Role of N-Acetylglucosamine within Core Lipopolysaccharide of Several Species of Gram-Negative Bacteria in Targeting the DC-SIGN (CD209)

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Role of N-Acetylg glucosamine within Core Lipopolysaccharide of Several Species of Gram-Negative Bacteria in Targeting the DC-SIGN (CD209)

Pei Zhang,* Scott Snyder,† Peter Feng,‡ Parastoo Azadi,† Shusheng Zhang,* Silvia Bulgheresi,§ Kenneth E. Sanderson,¶ Johnny He,‖ John Klena,‖ and Tie Chen‖*

Our recent studies have shown that the dendritic cell-specific ICAM nonintegrin CD209 (DC-SIGN) specifically binds to the core LPS of Escherichia coli K12 (E. coli), promoting bacterial adherence and phagocytosis. In this current study, we attempted to map the sites within the core LPS that are directly involved in LPS-DC-SIGN interaction. We took advantage of four sets of well-defined core LPS mutants, which are derived from E. coli, Salmonella enterica serovar Typhimurium, Neisseria gonorrhoeae, and Haemophilus ducreyi and determined interaction of each of these four sets with DC-SIGN. Our results demonstrated that N-acetylg glucosamine (GlcNAc) sugar residues within the core LPS in these bacteria play an essential role in targeting the DC-SIGN receptor. Our results also imply that DC-SIGN is an innate immune receptor and the interaction of bacterial core LPS and DC-SIGN may represent a primeval interaction between Gram-negative bacteria and host phagocytic cells. The Journal of Immunology, 2006, 177: 4002–4011.

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 are able to subvert several functions of DCs, such as Ag-presentation, production of different chemokines and cytokines, and phagocytosis (1, 2).

DCs express a C-type lectin called DC-specific ICAM-grabbing nonintegrin (DC-SIGN), CD209, which allows DCs to interact with microorganisms such as Helicobacter pylori, certain strains of Klebsiella pneumoniae, and Mycobacterium tuberculosis (3–7). DC-SIGN also serves as a receptor for the gpl20 Ag of HIV-1 and acts as a carrier for HIV-1 viruses, delivering them to target cells such as CD4 lymphocytes (8–10). Although the signal transduction pathway by which DC-SIGN mediates the uptake of microorganisms are not yet clear, the ITAM-like motif within the cytoplasmic domain might play a role in the uptake of bacteria or viruses (6).

DC-SIGN belongs to the mannose (Man) receptor family. Interactions between this family of receptors and pathogens usually occur through mannose-rich surface components such as the mannose-capped cell wall on M. tuberculosis and the mannose motif expressed in HIV. However, in another example, DC-SIGN binds to other sugar components that lack mannose-related epitopes, such as the Lex structure (Gal\(^1\)-\(1\)-4(Fuc\(^1\)-\(1\)-3)GlcNAc\(^\beta\)) (galactose-fucose-N-acetylg glucosamine). Recently, a detailed structural study and analysis of DC-SIGN has concluded that ligand structures containing ManGlcNAc and FucGlcNAc residues mediate the strongest binding to DC-SIGN (11). Interestingly, all three of these latter structures contain GlcNAc, suggesting that the GlcNAc rather than other sugar residues such as mannose may play a central role in mediating interaction of these ligands with DC-SIGN.

Recently, we have found that DC-SIGN promotes adherence and phagocytosis of a nonpathogenic Escherichia coli K12 strain and a lgtB mutant (Fig. 1) from Neisseria gonorrhoeae (GC) in both DCs and HeLa cells expressing human DC-SIGN Ag (HeLa-DC-SIGN) (12). We further demonstrated that it is the core LPS region of E. coli that interacts with DC-SIGN (13).

LPS is a unique bacterial glycolipid, consisting 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-Ag 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) (Fig. 1). Most enteric bacterial pathogens contain O-Ags as a component of their LPS, and O-Ags can promote resistance to serum killing and phagocytosis, enhancing the pathogenicity of Enterobacteriaceae such as E. coli, Shigella spp., and Salmonella spp (14–18).

Interestingly, many pathogenic Gram-negative nonenteric bacteria, such as N. gonorrhoeae, Haemophilus influenzae, Neisseria meningitidis, and Haemophilus ducreyi, do not contain O-Ag, and therefore their dominant outer membrane lipid is referred to as lipo-oligosaccharide (LOS) (Fig. 1). In the case of GC, the genes encoding the glycosyltransferases responsible for the addition of...
Table I. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotypes (Phenotype)</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>E. coli K-12(CS180)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS180</td>
<td>Wild type (rough)</td>
<td>27–34</td>
</tr>
<tr>
<td>CS2742</td>
<td>waaU</td>
<td>27–34</td>
</tr>
<tr>
<td>CS2198</td>
<td>waaR</td>
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<td>CS2488</td>
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<tr>
<td>CS1943</td>
<td>waaG</td>
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<tr>
<td>CS4249</td>
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<td>CS2210</td>
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<td>CS1861</td>
<td>CS180-O-Ag</td>
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<tr>
<td>43895</td>
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<tr>
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<td>lgtB</td>
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<tr>
<td>A77-pRSM1955</td>
<td>lgtB-A77</td>
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</table>

Adherence and phagocytosis assays

The assays for adherence and phagocytosis have been described previously (41, 45). Briefly, HeLa cells were plated in 24-well plates. Cells were suspended in RPMI 1640 with 2% FCS at a concentration of 4 × 10^5/ml. A total of 0.5 ml each of these cell suspensions was added to 24-well plates and after addition of 50 μl of bacterial suspensions at a concentration of 4 × 10^6 CFU/ml, the cells were allowed to incubate for 2.5 h at 37°C in the presence of 5% CO₂. The cell monolayers were then washed three times with PBS. The number of associated bacteria (adherent and internalized) per cell was quantified. For the inhibition assay, the saccharides including LPS and core LPS (hydrolyzed LPS oligosaccharide core) were added at a concentration of
FIGURE 1. Structures of inner- and outer-core regions of the LPS or LOS of E. coli K12, S. typhimurium, N. gonorrhoeae, and H. ducreyi 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 or still under investigation, are indicated by dashed lines. The abbreviations in this figure are: GlcNAc, N-Acetylglucosamine; Glc, glucose; Hep, heptose; Gal, galactose; P, phosphate; PPEtn, phosphoethanolamine; KDO, 2-keto-3-deoxyoctonate; PEA, phosphoethanolamine. It should be noted that E. coli K12, N. gonorrhoeae, and H. ducreyi do not possess O-Ag.

Purification of LPS and core LPS

A modified phenol/water extraction procedure adapted from Westphal and Jann (46) was used for LPS purification. Briefly, E. coli and N. gonorrhoeae were suspended in water and heated to 65°C. An equal volume of phenol heated to 65°C was added to the bacterial suspension and the mixture was incubated at 65°C for 1 h with stirring. After centrifugation, the aqueous phase was recovered and the interfacial/phenol phase material was re-extracted once with water. Aqueous phases were ultrafiltered three times through a 100-kDa molecular mass, ultrafiltration device (Millipore), ultracentrifuged at 100,000 × g for 6 h and lyophilized.

To produce core oligosaccharides (free of lipid A), a variation of a previously published method was used (47). Briefly, purified LPS was hydrolyzed in 0.1 M sodium acetate (NaOAc) (pH 4.5), for 4 h at 100°C. The mixture was centrifuged through a Dowex 50WX8 column (Scientelab, Inc.) to remove sodium. The eluted LPS mixture (including both core LPS and lipid A) was then ultrafiltered through a 100-kDa molecular mass ultrafiltration device to remove the lipid A molecule. The remaining material, considered core LPS, was lyophilized.

Expression, purification, and FITC conjugation of His-Mermaid

Mermaid is usually secreted by the Laxus oneistus onto the posterior, bacterium-associated region of this marine nematode. Recently, Mermaid has been identified as a DC-SIGN-like protein. It is expected that the interaction of Mermaid with bacteria induces symbiotic aggregation in seawater leading to symbiosis (48). His-Mermaid was expressed and purified as described previously (48). FITC-conjugated His-Mermaid (FITC-His-Mermaid) was obtained with the FITC Labeling Kit (Calbiochem) according to the manufacturer’s instructions. The molar ratio of FITC:His-Mermaid was 3:9 and FITC conjugation did not affect the cell agglutination properties of Mermaid (data not shown).

FITC-His-Mermaid-binding assay

E. coli CS180 and E. coli CS1861 suspended in PBS at an OD of 0.2 were incubated with 10 μg of FITC-His-Mermaid for 30 min and washed once with PBS. The binding ability of FITC-His-Mermaid to bacteria was measured with fluorescent intensity using flow cytometry.

Results

Rough strains of E. coli do not equally bind to DC-SIGN

We recently demonstrated that core LPS from E. coli is a ligand for DC-SIGN by showing that some rough strains of K-12 and uropathogenic E. coli, but not their isogenic smooth derivatives, interact with HeLa cells expressing DC-SIGN (HeLa-DC-SIGN) (13). We were subsequently interested in determining whether rough variants of other E. coli strains were able to interact with DC-SIGN. To test this, three smooth and rough pairs of E. coli strains (E. coli K12, CS1861/CS180; UPEC, GR-12/SMB-316; and EHEC, 43895/MA6) were examined for their ability to interact with HeLa-DC-SIGN. Fig. 2 shows that rough E. coli CS180 and SMB-316 promote strong DC-SIGN-mediated phagocytosis typical of our previous observations. All three smooth strains were resistant to phagocytosis mediated by DC-SIGN consistent with previous results. Unexpectedly, the rough derivative of the EHEC strain, MA6, was also resistant to phagocytosis. This result suggests that loss of the LPS O-Ag region in some E. coli isolates is not sufficient for the binding to DC-SIGN, and indicates that a specific epitope within the core LPS may be necessary for binding. It is known that the composition of core LPS, especially the outer core saccharide, can vary among different E. coli. It should be noted that most EHEC such as the 43895 and MA6 produce Shiga toxin, which could influence the ability of host cells to phagocytose the bacteria. However, we did not observe significant morphological changes, such as changes of cell shape and/or detachment of HeLa cells from plates after 2.5 h of incubation with these two bacterial strains. In addition, the results from CS1861/CS180 and GR-12/SMB-316 presented here were regarded as controls because similar data were published (13).

Examination of the interaction of core LPS mutants of E. coli with HeLa-DC-SIGN

To test the idea that a specific epitope within the core LPS might be involved in interaction with DC-SIGN, we attempted to define the core LPS epitope by using a set of core LPS mutants from E. coli K12. Adherence and phagocytosis of the core LPS mutants (Table I) with HeLa-DC-SIGN is depicted in Fig. 3. Four observations were made from these results.

1) The core LPS of E. coli mediates the interaction with DC-SIGN. Our previous data (13) and Fig. 3 confirm that the waaK mutant does not interact with DC-SIGN as well as its parent strain CS180, suggesting that it is the core LPS rather than other components on the E. coli surfaces interacting with DC-SIGN. Furthermore, the mutants waaO, waaG, and waaC, expressing decreasing lengths of LPS core, lose the ability to promote...
phagocytosis by HeLa-DC-SIGN. This is not observed with the LPS core side-chain mutants, waaB, waaQ, waaI, and waaP. The data suggest that the epitope for DC-SIGN resides in the main chain of the outer core of LPS.

2) WaaU does not play a role in the interaction. As shown above, the waaR mutant loses most of the ability to interact with DC-SIGN, indicating that either GlcNAc, GlcIII, or HepIV plays a role in interacting with DC-SIGN. If GlcNAc is an epitope, exposure of GlcNAc should increase interaction with DC-SIGN. To test that hypothesis, we examined the ability of the waaU mutant, which likely encodes the enzyme responsible for transfer of GlcNAc or HepIV to GlcIII, to interact with DC-SIGN. The result obtained for the waaU mutant was indistinguishable from that of the parent strain, CS180 in terms of phagocytosis by HeLa-DC-SIGN cells. There are two explanations for this result. First, the enzymatic product of the waaU gene has not yet unequivocally been shown to be a HepIV transferase that adds the $\beta$-GlcNAc on HepIV, although there is some biochemical data to support this assertion (49, 50). Therefore, it is possible that waaU does not code for HepIV transferase. Second, there may be other $\beta$-GlcNAc epitopes on the side chain of the core LPS between waaR and waaC of E. coli, as discussed below.

3) Interaction with DC-SIGN could be mediated by other epitopes on the side chain of core LPS. Although all three waaR, waaO, and waaG mutants are resistant to phagocytosis by HeLa-DC-SIGN, this resistance is not complete when compared with waaC, suggesting a role for some sugars in the core between heptose and the end of the LPS. It has been shown there is another $\beta$-GlcNAc epitope in E. coli R3 (49, 50), however, it is not clear whether E. coli K12 possesses the $\beta$-GlcNAc epitopes. Based on the E. coli LPS core R3 chemical structure, it is predicted that a $\beta$-GlcNAc residue is present between the core saccharide defined by the action of the WaaF and WaaR transferases, which could be demonstrated by testing a waaF mutant. Unfortunately, this mutant is unavailable for testing. However, the data from a S. typhimurium waaF strain presented below suggested this possibility. Interspecies complementation experiments transferring the waaF gene from E. coli to Salmonella restore wild-type phenotypes (J. Klena and A. Schnaitman, unpublished results).

4) The effect of length of core LPS on binding ability to DC-SIGN. The results from Fig. 3 show that the shorter the core LPS, the lesser interaction was observed with DC-SIGN, suggesting that the interaction of core LPS with DC-SIGN is not mediated by specific epitopes, but rather by a specific length of the core LPS. The data obtained using Salmonella core LPS mutants (discussed below) are also consistent with that speculation. However, the data obtained with the LOS mutants of GC do not seem to support this hypothesis (see below).

In short, from these E. coli mutants, while suggesting that an outer core saccharide such as GlcNAc is important for DC-SIGN interaction, the data are not sufficiently convincing to demonstrate that a specific epitope in the LPS core is responsible for the interaction with DC-SIGN.

The core of Salmonella LPS is also a ligand for DC-SIGN

We have established that the core LPS of E. coli interacts with DC-SIGN (13) and the results of Fig. 3 support those conclusions. To determine whether the core LPS of other Gram-negative bacteria can also interact with DC-SIGN, we examined the interaction of the rough strains of S. typhimurium with HeLa-DC-SIGN. In addition, because the core LPS of E. coli and S. typhimurium are similar and the LPS mutants of S. typhimurium are reasonably well defined (37, 38), we used a set of core LPS mutants from S. typhimurium to examine whether a specific epitope within core LPS that is responsible for interacting with DC-SIGN can be identified and how comparable these are to those observed for E. coli. Consistent with the results obtained for E. coli (Fig. 2) (13), the rough S. typhimurium strain (waaK mutant) promoted a typical DC-SIGN-mediated adherence and phagocytosis, while the wild-type strain resisted phagocytosis by HeLa-DC-SIGN (Fig. 4). Furthermore, the deletion of GlcNAc epitope in the waaK mutant reduced the ability of S. typhimurium to interact with DC-SIGN. Interestingly, phagocytosis of the S. typhimurium mutants by HeLa-DC-SIGN was consistent with the idea that the lengthier the core LPS is, the greater the ability to promote phagocytosis (WaaL > WaaK > WaaJ or I > WaaG > WaaF > WaaC = 0). However, it remains unclear whether or what kind of side-chain epitopes exist between the enzymatic products of WaaL and WaaC. In summary, data from S. typhimurium also indicated that GlcNAc plays a role in mediating interaction with DC-SIGN. However, without knowing the side chain of the core LPS in S. typhimurium, we could not rule out whether other components and mechanisms play a part in interaction with DC-SIGN. It should be noted that in comparison with E. coli (Fig. 3), S. typhimurium showed a limited invasion of HeLa cells, which is not mediated by core LPS-DC-SIGN interaction. Salmonella species are known to possess invasive properties that are part of their virulence factors. In addition, some mutants such as the waaC mutant, show an increase in their ability to bind, but not promote phagocytosis, to...
HeLa cells. This result is not surprising because the outer core may block access to surface structures that are exposed in a deep-rough defective LPS core (waaC). This could result in the increasing expression of other outer membrane proteins, which might be the ligand to some components in HeLa cells.

HeLa-DC-SIGN cells bind to and phagocytose the lgtB mutant of N. gonorrhoeae

We had attempted to use the most defined core LPS mutant series so far described for bacteria to determine the possible specific epitopes that interact with DC-SIGN. Although the data obtained from the E. coli and Salmonella experiments above indicate that GlcNAc plays a significant role in interacting with DC-SIGN, neither set of mutants could provide conclusive evidence that GlcNAc is the specific epitope. The major problem is that in both E. coli and S. typhimurium, GlcNAc is the last residue at the nonreducing end of the core saccharide of the rough strains, so that the deletion of the GlcNAc also shortens the core LPS. Therefore, it could be argued that the reduced binding to DC-SIGN may be due to the shortening of core LPS rather than deletion of specific sugar residues. To demonstrate that this is not the case, we examined the interaction of HeLa-DC-SIGN with another set of strains, N. gonorrhoeae. As shown in Fig. 1, the GlcNAc residue is centrally located within the outer core of the N. gonorrhoeae LOS. If the GlcNAc is necessary and sufficient for DC-SIGN binding, only LOS mutants, such as lgtB (Fig. 1) that has the GlcNAc epitope exposed should interact with DC-SIGN. Thus, we used the following genetic derivatives of GC strain F62: wild-type, lgtD, lgtB, lgtA, and lgtE, and strain MS11: wild-type and lgtF, to examine the interaction of GC LOS with HeLa-DC-SIGN. We also used two MS11GC strains because we did not have an access to lgtF mutant from F62. As expected, only GC strain lgtB mutant promoted DC-SIGN-mediated binding and phagocytosis, i.e., HeLa-DC-SIGN but not HeLa cells phagocytosed the lgtB strain (Fig. 5). This is consistent with results reported previously for Neisseria (12, 51).

In conclusion, these results strongly suggest that it is the GlcNAc epitope located within the main chain of the core LOS/LPS that mediates the interaction with DC-SIGN. The data also suggest that the overall core length is not critical as the position of the GlcNAc varies between the three Gram-negative bacteria tested.
1) Purified LPS and core LPS of lgtB mutant of N. gonorrhoeae reduce the core-LPS-DC-SIGN interaction. The purified LPS and core LPS from E. coli CS180, and a lgtB mutant of N. gonorrhoeae (Fig. 7A), were tested for their ability to inhibit the core-LPS-DC-SIGN interaction. Because the purified LPS may potentially affect either the bacteria or HeLa cells, the Opa I-expressing E. coli (pEXI) and CEACAM3 (CD66d) expressing HeLa cells (HeLa-CEACAM3) were used as controls. We have demonstrated previously that interaction of pEXI with HeLa-CEACAM3 promotes a typical phagocytosis of pEXI (42, 45). Fig. 7A showed that only purified LPS, especially the purified core of the lgtB (13) mutant of N. gonorrhoeae, inhibits core-LPS-DC-SIGN interaction but not the pEXI-HeLa-CEACAM3 interaction (Fig. 7B). However, no inhibition was evident when purified LPS and core LPS from E. coli CS180 was used. E. coli CS180 has exhibited a strong interaction with HeLa-DC-SIGN (Fig. 3) (12, 13).

2) The oligosaccharides containing the GlcNAc in a specific orientation, but not single sugar residues, inhibit the core-LPS-DC-SIGN interaction. We have proposed that GlcNAc-exposed core LPS interacts with DC-SIGN. To determine whether GlcNAc-containing oligosaccharides would inhibit the core-LPS-DC-SIGN interaction, four oligosaccharides (GlcNAc-Gal-Glc, Gal-GlcNAc, Fuc-GlcNAc, and GlcNAc-Gal-OMe), two single sugar residues (GlcNAc and GalNAc), and one sugar compound (glucan) were tested. Mannan, which inhibits core-LPS-DC-SIGN interaction as shown previously (12, 13), was used as a positive control for inhibition. Fig. 7A showed that GlcNAc-Gal-Glc and Fuc-GlcNAc inhibit the core-LPS-DC-SIGN interaction, while Gal-GlcNAc and GlcNAc-Gal-OMe marginally influence the interaction. However, we did not have an access to the GlcNAc-Gal-molecule, therefore we could not assess whether it is the specific methylation of this molecule that influences the outcome. It is also of considerable interest to note that the trisaccharide of GlcNAc-Gal-Glc is the same sequence as the terminal three residues of core LPS from lgtB (13) mutant of N. gonorrhoeae (Fig. 1). Fuc-GlcNAc-mediated inhibition of DC-SIGN has been reported previously (11).

3) A DC-SIGN-like molecule binds to the core LPS and inhibits the core-LPS-DC-SIGN interaction. The two experiments described above were conducted from the perspective of the ligand. We wanted to determine whether the purified DC-SIGN receptor plays a role in the core LPS-DC-SIGN interaction. However, DC-SIGN is a transmembrane protein and it is possible that purified DC-SIGN will not retain its biological functions. Recently, we contributed to the identification of Mermaid, a marine nematode Ca2+-dependent lectin whose carbohydrate recognition domain shares both structural and functional similarity with that of DC-SIGN (48). Therefore, we tested the ability of a recombinant form of Mermaid to bind CS180, and a mannose-binding lectin (MBL) (40), that is able to inhibit core-LPS-DC-SIGN interaction. As shown in Fig. 7A, FITC-His-Mermaid inhibits the core-LPS-DC-SIGN interaction. Furthermore, FITC-His-Mermaid binds to CS180 stronger than CS186l, indicating that this DC-SIGN-like protein is likely to directly interact with GlcNAc-exposing core LPS.

**Discussion**

We have previously demonstrated that transfected HeLa cells expressing DC-SIGN, but not untransfected HeLa cells, were able to bind and avidly internalize rough E. coli (12, 13). Using sets of core LOS/LPS mutants from four different Gram-negative bacteria, we have demonstrated that a GlcNAc residue within the main chain of the core LOS/LPS plays a major role in interacting with DC-SIGN. The dependence on GlcNAc is evident even though each of these bacteria has its own characteristic interaction with DC-SIGN.

**The purified LPS and core LPS of lgtB mutant of N. gonorrhoeae reduce the core-LPS-DC-SIGN interaction.** The purified LPS and core LPS from E. coli CS180, and a lgtB mutant of N. gonorrhoeae (Fig. 7A), were tested for their ability to inhibit the core-LPS-DC-SIGN interaction. Because the purified LPS may potentially affect either the bacteria or HeLa cells, the Opa I-expressing E. coli (pEXI) and CEACAM3 (CD66d) expressing HeLa cells (HeLa-CEACAM3) were used as controls. We have demonstrated previously that interaction of pEXI with HeLa-CEACAM3 promotes a typical phagocytosis of pEXI (42, 45). Fig. 7A showed that only purified LPS, especially the purified core of the lgtB (13) mutant of N. gonorrhoeae, inhibits core-LPS-DC-SIGN interaction but not the pEXI-HeLa-CEACAM3 interaction (Fig. 7B). However, no inhibition was evident when purified LPS and core LPS from E. coli CS180 was used. E. coli CS180 has exhibited a strong interaction with HeLa-DC-SIGN (Fig. 3) (12, 13).

**The role of purified LPS, oligosaccharide, and DC-SIGN-like molecule in core-LPS-DC-SIGN interaction**

The results presented so far were generated from interactions between bacteria expressing defined LPS mutations and DC-SIGN-expressing HeLa cells. We next addressed the interaction of the LPS and DC-SIGN using purified molecules (LPS, core LPS, and DC-SIGN).

**FIGURE 4.** The core region of LPS is required to mediate the S. typhimurium-DC-SIGN interaction. The adherence (A) and internalization (B) of the wild-type S. typhimurium and the core LPS mutants were examined and measured with the same procedures as in Fig. 2.

**Shielding the LOS core GlcNAc with other sugar residues blocks the interaction of H. ducreyi with DC-SIGN**

Although N. gonorrhoeae data clearly demonstrate that GlcNAc is an epitope promoting the interaction of GC with DC-SIGN, it is possible, when an LOS gene such as lgtB is deleted, the expression of other genes in the operon might also be affected, thus altering the binding to DC-SIGN. To address this concern, we used a set of H. ducreyi strains, A77 and A77-pRSM1955. The strain A77 naturally lacks galactosyltransferase II (39), exposing core LOS GlcNAc (a natural lgtB mutant). A77-pRSM1955 is the A77 strain containing the pRSM1955 plasmid expressing the N-acetyllactosamine portion of the LOS core from strain 35000HP (Fig. 1) (39). Based on the hypothesis above, A77 should interact with DC-SIGN, which would be inhibited when its GlcNAc epitope is covered. The result showed that A77 was phagocytosed by HeLa-DC-SIGN very well, but A77-pRSM1955 lost its ability to promote the phagocytosis by HeLa-DC-SIGN (Fig. 6). However, it should be noted that A77 interacts with HeLa cells and that expression of N-acetyllactosamine also inhibits DC-SIGN-independent interactions between A77 and HeLa cells.

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DC-SIGN belongs to the mannose receptor family. However, two of the three structure motifs: Le\(^\text{e}\)\(^{\text{a}}\) structure (Gal\(^{\text{b}}\)\(^1\)–4(Fuc\(^{\text{e}}\)\(^1\)–3) GlcNAc\(^{\text{a}}\)), ManGlcNAc, and FucGlcNAc that have previously been shown to bind to DC-SIGN (11), do not contain mannose epitopes but all three do contain GlcNAc. Similarly, the core LPS from \textit{E. coli} K12, \textit{H. ducreyi}, \textit{N. gonorrhoeae}, and \textit{S. typhimurium} do not contain either mannose or fucose (Fig. 1), but GlcNAc is a part of the core LOS/LPS region in each species. We have shown that when the GlcNAc LOS/LPS epitope is removed, the ability of the bacteria to interact with DC-SIGN is decreased or lost and therefore GlcNAc-containing LOS/LPS is critical in phagocytosis mediated by DC-SIGN. Furthermore, the role of GlcNAc has not been much considered in earlier studies (8, 11, 52, 53).

We have taken advantage of biologically, structurally, and genetically well-defined core LOS/LPS mutants to demonstrate the role of GlcNAc in DC-SIGN binding. In other systems, such as the glycosylated gp120 of HIV and DC-SIGN, it is technically challenging to create specific sugar synthesis null mutations, therefore complicating epitope identification. In addition, other studies have relied on the biosynthesis of specific sugar epitopes based on the possible structural features. The use of bacterial core LOS/LPS to investigate DC-SIGN interactions represents an important step in this area.

**FIGURE 5.** The exposure of \textit{N}-acetylglucosamine within the LOS of \textit{N. gonorrhoeae} results in interaction with DC-SIGN. The procedures for examining the adherence (A) and phagocytosis (B) of HeLa and HeLa-DC-SIGN with Neisserial strain F62 (lgtF mutant from MS11) and their isogenic LOS-mutated strains were performed as described in the legend of Fig. 2.

**FIGURE 6.** Blocking of GlcNAc epitopes blocks the interaction of \textit{H. ducreyi} with DC-SIGN. The interaction of strains of \textit{H. ducreyi}, A77 and A77-pRSM1955, with HeLa and HeLa-DC-SIGN was examined as described in the legend of Fig. 2. A. Adherence. B. Phagocytosis.
The question now becomes whether GlcNAc directly binds to DC-SIGN or forms a specific motif with other sugar residues that subsequently interacts with DC-SIGN. To answer this question, we used purified LPS and core LPS, four GlcNAc-containing oligosaccharides, GlcNAc, GalNAc, glucan, mannan (positive control) (12, 13), and the Mermaid (a DC-SIGN-like molecule) to block the interaction between the core LPS and DC-SIGN. The interaction of each molecule with DC-SIGN-expressing HeLa cells revealed that mannan most effectively competed with core LPS to inhibit phagocytosis, followed by Mermaid, GlcNAc-Gal-Glc/Fuc-GlcNAc, then the core LPS of I3, the LPS of I3/Gal-GlcNAc/GlcNAc-Gal-OMe, and glucan. The LPS of CS180, the core LPS of CS180, GlcNAc, and GalNAc showed no inhibition of phagocytosis. Collectively, we can deduce the following information from these data. 1) Oligosaccharides containing the GlcNAc with the correct configuration inhibit the core-LPS-DC-SIGN interaction as shown by GlcNAc-Gal-Glc and core LPS of I3, but not Gal-GlcNAc. It must be also recognized that the oligosaccharide sequence of GlcNAc-Gal-Glc is the same sequence as the terminal three residues of core LPS from I3. In addition, we believe that the Fuc-GlcNAc-mediated inhibition is due the fucose residue as demonstrated previously by other investigators (11). 2) Conformation of the individual saccharides may also play a role in the inhibition of core-LPS-DC-SIGN interaction. Both CS180 and I3 use their core LPS to bind to HeLa-DC-SIGN, however, only purified LPS and core LPS of I3 inhibited the core-LPS-DC-SIGN interaction. We speculate that the loss of the ability for inhibition is due to conformational changes during the purification processes. 3) A
compound with two and more oligosaccharides is necessary to be functional. Our data showed that GlcNAc-Gal-Glc and Fuc-GlcNAc rather than GlcNAc or GalNAc inhibit the core-LPS-DC-SIGN interaction. Similarly it is mann, made of multiple mannos residues rather than mannose alone, that inhibits most interactions promoted by the mannose receptor family, such as interaction inhibition of HIV with DC-SIGN (54).

The discovery of DC-SIGN as a receptor for core LPS of E. coli was fortuitous (12, 13). This observation with the results from the current study may explain why bacterial O-Ags are antiphagocytic. It is known that one major role of O-Ags in the pathogenicity of Enterobacteriaceae such as E. coli, Shigella, Klebsiella, and Salmonella is to promote resistance to serum killing and phagocytosis (14–18). Our results provide a potential mechanism, suggesting that O-Ags functions as an anti-phagocytic factor by simply shielding the ligand necessary for host cell contact. In the case of GC, only a lgtB mutant binds DC-SIGN, while the wild-type strain is poorly recognized by DC-SIGN, suggesting that GC may evolve mechanisms to avoid binding to DC-SIGN and killing by phagocytosis.

Using the various core LOS/LPS of these bacterial species to interact with host cells may represent an alternative approach to averting the innate immune system. For example, GC uses a frame-shifting mechanism at the genetic level, to express different LOS molecules at the phenotypic level. Several of these phenotypes mimic human cell surface structures such as gangliosides (23, 55, 56). Furthermore, GC LOS interacts with complement receptor 3 (CR3) from female human cervical epithelia (57, 58), and the asialoglycoprotein receptor on human sperm (59). In vivo, gonococcal LOS can switch from one phenotype to another in a poorly recognized by DC-SIGN, suggesting that GC may evolve mechanisms to avoid binding to DC-SIGN and killing by phagocytosis.

In contrast, N. gonorrhoeae, N. meningitidis, Moraxella catarrhalis, H. influenzae, E. coli, Campylobacter jejuni, and Salmonella can colonize mucosal surfaces, but quite often these events are asymptomatic, suggesting that innate immune defenses are able to control these pathogens. DC-SIGN could be one of the innate immune receptors because internalization via DC-SIGN is opsonin-independent phagocytosis. It is generally thought that ancestral bacteria possessed only LOS and that bacterial O-Ags, like adaptive immunity, were acquired as a consequence of pathogen-host coevolution. Bacteria appear to have evolved several independent mechanisms to avoid or exploit this recognition Therefore, from an evolutionary point of view, the interaction of bacterial core LOS/LPS and the innate immune receptor, DC-SIGN, may represent a primitive interaction between microbial pathogens and the professional phagocytic host cells.

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