IDENTIFICATION OF AN ACTIVE DISACCHARIDE UNIT OF A GLYCOCONJUGATE RECEPTOR FOR PNEUMOCOCCI ATTACHING TO HUMAN PHARYNGEAL EPITHELIAL CELLS*

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The high degree of tissue tropism of many infectious agents can, in part, be explained by their recognition of specific epithelial cell receptor sites. Binding to these receptors results in attachment (1–3). Streptococcus pneumoniae is a constituent of the flora in the healthy nasopharynx as well as a cause of localized or invasive infection (4–6). Pneumococcal isolates from the nasopharynx of patients with otitis media or healthy carriers adhered more avidly to human nasopharyngeal epithelial cells than isolates from patients with septicemia or meningitis (7). Adhesive capacity may thus localize pneumococci to that site. Consequently, classification of mechanisms of binding may provide tools to interfere with the bacterial colonization.

Pneumococci associate with numerous macromolecules (8–10). C-reactive protein binds the C-polysaccharide through the phosphorylcholine moiety (11). The collagen-binding region of human serum fibronectin can bind to as yet undefined pneumococcal surface structures.1 The binding of pneumococci to cells varies with species, individual, and tissue in a manner suggesting specific binding of bacterial adhesins and epithelial cell receptors (reference 7, and Andersson et al., unpublished observations). A proteinaceous pneumococcal adhesion was suggested since attachment was abolished by heating or protease treatment of the bacteria (7). Interactions of protein adhesins with epithelial cell receptors of a glycolipid nature were previously shown to explain the attachment of uropathogenic Escherichia coli to human uroepithelial cells (2, 12). More specifically, a Galα1→4Galβ-moiety was shown to be the minimal component sufficient (although not optimal) for binding (12, 13). This paper presents evidence that

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attaching pneumococci specifically bind glycoconjugate receptors containing the disaccharide GlcNAcβ1→3Galβ-.

Materials and Methods

**Bacteria.** The two strains of *S. Pneumoniae*, EF3114 and EF10276 (both of capsular type 6A, isolated from the nasopharynx of patients with otitis media) were used throughout the study. To test the generality of the proposed receptor structure the following strains were included: EF2987 (capsular type 23F), EF3030 (type 19F), EF3296 (type 4), EF3559 (type 14), isolated from patients with otitis, EF7509 (type 25) and EF1488 (type 15A) from patients with meningitis, and EF10175 (type 19F) isolated from the nasopharynx of a healthy carrier. The strains were kept lyophilized. For adherence testing the lyophils were transferred to blood agar plates and cultured over night, then grown for 18 h in 50 ml of the synthetic medium of van der Rijn and Kessler (14) supplemented with 2 g/l of ascorbic acid and as described in (15). Bacteria were harvested by centrifugation at 3,000 g for 20 min, and suspended in 1 ml of saline (7).

**Glycoconjugates.** The structures of the glycoconjugates and oligosaccharides used are listed in Table I. Human plasma fibronectin was isolated according to (22, 23). *E. coli* lipopolysaccharides (LPS) from smooth strains of serotypes 04 and 083 and from rough mutants with core structures R1, R2, R3, and R4 were prepared by hot phenol water extraction (24). The glycolipids neolactotetraosylceramide, *N*-acetylenuraminosylneolactotetraosylceramide, and globotetraosylceramide were isolated from human erythrocytes. The glycolipids were prepared by chloroform-methanol extraction, mild alkaline hydrolysis, dialysis, diethylaminoethyl-cellulose chromatography and silicic acid chromatography (25). The neutral glycolipids were further purified as their acetylated derivatives (26) and were at least 95% pure judged by thin layer chromatography. For the preparation of neolactotetraosylceramide, *N*-acetylenuraminosylneolactotetraosylceramide (the major ganglioside of human erythrocytes [27]) was isolated and degraded by *Vibrio cholerae* neuraminidase (Calbiochem-Behring Corp., San Diego, CA). The glycolipid structures were confirmed by mass spectrometry, nuclear magnetic resonance (NMR) spectroscopy and degradative methods (28-30 also references in 31). For testing, the glycolipids were taken from chloroform methanol solutions, cleared of solvent in a stream of nitrogen, desiccated over night, and suspended in NaCl by sonication in a water bath.

The tetrasaccharides neolactotetraose and lactotetraose were isolated from human milk (20). The octasaccharide was isolated from the urine of a patient with GM1-gangliosidosis (21). The structures were confirmed by mass spectrometry and inferred by paper chromatography.

The following compounds (c.f. Table I) were prepared by synthesis (to be reported elsewhere). The structures were confirmed by NMR and physical methods. Briefly:

**Ethyl 4-O-β-D-galactopyranosyl-β-D-glucopyranoside (9):** [α]_D^25 + 1.7° (c 1.0, water); NMR data: 1H (DMSO-d_6, 50°C, plus D_2O) δ 4.28-4.16 (3H, inter alia H-1 and H-1'), 1.14 (t, 3H, J 7 Hz). 13C (D_2O, TSP) δ 105.8, 104.6 (2d, J 158 and 155 Hz, C-1 and C-1'), 17.1 (CH_3).

**Methyl 2-acetamido-2-deoxy-4-O-(13-O-galactopyranosyl)-β-D-glucopyranoside (11):** m.p. decomposition, [α]_D^20 - 16° (c 1, water); Lit. (32): m.p. decomp., [α]_D^20 - 16.7° (c 0.3, water).

**Ethyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-β-D-glucopyranoside (12):** was reported earlier (33).

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**Abbreviations used in this paper.** EID<sub>75</sub>, effective inhibitory dose reducing adherence to 25% of the saline control (75% inhibition); LPS, lipopolysaccharide; m.p., melting point; NMR, nuclear magnetic resonance spectroscopy.
### TABLE I

**Chemical Structure of Characteristic Parts of Glycoconjugates and Saccharides Tested for Receptor Activity**

| Compound                      | Source          | Number | Chemical structure of characteristic saccharide part                                                                 | Reference |
|-------------------------------|-----------------|--------|----------------------------------------------------------------------------------------------------------------------|-----------|
| **Glycoconjugates:**          |                 |        |                                                                                                                      |           |
| Human fibronectin             | Human blood     | 1      | NeuNAc$_2$$\rightarrow$$4$(6)Gal$_1$$\rightarrow$$4$GlcNAc$_1$$\rightarrow$$2$Man$_1$                           | (19)      |
| Neolactotetraosylceramide     |                 | 2      | Gal$_1$$\rightarrow$$4$GlcNAc$_1$$\rightarrow$$3$Gal$_1$$\rightarrow$$4$Glc-Cer                                      | (20)      |
| Sialyneolactotetraosylceram-  |                 | 3      | NeuNAc$_2$$\rightarrow$$3$Gal$_1$$\rightarrow$$4$GlcNAc$_1$$\rightarrow$$3$Gal$_1$$\rightarrow$$4$Glc-Cer       | (20)      |
| ide                           |                 |        |                                                                                                                      |           |
| Globotetraosylceramide        | Human milk      | 4      | GalNAc$_1$$\rightarrow$$3$Gal$_1$$\rightarrow$$4$Gal$_1$$\rightarrow$$4$Glc-Cer                                  | (20)      |
| **Natural oligosaccharides:** |                 |        |                                                                                                                      |           |
| Neolactotetraose              | Human milk      | 5      | Gal$_1$$\rightarrow$$4$GlcNAc$_1$$\rightarrow$$3$Gal$_1$$\rightarrow$$4$Glc                                      | (21)      |
| Lactotetraose                 |                 | 6      | Gal$_1$$\rightarrow$$4$GlcNAc$_1$$\rightarrow$$3$Gal$_1$$\rightarrow$$4$Glc                                      | (21)      |
| "Octasaccharide"             | Human urine     | 7      | Gal$_1$$\rightarrow$$4$GlcNAc$_1$$\rightarrow$$3$Gal$_1$$\rightarrow$$4$Glc                                      | (22)      |
| **Synthetic oligosaccharides:** | Milk           | 8      | Gal$_1$$\rightarrow$$4$Glc                                                                                           |           |
| β-Ethyl-lactose               | Synthetic       | 9      | Gal$_1$$\rightarrow$$4$Glcβ-O-Et                                                                                  |           |
| α-Methyl-N-acetyl-lactosae-   | *               | 10     | Gal$_1$$\rightarrow$$4$GlcNAc$_1$-O-Me                                                                             |           |
| mine                          |                 |        |                                                                                                                      |           |
| β-Methyl-N-acetyl-lactosae-   | *               | 11     | Gal$_1$$\rightarrow$$4$GlcNAc$_1$-O-Me                                                                             |           |
| mine                          |                 |        |                                                                                                                      |           |
| β-Ethyl-N-acetyl-lactosamine  | *               | 12     | Gal$_1$$\rightarrow$$4$GlcNAc$_1$-O-Et                                                                              |           |
| N'-Acetyl-lactosamine         | *               | 13     | Gal$_1$$\rightarrow$$4$GlcNAc                                                                                       |           |
| *                             | 14               |        | GlcNAc$_1$$\rightarrow$$4$Gal$_1$-O-Me                                                                            |           |
| *                             | 15               |        | GlcNAc$_1$$\rightarrow$$3$Gal$_1$-O-Me                                                                            |           |

Nomenclature as recommended in references 17 and 18. Numbers are referred to in the text by italics.

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2-Acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-D-glucose (13): Physical data were determined for the α-per-O-acetate: m.p. 224-225°C, \( [\alpha]_D^{25} + 58.6^\circ \) (c 0.9, chloroform); Lit. (34): m.p. 224-225°C, \( [\alpha]_D^{25} + 58^\circ \) (c 1, chloroform).

Methyl 4-O(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (14): \( [\alpha]_D^{23} + 19^\circ \) (c 0.3, water); NMR data: \( ^1H (D_2O) \delta 4.70 \) (d, J 8.3 Hz, H-1'), \( 4.30 \) (d, J 7.9 Hz, H-1), 3.53 (s, OMe), 2.04 (s, COMe); \( ^13C (D_2O) \delta 106.5, 104.9, 25.1 \) (COCH3).

Methyl 3-O(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (15): \( [\alpha]_D^{25} + 3.6^\circ \) (c 0.4, water); NMR data: \( ^1H (D_2O) \delta 4.69 \) (d, J 8.5 Hz, H-1'), \( 4.30 \) (d, J 7.8 Hz, H-1), 3.56 (s, OMe), 2.03 (s, COMe); \( ^13C (D_2O) \delta 106.7 \) (C-1), 105.5 (C-1'), 60.0 (OCH3), 58.5 (C-2'), 25.0 (COCH3).

**Bacterial Binding Assays**

**Epithelial Cell Adherence.** The binding to human nasopharyngeal epithelial cells was assayed in vitro as previously described (7). Saline suspension of bacteria (10^9/ml) and pharyngeal epithelial cells from a healthy male donor (10^4/ml) were mixed to an end volume of 200 µl, centrifuged at 1,500 g for 10 min, and incubated at 37°C for 30 min. After washing twice in 2.5 ml of saline to separate epithelial cells from unattached bacteria the number of bacteria adhering to 40 epithelial cells were counted and the mean number per cell was determined.

**Hemagglutination.** Adhering bacteria may be expected to bind to any target cell or surface where receptors are present. Bacterial binding to erythrocytes, resulting in hemagglutination has been a useful tool to approach mechanisms of binding. Thus, the ability of the adhering pneumococci to agglutinate erythrocytes was investigated. Freshly drawn citrated blood was washed and 5% suspensions in saline of human, bovine, rabbit, and guinea pig erythrocytes were prepared. Human O, A, B, AB, and cord blood samples were provided by the blood bank of Sahlgren’s Hospital, Göteborg, Sweden. For enzymatic treatment the 5% suspensions were mixed with Papain (Kebo Grave, Stockholm, Sweden) or neuraminidase (Sigma Chemical Co., St. Louis, MO) to a final concentration of 1 mg/ml and rotated at 37°C for 30 min. After washing twice the suspension was readjusted to 5%. Bacterial and erythrocyte suspensions (25 µl of each) were mixed on a microscope slide, and agglutination read by the naked eye.

**Screening for Receptor Activity of the Glycoconjugates**

Receptor activity of glycoconjugates or free oligosaccharides, was assayed in two main ways.

(a) Inhibition of binding by excess of the free sugar. The bacterial inoculum was preincubated with dilutions of the compounds with potential receptor activity, for 15 min at 37°C. Subsequently epithelial cells were added and adhesion testing proceeded as described. The adhesion of the treated samples is given in per cent of the saline control. The variation between experiments is described by the range. The effective inhibitory oligosaccharide dose, EI75%, required for 75% inhibition of adhesion is given.

(b) Induction of binding by coating with receptor. Association of receptor-active glycolipids with epithelial cells or erythrocytes might increase the bacterial binding to epithelial cells already containing the receptor or induce binding to cells previously lacking the receptor (12). Rabbit erythrocytes (100 µl of a 5% suspension) were mixed with neolactotetraosylceramide, sialyneolactotetraosylceramide, or globotetraosylceramide (500 µl of suspensions of 200 µg/ml) and incubated at 37°C for 3 h during rotation (12). After elimination of unbound glycolipid by repeated washing, the erythrocyte concentration was readjusted to 5% and used for hemagglutination. In parallel with the adherence inhibition assays the human nasopharyngeal epithelial cells were pretreated with decreasing concentrations of glycolipid, incubated for 2 h at 37°C, washed, and used for adherence testing.

**Results**

**Initial Studies.** The focus on the neolactoseries of glycolipids as receptor candidates resulted from work with other glycoconjugates. Human plasma fibro-
nectin inhibited adhesion (42% of saline control at 100 μg/ml). Increased attachment was obtained after pretreatment of the epithelial cells with 1 mg/ml of fibronectin (Table II). *E. coli* LPS of serotypes 04 and 083 completely inhibited adhesion at 1 mg/ml. Partial inhibition resulted after pretreatment with R₃ LPS at 1 mg/ml; R₁, R₂, and R₄ were not inhibitory. No decrease in pneumococcal viability after treatment with glycoconjugates was detected by viable counts on 10-fold saline dilutions.

Compounds with potential receptor activity were selected for testing because of structural similarity to the known oligosaccharide sequences of fibronectin and R₃ LPS.

Glycolipids. The receptor activity of the glycolipids is shown in Table II. Pretreatment of the pneumococci with neolactotetraosylceramide inhibited adhe-

### Table II
**Effect of Glycoconjugates on the Adhesion of Pneumococci to Human Pharyngeal Epithelial Cells**

| Compound                        | Concentration | Adhesion* | No. | Concentration | Adhesion* | No. |
|---------------------------------|---------------|-----------|-----|---------------|-----------|-----|
|                                 | μg/ml         | % of saline control | Mean | Range | μg/ml         | % of saline control | Mean | Range | exp. | No. | exp. |
| Fibronectin                     | 100           | 42        | 23-50 | 3     | 1,000        | 234       | —   | 1     |
| Neolactotetraosylceramide       | 200           | 59        | 22-100| 6     | 200          | 190       | 85-244| 4     |
| Sialyneolactotetraosylceramide  | 200           | 100       | 93-108| 3     | 200          | 94        | 65-127| 3     |
| Globotetraosylceramide          | —             | 200       | 100   | —     | 1            |

* Bacteria were preincubated with the glycoconjugates before addition of epithelial cells (competitive inhibition).

* Epithelial cells were preincubated with glycoconjugate, washed and used for adherence testing (coating).

* More data in reference 12.

### Table III
**Inhibition of Pneumococcal Adhesion by Natural Oligosaccharides**

| Oligosaccharide | No. in Table I | Concentration | Adhesion | No. exp. |
|-----------------|----------------|---------------|----------|----------|
|                 | mg/ml          | % of saline control | Mean | Range | EID₇₅* |
| Neolactotetraose| 5              | 10            | 1        | 0-2     | 3       |
|                 | 5              | 0             | 0-0      | 4       |
|                 | 1              | 28            | 14-56    | 1.5     | 5       |
|                 | 0.1            | 52            | 36-63    | 4       |
| Lactotetraose   | 6              | 5             | 4        | 2-5     | 3       |
|                 | 1              | 25            | 14-45    | 1.0     | 5       |
|                 | 0.1            | 68            | 55-81    | 2       |
| *Octasaccharide*| 7              | 5             | 71       | 43-116  | 3       |
|                 | 1              | 62            | 53-78    | >10      | 3       |
|                 | 0.1            | 141           | 47-235   | 2       |

* EID₇₅ = effective inhibitory dose reducing adherence to 25% of the saline control (75% inhibition).
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TABLE IV

Inhibition of Pneumococcal Adhesion by Synthetic Disaccharides

| Disaccharide            | No. in Table I | Concentration mg/ml | Adhesion % of saline control | EID75 mg/ml | No. exp. |
|-------------------------|----------------|---------------------|-----------------------------|-------------|----------|
| Galβ1→4Glc              | 8              | 10                  | 101                          | 100-102     | >10      | 2        |
|                         |                | 5                   | 107                          | 100-111     |          | 2        |
|                         |                | 1                   | 97                           | 76-118      |          | 2        |
| Galβ1→4Glcβ1-O-Et       | 9              | 10                  | 65                           | 48-77       | >10      | 2        |
|                         |                | 5                   | 78                           | 69-86       |          | 2        |
|                         |                | 1                   | 85                           | 83-86       |          | 2        |
| Galβ1→4GlcNAca1-O-Me    | 10             | 10                  | 129                          | 111-146     | >10      | 2        |
|                         |                | 5                   | 98                           | 71-127      |          | 2        |
|                         |                | 1                   | 96                           | —           |          | 1        |
| Galβ1→4GlcNAcβ1-O-Me    | 11             | 10                  | 29                           | 26-35       | >10      | 3        |
|                         |                | 5                   | 45                           | 32-53       |          | 4        |
|                         |                | 1                   | 96                           | 88-103      |          | 2        |
| Galβ1→4GlcNAcβ1-O-Et    | 12             | 10                  | 31                           | 28-34       | >10      | 2        |
|                         |                | 5                   | 38                           | 21-54       |          | 2        |
|                         |                | 1                   | 67                           | 59-74       |          | 2        |
| Galβ1→4GlcNAc          | 13             | 10                  | 127                          | 12-251      | >10      | 4        |
|                         |                | 5                   | 144                          | 58-335      |          | 5        |
|                         |                | 1                   | 95                           | 67-123      |          | 2        |
| GlcNAcβ1→4Galβ-O-Me     | 14             | 10                  | 75                           | 57-92       | >10      | 2        |
|                         |                | 5                   | 73                           | 65-81       |          | 2        |
|                         |                | 1                   | 72                           | 71-72       |          | 2        |
|                         |                | 0.1                 | 65                           | 64-66       |          | 2        |
| GlcNAcβ1→3Galβ-O-Me     | 15             | 10                  | 4                            | 0-11        | 4.5      | 3        |
|                         |                | 5                   | 15                           | 0-31        |          | 3        |
|                         |                | 1                   | 105                          | 80-136      |          | 2        |
|                         |                | 0.1                 | 66                           | 65-66       |          | 2        |

sion (59% of saline control at 200 μg/ml). Sialyolactotetraosylceramide had no effect (Table II). Coating of pharyngeal epithelial cells with 200 μg/ml of neolactotetraosylceramide increased adhesion (Table III) while sialyolactotetraosylceramide or globotetraosylceramide had no effect at the same concentration. The structures of these compounds are shown in Table I.

Oligosaccharides. Of the purified oligosaccharides tested, neolactotetraose and lactotetraose were the best inhibitors, with an EID75 of 1.5 and 1.0 mg/ml (Table III). The “octasaccharide” from human urine had no effect. Lactose was inactive. Subsequent studies were concentrated on defining the active site in the tetrasaccharide sequence. N-acetyl-lactosamine and α-methyl-N-acetyl-lactosamine were inactive. Even in high concentrations β-methyl and β-ethyl-N-acetyl-lactosamine only partially reduced adhesion (EID75 > 10 mg/ml). GlcNAcβ1→3Galβ-O-Me abolished adhesion at 10 mg/ml with an EID75 of 4.5 mg/ml. The disaccharide was, thus, three times less active than the tetrasaccharides on a weight basis. The carbohydrate pneumococcal receptor was thus proposed to reside in the trisaccharide unit Galβ1→4GlcNAcβ1→3Galβ- with
Hemagglutination of Erythrocytes by an Adhesive Pneumococcal Strain (EF 3114)

| Erythrocyte species      | Enzyme treated | Glycoconjugate coated | Uncoated |
|--------------------------|----------------|-----------------------|----------|
|                          |                | 2 3 4                 |          |
| Human blood group O, A, B, or AB |                |                       |          |
| Human cord blood         |                |                       |          |
| Bovine                   | (+)            | + ++                  | ND       |
| Rabbit                   | (+)            | + ++                  | (++)     |
| Guinea pig               | ++             | ND                    | ND       |
| Sheep                    |                |                       | ND       |

* The hemagglutination was read: (+), some agglutinated and many lysed erythrocytes; +, only few agglutinated erythrocytes; ++, larger aggregates with some free erythrocytes; ++++, most of the erythrocytes agglutinated.
+ Rabbit erythrocytes were incubated with glycolipids. The numbers indicate structures in Table I.

Inhibition of the Adhesion of Various Strains of S. Pneumoniae by Lactotetraose

| Strain   | Capsular type | Concentration of saccharide | Adhesion | No. of exp. |
|----------|---------------|-----------------------------|----------|-------------|
|          |               | mg/ml | % of saline control | Mean | Range |          |
| EF1488   | 15A           | 10   | 17 | 9-25 | 2 |
| EF2987   | 23F           | 10   | 75 | 65-85 | 2 |
| EF3030   | 19F           | 10   | 51 | 48-53 | 2 |
| EF3296   | 4             | 10   | 7  | 6-7   | 2 |
| EF3559   | 14            | 10   | 1  | 0     | 1 |
| EF7509   | 25            | 10   | 17 | 12-21 | 2 |
| EF10175  | 19F           | 10   | 16 | 6-36  | 3 |

GlcNAcβ1→3Galβ as the principal binding site.

To test the generality of binding to Galβ1→3GlcNAcβ1→3Galβ1→4Glc as a mechanism of pneumococcal adhesion, seven strains with different capsular types and high adhesive capacity were used. Adhesion of all the strains tested was inhibited after pretreatment of the bacteria with lactotetraose (Table VI). No correlation between inhibition and the capsular type of the pneumococcal strain was observed.

Hemagglutination. The pneumococcal strains did not agglutinate any of the erythrocyte species tested. After papain or neuraminidase treatment guinea pig and to a smaller extent bovine erythrocytes became agglutinable (Table V). After coating with neolactotetraosylceramide (200 μg/ml) the previously inactive rabbit erythrocytes became agglutinable. Sialylneuolactotetraosylceramide and glo-
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botetraosylceramide were inactive.

Discussion

This study demonstrates specific attachment of pneumococci to glycoconjugate receptors where the disaccharide GlcNAcβ1→3Galβ- is shown to be the main binding site. The high degree of specificity of this binding is shown by the lack of activity of GlcNAcβ1→4Galβ-O-Me (Table III). The importance of the β-configuration of the N-acetylgalactosaminosyl group was demonstrated by the inhibitory activity of β-methyl but not α-methyl-β-N-acetyl-lactoseamine.

The identification of the receptor was done experimentally in several steps using inhibition and induction of binding. In analogy with Streptococcus pyogenes, the pneumococci bound human plasma fibronectin. In contrast to S. pyogenes, the pneumococci bound at the collagen-binding region of fibronectin. The partly characterized oligosaccharide sequence at that site contains the "octasaccharide" (see 7 in Table I), that did not inhibit adhesion. This does not contain the active disaccharide and did not inhibit adhesion (Table III). Accordingly, this part of the fibronectin structure probably is not the pneumococcal binding site. The fibronectin-binding may involve other adhesion receptor interactions.

Receptor activity of the neolacto-series of glycolipids was indicated by the inhibition of adhesion obtained after pretreatment of bacteria with neolactotetraosylceramide and the increased binding after coating of epithelial cells and erythrocytes. Receptor activity of the β-O linkage per se was unlikely, since lactose-β-O-Me did not inhibit adhesion. Receptor activity of the ceramide portion was ruled out, since e.g. sialyneolactotetraosylceramide was inactive. The complete inhibition of pneumococcal adhesion obtained with neolactotetraose and lactotetraose suggested that the receptor was a common feature of these tetrasaccharides. Since lactose, lactoseamine, and the "octasaccharide" (which contains two terminal lactoseamine residues) are all inefficient as inhibitors, it was thought that the central disaccharide portion of lactoneotetraose should be the active receptor. Accordingly, this compound (15 in Table I) was synthesized in the form of its β-methyl glycoside. Testing of the inhibitory activity supported this assumption.

The varying ability to agglutinate erythrocytes of different species has been useful for classifying bacterial adhesins (35). The GlcNAcβ1→3Gal has been identified as part of the saccharide chains of many glycolipids and glycoproteins including long chains containing repeating units of (→GlcNAcβ1→3Galβ→) (36, 37). It has been identified in human (36, 37), ox (38, 39), rabbit (40), and sheep (41) erythrocytes, but so far not in guinea pig erythrocytes (42). Thus a correlation between presence of receptor-active oligosaccharide and pneumococcal hemagglutination was not apparent (Table V) as found between hemagglutination by uropathogenic E. coli and the presence of the Galα1→4Gal sequence (2). The receptor activity may depend not only on the presence but also on availability of the receptor in the cell surface micro-environment (36).

The identification of the interaction of pneumococci with specific saccharides is interesting from many biological and medical points of view. The disaccharide GlcNAcβ1→3Galβ- (substituted at various positions) is part of many saccharide chains active as blood group ABH, Lewis, or Li antigens (37). Its presence and
availability therefore may be subject to variations between individuals, and may affect the susceptibility to pneumococcal infection. Compare the relation between P blood group phenotype and urinary tract infection (43). Our preliminary results indicate that epithelial cells from patients with frequent attacks of otitis media have an increased receptivity for adhering pneumococci compared with cells from age matched controls (44). The inhibitory effect of receptor oligosaccharides in vitro might be used to inhibit bacterial adhesion in vivo. The principal usefulness of this approach has been demonstrated in other infections (45, 46). Furthermore pneumococcal adhesion was also inhibited by a fraction of human milk rich in oligosaccharides and from which antibodies had been removed. The natural presence of neolacto- and lactotetraose in human milk and colostrum (20) may interfere with pneumococcal colonization in the newborn, and could in part explain the suggested positive effect of breast feeding on otitis media (47, 48).

Summary

Glycoconjugates containing the disaccharide unit GlcNAcβ1→3Galβ were suggested as receptors for pneumococci adhering to human pharyngeal epithelial cells. The receptor activity was detected both by inhibition of adhesion by an excess of free oligosaccharide and by induction or increase of adhesion after coating of target cells with glycolipid. Studies with free natural and synthetic oligosaccharides identified the disaccharide GlcNAcβ1→3Galβ as one critical binding site. The specificity of recognition was shown inter alia by the lack of inhibitory activity of GlcNAcβ1→4Galβ, which differs only in the linkage of the two sugars. Specific interference with pneumococcal adhesion by administration of soluble receptor sugar may improve our understanding of the role of adhesion in vivo.

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References

1. Gibbons, R. J., and J. van Houte. 1971. Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. Infect. Immun. 3:567.
2. Leffler, H., and C. Svanborg Edén. 1980. Chemical identification of a glycosphingolipid receptor for Escherichia coli attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 8:127.
3. Simpson, W. A., and E. H. Beachey. 1983. Adherence of group A streptococci to fibronectin on oral epithelial cells. Infect. Immun. 39:275.
4. Finland, M. 1979. Pneumonia and pneumococcal infections, with special reference to pneumococcal pneumonia. Am. Rev. Respir. Dis. 120:481.
5. Riley, I. D., and R. M. Douglas. 1981. An epidemiologic approach to pneumococcal disease. Rev. Infect. Dis. 3:233.
6. Klein, J. O. 1981. The epidemiology of pneumococcal disease in infants and children. Rev. Infect. Dis. 3:246.
7. Andersson, B., B. Eriksson, E. Falsen, A. Fogh, L. Å. Hanson, O. Nylén, H. Peterson, and C. Svanborg Edén. 1981. Adhesion of Streptococcus pneumoniae to human pharyngeal epithelial cells in vitro: differences in adhesive capacity among strains isolated from subjects with otitis media, septicemia, or meningitis or from healthy carriers. Infect. Immun. 32:311.
8. Winkelstein, J. A. 1981. The role of complement in the host’s defense against the Streptococcus pneumoniae. Rev. Infect. Dis. 3:289.
9. Tomasz, A. 1978. Crossing of cellular boundaries by nucleic acids in bacteria. In Transport of macromolecules in cellular systems. Dahlem Konferenzen, Berlin. 21.
10. Stephens, C. G., W. P. Reed, G. Kronvall, and R. C. Williams Jr. 1974. Reactions between certain strains of pneumococci and Fc of IgG1. J. Immunol. 112:1955.
11. Volonakis, J. E., and M. D. Kaplan. 1971. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. Proc. Soc. Exp. Biol. 136:612.
12. Leffler, H., and C. Svanborg Edén. 1981. Glycolipid receptors for uropathogenic Escherichia coli on human erythrocytes and uroepithelial cells. Infect. Immun. 34:920.
13. Källenius, G., R. Möllby, S. B. Svensson, J. Winberg, A. Lundblad, S. Svensson, and B. Cedergren. 1980. The P antigen as receptor for the hemagglutination of pyelonephritis Escherichia coli. FEMS (Fed Eur. Microbiol. Soc.) Microbiol. Lett. 7:297.
14. van de Rijn, I., and R. E. Kessler. 1980. Growth characteristics of group A streptococci in a new chemically defined medium. Infect. Immun. 27:444.
15. Rane, L., and Y. Subbarow. 1940. Choline, pantothenic acid and nicotinic acid as essential growth factors for pneumococcus. J. Biol. Chem. 134:455.
16. IUPAC-IUB Combined Commission on Biochemical Nomenclature (CBN). Abbreviations and symbols for chemical names of special interest in biological chemistry. 1967. Eur. J. Biochem. 1:259.
17. IUPAC-IUB Commission on Biochemical Nomenclature. The nomenclature of lipids. 1978. Biochem. J. 171:21.
18. Wrann, M. 1978. Methylation analysis of the carbohydrate portion of fibronectin from human plasma. Biochem. Biophys. Res. Commun. 84:269.
19. Macher, B. A., and C. C. Sweeley. 1978. Complex carbohydrates. Methods Enzymol. 50:236.
20. Kobata, A. 1977. Milk glycoproteins and oligosaccharides. In The Glycoconjugates. M. I. Horowitz, and W. Pigman, editors. Academic Press, London. I:423.
21. Lundblad, A., S. Sjöblad, and S. Svensson. 1978. Characterization of a penta- and octasaccharide from urine of a patient with juvenile GM1-gangliosiosis. Arch. Biochem. Biophys. 188:130.
22. Vuento, M., and A. Vaheri. 1979. Purification of fibronectin from human plasma by affinity chromatography under non-denaturing conditions. Biochem. J. 183:331.
23. Simpson, W. A., D. L. Hasty, J. M. Mason, and E. H. Beachey. 1982. Fibronectin mediates the binding of group A streptococci to human polymorphonuclear leukocytes. Infect. Immun. 37:805.
24. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with hot phenol-water and further application of the procedure. Methods Carbohydr. Chem. R. C. Whistler, editor. Academic Press, New York. 5:83.
25. Karlsson, K.-A., B.-E. Samuelsson, and G. O. Steen. 1973. The sphingolipid composition of bovine kidney cortex, medulla and papilla. Biochem. Biophys. Acta. 316:317.
26. Handa, S. 1963. Blood group active glycolipid from human erythrocytes. Jpn. J. Exp.
27. Sweeley, C. C., and B. Siddiqui. 1977. Chemistry of mammalian glycolipids. In The Glycoconjugates. M. I. Horowitz and W. Pigman, editors. Academic Press, New York. 1:459.

28. Falk, K.-E., K.-A. Karlsson, and B.-E. Samuelsson. 1979. Proton nuclear magnetic resonance analysis of anomic structure of glycosphingolipids. The globo series (one to five sugars) Arch. Biochem. Biophys. 192:164.

29. Falk, K.-E., K.-A. Karlsson, and B.-E. Samuelsson. 1979. Proton nuclear magnetic resonance analysis of anomic structure of glycosphingolipids. Blood group ABH active substances. Arch. Biochem. Biophys. 192:177.

30. Karlsson, K.-A. 1978. Mass spectrometric sequence studies of lipid-linked oligosaccharides, blood group fucolipids, gangliosides and related cell surface receptors. Progr. Chem. Fats Other Lipids. 16:207.

31. Breimer, M. E., G. C. Hansson, K.-A. Karlsson, H. Leffler, W. Pimlott, and B.-E. Samuelsson. 1979. Selected ion monitoring of glycolipid mixtures. Determination of the structure of 8 different blood group type glycolipids in the small intestine of an individual rabbit. Biomed. Mass Spectrom. 6:231.

32. Takamura, T., T. Chiba, and S. Tejima. 1981. Chemical modification of lactose. XIV. Synthesis of Lacto-N-neo-hexaose. Chem. Pharm. Bull. (Tokyo). 29:2270.

33. Dahmen, J., T. Frejd, G. Grönberg, T. Lave, G. Magnusson, and G. Noori. 1983. 2-Bromoethylglycosides. Applications in the synthesis of spacer-arm glycosides. Carbohydr. Res. In press.

34. Jaquinet, J. C., and P. Sinay. 1976. Une synthèse du 2-acetamido-2-desoxy-4-O-β-o-galactopyranosyl-α-D-glucopyranose (N-acetyllactosamine). Carbohydr. Res. 46:138.

35. Duguid, J. P., and D. C. Old. 1980. Adhesive properties of enterobacteriaceae. In Bacterial adherence. Receptors and recognition, series B. E. H. Beachey, editor. Chapman and Hall, London. 6:6185.

36. Hakomori, S-i. 1981. Glycosphingolipids in cellular interaction, differentiation and oncogenesis. Annu. Rev. Biochem. 50:733.

37. Hakomori, S-i. 1981. Blood group ABH and Ii antigens of human erythrocytes: chemistry polymorphism and their developmental change. Semin. Hematol. 18:39.

38. Uemura, U.-I., M. Yuzawa, and T. Takeyomi. 1978. Characterization of major glycolipids in bovine erythrocyte membrane. J. Biochem. 83:463.

39. Hamada, A., M. Tomita, K. Fukuda, and I. Kawashima. 1981. Structural differences in sugar chains of glycoporphins from various animals. In Proceedings of the Sixth International Symposium on Glycoconjugates. T. Yamakawa, T. Osawa, and S. Handa, editor. Japan Scientific Societies Press, Tokyo. 280.

40. Eto, T., Y. Ichikawa, K. Nishimura, K. Audo, and T. Yamakawa. 1968. Occurrence of ceramide pentasaccharide in the membrane erythrocytes and reticulocytes of rabbit. J. Biochem. 64:205.

41. Mowoi, M., and T. Yamakawa. 1978. Glucosamine-containing sphingoglycolipids from sheep erythrocytes. J. Biochem. 84:317.

42. Seyama, Y., and T. Yamakawa. 1974. Chemical structures of glycolipid of guinea pig red blood cell membrane. J. Biochem. 75:837.

43. Lomberg, H., U. Jodal, C. Svanborg Edén, H. Leffler, and B. Samuelsson. 1981. P blood group and urinary tract infection. Lancet. 1:946.

44. Andersson, B. A., F. Fogh, S. Jorgensen, H. Leffler, G. Magnusson, O. Nylén, C. V. Sjödow, and C. Svanborg Edén. 1983. Attachment of Streptococcus pneumoniae to human pharyngeal epithelial cells in vitro—mechanism of binding. Proceedings of American Academy of Otalaryngology. Head Neck Surg. In press.

45. Svanborg Edén, C., R. Freter, L. Hagberg, R. Hull, S. Hull, H. Leffler, and G.
Schoolnik. 1982. Inhibition of experimental ascending urinary tract infection by receptor analogues. *Nature (Lond.)*. 298:560.

46. Aronson, M., O. Medalia, L. Schori, D. Mirelman, N. Sharon, and I. Ofek. 1979. Prevention of colonization of the urinary tract of mice with *Escherichia coli* by blocking of bacterial adherence with methyl α-D-mannopyranoside. *J. Infect. Dis.* 139:329.

47. Cunningham, A. S. 1979. Morbidity in breast-fed and artificially fed infants II. *J. Pediatr.* 95:685.

48. Dele, D. W., J. O. Klein, B. Rosner, and The Greater Boston Collaborative Otitis Media Program. 1980. Beneficial effects of breast feeding on duration of middle ear effusion (MEE) after first episode of acute otitis media (AOM). *Pediatr. Res.* 14:494.