

# Sialylation of Lipooligosaccharides Is Dispensable for the Virulence of *Haemophilus ducreyi* in Humans

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Sialylated glycoconjugates on the surfaces of mammalian cells play important roles in intercellular communication and self-recognition. The sialic acid preferentially expressed in human tissues is *N*-acetylneuraminic acid (Neu5Ac). In a process called molecular mimicry, many bacterial pathogens decorate their cell surface glycolipids with Neu5Ac. Incorporation of Neu5Ac into bacterial glycolipids promotes bacterial interactions with host cell receptors called Siglecs. These interactions affect bacterial adherence, resistance to serum killing and phagocytosis, and innate immune responses. *Haemophilus ducreyi*, the etiologic agent of chancroid, expresses lipooligosaccharides (LOS) that are highly sialylated. However, an *H. ducreyi* sialyltransferase (*lst*) mutant, whose LOS contain reduced levels of Neu5Ac, is fully virulent in human volunteers. Recently, a second sialyltransferase gene (*Hd0053*) was discovered in *H. ducreyi*, raising the possibility that *Hd0053* compensated for the loss of *lst* during human infection. CMP-Neu5Ac is the obligate nucleotide sugar donor for all bacterial sialyltransferases; LOS derived from an *H. ducreyi* CMP-Neu5Ac synthetase (*neuA*) mutant has no detectable Neu5Ac. Here, we compared an *H. ducreyi neuA* mutant to its wild-type parent in several models of pathogenesis. In human inoculation experiments, the *neuA* mutant formed papules and pustules at rates that were no different than those of its parent. When grown in media with and without Neu5Ac supplementation, the *neuA* mutant and its parent had similar phenotypes in bactericidal, macrophage uptake, and dendritic cell activation assays. Although we cannot preclude a contribution of LOS sialylation to ulcerative disease, these data strongly suggest that sialylation of LOS is dispensable for *H. ducreyi* pathogenesis in humans.

Sialic acids are 9-carbon sugars that decorate glycoconjugates found on the surfaces of eukaryotic cells. Sialylated glycoconjugates bind to receptors called sia-recognizing immunoglobulin superfamily lectins (Siglecs). Some Siglecs contain cytoplasmic signaling domains that modulate immune responses; others lack signaling domains and function in intercellular communication (20, 58). The sialic acids found in most mammalian tissues are *N*-acetylneuraminic acid (Neu5Ac) and its derivative *N*-glycolylneuraminic acid (Neu5Gc) (58, 60). Due to a mutation that occurred prior to the emergence of humans in a gene encoding an enzyme that catalyzes the conversion of Neu5Ac to Neu5Gc, Neu5Ac is expressed almost exclusively on human cells (58, 60). Not surprisingly, human Siglecs evolved to preferentially bind Neu5Ac over Neu5Gc (58).

Many bacterial pathogens incorporate Neu5Ac into their surface glycolipids, which can then bind to human Siglecs (58). These interactions either dampen immune responses and promote pathogen survival or activate immune responses and foster auto-immune responses (13, 20, 31). Incorporation of Neu5Ac into lipopolysaccharides, lipooligosaccharides (LOS), or capsular structures affects bacterial adherence to human cells, biofilm formation, resistance to complement-mediated killing, neutrophil function, and dendritic cell (DC) activation (13, 19, 20, 30, 49, 61). In animal models of infection, some pathogens require sialylation of LOS for virulence (11, 30).

*Haemophilus ducreyi* causes chancroid, a sexually transmitted genital ulcer disease endemic in resource-poor areas of Asia and Africa (51). *H. ducreyi* also causes a chronic lower-limb ulceration syndrome that is not sexually transmitted and is reported over a 4,800-mile range of the South Pacific from New Guinea to Samoa (38, 43, 56). In addition to causing its own morbidity, chancroid

facilitates the sexual transmission and acquisition of HIV-1 (51). Clinical isolates of *H. ducreyi* are currently grouped into 2 classes, based on variations in their LOS structures and outer membrane proteins. Recent evidence suggests that these classes form distinct species that diverged from each other approximately 5 million years ago (46). While the LOS of class II strains lack sialic acid, the majority of clinical isolates belong to class I and express LOS that are highly sialylated (39, 45).

The major glycoform of the LOS of class I strains is Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Hep $\alpha$ 1-6Glc $\beta$ 1-4Hep $\alpha$ 1-KDO, which resembles paragloboside, a precursor of major human blood group antigens (45, 64). As is the case with paragloboside, Neu5Ac is added to the terminal galactose of this structure by a  $\alpha$ 2,3-sialyltransferase encoded by *lst* (12). *H. ducreyi* does not synthesize Neu5Ac and uses an ABC transporter system to scavenge environmental Neu5Ac (44, 47). When *H. ducreyi* is grown on chocolate agar, which contains approximately 0.5  $\mu$ M Neu5Ac, up to 15% of the major LOS glycoform is sialylated (18, 47). When cultured on agar supplemented with 1 mM Neu5Ac, up to 62% of the major LOS glycoform is sialylated; supplementation with higher concentrations of Neu5Ac does not result in additional sialic acid incor-

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TABLE 1 Bacterial strains used in this study

<i>H. ducreyi</i> strain	Description	Reference
35000HP	Human passaged variant of strain 35000	3
35000HP-RSM208	35000HP <i>neuA::kan</i> (nonpolar) insertion mutant	12
35000HP-RSM203	35000HP <i>lst::Ω kan</i> insertion mutant	12
FX517	35000 <i>dsrA::cat</i> insertion mutant	14

poration (18, 47). Addition of Neu5Ac to cocultures of *H. ducreyi* and human foreskin fibroblasts (HFF) significantly increases the adherence of *H. ducreyi* to HFF (2). However, an *H. ducreyi lst* mutant, whose LOS contain only 3% of the wild-type level of Neu5Ac, is fully virulent in experimentally infected human volunteers, suggesting that sialylation of LOS is dispensable for pathogenesis (12, 64).

Recently, a gene (*Hd0053*) that encodes a second  $\alpha$ 2,3-sialyltransferase was discovered in *H. ducreyi* (35), raising the possibility that *Hd0053* may have compensated for the loss of *lst* during human infection. The obligate nucleotide sugar donor for all bacterial sialyltransferases is CMP-Neu5Ac. In contrast to the *lst* mutant, LOS derived from an *H. ducreyi* CMP-Neu5Ac synthetase (*neuA*) mutant contain no detectable Neu5Ac (12, 40, 60).

Since sialic acid plays many key roles in bacterial pathogenesis, here we reexamined whether the ability of a class I strain of *H. ducreyi* to sialylate its LOS contributes to virulence. We compared the *neuA* mutant to its parent in human inoculation experiments. Given that DC responses and resistance to serum killing and phagocytosis are important features of *H. ducreyi* pathogenesis (8, 10, 24), we also compared the strains grown with and without Neu5Ac supplementation in DC activation, bactericidal, and macrophage uptake assays.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The bacterial strains used in this study are included in Table 1. *H. ducreyi* strains were grown on chocolate agar plates supplemented with 1% IsoVitaleX in the presence of 5% CO<sub>2</sub>. In some experiments, the chocolate agar plates were supplemented with 1 mM Neu5Ac (Calbiochem, La Jolla, CA) (44). For the human inoculation experiments, *H. ducreyi* was grown in a proteose peptone broth-based medium with 5% heat-inactivated fetal calf serum (27). All cultures were grown at 33°C. Where appropriate, the media were supplemented with chloramphenicol or kanamycin at 0.3 µg/ml or 20 µg/ml, respectively.

**Analysis of LOS.** 35000HP and the *neuA* mutant were grown in the broth described above with and without 50 µg/ml CMP-Neu5Ac (Sigma-Aldrich) (48). Bacteria were harvested at mid-log phase, and LOS was prepared by proteinase K digestion followed by phenol extraction and analyzed on 14% polyacrylamide gels as described previously (4).

**Human inoculation experiments.** Dedicated stocks of 35000HP and 35000HP-RSM208 were prepared according to FDA guidelines under BB-IND 13064. Five healthy adult volunteers (four females and one male; 4 white and 1 black; mean age ± standard deviation [SD], 34.2 ± 13.7 years) over 21 years of age participated in the study. Subjects gave informed consent for participation and for HIV serology, in accordance with the human experimentation guidelines of the Institutional Review Board of Indiana University-Purdue University of Indianapolis and the U.S. Department of Health and Human Services. The preparation and inoculation of the bacteria, calculation of the estimated delivered dose (EDD), clinical observations, surface cultures, definitions of clinical endpoints, biopsies, and antibiotic treatment of the volunteers were carried out exactly as described previously (28). To confirm that the bacteria isolated

from the inocula, surface cultures, or biopsy specimens had the intended phenotype, colonies were screened for susceptibility to Kan (28).

In the human model, site outcomes within a subject are not independent. To account for the correlation among site outcomes within an individual, comparison of papule and pustule formation rates for the two strains was performed using a logistic regression model with generalized estimating equations (GEE) (54). The GEE sandwich estimate for the standard errors was used to calculate 95% confidence intervals (95% CI) for these rates.

**Bactericidal assays.** Bactericidal assays were done using normal human serum (NHS) obtained from a single healthy donor (1, 6, 53). In the first set of experiments, we compared the survival rates in 50% NHS of 35000HP and its isogenic *neuA*, *lst*, and *dsrA* mutants, using bacteria grown on chocolate agar for 16 h. In the second set of experiments, we compared the survival rates of 35000HP, the *neuA* mutant, and the *dsrA* mutant grown on chocolate agar supplemented with Neu5Ac with that of the *dsrA* mutant grown without supplementation. Data were reported as percent survival in active NHS compared to that in heat-inactivated serum [(geometric mean CFU in active NHS/geometric mean CFU in heat-inactivated NHS) × 100]. Each experiment was repeated five times, and the arithmetic mean and standard deviation (SD) of the percent survival were calculated. Comparison of the strains was performed using paired Student's *t* tests. With the Bonferroni adjustment for multiple comparisons, a *P* value of <0.0083 was considered significant for these assays.

**Macrophage assays.** Peripheral blood mononuclear cells (PBMC) were isolated from leukopacks obtained from 8 anonymous donors from the Central Indiana Regional Blood Center or from blood obtained from 4 healthy uninfected adult volunteers by Ficoll-Paque Plus gradient centrifugation. Informed consent was obtained from the volunteers according to institutional and federal guidelines. CD14<sup>+</sup> monocytes were purified from PBMC by positive selection using magnetic CD14 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. CD14<sup>+</sup> cells were differentiated into monocyte-derived macrophages (MDM) in X-vivo 15 medium (Lonza) supplemented with 1% heat-inactivated human AB serum (Invitrogen) for 5 days. MDM were harvested and seeded onto glass coverslips in 24-well tissue culture plates at a density of 2.5 × 10<sup>5</sup> cells/well and grown for 24 h. 35000HP and the *neuA* mutant were grown on chocolate agar plates with and without Neu5Ac supplementation for 18 h, harvested, and suspended in Hanks buffered salt solution (HBSS). The bacterial suspension was vortexed for 30 s, and clumps were allowed to settle for 2 min. The bacteria were either not opsonized or opsonized with 100% complement-replete autologous serum for 20 min at room temperature. Approximately 2.5 × 10<sup>6</sup> CFU was added to wells containing MDM, centrifuged at 180 × *g* for 10 min, and incubated for 30 min at 35°C in 5% CO<sub>2</sub>. Nonadherent bacteria were removed by washing three times with HBSS. To determine the number of MDM-associated bacteria, wells were incubated with HBSS containing 0.2% saponin for 15 min at room temperature and quantitatively cultured. To measure the number of internalized bacteria, other wells were incubated in HBSS containing gentamicin (50 µg/ml) for 30 min, washed three times with HBSS, treated with HBSS containing 0.2% saponin, and quantitatively cultured. The percentages of associated and internalized bacteria were calculated as the ratios of MDM-bound CFU and gentamicin-protected CFU, respectively, to the initial CFU added per well.

**DC activation assays.** DC were differentiated from CD14<sup>+</sup> cells in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 µM beta-mercaptoethanol, 10% heat-inactivated fetal bovine serum, and 5 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) (R&D Systems) for 7 days (7). DC were collected, washed once, and suspended in the DC growth medium without GM-CSF and IL-4 in 48-well tissue culture plates. 35000HP and the *neuA* mutant grown on chocolate agar plates with and without Neu5Ac for 18 h were centrifuged onto wells containing 4 × 10<sup>5</sup> DC at a multiplicity of infection (MOI) of 10:1; control wells were not infected.

TABLE 2 Response to inoculation with live *H. ducreyi*

Volunteer <sup>a</sup> (gender)	Observation period (days)	Strain <sup>b</sup>	Dose (CFU) <sup>c</sup>	No. of initial papules	Final outcome of papule	
					No. resolved	No. of pustules
395 (F)	7	P	32	3	1	2
		M	24–97	3		3
396 (F)	13	P	32	1	1	
		M	24–97	2	2	
397 (F)	8	P	32	3	3	
		M	24–97	3	1	2
398 (M)	6	P	63	3		3
		M	23–91	2	1	1
400 (F)	7	P	63	3	1	2
		M	23–91	3	3	

<sup>a</sup> Volunteers 395, 396, and 397 were inoculated in the first group; volunteers 398 and 400 were inoculated in the second group. F, female; M, male.

<sup>b</sup> P, parent strain 35000HP; M, mutant strain 35000HP-RSM208.

<sup>c</sup> Doses are indicated as follows: 24–97, one dose each of 24, 48, and 97 CFU; 23–91, one dose each of 23, 46, and 91 CFU.

After 90 min of incubation at 35°C, gentamicin was added at a final concentration of 50 µg/ml, and the DC were incubated at 37°C for 22.5 h.

Uninfected and *H. ducreyi*-infected DC were stained with allophycocyanin-conjugated antibodies to CD40 or CD86, phycoerythrin-conjugated anti-CD80 or anti-CD83 antibodies, and isothiocyanate-conjugated anti-HLA-DR antibodies (BD Biosciences). Samples were analyzed using a FACSCalibur flow cytometer and BD CellQuest Pro version 4.0.1 software (BD Biosciences). DC culture supernatants were collected and frozen at –80°C. The levels of IL-6, IL-10, IL-12, and tumor necrosis factor alpha (TNF-α) were measured by cytokine enzyme-linked immunosorbent assay (ELISA) kits from BD Biosciences according to the manufacturer's instructions. All comparisons between groups in the macrophage and DC assays were made using paired Student's *t* tests.

## RESULTS

**An *H. ducreyi neuA* mutant is not impaired in its ability to infect human volunteers.** We had shown previously that an *H. ducreyi lst* mutant is fully virulent in experimentally infected human volunteers (64). A gene (*Hd0053*) that encodes a second α2,3-sialyltransferase was subsequently discovered in *H. ducreyi* (35), raising the possibility that *Hd0053* may have compensated for the loss of *lst* *in vivo*. As CMP-Neu5Ac is the obligate nucleotide sugar donor for all sialyltransferases, LOS derived from an *H. ducreyi neuA* mutant contain no detectable Neu5Ac (12). The LOS of the *neuA* mutant lack the Neu5Ac a-branch and contain a lactosamine b-branch on the major glycoform; the parental LOS profile is restored by complementation (12). The outer membrane protein profiles and growth rates of 35000HP and the *neuA* mutant in broth are identical (12).

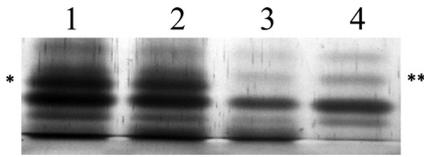
To test whether sialylated LOS are required for *H. ducreyi* infection in humans, we inoculated 2 groups of volunteers. The first group, with 3 participants, was inoculated with an EDD of 32 CFU of 35000HP at 3 sites on one arm and with EDDs of 24, 48, and 97 CFU of the *neuA* mutant at 3 sites on the other arm. The second group, with 2 participants, was inoculated with an EDD of 63 CFU of 35000HP at 3 sites on one arm and with EDDs of 23, 46, and 91 CFU of the *neuA* mutant at 3 sites on the other arm. Overall, papules formed at 86.7% (95% CI, 63.3 to 99.9%) of parent-inoculated sites and at 86.7% (95% CI, 72.4 to 99.9%) of mutant-inoculated sites ( $P = 1.0$ ) (Table 2). After 24 h of infection, the mean area of the papules was  $18.5 \pm 10.7$  mm<sup>2</sup> at parent sites and  $16.9 \pm 17.6$  mm<sup>2</sup> at mutant sites ( $P = 0.69$ ). Pustules formed at 46.7% (95% CI, 11.6 to 81.7%) of parent sites and 40% (95% CI, 5.9 to 74.1%) of mutant sites ( $P = 0.39$ ).

At least one positive surface culture for *H. ducreyi* was obtained during follow-up visits from 20% of the parent-inoculated and 7% of the mutant-inoculated sites. All colonies recovered from the surface cultures of parent sites ( $n = 93$ ) and mutant sites ( $n = 30$ ) had the expected kanamycin resistance phenotype. Of 3 biopsy specimens cultured from parent-inoculated sites, 2 yielded *H. ducreyi*. Of 3 biopsy specimens cultured from mutant-inoculated sites, 2 yielded *H. ducreyi*. All colonies recovered from biopsy specimens of parent ( $n = 24$ ) and mutant ( $n = 75$ ) sites had the expected kanamycin resistance phenotype, as did all colonies tested from the parent ( $n = 72$ ) and mutant ( $n = 69$ ) inocula. Thus, there was no evidence of cross-contamination between mutant-inoculated and parent-inoculated sites.

Two subjects (395 and 398) developed pustules at both mutant-inoculated and parent-inoculated sites. One mutant- and one parent-inoculated site were biopsied from each subject and stained with hematoxylin-eosin and anti-CD3 antibodies as described previously (64). Both samples contained micropustules in the epidermis and a dermal infiltrate of perivascular CD3<sup>+</sup> cells and were indistinguishable (data not shown). Taken together, the data indicate that the inability of *H. ducreyi* to incorporate Neu5Ac into its LOS did not impair its virulence in humans.

**The *neuA* mutant does not sialylate its LOS in media containing CMP-Neu5Ac.** Some bacterial pathogens scavenge CMP-Neu5Ac from the host and sialylate their LOS using an extracellular sialyltransferase (60). To exclude the possibility that the *neuA* mutant could sialylate its LOS by this mechanism, we analyzed LOS prepared from 35000HP and the *neuA* mutant grown in broth with and without CMP-Neu5Ac supplementation. LOS purified from 35000HP contained the Neu5Ac a-branch; supplementation with CMP-Neu5Ac did not increase the level of expression of the a-branch (Fig. 1). Under both growth conditions, LOS purified from the *neuA* mutant lacked the a-branch and contained the lactosamine b-branch (Fig. 1). Thus, it is unlikely that the *neuA* mutant can utilize host-derived CMP-Neu5Ac to sialylate its LOS.

**Sialylation of LOS does not affect the serum susceptibility of *H. ducreyi*.** Resistance to the killing of normal human serum plays a major role in *H. ducreyi* pathogenesis and is mediated primarily by the outer membrane protein DsrA (10, 28). Sialylation of LOS affects the ability of several organisms to resist serum killing (30, 61). Thus, we compared the survival rates of 35000HP and its

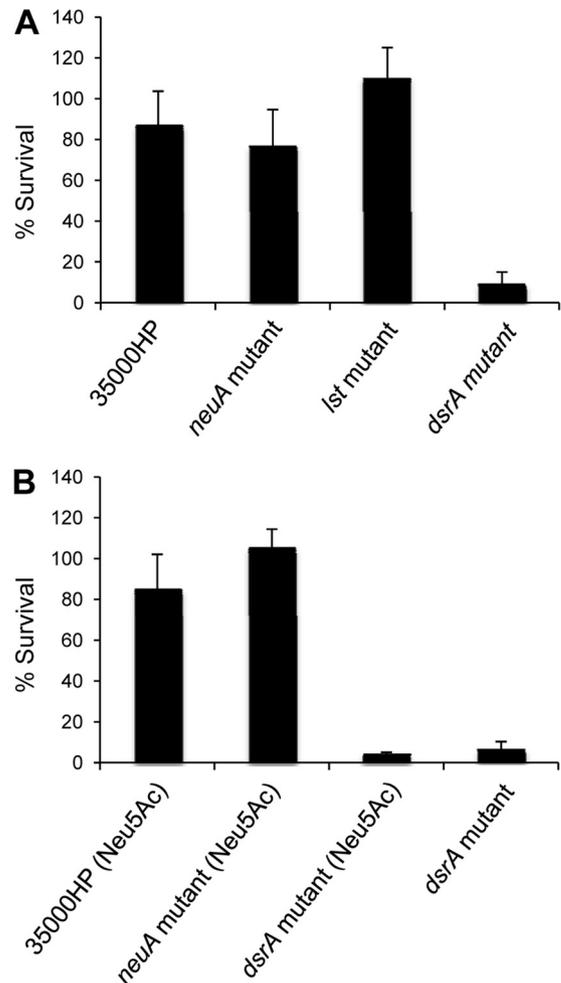


**FIG 1** Silver-stained gel of LOS isolated from 35000HP (lanes 1 and 2) and the *neuA* mutant (lanes 3 and 4). The bacteria were grown in broth without (lanes 1 and 3) and with (lanes 2 and 4) CMP-Neu5Ac supplementation. Note that under both growth conditions LOS derived from 35000HP contains the a-branch (\*), while LOS derived from the *neuA* mutant contains only the b-branch (\*\*).

isogenic *neuA*, *lst*, and *dsrA* mutants in 50% NHS (6). When the bacteria were grown without Neu5Ac supplementation, the mean  $\pm$  SD percent survival rates were  $86\% \pm 17\%$  for 35000HP,  $76\% \pm 18\%$  for the *neuA* mutant,  $110\% \pm 15\%$  for the *lst* mutant, and  $9\% \pm 7\%$  for the *dsrA* mutant (Fig. 2A). Only the percent survival of the *dsrA* mutant was significantly different from results for the other 3 strains (Fig. 2A). In the next set of experiments, we compared the survival rates of 35000HP, the *neuA* mutant, and the *dsrA* mutant grown in media supplemented with 1 mM Neu5Ac with that of the *dsrA* mutant grown in the absence of supplementation. The mean  $\pm$  SD percent survival rates were  $87\% \pm 19\%$  for 35000HP,  $105\% \pm 11\%$  for the *neuA* mutant,  $4\% \pm 1\%$  for the *dsrA* mutant grown with Neu5Ac supplementation, and  $7\% \pm 4\%$  for the *dsrA* mutant grown without Neu5Ac supplementation (Fig. 2B). Only the percent survival of the *dsrA* mutant grown with and without Neu5Ac supplementation was significantly different from results for the other 2 strains (Fig. 2B). Thus, the ability of *H. ducreyi* to sialylate its LOS has no effect on serum susceptibility.

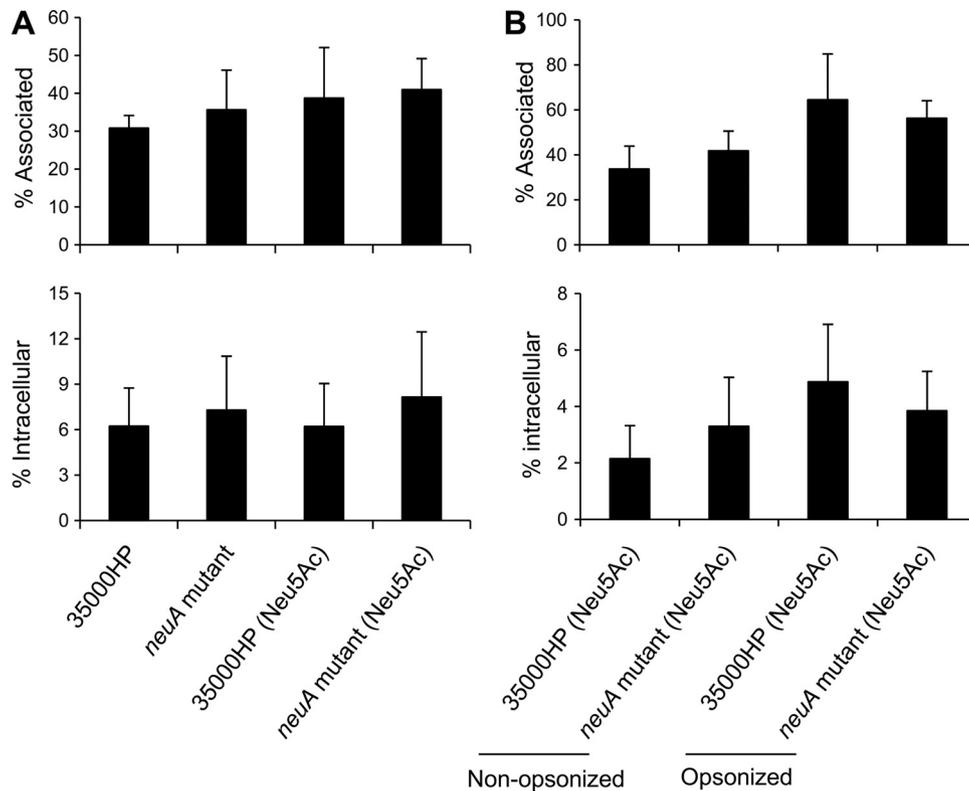
**Sialylation of LOS does not modulate *H. ducreyi* association with or uptake by macrophages.** Human and murine macrophages express a sialoadhesin (Sn, siglec-1) that promotes the uptake of sialylated viruses and bacteria (29, 58). *H. ducreyi* resists phagocytosis by human macrophage-like cell lines and primary human MDM (52, 57, 62). We examined whether sialylation of *H. ducreyi* LOS affects the ability of the bacterium to associate with or be taken up by macrophages. 35000HP and the *neuA* mutant were grown with and without Neu5Ac supplementation and used to infect primary human MDM. After 30 min of infection, approximately 30 to 40% of the nonopsonized bacteria were associated with the MDM; under both growth conditions, there were no significant differences in the percentages of associated bacteria between the wild type and the *neuA* mutant strain (Fig. 3A, top). In gentamicin protection experiments, under both growth conditions there were also no significant differences in the percentages of ingested bacteria between the nonopsonized wild-type and mutant strains (Fig. 3A, bottom). Next, we grew 35000HP and the *neuA* mutant with sialic acid supplementation and compared uptake with and without opsonization with 100% autologous complement-replete serum. Again, we found no differences in adherence (Fig. 3B, top) or uptake (Fig. 3B, bottom) of the strains under opsonized and nonopsonized conditions. Thus, the ability to incorporate Neu5Ac into LOS did not affect the ability of opsonized and nonopsonized *H. ducreyi* to associate with or be taken up by macrophages.

**DC activation is not affected by *H. ducreyi* sialylation.** Sialylation of LOS can enhance the activation of DC as measured by



**FIG 2** Bactericidal assays. (A) Percent survival of 35000HP, 35000HP-RSM208 (*neuA* mutant), 35000HP-RSM203 (*lst* mutant), and FX517 (*dsrA* mutant) in 50% NHS, calculated as follows: [(geometric mean CFU in active NHS/geometric mean CFU in heat-inactivated NHS)  $\times$  100]. *P* values are as follows: for 35000HP versus 35000HP-RSM208, *P* = 0.22; for 35000HP versus 35000HP-RSM203, *P* = 0.25; for 35000HP versus FX517, *P* = 0.001; for 35000HP-RSM208 versus 35000HP-RSM203, *P* = 0.057; for 35000HP-RSM208 versus FX517, *P* = 0.0004; and for 35000HP-RSM203 versus FX517, *P* = 0.0002. (B) Percent survival of 35000HP, 35000HP-RSM208, and FX517 grown in media supplemented with 1 mM Neu5Ac and FX517 grown without Neu5Ac supplementation in 50% NHS, calculated as described for panel A. *P* values are as follows: for 35000HP versus 35000HP-RSM208, *P* = 0.059; for 35000HP versus FX517 grown with Neu5Ac, *P* = 0.006; for 35000HP versus FX517 grown without Neu5Ac, *P* = 0.0001; for 35000HP-RSM208 versus FX517 grown with Neu5Ac, *P* < 0.0001; for 35000HP-RSM208 versus FX517 without Neu5Ac, *P* < 0.0001; and for FX517 grown with Neu5Ac versus FX517 grown without Neu5Ac, *P* = 0.16. For both panels, values are means  $\pm$  SDs from 5 independent experiments.

surface markers and cytokine production (31). *H. ducreyi* infection of human monocyte-derived DC leads to secretion of both proinflammatory and anti-inflammatory cytokines and upregulation of several DC surface activation markers (7, 24). To examine whether sialylation of *H. ducreyi* LOS plays a role in modulating DC activation, 35000HP and the *neuA* mutant grown with and without Neu5Ac supplementation were used to infect DC for 24 h. Compared to uninfected DC, infected DC



**FIG 3** Percentages of *H. ducreyi* that were associated with (top) or internalized by (bottom) macrophages. (A) 35000HP and 35000HP-RSM208 (*neuA* mutant) grown on chocolate agar plates with and without Neu5Ac supplementation were incubated with macrophages at an MOI of 10:1 for 30 min without opsonization, followed by 30 min of treatment with gentamicin. Percent association or internalization was calculated as the ratio of bacteria recovered without or with gentamicin treatment to initial CFU added. The values represent the means  $\pm$  SDs from assays done with macrophages from 5 (top) or 6 (bottom) donors. There were no significant differences among the groups in either assay. (B) 35000HP and 35000HP-RSM208 were grown on chocolate agar plates with Neu5Ac supplementation and were either not opsonized or opsonized with 100% autologous serum prior to performance of the assays outlined for panel A. The values in both panels represent the means  $\pm$  SDs from assays done with macrophages from 4 donors. There were no significant differences between the *neuA* mutant and the parent strain in either assay.

significantly upregulated the percent positive expression (Table 3) and mean fluorescence intensity (MFI) (Table 4) of the surface activation markers CD40, CD80, CD83, and CD86 and the MFI of HLA-DR (all  $P < 0.018$ ). However, after adjustment for multiple comparisons, the levels of activation did not significantly differ between the parent and the mutant strains (Tables 3 and 4). *H. ducreyi*-infected DC secreted proinflammatory cytokines IL-6, IL-12, and TNF- $\alpha$  and the anti-inflammatory cytokine IL-10. However, the levels of cytokine production did not differ between the parent strain and the *neuA* mutant grown in the presence or absence of Neu5Ac sup-

plementation (Fig. 4). Thus, we found little evidence that the ability of *H. ducreyi* to sialylate its LOS affected DC activation.

## DISCUSSION

Most clinical isolates of *H. ducreyi* belong to class I and express highly sialylated LOS (39, 45). In this study, we showed that a class I *neuA* mutant, which is incapable of LOS sialylation, is fully virulent in human volunteers. Even under growth conditions that favor maximal sialylation of the LOS in the wild type (18, 47), the *neuA* mutant and its parent had indistinguishable phenotypes in bactericidal, macrophage adherence and uptake, and DC activa-

**TABLE 3** Effect of *H. ducreyi* sialylation on percent positive expression

Treatment <sup>a</sup>	Percent positive expression <sup>b</sup>				
	HLA-DR	CD40	CD80	CD83	CD86
Media	93.5 (4.0)	11.1 (5.3)	42.2 (7.9)	1.3 (0.8)	21.8 (14.4)
35000HP	94.5 (3.4)	76.2 (7.1)	87.2 (13.1)	36.3 (10.7)	83.7 (7.34)
35000HP-RSM208	94.3 (2.1)	74.4 (7.9)	86.5 (12.4)	38.12 (9.6)	82.7 (6.9)
35000HP (Neu5Ac) <sup>c</sup>	94.0 (1.9)	75.9 (6.5)	87.4 (11.5)	36.1 (11.9)	81.9 (8.0)
35000HP-RSM208 (Neu5Ac) <sup>c</sup>	94.2 (1.9)	74.7 (9.2)	85.6 (12.2)	36.4 (11.5)	82.1 (7.9)

<sup>a</sup> Samples were incubated with media or with *H. ducreyi* strains for 24 h.

<sup>b</sup> Values are means (SDs) of the percentages of cells expressing the surface markers over that of isotype-matched control antibodies and represent data from 6 donors.

<sup>c</sup> Strains were grown with Neu5Ac supplementation.

TABLE 4 Effect of *H. ducreyi* sialylation on MFI

Treatment <sup>a</sup>	Geometric MFI <sup>b</sup>				
	HLA-DR	CD40	CD80	CD83	CD86
Media	27.9 (14.8)	3.2 (2.2)	3.9 (0.3)	1.0 (0.1)	2.8 (1.1)
35000HP	41.4 (17.3)	10.2 (3.9)	16.6 (6.5)	3.1 (0.9)	12.6 (3.4)
35000HP-RSM208	39.0 (18.0)	9.9 (3.2)	16.9 (5.8)	3.2 (1.0)	12.7 (2.9)
35000HP (Neu5Ac) <sup>c</sup>	39.0 (18.0)	9.8 (3.7)	15.6 (5.2)	3.0 (1.0)	11.9 (3.1)
35000HP-RSM208 (Neu5Ac) <sup>c</sup>	40.0 (18.8)	9.7 (3.6)	16.1 (6.5)	3.1 (1.1)	12.0 (3.4)

<sup>a</sup> Samples were incubated with media or with *H. ducreyi* strains for 24 h.

<sup>b</sup> Values are means (SDs) of the geometric mean fluorescence intensity (MFI) expressed as fold change over that of isotype-matched control antibodies and represent data from 6 donors.

<sup>c</sup> Strains were grown with Neu5Ac supplementation.

tion assays. Taken together with the observation that the LOS of class II strains lack Neu5Ac (45), these data strongly suggest that sialylation of LOS is dispensable for *H. ducreyi* pathogenesis in humans.

The human challenge model has several limitations, including an artificial route of inoculation. Delivery of the bacteria by tines into the skin could mask the role of sialylation in an initial stage of pathogenesis, such as adherence. However, *ncaA*, *fgbA*, and *flp-tad* operon mutants are attenuated for pustule formation in human volunteers (9a, 16, 28a, 54), indicating that the model detects contributions of adherence factors to pathogenesis. Of the 28 mutants tested in the model, 9 are fully attenuated and 5 are partially attenuated for pustule formation (28, 28a, 32, 33, 42, 53); a partial role of sialylation in virulence should have been detected by the model. For safety reasons, the model is restricted to the papular

and pustular stages of infection. Although the model cannot directly assess the contribution of a virulence determinant to the ulcerative stage, *H. ducreyi* maintains similar relationships with host cells in experimental and natural infection (8, 9). Neu5Ac is abundant in human tissues; it is likely that wild-type LOS become highly sialylated following inoculation. Thus, it seems unlikely that sialylation of LOS would contribute only to the ulcerative stage.

Sialylation of LOS can block the binding of antibodies directed at terminal LOS epitopes (36). We evaluated the ability of *H. ducreyi* to resist serum killing and uptake by macrophages using complement-replete sera obtained from uninfected donors. Antibody to *H. ducreyi* LOS is detectable in sera obtained from 88% and 98% of healthy individuals from areas where the related disease is nonendemic and endemic, respectively (15). Thus, serum

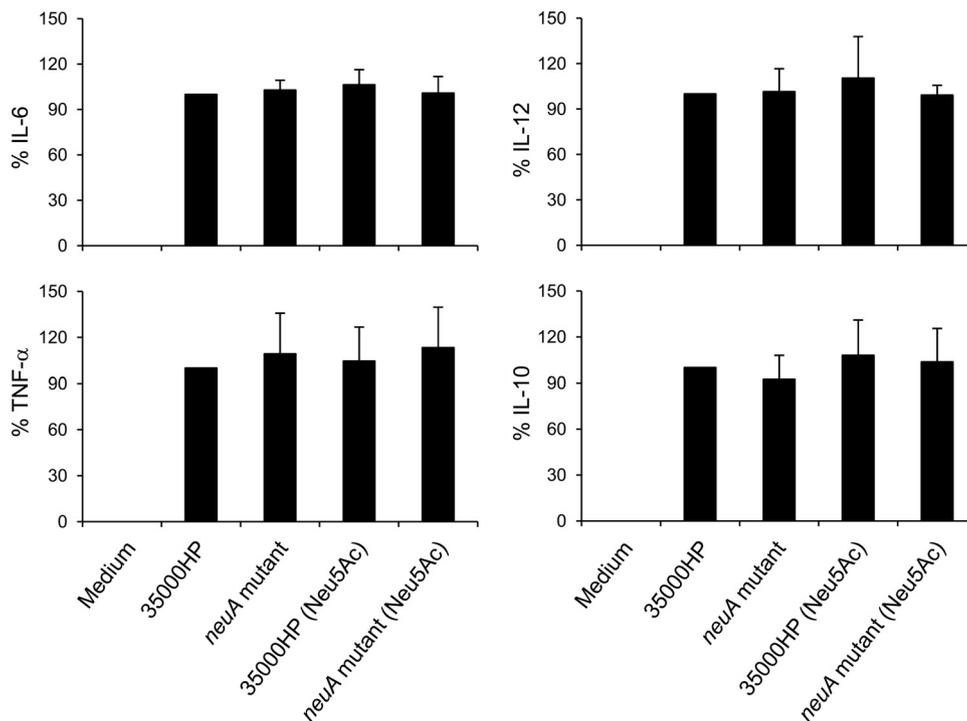


FIG 4 Cytokine production by DC. DC were uninfected or infected with 35000HP and 35000HP-RSM208 (*neuA* mutant) grown on chocolate agar plates with and without 1 mM Neu5Ac supplementation at an MOI of 10:1 for 24 h. Culture supernatants were assayed for IL-6 ( $n = 7$ ), IL-12 ( $n = 4$ ), TNF- $\alpha$  ( $n = 7$ ), and IL-10 ( $n = 7$ ) by ELISA. Due to donor-to-donor variation in cytokine production, cytokine levels were normalized to that of DC incubated with 35000HP grown in the absence of 1 mM Neu5Ac, which was set at 100%. Bars are means  $\pm$  SDs. There were no significant differences in the levels of production of each cytokine among the groups in these assays.

from uninfected donors is appropriate to study potential effects of sialylation on serum resistance and phagocytosis.

Members of the *Pasteurellaceae* do not synthesize Neu5Ac and rely on ATP-dependent or ATP-independent transporter systems to scavenge sialic acid from the host (49). Disease-associated isolates of the bovine pathogen *Histophilus somni* express sialylated LOS, while commensal strains do not; LOS sialylation enhances the serum resistance of this organism (25). Addition of Neu5Ac into the LOS of nontypeable *Haemophilus influenzae* is required for serum resistance, biofilm formation, and virulence in experimental otitis media in animal models of infection (11, 30, 50). The role of sialylation of *H. influenzae* LOS in human disease is unknown. However, the data suggest that LOS sialylation may differ in importance among the human pathogens in this family.

In humans, the role of LOS sialylation has been studied most extensively in the pathogenesis of *Neisseria gonorrhoeae* infection in men. *N. gonorrhoeae* differs from *H. ducreyi* in that it lacks CMP-Neu5Ac synthetase, scavenges CMP-Neu5Ac from the host, and substitutes Neu5Ac on its LOS with an extracellular sialyltransferase encoded by *lst* (60). In urethral exudates obtained from naturally infected males, gonococci found within polymorphonuclear leukocytes (PMN) express LOS that are sialylated (5). However, *N. gonorrhoeae* grown in media supplemented with CMP-Neu5Ac sialylates its LOS and is impaired in its ability to invade urethral epithelial cells (19) and to infect male volunteers (48). In male volunteers, an *lst* mutant in the serum-resistant FA1090 background is as virulent as its parent; an *lst* mutant in the serum-sensitive MS11mkC background is infectious but trends to lower infectivity (22). *In vivo*, *N. gonorrhoeae* circulates between intracellular and extracellular compartments. Nonsialylated gonococci may be more efficient at invading urethral epithelial cells, while sialylated gonococci may have survival advantages within PMN or in extracellular compartments that contain serum transudates (22). Although the latter hypothesis has not been tested in humans, an MS11 *lst* mutant is impaired in its ability to resist killing by murine PMN and to survive in the murine model of female genital tract infection (63). In contrast to *N. gonorrhoeae*, *H. ducreyi* appears to reside only in an extracellular compartment; sialylation of its LOS may have a minor role in its ability to resist complement-mediated killing and phagocytic uptake, as discussed below.

Incorporation of Neu5Ac into the LOS of otherwise serum-susceptible strains of *N. gonorrhoeae*, such as MS11mkC, promotes the binding to the bacterium of factor H, an inhibitor of the alternative complement pathway, enabling the organism to become serum resistant (61). Other strains of *N. gonorrhoeae*, such as FA1090, are intrinsically serum resistant because their porin proteins bind factor H regardless of the sialylation state of their LOS (61). *H. ducreyi* is serum resistant primarily due to the expression of the outer membrane protein DsrA, which does not affect factor H binding but does prevent the binding of IgM and activation of the classical complement pathway (1). *H. ducreyi* also expresses two additional outer membrane proteins, MOMP and DltA, which have partial roles in serum resistance (21, 26, 34). Thus, sialylation of LOS likely does not affect the serum resistance of *H. ducreyi* due to the fact that the organism is intrinsically serum resistant.

Macrophages express the siglec Sn, which binds Neu5Ac in  $\alpha 2,3$  or  $\alpha 2,8$  linkages (58). *Campylobacter jejuni* strains that express sialylated LOS do not adhere to wild-type CHO cells but do

adhere to CHO cells transfected with Sn (20). Meningococci that express sialylated LOS are ingested more efficiently by bone marrow-derived macrophages obtained from wild-type mice than by those from Sn-deficient (Sn<sup>-/-</sup>) mice (29). Although these data suggest that sialylation of LOS can facilitate bacterial adherence to and uptake by macrophages, sialylation of *H. ducreyi* LOS did not affect adherence to or uptake by macrophages. *H. ducreyi* secretes two proteins, LspA1 and LspA2, which are required for virulence in humans and inhibit the activation of Src family protein kinases and phagocytic signaling of macrophage-like cell lines (27, 41). Expression of these antiphagocytic proteins may interfere with the ability of sialylated *H. ducreyi* to be taken up by macrophages.

*C. jejuni* strains associated with the development of Guillain-Barré syndrome express sialylated LOS (20). Compared to unsialylated LOS, sialylated LOS from *C. jejuni* enhance DC activation through a Toll-like receptor 4 (TLR4)-dependent mechanism, which promotes B cell proliferation and may contribute to the development of antiganglioside antibodies (31). In contrast, sialylation of meningococcal LOS has little effect on the secretion of cytokines by DC (55), while sialylated LOS from *H. somni* induce less NF- $\kappa$ B activation and chemokine response from bone marrow-derived macrophages than desialylated LOS (23). We found little evidence that sialylated *H. ducreyi* LOS affected the expression of surface activation markers or cytokine production by DC compared to unsialylated LOS. Taken together, the data suggest that sialylation of LOS makes different contributions to activation of DC or macrophages in different organisms.

Some organisms, including *Streptococcus pneumoniae* and *H. influenzae*, utilize Neu5Ac as a carbon source, which aids in colonization of the host (37, 59). *H. ducreyi* contains homologues of several of the genes encoding proteins required for sialic acid catabolism but does not have an identified homologue of NanK (44). As the second step in the catabolism pathway, NanK phosphorylates *N*-acetylmannosamine; lack of a NanK homologue suggests that *H. ducreyi* may not be able to utilize sialic acid as a carbon source (44). As defined media for the growth of *H. ducreyi* are not available, it remains to be determined experimentally whether the organism utilizes Neu5Ac for growth *in vitro* or *in vivo*; this study does not preclude that possibility.

In summary, we conclude that sialylation of *H. ducreyi* LOS does not play a major role in the pathogenesis of chancroid. Addition of Neu5Ac to its LOS may initially have aided *H. ducreyi* in its adaptation to the human host as it diverged from its closest relatives, which are animal pathogens (17). The bulk of the evidence suggests that expression of other virulence determinants has supplanted potential roles for sialylated *H. ducreyi* LOS *in vivo*.

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