The Core Lipopolysaccharide of *Escherichia coli* Is a Ligand for the Dendritic-Cell-Specific Intercellular Adhesion Molecule Nonintegrin CD209 Receptor

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The dendritic-cell-specific intercellular adhesion molecule nonintegrin (DC-SIGN) CD209 is a receptor for *Escherichia coli* K-12 that promotes bacterial adherence and phagocytosis. However, the ligand of *E. coli* for DC-SIGN has not yet been identified. In this study, we found that DC-SIGN did not mediate the phagocytosis of several pathogenic strains of *E. coli*, including enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, and uropathogenic *E. coli*, in dendritic cells or HeLa cells expressing human DC-SIGN antigen. However, we showed that an outer core lipopolysaccharide (LPS) (rough) mutant, unlike an inner core LPS (deep rough) mutant or O-antigen-expressing recombinant of *E. coli* K-12 was phagocytosed. These results demonstrate that the host cells expressing DC-SIGN can phagocytose *E. coli* in part by interacting with the complete core region of the LPS molecule. These results provide a mechanism for how O antigen acts as an antiphagocytic factor.

Dendritic cells (DCs) are professional phagocytic cells that play an essential role in host defense against invading pathogens and help control and maintain innate and adaptive immunity. Microbial pathogens could subvert functions of DCs, such as antigen presentation, production of different chemokines and cytokines, and phagocytosis of microorganisms (1, 31).

Lipopolysaccharide (LPS) is a uniquely bacterial glycolipid. It consists of three structurally distinct domains; lipid A, the membrane anchor and endotoxic portion of LPS; the core saccharide consisting of a branched chain of nonrepeating hexose and heptose sugars; and the O-antigen side chain, a repeating unit of sugars that extends into the extracellular milieu. The core can be further divided into the outer core (semivariable composition of sugars and linkages among bacterial species and strains) and the inner core (considerably conserved among bacterial species and strains). Most enteric bacterial pathogens contain O antigens as a component of their LPS, and O antigens can promote resistance to serum killing and phagocytosis, enhancing the pathogenicity of members of the family *Enterobacteriaceae*, such as *Escherichia coli*, *Shigella* spp., and *Salmonella* (2, 6, 20, 21, 28).

DCs uniquely express a C-type lectin called DC-specific intercellular adhesion molecule-grabbing nonintegrin, CD209 (dendritic-cell-specific intercellular adhesion molecule nonintegrin [DC-SIGN]). DCs can interact with microorganisms by using DC-SIGN. Bacteria, such as *Helicobacter pylori* and certain strains of *Klebsiella pneumoniae*, interact with DC-SIGN through LPS structures that contain Le^x [Galβ1–4(Fucα1–3)GlcNAcβ] or mannose sugar residues (18, 34, 35, 38). *Mycobacterium tuberculosis* uses its mannose-capped cell wall component to interact with DC-SIGN to promote its own internalization, which in turn inhibits DC functions (9, 34). DC-SIGN also serves as a receptor for the gp120 antigen of human immunodeficiency virus type 1 (HIV-1) and acts as a carrier for HIV-1, delivering virus to target cells, such as CD4 lymphocytes (7, 8, 19). Although signal transduction mechanisms for how DC-SIGN mediates the uptake of microorganisms are not yet clear, the immunoreceptor tyrosine-based activation motif (ITAM)-like motif on the cytoplasmic domain might play a role in the uptake of bacteria or viruses (35).

Recently, we demonstrated that DC-SIGN promotes adherence and phagocytosis of a nonpathogenic *E. coli* K-12 strain in both DCs and HeLa cells expressing human DC-SIGN antigen (HeLa-DC-SIGN) (39). In this study, we investigated whether DC-SIGN also served as a receptor for pathogenic *E. coli* and defined a region of the core LPS as a ligand on *E. coli* for DC-SIGN.

MATERIALS AND METHODS

Bacterial strains, antibodies, reagents, and culture cell lines. *E. coli* HB101, a laboratory-derived isolate (hybrid of *E. coli* K-12) that lacks O antigen, has recently been demonstrated to bind DC-SIGN (39). *E. coli* K-12 strain CS180 contains core LPS but lacks O antigen (29). Two derivatives of CS180, CS1861 (CS180 harboring pSS37, a plasmid containing all of the genes necessary for the expression of the *Shigella dysenteriae* 1 O antigen) and a deep rough mutant CS2429 (*waaC*) (lacking both O antigen and most of the core) were used to assess the role of LPS in bacterium-DC interactions (11, 13, 29). Strains CS2198, CS2210, CS2274, and CS2774, isogenic mutants of CS180, contain mutations in the *waaR*, *waaP*, *waaR waaB* (double mutant), and *waaQ* genes, respectively (12, 24–27) (Fig. 1). These strains produce intermediate-length core LPS and were

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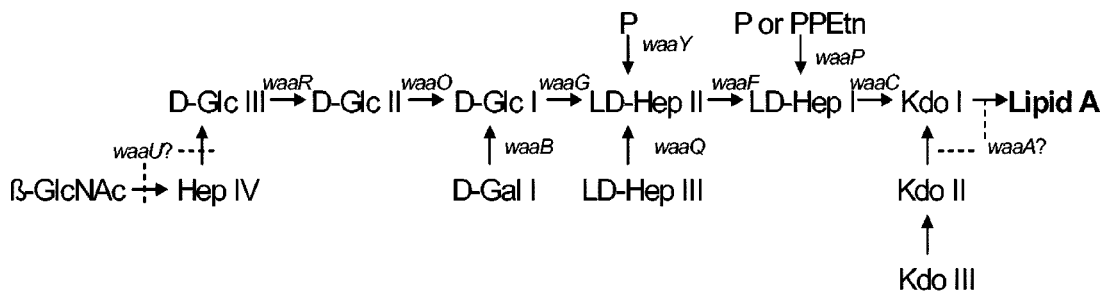
FIG. 1. Structures of inner and outer core regions of *E. coli* K-12 LPS and the genes involved in their synthesis. Genes involved in the biosynthesis of core LPS are shown at their approximate site of action (solid line). The sites, which are variably substituted, are indicated by broken lines. Abbreviations: GlcNAc, *N*-acetylglucosamine; Glc, glucose; Hep, heptose; Gal, galactose; P, phosphate; PPEtn, phosphoethanolamine; Kdo, 2-keto-3-deoxyoctonate.

used to define the minimum *E. coli* K-12 LPS structure necessary to act as a DC-SIGN ligand.

Enteropathogenic *E. coli* (EPEC) (ATCC 49106), enterohemorrhagic *E. coli* (EHEC) (ATCC 438950), and enterotoxigenic *E. coli* (ETEC) (BAA-454) were purchased from American Type Culture Collection. The uropathogenic *E. coli* (UPEC) (116) strain was isolated from the urinary tract of a patient during an outbreak in a nursing home in New Zealand (unpublished data). *E. coli* GR-12, an UPEC, was originally isolated from a patient with pyelonephritis (33). GR-12-SMB-316, a derivative from *E. coli* GR-12, is a rough strain, in which the genes for O-antigen syntheses have been deleted (2). Bacteria were cultured on Luria-Bertani medium (LB) supplemented with 1.5% agar at 37°C overnight.

Anti-CD209 monoclonal antibody (MAb), specific for DC-SIGN, was purchased from Pharmingen, San Diego, Calif. YTH71.3., an anti-CD66 antibody, which recognizes CEACAM1, CEACAM6, and CEACAM3 (CD66acd) (23), was purchased from Harlan Bioproducts (Indianapolis, Ind.). Mannan, the ligand antagonist of human mannose receptor, was purchased from Sigma (Steinheim, Germany).

HeLa-DC-SIGN cells were constructed by transfecting HeLa cells with human DC-SIGN cDNA and selected for surface antigen expression as described previously (22, 30).

Preparation of DCs. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from the Indiana Blood Center (Indianapolis, Ind.) by density gradient centrifugation over FicollPaque^plus (1.077 g/ml; Pharmacia, Piscataway, N.J.) (17). Acquisition of human blood was approved by the Institutional Review Board (IRB) and Study Committees at Indiana University School of Medicine (IUPUI and Clarian). Buffy coats were diluted 1:4 with phosphate-buffered saline (PBS) and loaded in a 1:1 (vol/vol) ratio on Ficoll and centrifuged without stopping for 30 min. PBMC were washed four times with PBS, and monocytes were purified from PBMC using CD14 microbeads (Miltenyi Biotec, Auburn, Calif.) as previously described (17). To increase purity, cells were passed over a second CD14 microbead column. The final purity of the isolated monocytes was >98% as assessed by labeling with a fluorescein isothiocyanate (FITC)-conjugated CD14 antibody (Caltag Laboratories, Burlingame, Calif.) and flow cytometric analysis. Purified CD14$^+$ monocytes (5×10^5 cells/ml) were cultured for 6 days to promote differentiation of immature monocyte-derived DCs in culture medium consisting of RPMI 1640 (BioWhittaker, Walkersville, Md.), 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), 100 U of penicillin per ml, and 100 μg of streptomycin per ml in the presence of 20 ng of recombinant human granulocyte-macrophage colony-stimulating factor (Immunex, Seattle, Wash.) per ml and 10 ng of recombinant human interleukin 4 (Peprotech, Rock Hill, N.J.) per ml. The DCs derived from these cultured monocytes display typical dendrites, and in mixed lymphocyte cultures, they promote activation of alloreactive T cells. The phenotypes of these cells are HLA-DR$^+$ CD1a$^+$ CD86$^+$ CD40$^+$ CD14$^-$ (17). Upon LPS stimulation, these DCs express CD83 (40, 41).

Adherence and phagocytosis assays. The assays for adherence and phagocytosis have been described previously (3–5). Briefly, DCs and HeLa cells were plated into the wells of 24-well plates, and each well was covered with a coverslip. DCs were suspended in RPMI 1640 with 2% fetal calf serum (FCS) at a concentration of 4×10^5/ml. A 0.5-ml sample of each of these cell suspensions was added to each well in 24-well plates, and after the addition of 50 μl of bacterial suspensions at a concentration of 4×10^7 CFU/ml, the cells were incubated at 37°C for 2.5 h in the presence of 5% CO$_2$. The DC monolayers were washed twice with PBS with a cytospin and fixed with 2% paraformaldehyde in PBS

containing Giemsa stain. The number of associated bacteria (adherent and internalized) per DC was determined by microscopy by counting the bacteria associated with 100 cells on the coverslips. For the HeLa cells, the associated bacteria were quantified by washing the cells three times with RPMI 1640 with 2% FCS and plating the culture after the cells were lysed by 0.5% saponin (Calbiochem Corp., San Diego, Calif.).

To determine the internalization of bacteria, gentamicin, which kills extracellular bacteria but cannot penetrate into host cells, was added into each well to a final concentration of 100 μg/ml, and the cultures were incubated for 90 min. The cells were suspended in PBS containing 0.5% saponin, diluted, and plated on LB plates. The level of internalization of bacteria in DCs and HeLa cells was calculated by determining the CFU recovered from lysed cells. All experiments were performed in triplicate, and data were expressed as means ± standard errors. Statistical significance was calculated using the Student's *t* test. It should be noted that the infection time selected in our experiment is based on our preliminary experiments and a balance between the numbers of bacteria killed by host cells and the time needed for bacterial entry.

RESULTS

Neither anti-DC-SIGN antibody nor mannan inhibit the phagocytosis of pathogenic *E. coli* by DCs. We recently showed that DCs phagocytose *E. coli* HB101, an avirulent *E. coli* strain. We also showed that this interaction was reduced by an anti-DC-SIGN MAb and by mannan. To investigate whether a similar interaction occurs between pathogenic *E. coli* and DCs, we performed the same phagocytosis assay with four pathogenic *E. coli* strains, EPEC, ETEC, EHEC, and UPEC strains. All of these pathogenic *E. coli* strains were phagocytosed by DCs to some extent, although there was less uptake than with HB101 (Fig. 2A). Phagocytosis of UPEC (Fig. 2B) and HB101 (39) was confirmed by electron microscopy. In contrast, phagocytosis of these pathogenic *E. coli* by DCs was not reduced by the addition of anti-DC-SIGN antibody or mannan, suggesting that DCs use different phagocytic mechanisms to internalize HB101 and the pathogenic *E. coli*.

DC-SIGN does not mediate phagocytosis of pathogenic *E. coli*. To further investigate the interaction of DCs with pathogenic *E. coli*, a stably transfected DC-SIGN HeLa cell line (HeLa-DC-SIGN) was used to test its ability to bind and phagocytose the panel of pathogenic *E. coli* strains. *E. coli* HB101 bound to and was phagocytosed by HeLa-DC-SIGN (Fig. 3A and B), but not to HeLa cells, as shown previously (39). In contrast, all pathogenic *E. coli* strains bound to both HeLa and HeLa-DC-SIGN. Surprisingly, none of the pathogenic *E. coli* strains could be taken up by HeLa-DC-SIGN. In addition, the UPEC-mediated adherence was not inhibited by
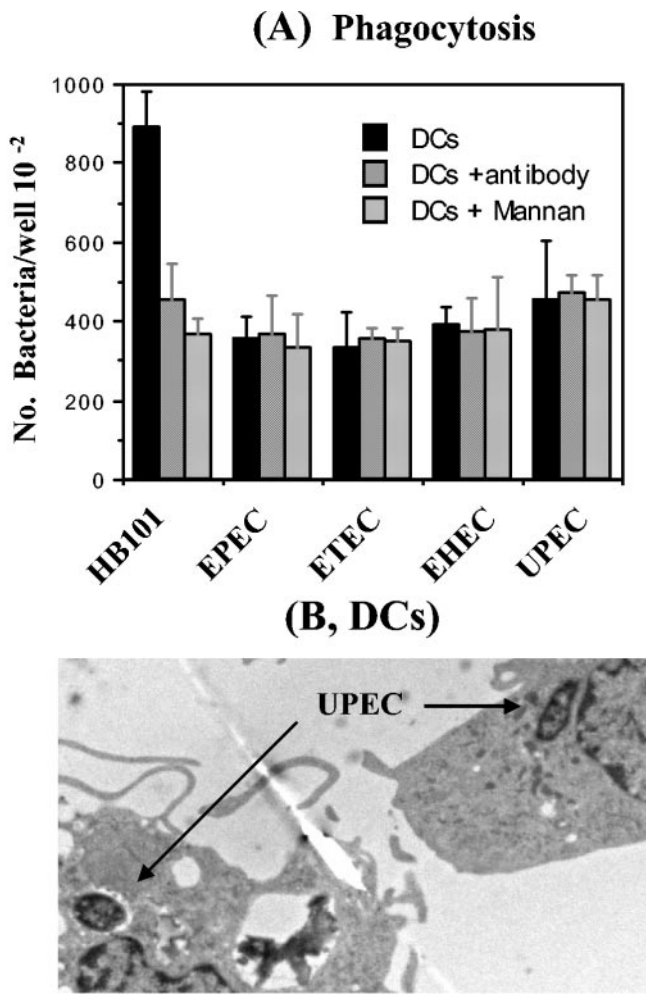
(A) Phagocytosis

(B, DCs)

FIG. 2. DCs phagocytose pathogenic *E. coli* in a DC-SIGN-independent manner. Four pathogenic *E. coli* strains were examined for phagocytosis by DCs alone or in the presence of anti-DC-SIGN or mannan. DCs were incubated for 2.5 h with these *E. coli* strains, and the internalization of *E. coli* by DCs was measured by killing the extracellular bacteria with gentamicin (100 μg/ml [final concentration]) (A) and confirmed by electron microscopy (B), showing the phagocytosed UPEC. The number of phagocytosed bacteria was determined by counting CFU recovered after gentamicin treatment.

anti-DC-SIGN antibody or mannan (Fig. 3C), which blocked the interaction of HB101 with HeLa-DC-SIGN (39). These results demonstrate that DC-SIGN is not a receptor for these pathogenic strains of *E. coli*.

The core region of LPS is required to mediate the *E. coli*-DC interaction. DC-SIGN belongs to the mannose receptor family, and interactions with bacteria usually occur through the mannose-rich components on the bacterial surface. Several bacteria target mannose receptors by using the mannose components on O antigens (35, 38). As described above, *E. coli* HB101 used in this study lacks O antigen. To examine which domains of the *E. coli* LPS may interact with DC-SIGN, two sets of strains were used to examine their interaction with DCs and HeLa-DC-SIGN. The first set includes three *E. coli* K-12 strains: CS180, a naturally rough strain; its isogenic deep rough *waaC* mutant, CS2429, containing only lipid A and two 2-keto-

(A) Adherence
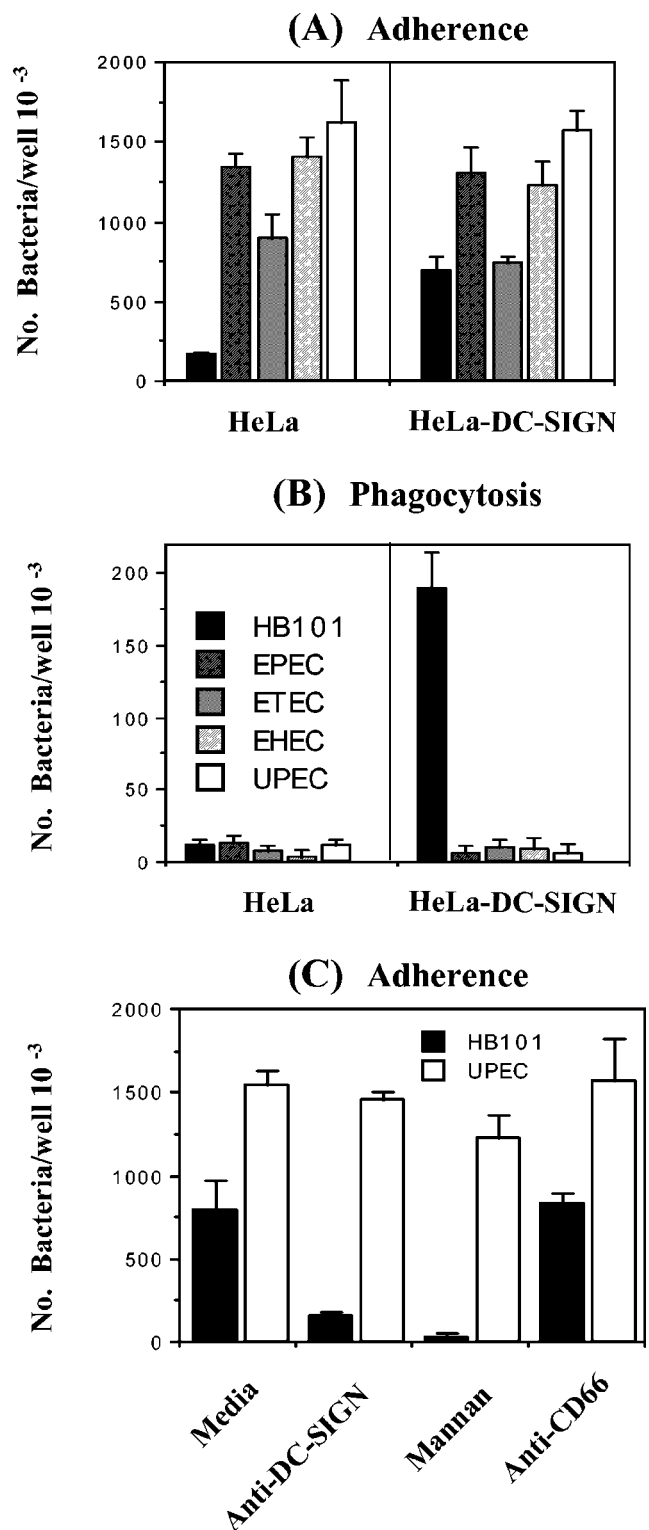

(B) Phagocytosis

(C) Adherence

FIG. 3. HeLa-DC-SIGN cells phagocytose *E. coli* HB101, but not the pathogenic *E. coli* strains. The adherence (A) and phagocytosis (B) of these pathogenic *E. coli* strains with HeLa and HeLa-DC-SIGN were performed as described previously (39). *E. coli* HB101 serves as a positive control. Anti-DC-SIGN antibody (5 μg/ml) and mannan (0.5 mg/ml) were also used to inhibit the adherence of HB101 and UPEC with HeLa-DC-SIGN (C). Anti-DC66 antibody (5 μg/ml) was used as a negative control. The phagocytic rates values for HB101 and UPEC were statistically significantly different (*P* < 0.001).

3-deoxyoctonate (KDO) molecules (Fig. 1); and CS1861, the CS180 strain expressing the full-length O antigen of *S. dysenteriae* 1 from pSS37 (11, 13, 29). The second set of strains consists of a smooth UPEC (GR-12) and its rough isogenic derivative, SMB-316. Strains CS180 and SMB-316 promoted typical DC-SIGN-mediated adherence and phagocytosis (Fig. 4A and B), but strains CS2429 and CS1861 were resistant to both DCs (Fig. 4C) and HeLa-DC-SIGN (Fig. 4B). Strain GR-12 was also completely resistant to phagocytosis via HeLa-DC-SIGN (Fig. 4B). It was phagocytosed to a certain level by DCs (Fig. 4C). This phagocytosis was probably not mediated by DC-SIGN for the reasons described above. This result demonstrated that the ligand of *E. coli* K-12 for DC-SIGN is in the core region of LPS and suggests a role for O antigen in the bacterial resistance of phagocytosis by DCs.

Examination of the sugars responsible for core LPS–DC-SIGN interaction. To examine which sugar residues in the core LPS are responsible for the interaction with DC-SIGN in *E. coli*, additional *E. coli* K-12 core LPS mutants, *waaR*, *waaR waaB*, *waaP*, and *waaQ* mutants, were used. The *waaC* mutant and strain CS180 were used here as the negative and positive controls, respectively. All of these strains were tested for their ability to adhere to and be phagocytosed by HeLa and HeLa-DC-SIGN. As shown in Fig. 5, the *waaR* and *waaR waaB* mutants demonstrated the same level of intermediate resistance to phagocytosis by HeLa-DC-SIGN as the *waaC* mutant did. However, the *waaP* and *waaQ* mutants behaved like their isogenic parent strain, CS180, in that they were fully sensitive to phagocytosis by HeLa-DC-SIGN. These results in combination with data from rough strain SMB-316 strongly suggest that it is the core LPS itself, rather than other components, that bind to DC-SIGN. It is possible that the ligand could be other outer membrane proteins, since the O antigen blocks access to many surface structures which are exposed in rough mutants, and the deep rough defects in the LPS core might result in defective (or decreased) expression of other outer membrane proteins, which could be the ligand. However, because the *waaR* mutant is also resistant to the phagocytosis and is not a deep rough mutant, we conclude that the ligand for DC-SIGN is from the core LPS region.

DISCUSSION

Our previous study demonstrated that DC-SIGN could be a receptor for the phagocytosis of *E. coli* HB101 (39). Transfected HeLa cells expressing DC-SIGN, but not untransfected HeLa cells, were able to bind and avidly internalize *E. coli*. This interaction was blocked by mannan, an antagonist of human mannose receptors, including DC-SIGN, and by antibody specific for DC-SIGN (39).

In this study, we examined the roles of DCs and DC-SIGN in interactions with several pathogenic *E. coli* strains, including EPEC, EHEC, ETEC, and UPEC and found that all pathogenic *E. coli* strains were phagocytosed to some degree by DCs. However, unlike nonpathogenic *E. coli* HB101, phagocytosis of these pathogenic *E. coli* strains was not mediated directly by DC-SIGN. Since *E. coli* HB101 is a rough strain that does not contain the O-antigen polysaccharide on its LPS, which most pathogenic *E. coli* strains possess, we speculated that the li-
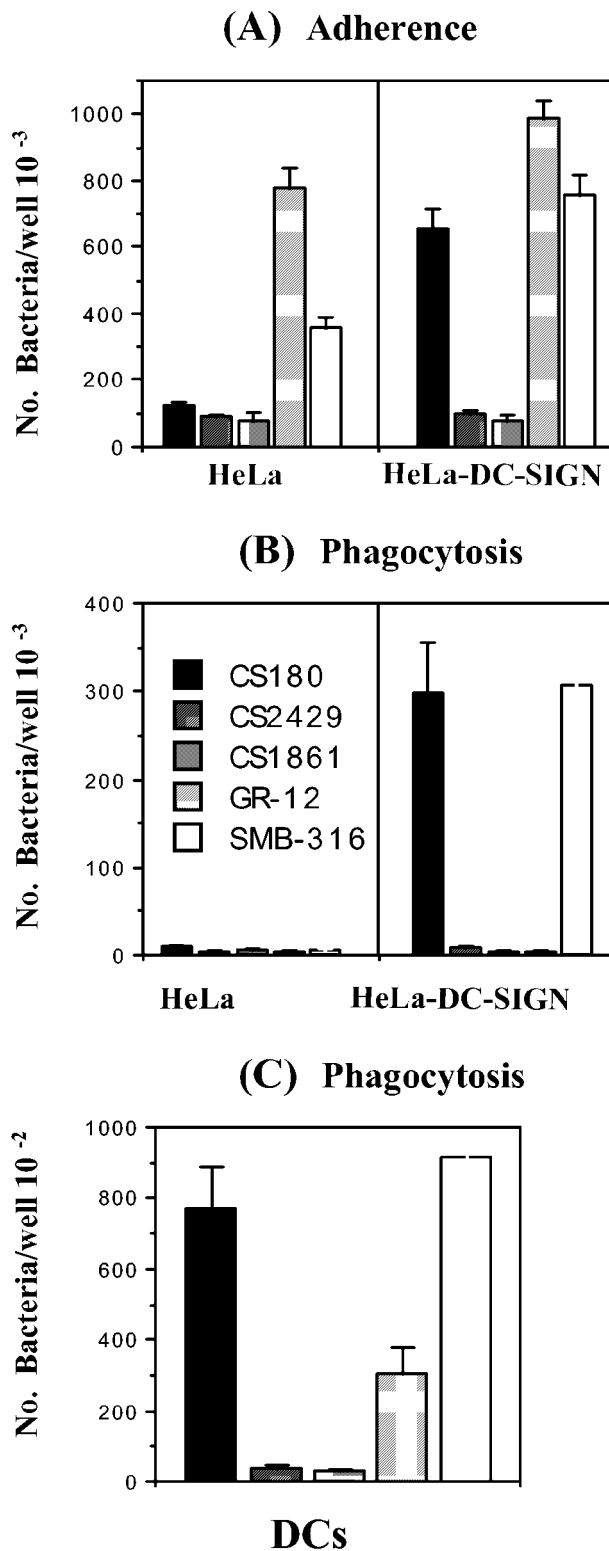

FIG. 4. HeLa-DC-SIGN and DCs cannot phagocytose the deep rough mutant of *E. coli* and the *E. coli* expressing O antigen. The adherence (A) and internalization (B) of *E. coli* K-12 CS180, its derivatives, CS2429 and CS1861, *E. coli* UPEC GR-12 and its derivative by HeLa, HeLa-DC-SIGN, and DCs (C) were performed as described in the legend to Fig. 2. The number of phagocytosed bacteria was determined by the recovery of bacteria from the gentamicin protection assay.

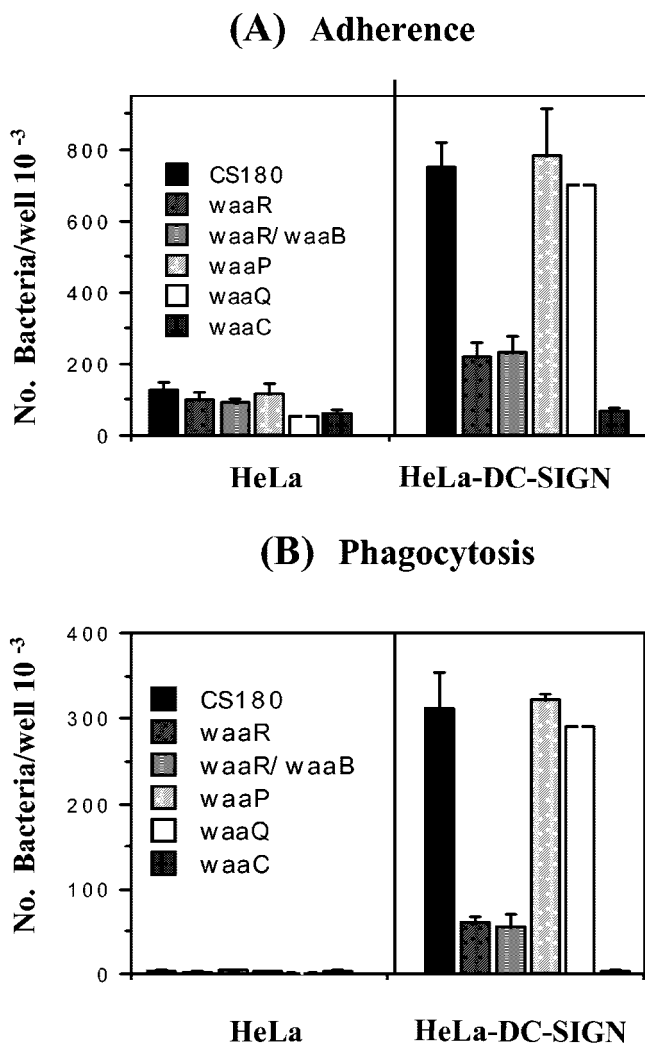## (A) Adherence

(B) Phagocytosis

FIG. 5. Determination of the epitopes on core LPS binding to DC-SIGN. The adherence (A) and internalization (B) of the core LPS mutants (the *waaR*, *waaP*, *waaB*, *waaQ*, and *waaC* mutants and the *waaR waaB* double mutant) and their parent strain, CS180 (Fig. 1), were examined and measured as described in the legend to Fig. 2.

gand(s) of DC-SIGN receptors in *E. coli* could be sterically hindered in these pathogenic *E. coli* strains by O antigens.

E. coli K-12 (HB101 and CS180 strains used in this study) are rough strains, lacking the polysaccharide O-side chain. Therefore, if *E. coli* LPS is involved in binding to DC-SIGN, it should be the core or lipid A region. To test the hypothesis, we used a deep rough *E. coli* K-12 strain, CS2429, which lacks the O antigen and the majority of the core region, and strain CS1861, which covers the core region with the O antigen of *S. dysenteriae* 1. It should be noted that *E. coli* and *S. dysenteriae* are very closely related genetically and have homologous sequences for many genes involved in the synthesis of LPS (15, 16, 36). We also used a pathogenic *E. coli*, UPEC (GR-12) and its O-antigen deletion mutant. Our findings demonstrate that the core region of LPS is required to mediate the *E. coli*–DC-SIGN interaction.

We attempted to determine which specific sugar residues in the core region mediate the binding between DC-SIGN and *E.*

coli K-12 using several core LPS mutants as shown in Fig. 1. Although we were unable to acquire all of the core LPS mutants to systematically analyze their involvement, based on the results in Fig. 5, we propose the following scenario. The fact that the *waaP* and *waaQ* mutants did not influence the adherence and phagocytosis by HeLa-DC-SIGN suggests that the inner core branch constituents added by the WaaP enzyme (a branch phosphoethanolamine or phosphate) and the WaaQ enzyme (a branch heptose III) are not involved in the interaction. The most significant results were the results for the *waaR* mutant and *waaR waaB* double mutant, which are resistant to phagocytosis by HeLa-DC-SIGN, indicating that it is these specific sugar residues that determine the core–DC-SIGN interaction, since both mutants are rough, rather than deep rough, mutants. This result also indicated that D-Gal I might not participate in the interaction with DC-SIGN. Because *waaU* is proposed to encode Hep IV transferase, it is not well documented whether the core LPS of *E. coli* K-12 contains β-GlcNAc and Hep IV, although there are some biochemical data to support the existence of the Hep IV position at the terminal Glc in the chain (10, 37). Therefore, the specific molecule in core LPS that is responsible for interacting with DC-SIGN could be either β-GlcNAc or D-Glc III. Moreover, a recent report on core LPS of *Neisseria meningitidis* (32) and our preliminary data from *Neisseria gonorrhoeae* (data not shown) suggested that the epitope may be β-GlcNAc that binds to DC-SIGN. We are in the process of analyzing each step in the biosynthesis of the LPS core to determine the precise biochemical ligand for DC-SIGN.

One major role of O antigens in the pathogenicity of members of the family *Enterobacteriaceae*, such as *E. coli*, *Shigella*, *Klebsiella*, and *Salmonella*, is promoting resistance to serum killing and phagocytosis (2, 6, 20, 21, 28), but the mechanism of how these gram-negative enteric bacilli resist phagocytosis by host cells is not clear (2, 6). Our results provide a potential mechanism, suggesting that O antigen functions as an antiphagocytic factor simply by shielding the ligand necessary for host cell contact. Very interestingly, a similar observation was obtained for plasminogen activation in *Yersinia pestis* and *Salmonella enterica* (14). Since the LPS cores of members of the family *Enterobacteriaceae* are similar, our results suggest that DC-SIGN may also serve as a receptor for LPS cores of *Salmonella*, *Yersinia*, and *Shigella*. As indicated above, some core LPS mutants from either *N. meningitidis* (32) or *N. gonorrhoeae* (data not shown) bind to DC-SIGN.

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