefly summarized as follows. A 5- to 50-μl volume of a bacterial overnight culture was added to 2 ml of fresh LB medium and incubated until it reached the mid-logarithmic growth phase (optical density at 600 nm ≈ 0.4 to 0.6). The bacteria (1×10^5 to 5×10^5) were added to a confluent monolayer of epithelial cells and incubated for up to 3 h at 37°C in a 5% CO_2–95% air atmosphere (i.e., invasion period). The exact value for each inoculum was determined by a quantitative plate count. The monolayer was washed once with Earl's balanced salt solution after the initial invasion period, and fresh medium containing 100 μg of gentamicin per ml was added to kill extracellular bacteria. After the 1-h kill period, the monolayer was washed twice with Earl's balanced salt solution and lysed with 0.2% sodium deoxycholate for 4 min. The released intracellular bacteria were enumerated by a quantitative plate count. The bacterial viability of all the strains used in this study was not affected by the 0.2% sodium deoxycholate treatment. Invasiveness was expressed as the percentage of the inoculum recovered after gentamicin treatment. Each assay was conducted in duplicate and was independently repeated at least three times. The results are expressed as the mean of all replicate experiments. In control studies in the absence of epithelial cells, using equivalent bacterial numbers, treatment with gentamicin (100 μg/ml) for 1 h killed all bacteria of all strains included in this study. Epithelial cell viability and monolayer integrity were routinely controlled by the addition of trypan blue (Hazleton Biologics, Lenexa, Kans.) and light microscopic analysis.

Invasion assays in the presence of inhibitors. Cytoskeletal inhibitors (Sigma Chemical Co., St. Louis, Mo.) were added 1 h prior to the addition of bacteria to the monolayer and were present during the invasion period. Microfilaments were depolymerized with 2 μM cytochalasin D. Microtubules were depolymerized with 10 μM colchicine. The effectiveness of the inhibitors was demonstrated by direct immunofluorescence with fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma) or indirect immunofluorescence with mouse anti α-tubulin antibodies (Sigma) and tetramethylrhodamine-5-isothiocyanate (TRITC)-labeled secondary anti-mouse antibodies (Sigma) as described previously (23). Lectins (5 to 40 μg/ml) and purified OmpA (30 μg/ml) were added to the cell monolayers 1 h prior to the addition of bacteria and incubated under cell culture conditions (32). The following lectins were used: wheat germ agglutinin (WGA) (*Triticum vulgare*), concanavalin A (ConA) (*Canavalia ensiformis*), DSA (*Datura stramonium*), GMA (*Glycine max*), LCA (*Lens culinaris*), and AAA (*Aleuria aurantia*). They were also present during the invasion period. Bacteria were incubated for 1 h on ice with *N*-acetylglucosamine (GlcNAc) (Sigma), chitin hydrolysate (Vectra), *N*-acetylneuraminic acid, various carbohydrates (Roth; Sigma), and rabbit anti-OmpA serum before being added to the monolayer after the tissue culture medium had been aspirated (33). The carbohydrates used were L-glucose, L-galactose, L-mannose, and L-fucose. All the carbohydrates were directly dissolved in the appropriate cell culture medium at 100 mM, except for chitin hydrolysate (0.6 mg/ml) and *N*-acetylneuraminic acid (5 mg/ml). Inhibition of internalization by GlcNAc in a dose-dependent manner was analyzed at concentrations of 10 to 50 mg/ml (i.e., 40 to 200 mM). Control studies under identical conditions to those in inhibitor assays with equivalent bacterial numbers but in the absence of epithelial cells demonstrated that neither lectins, cytochalasin D, colchicine, nor any of the carbohydrates used adversely affected bacterial viability. Trypan blue staining was performed to verify monolayer integrity under the assay conditions.

The role of oligosaccharides at the surface of epithelial cells in the internalization process was analyzed by using different enzymes and inhibitors. Monolayers of epithelial cells were incubated for 5 min with neuraminidase (0.1 U/ml) and for 1 h with *N*-acetyl-β-D-glucosaminidase (1 U/ml) or endo-β-galactosidase (10 mU/ml) before the bacteria were added. A longer incubation time or higher concentrations disrupted the monolayer. All the enzymes were purchased from Boehringer Mannheim. Tunicamycin (Sigma) prevents the N glycosylation of proteins in the endoplasmic reticulum. *N*-Glycosidase F (Boehringer Mannheim) cleaves off asparagine-linked oligosaccharides from glycoproteins. For treatment with these reagents, epithelial cells were seeded and each reagent was added after ca. 12 h, when the monolayer had reached about 80% confluency. The cells were cultivated overnight for another 15 h and then used for the invasion assays as described above. Control experiments under assay conditions with and without inhibitors but in the absence of epithelial cells demonstrated that the reagents in the concentrations used had no adverse effect on bacterial viability. Epithelial cell viability and monolayer integrity in the presence and absence of inhibitors were controlled by the addition of trypan blue and the use of light microscopic analysis. No effect of the inhibitors on viability and monolayer integrity was observed under the assay conditions.

Fluorescence microscopy. Fluorescently labeled lectins (Sigma) at 40 μg/ml were added to confluent monolayers of epithelial cells and incubated under tissue culture conditions for 1 h. ConA and WGA were labeled with FITC. The digoxigenin-labeled AAA was visualized by adding an FITC-labeled anti-digoxigenin antibody. After a 1-h incubation, the monolayer was fixed with a solution of 3.8% freshly prepared paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and covered with Fluoprep (bio-Merieux). Labeled cells were viewed at a wavelength of 530 to 580 nm with an epifluorescence microscope (Axioplan; Zeiss). No fluorescence was observed if only the secondary antibody had been applied, and fixed cells did not show autofluorescence.

Indirect immunofluorescence for the detection of G-pilus expression was performed as follows. Bacteria were grown for 2 h until they reached the middle of the logarithmic growth phase. Then the bacterial culture (100 μl) was centrifuged, and the sediment was suspended in 30 μl of freshly prepared 3.8% PFA and dried on a slide at 37°C. Nonspecific binding was prevented by blocking with 1% bovine serum albumin in PBS for 5 min. The specimens were treated with rabbit anti-G fimbrial serum (1:10) for 45 min, washed twice with PBS, and visualized by incubation with an FITC-labeled anti-rabbit antibody for an additional 45 min. Finally, the material was covered with Fluoprep and analyzed with an epifluorescence microscope. Strain HB101/pRR5 was used as positive control (35), and the secondary antibody alone was used to demonstrate the specificity of the staining.

Determination of the presence of GafD, CF29K, OmpA, and type 3 fimbriae. To determine the presence of the genes encoding GafD, CF29K, OmpA, and type 3 fimbriae, Southern blot and PCR analyses were carried out by the method described by Sambrook et al. (39) and Saiki et al. (37). DNA isolation was performed as described by Sambrook et al. (39). The sequences of the primers (MWG-Biotech) used for PCR detection were as follows: *gafD*, 5′-TAA GGT CTG TCT GGC TGT and 5′-TCC GTT AAC CGT ACC CGG; *cf29k*, 5′-GCG TGG ACC ACT GGT GAT and 5′-TGC CAC GTT CAG CGG AGC; *ompA*, 5′-ACA GCT ATC GCG ATT GCA and 5′-GGC TGA GTT ACA ACG TCT; *mrkA*, 5′-AAG GTT CTT CTC TCT GCT GC and 5′-TTT CGT AAG TCG CGT AGC TG; and *mrkD*, 5′-TAT TGG CTT AAT GGC GCT GG and 5′-GCA TAT TGA TAG AAG CGC GC.

For Southern blot analysis, the presence of *gafD* was determined by using an *Eco*RI-*Sph*I fragment (1.1 kb) of plasmid pRR5 as the probe (36). A 1-kb *Pst*I fragment of the CF29K gene from pCFF15 was labeled for CF29K detection. The PCR products obtained with the indicated primer pairs specific for *mrkA* and *mrkD* were used as probes for the detection of *mrkA* and *mrkD* by Southern hybridization, respectively. The hybridization was carried out with the nonradioactive enhanced chemiluminescence direct nucleic acid labeling and detection system from Amersham as specified by the manufacturer (Amersham, Braunschweig, Germany). Positive controls were HB101/pRR5 for G-pili and IA565 for type 3 pili and C600/pCFF15 for the nonfimbrial adhesin CF29K. *E. coli* 536 (O6:K15:H31) was used as the positive control for *ompA*.

RESULTS

Invasion efficiencies of various *K. pneumoniae* urinary tract isolates. Recently, we reported the invasiness of *K. pneumoniae* 3091 in cultured human epithelial cells (29). To determine if other clinical isolates of *K. pneumoniae* possessed the ability to invade epithelial cells, the invasiveness of four clinical urinary tract isolates obtained from patients with UTI either by renal puncture or from indwelling-catheter cultures was compared to that of the prototypic strain 3091 (Table 1). These isolates had been randomly selected from our *K. pneumoniae* strain collection and represented different capsule serotypes (Table 1). As shown in Table 1, the highest invasion efficiencies for all of the *K. pneumoniae* strains were obtained with ileocecal HCT8 cells compared to those obtained with bladder T24 cells and the lung alveolar cell line, A549 (data not shown). The percent invasion rates for the *Klebsiella* strains internalized by HCT8 cells ranged from 10.1 to 48.6%, and no significant difference was seen among the isolates with respect to source or capsule type. In contrast, the percent invasion rate for the highly invasive *S. typhimurium* C17 and the noninvasive *E. coli* DH5α control strains were 65.8 and 0.5%, respectively. With T24 cells, *K. pneumoniae* invasion rates ranged from 1.6 to 14.6% and the positive and negative controls exhibited invasion rates of 34.5 and 0.1%, respectively. Because the internalization ability of A549 and T24 cells was much smaller than that of HCT8 cells, the *K. pneumoniae* internalization studies, described in this report, were carried out with only HCT8 cells. Interestingly, strain 706, the only unencapsulated strain, exhibited the highest invasion rate among the *Klebsiella* strains for HCT8 cells. Although these results are preliminary, they do correlate with similar data obtained from studies under way (data not shown), which suggest that the absence of a capsule allows for greater internalization of *Klebsiella* strains.

Cytoskeleton involvement in the internalization of *K. pneumoniae* strains. To determine if the other four clinical *K. pneumoniae* strains used similar internalization mechanisms to that used by strain 3091, invasion assays incorporating cytoskeleton inhibitors, cytochalasin D, and colchicine were performed. All of the *K. pneumoniae* strains showed at least a 99% reduction in invasion efficiency after treatment of the epithelial cells with cytochalasin D (microfilament depolymerization). The internalization efficiency of the microfilament-dependent control strain, *S. typhimurium* C17, was also significantly inhibited by cytochalasin D (93%). Pretreatment of the monolayer with colchicine (microtubule depolymerization) resulted in a reduction of invasion rates of 80 to 89% among the *Klebsiella* strains. The invasion efficiency of the microtubule-dependent control strain, *Citrobacter freundii* 3009 (27), was similarly reduced (70%). There were no differences in internalization mechanisms observed among the *Klebsiella* strains with respect to source or capsule type.

Effect of lectins on *K. pneumoniae* 3091 internalization. Lectins with known binding specificities were used to determine the inhibitory effects of lectins on the invasion of *K. pneumoniae* into HCT8 cells. ConA and WGA, both known for their specificity to recognize GlcNAc residues, inhibited 3091 uptake the most efficiently, causing a greater than 95% reduction (Fig. 1). Similarly, DSA, with a specificity for Gal β1-4 GlcNAc and terminal GlcNAc residues, showed an inhibitory effect on 3091 uptake, causing a 75% reduction. In contrast, GMA exhibited a similar reduction in relative internalization without having a known GlcNAc specificity. This result suggests that either the *Klebsiella* invasion receptor may be a complex glycoprotein composed of more than one carbohydrate moiety or there may be more than one receptor involved.

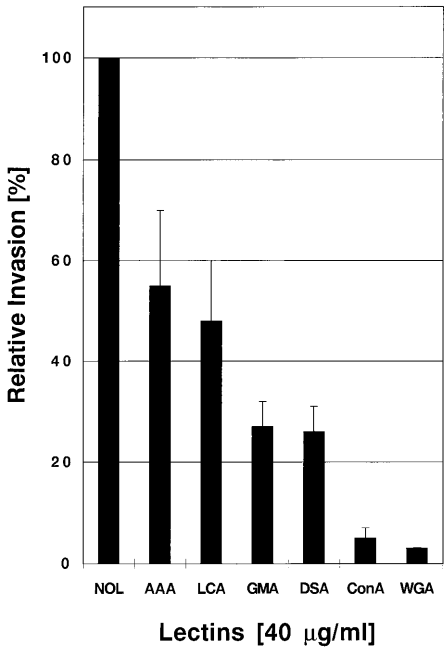

FIG. 1. Relative efficiency of invasion into HCT8 cells of *K. pneumoniae* 3091 in the absence (NOL) and presence of lectins (40 μg/ml). The lectins were added 1 h before infection to the epithelial cells, and invasion assays were performed in their presence.

Other lectins such as AAA and LCA did show some inhibition of *Klebsiella* uptake compared to the untreated control. However, inhibition of internalization was much less dramatic, corresponding to their lower GlcNAc specificity. ConA and WGA were shown to inhibit the entry of 3091 into HCT8 cells in a dose-dependent manner (Fig. 2).

The fluorescence-labeled derivates of the same lectins used in invasion assays were used to show the binding of these lectins to the epithelial cells and/or the bacteria. Fluorescence labeling of epithelial cells was achieved with all the lectins, regardless of whether they showed a strong inhibitory effect. In contrast, incubation of *K. pneumoniae* 3091 with any of the fluorescence-labeled lectins did not result in fluorescent staining of the bacteria (data not shown). Therefore, the inhibitory effect of ConA and WGA appears to be the result of binding of these lectins to the epithelial cells and not to the bacteria.

Inhibition of *K. pneumoniae* internalization by GlcNAc. The effect of GlcNAc on the invasion efficiency of all five *K. pneumoniae* isolates was determined. Preincubation of the bacteria with GlcNAc followed by an invasion assay in the presence of GlcNAc resulted in up to 65% inhibition of *Klebsiella* internalization into HCT8 cells (Table 2). This inhibitory effect of GlcNAc on *Klebsiella* uptake was dose dependent (data shown only for 3091) (Fig. 3). Furthermore, GlcNAc not only inhibited entry of *Klebsiella* into HCT8 cells but also reduced the internalization of *Klebsiella* into T24 and A549 cells (data shown only for HCT8 cells). Chitin hydrolysate, a GlcNAc polymer, and a variety of other carbohydrates, all present on mammalian glycolipids and/or glycoproteins, were also used in invasion assays. Chitin hydrolysate exhibited a greater inhibitory effect (98%) than did GlcNAc on *K. pneumoniae* invasion (Fig. 4). All the other carbohydrates tested showed no effect on *K. pneumoniae* uptake (Fig. 4). *S. typhimurium* internalization was not inhibited by GlcNAc or by any of the other carbohydrates.

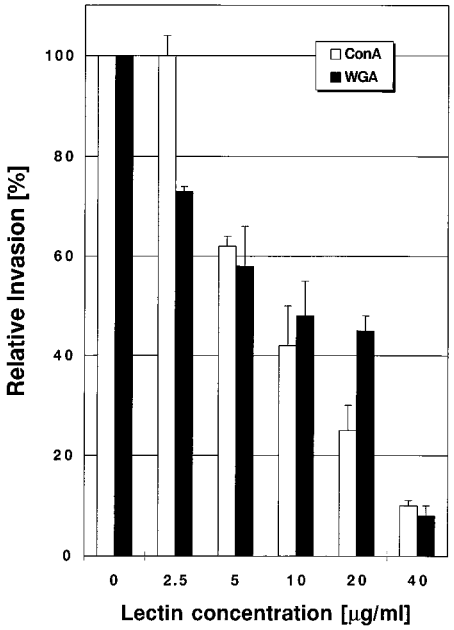

FIG. 2. Dose-dependent inhibition of *K. pneumoniae* 3091 internalization into HCT8 cells by the lectin ConA or WGA. Epithelial cell monolayers were pretreated for 1 h with the appropriate lectin concentration before the bacteria were added. The lectins were present during the invasion period.

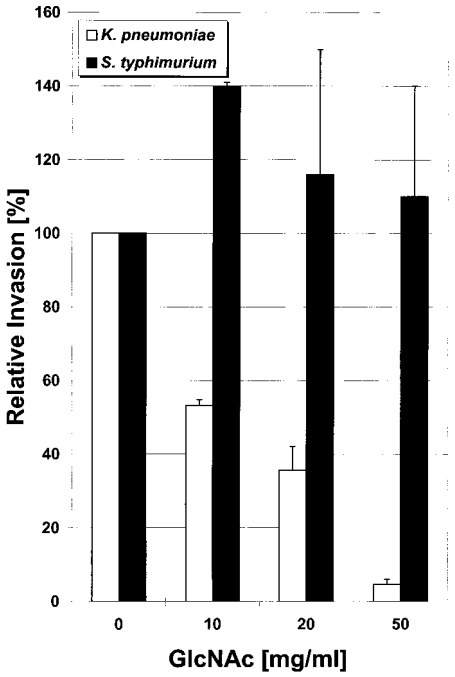

FIG. 3. Relative invasion into HCT8 cells of *K. pneumoniae* 3091 and *S. typhimurium* C17 in the presence of different GlcNAc concentrations. Bacteria were incubated for 1 h on ice in the presence of GlcNAc at the indicated concentrations, and invasion assays were performed in the presence of GlcNAc.

The entry of *Klebsiella* into HCT8 cells was not inhibited when the epithelial cells instead of the bacteria were pretreated with any of the carbohydrates and removed before the addition of the inoculum to the cell monolayer. Additionally, *Klebsiella* uptake was not inhibited when GlcNAc was present only during the period when gentamicin was present. Washing the bacteria after the GlcNAc pretreatment, before adding them to the monolayer, caused a slightly lower inhibition, probably due to a dilutional effect caused by an increase in the proportion of daughter cells not bound to GlcNAc in the inoculum (data not shown).

Effects of tunicamycin and endoglycosidases. The very first step of N glycosylation of proteins can be inhibited by tunicamycin (6). Pretreatment of the HCT8 cell monolayers with 1 μg of tunicamycin per ml before infection with *K. pneumoniae* resulted in a reduction of internalization by more than 93% (Fig. 5). In contrast, the uptake of *S. typhimurium* by HCT8 cells was not reduced at all by tunicamycin treatment (Fig. 5). Another approach was to treat HCT8 cells with glycosidases. The enzyme *N*-glycosidase F hydrolyzes all types of N-glycan chains from surface-exposed glycoproteins of mammalian cells. The removal of these N-glycan chains severely inhibited the internalization of *K. pneumoniae* but not that of *S. typhimurium* (Fig. 5). In contrast, other glycosidases, such as endo-β-galactosidase, *N*-acetylglucosaminidase, and neuraminidase, showed no inhibitory effect on *K. pneumoniae* entry in invasion assays (Fig. 5).

TABLE 2. Effect of GlcNAc on internalization of various invasive *K. pneumoniae* isolates by HCT8 cells[a]

Strain	Relative invasion (%)[b]
K. pneumoniae 3091	35.3 ± 5.5
K. pneumoniae IA565	62.3 ± 4.2
K. pneumoniae 375	48.3 ± 2.1
K. pneumoniae 625	44.5 ± 9.4
K. pneumoniae 706	54.4 ± 13.7
S. typhimurium C17	113.4 ± 14.1

[a] The bacteria were pretreated with 100 mM GlcNAc for 1 h on ice, and GlcNAc was present in the same concentration during the invasion period.

[b] Invasion efficiency is expressed as relative invasion (mean ± standard deviation). The invasion efficiency of bacteria not treated with GlcNAc is defined as 100%.

Presence and involvement of common *Enterobacteriaceae* GlcNAc-binding surface components in *K. pneumoniae* inter-

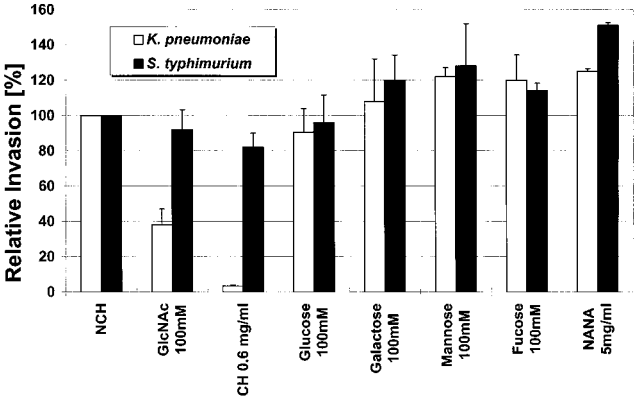

FIG. 4. Effect of various carbohydrates on internalization of *K. pneumoniae* 3091 and *S. typhimurium* C17 into HCT8 cells. Invasion assays were performed with untreated (no carbohydrates; NCH) or with bacteria pretreated for 1 h with carbohydrates at concentrations as indicated. The invasion period of assays with the pretreated bacteria took place in the presence and at the same concentration of the respective carbohydrates. Abbreviations: NANA, *N*-acetylneuraminic acid; CH, chitin hydrolysate.

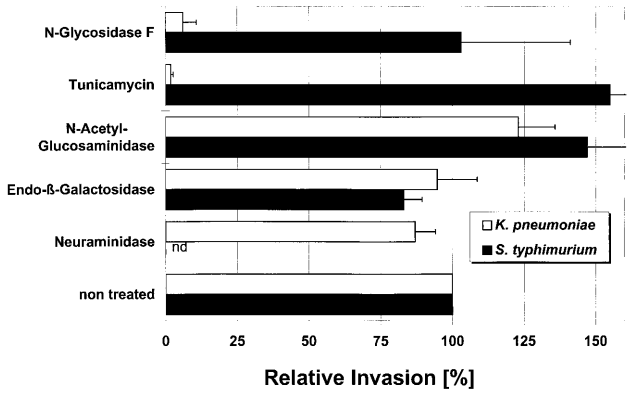

FIG. 5. Effects of different glycosidases and the N-glycosylation inhibitor tunicamycin on the efficiency of invasion into HCT8 cells of *K. pneumoniae* 3091 and *S. typhimurium* C17. Epithelial cell monolayers were treated with either compound and were present during the invasion period. The following concentrations were used: neuraminidase, 0.1 U/ml for 5 min; endo-β-galactosidase, 10 mU/ml for 1 h; *N*-acetylglucosaminidase, 1 U/ml for 1 h; tunicamycin, 1 μg/ml for 15 h; *N*-glycosidase F, 5 U/ml for 15 h. nd, not determined.

nalization. Three surface components of members of the family *Enterobacteriaceae* are well known to recognize GlcNAc present on eukaryotic cell surfaces (32, 33, 35, 36). These are the outer membrane protein OmpA of meningopathic *E. coli* (32); the GafD adhesin of G-pili expressed by bovine enteropathogenic and septicemic *E. coli* (35, 36), and the nonfimbrial adhesin CF29K of *Klebsiella* strains (5), isolated from patients with nosocomial *Klebsiella* infections. The presence of *gafD* and *cf29k* in *Klebsiella* was excluded by PCR and Southern blot analysis. Additionally, immunofluorescence microscopy with GafD-specific antiserum did not label any surface structures expressed by *K. pneumoniae* 3091. However, the *ompA* gene in *Klebsiella* was detected by PCR (data not shown). The involvement of OmpA in internalization of *K. pneumoniae* was determined by inhibition studies with specific OmpA-antibodies and purified OmpA in invasion assays. Preliminary results showed that neither the antibody nor OmpA protein inhibited *K. pneumoniae* invasion (data not shown).

Other adherence factors. Besides the nonfimbrial adhesin CF29K, fimbrial adhesins commonly expressed by clinical isolates of *K. pneumoniae* are the type 1 and type 3 fimbriae. By PCR and Southern hybridization analyses, the genes encoding the major subunit (*mrkA*) and adhesin (*mrkD*) of type 3 fimbriae were detected only in strain IA565 (type 3 fimbriae prototypic strain) and the *mrkD* gene was detected solely in strain 375. The other *Klebsiella* strains did not possess these genes (references 1, 17, and 41 and data not shown). Internalization of *K. pneumoniae* in the presence of mannose was not inhibited, suggesting that type 1 fimbriae are not involved in the internalization of *K. pneumoniae* (Fig. 4).

DISCUSSION

K. pneumoniae is considered to be a primary pathogen of nosocomial UTIs, with serious illness occurring more frequently (~50%) in patients with indwelling urinary catheters (26, 38). The indwelling catheter provides direct access to the uroepithelium, making bacteriuria and more serious sequelae such as cystitis, pyelonephritis, bacteremia, and urosepsis inevitable (38). For bacteria to cause UTIs, they must first gain access, colonize the uroepithelium, induce inflammation, and cause tissue damage. In a clinical study described by Menon and Tan (26), *K. pneumoniae* was reported to be the most frequent organism cultured from the urine of catheterized patients and represented 25% of all uroseptic organisms recovered.

The mechanisms and associated adherence factors by which bacteria colonize the uromucosal cells vary extensively. One common theme, however, is that the bacterial adherence factors first must interact with a receptor on the uromucosal cell surface. In fact, P-pili expressed by uropathogenic *E. coli* strains appear to be a risk factor in the progression of kidney infections to urosepsis (30). Known *K. pneumoniae* adherence factors include type 1 and type 3 fimbriae and the nonfimbrial adhesin CF29K (5, 26, 41).

Adherence is thought to be a prerequisite for triggering the internalization of bacteria by epithelial cells (13). Recently, Oelschlaeger and Tall have described, as a probable new virulence factor, the ability of *K. pneumoniae* to invade cultured epithelial cells (29). Characterization and identification of the invasion receptor on the epithelial cells are crucial for understanding the internalization mechanism(s) induced by these invasive urinary tract pathogens. The surface of the uroepithelial cells is thought to be covered with lipids and proteins, many of which are glycosylated. These surface carbohydrate structures are most probably the first eukaryotic components of receptors recognized by *K. pneumoniae* to initiate internalization. Therefore, the study reported here was designed to characterize the host cell surface structure(s) involved in *K. pneumoniae* invasion. To investigate this question, invasion assays with infected monolayers, pretreated with a variety of lectins with known carbohydrate specificities, were used. Although all the lectins studied bound to the epithelial cells and induced a relative reduction of internalization, only lectins that specifically recognized GlcNAc significantly inhibited *K. pneumoniae* internalization.

Immunofluorescence labeling revealed that the lectins did not bind to the bacteria. Of the six lectins tested, WGA (specific for GlcNAc) and ConA (specific for methyl-α-D-mannose and GlcNAc) most significantly reduced the uptake of *Klebsiella* (~95%) by HCT8 cells. They were followed by DSA (specific for Gal β1-4 GlcNAc and terminal GlcNAc residues), which showed a 75% diminution of bacterial internalization. However, GMA (specific for GalNAc and methyl-α-D-galactopyranoside) also showed a similar inhibitory effect, suggesting that there might be more than one glycosylated receptor involved or that the receptor might contain carbohydrate moieties other than GlcNAc. The weak inhibitory effect exhibited by LCA and AAA was most probably due to their low GlcNAc binding capacity. Additionally, steric inhibition or other pleiotropic effects cannot be ruled out as a possible explanation for the inhibitory effect of these two lectins and, for that matter, the GMA effect on *Klebsiella* uptake. Interestingly, the inhibitory effect observed for ConA and WGA was shown to be dose dependent, suggesting that there might be a finite number of cell surface internalization receptors.

The findings described here strongly indicated the existence of a bacterial ligand(s) with a binding specificity for GlcNAc residues, which is essential for the efficient internalization of *Klebsiella*. To further support this conclusion, bacteria were pretreated with, and invasion assays were performed in the presence of, a variety of sugars. As expected, GlcNAc efficiently inhibited *Klebsiella* invasion, and the oligomer of GlcNAc, chitin hydrolysate, exhibited an even more pronounced effect. All the other sugars showed no inhibition of *Klebsiella* uptake. Since *Salmonella* uptake was not affected by GlcNAc or chitin hydrolysate, a specific GlcNAc-requiring uptake mechanism for *K. pneumoniae* can be suggested.

These results indicated an important role for GlcNAc residues on the surface of epithelial cells involved in the internalization of *K. pneumoniae*. To test the requirement for GlcNAc residues on the epithelial cell surface and to find whether such residues are part of an internalization receptor, uptake by infected epithelial cells, pretreated with either tunicamycin or *N*-glycosidase F, was analyzed. Both of these treatments significantly inhibited *K. pneumoniae* internalization. In contrast, the uptake of *S. typhimurium* was not affected, demonstrating that the treatments did not interfere nonspecifically with internalization. Another endoglycosidase, endo-β-galactosidase (which hydrolyzes internal β-galactosidic linkages of the lactosamine type), had no effect on *K. pneumoniae* uptake. Additionally, the exoglycosidases *N*-acetylglucosaminidase (cleaves off terminal GlcNAc residues) and neuraminidase (cleaves off terminal sialic acid residues) had no inhibitory effect on *Klebsiella* invasion. Taken together, these findings demonstrate that a glycoprotein, rather than a glycolipid as a putative invasion receptor, is essential for the efficient uptake of *K. pneumoniae*. Additionally, GlcNAc residues seem to be one component of this receptor(s). The fact that the exoglycosidases showed no inhibitory effect on internalization most probably suggests that the GlcNAc residues are internal and are not the terminal sugars of the glycan receptor(s).

In contrast to the variety of identified bacterial ligands responsible for invasion, only a very limited number of corresponding eukaryotic cell surface receptors have been characterized (11, 13, 18, 31). For example, the epidermal growth factor receptor and other receptors have been reported to be involved in *Salmonella* internalization (14). Recently, Mengaud et al. (25) reported that the invasion receptor for *Listeria monocytogenes* had been identified as cadherin. The receptor for the invasin of *Yersinia* was identified as a subset of GlcNAc-substituted integrins (19, 34, 44). However, studies reported by van Putten and Paul suggest that the GlcNAc residues are not involved in the interaction with the bacterial ligand, since tunicamycin treatment of epithelial cells did not reduce the uptake of *E. coli* expressing the *inv* gene (42). Another bacterial invasion receptor which recognizes the Opa30 of *Neisseria gonorrhoeae* seems to be a member of the syndecan family of proteoglycans (42). Again, N-linked glycan chains of glycoproteins appear not to be involved in internalization of *N. gonorrhoeae*, as demonstrated by the lack of inhibition of *N. gonorrhoeae* uptake into tunicamycin-treated epithelial cells (42). These studies support the conclusions of Deal and Krivan (4) that the invasion receptor for *N. gonorrhoeae* is glycolipid, not glycoprotein, in composition. Therefore, the known GlcNAc-containing receptors for *Neisseria* and *Yersinia* invasion can be excluded for *K. pneumoniae* invasion.

It is important to note that all these receptors, which have been identified as essential for the internalization of bacteria, are transmembrane proteins. Therefore, it is highly likely that they are not just binding sites for bacterial ligands but are also responsible for initiating the signal transduction process, a prerequisite for internalization. Additionally, these receptors are connected to the microfilament part of the cytoskeleton, with the uptake of the corresponding bacterial species demonstrated to be heavily dependent on intact microfilaments (7, 8, 10, 12, 16).

The N-glycosylated GlcNAc-containing glycoprotein(s) of epithelial cells used by *Klebsiella* as a receptor for invasion should be different from those of neonatal meningitis-causing *E. coli* (MENEC) because, in contrast to internalization of *Klebsiella*, uptake of MENEC into brain microvascular endothelial cells was not reduced by treatment of the host cells with the lectin ConA (33). Furthermore, uptake of MENEC could not be blocked by GlcNAc monomers. Only chitooligomers were able to interfere with entry of these *E. coli* into brain microvascular endothelial cells (33).

The bacterial ligand(s) responsible for interaction with the GlcNAc-containing eukaryotic receptor(s) and subsequent internalization of *Klebsiella* has not yet been identified. Currently, there are three groups of bacterial ligands expressed by members of the *Enterobacteriaceae* that are known to recognize GlcNAc residues. These are the outer membrane protein OmpA of MENEC (32), fimbriae of the G-pilus family of *E. coli* strains that cause diarrhea and septicemia in newborn calves (35, 40), and the nonfimbrial *K. pneumoniae* adhesin CF29K (5). However, none of these GlcNAc binding proteins were involved in *K. pneumoniae* internalization. Furthermore, type 1 and type 3 pili are also not involved in internalization of the investigated *Klebsiella* strains. Although our OmpA results are preliminary, one possible explanation of why these studies were inconclusive was that in our studies we used epithelial cells, not endothelial cells, as published by Prasadarao et al. (32). Another possibility was that although *ompA* was present, it might not be expressed. Taken together, these results suggest the possibility that a yet to be identified gene product(s) expressed by *Klebsiella* is involved in the recognition of the GlcNAc-containing glycoprotein receptor(s) on epithelial cells, with the subsequent induction of internalization.

In conclusion, these studies suggest that an N-glycosylated protein receptor is involved in the internalization of *K. pneumoniae* by human epithelial cells in vitro. The results also indicate that internal GlcNAc residues might be a carbohydrate component of the receptor. Studies to clone the invasion genes of *Klebsiella* and genetically and biochemically characterize the genes and their products are in progress. Additional work aims are to isolate the eukaryotic GlcNAc-containing glycoprotein receptor(s) of epithelial cells responsible for invasion of *Klebsiella*.

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