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Sulfatide and Its Synthetic Analogues Recognition by *Moraxella catarrhalis*

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***Abstract*: *Moraxella catarrhalis* is one of the major pathogens of respiratory and middle ear infections. Attachment of this bacterium to the surface of human pharyngeal epithelial cells is the first step in the pathogenesis of infections. This study revealed that sulfatide might act as a binding molecule for the attachment of *M. catarrhalis* to human pharyngeal epithelial cells. Furthermore, six different synthetic sulfatides were found to inhibit the attachment of *M. catarrhalis* significantly at an optimum concentration of 10 μg/ml. Synthetic sulfatides may have the potential to be used as a therapy to prevent *M. catarrhalis* infections.**

***Key words*: Attachment, *Moraxella catarrhalis*, Sulfatide, Human pharyngeal epithelial cell**

Moraxella catarrhalis is one of the major pathogens of respiratory and middle ear infections. β-Lactamase production is the principal mechanism of antibiotic resistance in this bacterium and in several countries 100% of the *M. catarrhalis* is a β-lactamase producer (10). Although the bacterium does not produce any enzymes that compromise the host mucosal defenses, the attachment of *M. catarrhalis* to host cells plays an important role in the pathogenesis of infections. Thus therapeutic use of anti-attachment molecules for *M. catarrhalis* infections may be an option that needs proper evaluation before considering using *in vivo*. To human respiratory cells *M. catarrhalis* carries two different binding specificities, one in the recognition of GalNAcβ1-4(NeuAcα2-3) present in different gangliosides, and the other is asialoganglioside GM1 (1, 2). It is not uncommon for bacteria to use multiple receptors on the host cells for firm attachment (8). Since sulfatides are found in the extracellular matrix, in mucus and on the surface of epithelial cells of human trachea and lungs (6), there is a possibility that *M. catarrhalis* might use them as an attachment molecule. Therefore this study was done to define the role of sulfatide as a molecule for attachment of *M. catarrhalis* to human airway epithelial cells and to evaluate the potential of its synthetic analogues as an anti-attachment agent.

M. catarrhalis strain B-88-152 was used in this study. Only in thin layer chromatography (TLC) an additional four strains of *M. catarrhalis*, B-87-69, B-87-75, B-87-94 and B-87-133 were used. All strains were isolated from the sputum of patients with respiratory infections and the culture conditions are described elsewhere (1).

The glycoconjugates used in this study were a natural

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Abbreviations: AA, polyacrylamide; BSA, bovine serum albumin; GalCer, galactosylceramide; HPECs, human pharyngeal epithelial cells; HRP, horseradish peroxidase; mAb, monoclonal antibody; PBS, phosphate buffered saline; *p*NP, *p*-nitrophenol; TLC, thin layer chromatography.

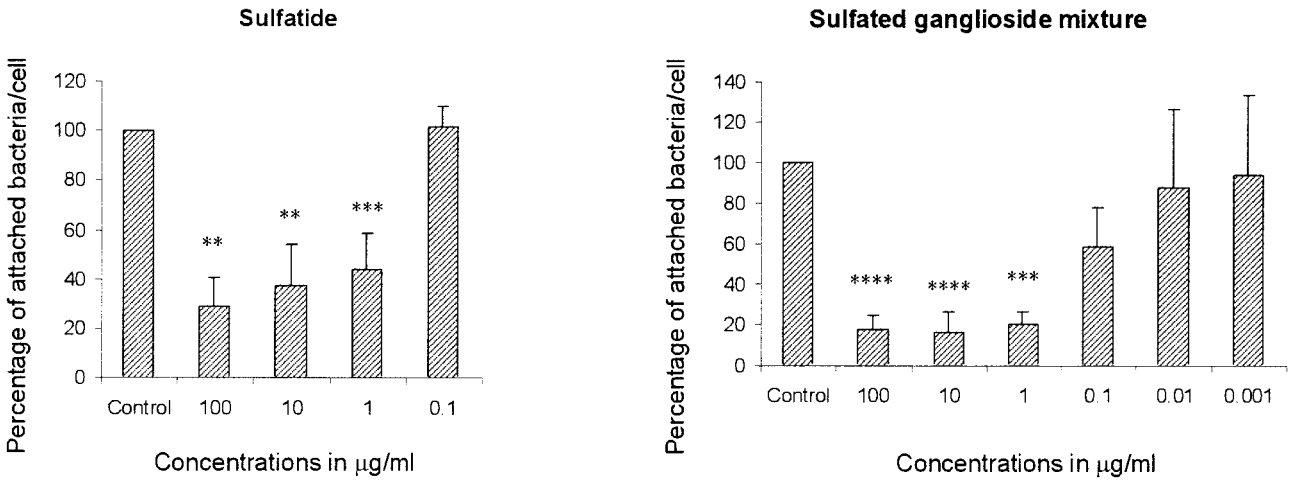

Fig. 1. Results of attachment inhibition assay using strain B-88-152 are shown in two different panels. The left and right panels are for sulfatide and sulfated ganglioside mixture, respectively. The concentrations of glycoconjugates in μg/ml are shown in the *X* axis. *Y* axis indicates the attachment of bacteria per epithelial cell expressed as percentage of the respective control. *P* values of <0.01, <0.005 and <0.001 are indicated by **, *** and ****, respectively.

sulfated gangliosides mixture, sulfatides (3-SO_3-GalCer), galactosylceramide (GalCer) and synthetic sulfatides. The sulfated gangliosides mixture (GM1a, GD1a, GD1b and GT1b) was prepared from gangliosides purified from bovine brains (7). The structures of the per-*O*-sulfated gangliosides were supported by the positive ion fast bombardment mass spectrometry (JMS-SX102, JEOL, Tokyo). Sulfatide was isolated from fresh bovine brain by procedures as described elsewhere (14). Galactosylceramide was a desulfated form of sulfatide.

In this study, the following synthetic sulfatide analogues were also used: polymeric poly-(3-SO_3-Gal) [sugar: AA (polyacrylamide)=36:64], poly-(6-SO_3-Gal) [sugar:AA=6:94] and poly-(3,6-SO_3-Gal) [sugar: AA=6:94], and monomeric 6-SO_3-Gal-*p*NP (*p*-nitrophenyl) and 3,6-SO_3-Gal-*p*NP. They were prepared according to the methods described previously (9, 15–17). Na_2SO_3 (sodium sulfite) was purchased from Sigma (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

A mouse anti-sulfatide mAb, GS-5 (IgM), was prepared as described previously (13). The culture supernatant from hybridoma cells was obtained and stored at −80 C until used. The GS-5 mAb was found to recognize sulfatide and seminolipid (HO_3S-3Galβ1-alkylacylglycerol) and not other related sulfated glycosphingolinpids, such as lysosulfatide, SM3 (HO_3S-3Galβ1-4Glcβ1-1'ceramide), SM2 [GalNAcβ1-4(HO_3S-3)Galβ1-4Glcβ1-1'ceramide], SB2 [HO_3S-3GalNAcβ1-4(HO_3S-3)Galβ1-4Glcβ1-1' ceramide] and SB1a [HO_3S-3Galβ1-3GalNAcβ1-4(HO_3S-3) Galβ1-4Glcβ1-1' ceramide] (13).

An attachment inhibition assay was done with two different established approaches (1, 2): one was to determine the effect of different glycoconjugates suspended in 1/15 mol phosphate buffer, pH 7.2, and the other was to determine the effect of anti-sulfatide antibody on human pharyngeal epithelial cells (HPECs). The mean of the duplicate experiment was determined in each experiment. At least three experiments were done for each potential inhibitor and its control. Student's *t* test was done to find the significance; a *P* value of <0.05 was considered significant. After the treatment with glycoconjugates at a concentration of 100 μg/ml the viability of *M. catarrhalis* was determined by quantitative culture (1). TLC was performed with sulfatide and GalCer dissolved in methanol according to the methods described previously (2).

Compared to the control there was a significant dose dependent inhibition of attachment of *M. catarrhalis* (28.9±11.4, 37.4±16.8 and 43.9±14.4% of the control) when the bacteria were treated with 100, 10, 1 μg/ml of sulfatide (Fig. 1). However no significant effects were observed at a dose of 0.1 μg/ml (101.7±7.9% of control). Again compared to the control there was a significant decrease of attachment (17.5±7.2, 16.5±9.6 and 19.7±6.3% of the control) when *M. catarrhalis* was treated with 100, 10 and 1 μg/ml of sulfated gangliosides (Fig. 1). However there was no significant decrease of attachment (58.5±19.4, 87.9±38.0 and 94.2±38.7% of the control) when *M. catarrhalis* was treated with 0.1, 0.01 and 0.001 μg/ml of sulfated gangliosides. A dose dependent effect was seen in this attachment inhibition assay. Compared to the control there was no significant difference when *M. catarrhalis* was treated with 100 and 10 μg/ml of Na_2SO_3 (100.6±22.6 and 96.0±43.5% of the control) and GalCer (95.7±8.1 and 77.6±19.6% of the control).

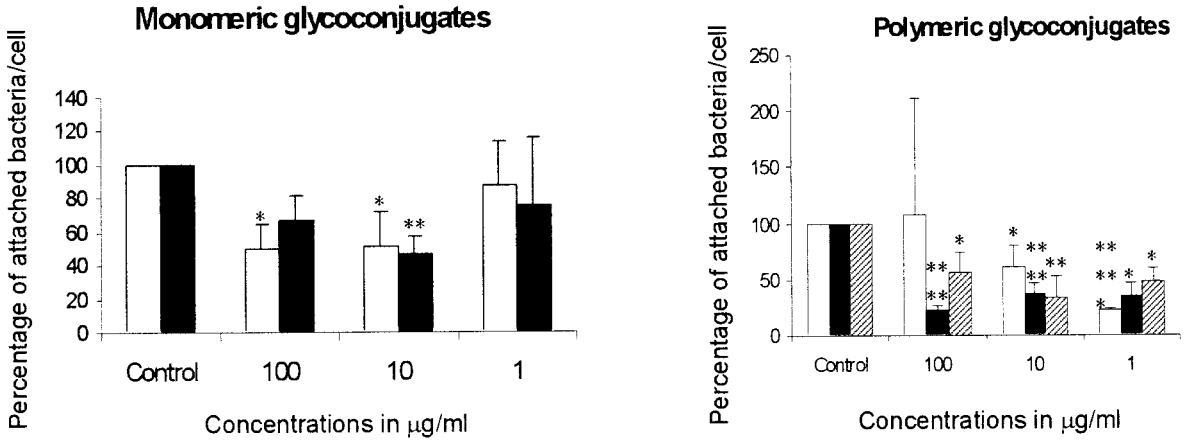

Fig. 2. Results of attachment inhibition assay using strain B-88-152 are shown in two different panels. The left panel is for monomeric glycoconjugates, 6-SO_3-Gal-*p*NP (*p*-nitrophenyl) and 3,6-SO_3-Gal-*p*NP are indicated by open and closed column, respectively. The right panel is for polymeric glycoconjugates, poly-(3-SO_3-Gal) [sugar:AA (polyacrylamide)=36:64], poly-(6-SO_3-Gal) [sugar:AA=6:94], poly-(3,6-SO_3-Gal) [sugar:AA=6:94] are indicated by open, closed and hatched column, respectively. The concentrations of glycoconjugates in μg/ml are shown in the *X* axis. *Y* axis indicates the attachment of bacteria per epithelial cell expressed as percentage of the respective control. *P* values of <0.05, <0.01, <0.001 and <0.0005 are indicated by *, **, $\overset{**}{*}$ and $\overset{***}{**}$, respectively.

Figure 2 shows the effects of different synthetic sulfatides at concentrations of 100, 10 and 1 μg/ml, on the attachment inhibition of *M. catarrhalis*. There was a significant decrease of attachment except for *p*NP 6-SO_3-Gal, *p*NP 3,6-SO_3-Gal and poly-(3-SO_3-Gal) at concentrations of 1, 1 and 100 μg/ml, respectively. At a concentration of 100 μg/ml *p*NP 6-SO_3-Gal showed a borderline significant ($P<0.06$) decrease of attachment. Compared to the untreated control, there was no difference of viability after bacteria were treated with different glycoconjugates.

When HPECs were treated with 1:100 and 1:1,000 dilution of mAb, the attachment of *M. catarrhalis* was 23.1 ± 4.1 and $66.7\pm43.5\%$ of the control, respectively. Statistical significance ($P<0.0005$) was obtained at 1:100 dilution only. For all strains, a dark band becomes visible on the TLC plate signifying a strong positive interaction of *M. catarrhalis* with sulfatide, while for GalCer the interaction varied from weak to strong for different strains at separate experiments.

A key virulence element of *M. catarrhalis* is its ability to bind with the molecules present on host cells. This study provided evidence that sulfatide can act as a molecule for the attachment of *M. catarrhalis* to HPECs. No significant inhibition was observed when bacteria were treated with sodium sulfite indicating the interaction of sulfatide is specific and not an ionic interaction. Utilizing different types of binding molecules is not uncommon in microorganisms; many bacteria such as *Bordetella pertussis* (4) attach with sulfatide as well as with asialo GM1. The parallel occurrence of sialic acid and sulfate group specificities has been observed with other lectins also (11). This shows the use of multiple receptors might reflect a specialized adaptation of *M. catarrhalis* to the human respiratory tract. It provides an advantage to this bacterium to bind strongly with the cell surface to overcome the normal cleansing mechanism in respiratory organs.

Sulfated derivatives of polysaccharides have been reported to have much stronger activities than non-sulfated polysaccharides (7); therefore sulfated gangliosides were examined in this study to find out their effects on the attachment inhibition of *M. catarrhalis*. Sulfated gangliosides showed a remarkable decrease of attachment possibly due to their possession of sialic acid as well as sulfatide part. A past report indicated that sialic acid is important for the attachment of *M. catarrhalis* to HPECs (1).

In the present study *M. catarrhalis* showed interaction, although occasionally weak, with GalCer on TLC plate. This disparity from our earlier report (2) is due to different culture conditions used in these two experiments. Most importantly in attachment inhibition assays, GalCer could not inhibit the attachment of *M. catarrhalis* to HPECs. TLC enabled detection of low-affinity cooperative multi-site interactions that would escape detection by soluble univalent receptors in attachment inhibition experiments (12). Therefore interaction of *M. catarrhalis* with GalCer on the cell surface is probably of too low avidity to be important for pathogenesis. Sulfatide mediated attachment may ultimately guarantee the bacteria for intimate contact necessary for pathogenesis as its interaction occurring both polyvalent and monovalent presentation.

The interaction of *M. catarrhalis* with different synthetic glycoconjugates was variable. For example even at a lower concentration, polymeric glycoconjugates showed stronger inhibition of bacterial attachment. This is because increasing carbohydrate ligand concentration does not always lead to an increase in binding with the complementary protein (3). There is also a possibility that the interaction of synthetic glycoconjugate with respective adhesin leads to some conformational changes (5) of other adhesin molecules of *M. catarrhalis* thus caused variable results. Among the synthetic glycoconjugates tested in this study, *p*NP 3,6-SO_3-Gal seems to be a promising candidate since it showed dose dependent attachment inhibition. Otherwise all the synthetic glycoconjugates showed a significant decrease of attachment at 10 μg/ml; therefore this concentration is optimal to inhibit the attachment of *M. catarrhalis* to HPECs. In the present study cells were directly obtained from the human pharynx which reflects the behavior of pathogen-host interaction faithfully. We hope that these results will help in the future to conduct *in vivo* studies to find out the effects of these synthetic glycoconjugates in the prevention of *M. catarrhalis* infection.

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