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SPECIFICITY OF SALIVARY-BACTERIAL INTERACTIONS: II. EVIDENCE FOR A LECTIN ON STREPTOCOCCUS SANGUIS WITH SPECIFICITY FOR A NeuAcα2,3Galβ1,3GalNAc SEQUENCE

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SUMMARY: Evidence is presented for the presence of a lectin on Streptococcus sanguis with specificity towards the major acidic oligosaccharide of human salivary mucin. Based upon hemagglutination inhibition studies, the strongest inhibitor was NeuAcα2,3Galβ1,3GalNAcol >> NeuAcα2,3Galβ1,4Glc >> NeuAc > Gal. Interactions were not heat sensitive or charge dependent, and were not affected by the presence of bacterial cell associated neuraminidase. The lectin could be extracted from Streptococcus sanguis with lithium 3,5-diiodosalicylate (LIS). Incubation of LIS extracts with carbohydrate ligands demonstrated that the specificity of binding was NeuAcα2,3Galβ1,3[^{3}H-]GalNAcol >> Galβ1,3[^{3}H-]GalNAcol.

INTRODUCTION

Dental caries and periodontal disease are among the most prevalent infections in man. They are initiated by colonization of Streptococcus sanguis, Streptococcus mitis, and Gram-positive filaments onto the tooth surface (1). To combat adherence, saliva functions to inhibit attachment by direct interaction with bacteria. Such interaction facilitates clearance of bacteria from the oral cavity by mastication, movement of the tongue and cheeks, and swallowing. It is now recognized that salivary mucins are involved in the clearance of Streptococcus sanguis (2,3). Recent investigations have shown that the sialic acid residues of salivary mucin are in-

Abbreviations: NeuAc, N-Acetylneuraminic acid; Gal, galactose; GalNAc, N-Acetylgalactosamine; GalNAcol, N-Acetylgalactosaminitol; LIS, lithium 3,5-diiodosalicylate; RBC, erythrocytes; PMSF, phenylmethylsulfonylfluoride; sialyllactose, N-Acetylneuramin-lactose; PBS, phosphate buffered saline.

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volved in these interactions (2,4). It is postulated that mucin's sialic acid can bind to bacteria by several mechanisms. One involves calcium bridges with negatively charged bacterial components (5). Another mechanism includes interactions with bacterial lectins and/or enzymes (3,6). Recently, we have shown that the major acidic oligosaccharide of salivary mucin (M_r:200-250,000 daltons) has the structure: NeuAcα2,3Galβ1,3GalNAc (7). The present communication describes the presence of a lectin on Streptococcus sanguis which displays specificity for this acidic trisaccharide.

MATERIAL AND METHODS

Bacteria and Culture Conditions: Reference strains of Streptococcus sanguis included ATCC 10556, ATCC 10557, and G9B (kindly provided by Dr. B. Rosan, Univ. Pennsylvania). Fresh strains of Streptococcus sanguis were isolated from 24 h human dental plaque using Mitis-Salivarius agar (Difco). Preliminary identification of Streptococcal strains was based upon colony morphology on Mitis-Salivarius agar, type of hemolysis, Gram-stained smears, and a negative catalase test. Subsequent characterization of strains was based upon serological (8) and physiological (9) properties. Bacterial cells for all hemagglutination experiments were grown aerobically to early stationary growth (18 h) in chemically defined FMC medium, supplemented with freshly prepared sodium carborate (10). The organisms were harvested by centrifugation (6800 x g for 15 min at 4°C) and washed 3X with cold 0.01M sodium phosphate buffer, pH 7.2, with 0.154 M NaCl and 0.02% sodium azide (PBS). For study, cells were diluted to a concentration of 2 x 10^9 Streptococci/ml with PBS. In some cases, bacterial suspensions were heat treated at 80°C for 45 min to inactivate cell associated neuraminidase.

Hemagglutination Tests: For the majority of studies, freshly drawn citrated guinea pig erythrocytes were stored in Alsevier's solution (1:1) for up to 1 wk. Human (A,B, and O), rabbit, chicken, sheep, and rat RBC were also screened in preliminary studies. Prior to use, RBC were washed 3X in cold PBS and then diluted with PBS to a 4% (vol/vol) suspension. Visual assays for bacterial mediated hemagglutination were performed in round bottom microtiter plates (Linbro/Titertek No. 76-311-05) at room temperature. Twofold serial dilutions of RBC (25 µl) were added to 25 µl of serial dilutions of bacterial suspensions in a checkerboard fashion. For inhibition studies, 25 µl of serial dilutions of inhibitor (33-100[mM] in PBS with pH adjusted to 7.2) and 25 µl of bacterial suspension were gently shaken for 1 h and then 25 µl of a 1% (vol/vol) RBC suspension added. Hemagglutination was assessed from 0 to 4+ after shaking the plates for 1 h and then letting them sit for an additional hour. Inhibition activity was expressed as the mM concentration needed to inhibit hemagglutination by 50%.

Asialo-RBC (guinea pig) were prepared by incubating 1 ml of packed cells (in 0.1 M Tris/HCl pH 7.2, with 0.154 M NaCl and 1mM $CaCl_2$) with 3 U neuraminidase (Vibrio cholerae, Gibco) for 1 h at 37°C. The cells were then washed 3 times with incubation buffer and then stored in Alsevier's solution until use. Removal of sialic acid was tested by hemagglutination (11) of treated and untreated cells with Limulin lectin (Sigma Chem. Co.) at a concentration of 4 µg/ml.

Extraction of Bacteria: Streptococcus sanguis strain KS32AR (18 h cultures) was washed 3X with cold 0.05 M Tris/HCl buffer pH 7.5 and resuspended in this buffer with 0.3 M LIS and 100 mM PMSF to a concentration of 2 x 10^{10}

cells/ml. The suspension was mixed at room temperature for 1 h and then diluted 3 fold with 0.05 M Tris/HCl, pH 7.5 with 100 mM PMSF and gently stirred overnight at 4°C. The extract was centrifuged (12,000 x g, 15 min, 4°C) and then the supernatant dialyzed against distilled water until the absorbance (323 nm) of the dialysate was < 0.05. The retentate was recovered by lyophilization. Phase contrast microscopy showed that this extraction method did not rupture cell walls.

Lectin Activity of LIS Extracts: LIS extracts were reconstituted in 0.1 M Tris/HCl, pH 7.2 (3 mg protein/ml) and incubated with 12 nmol of [^{3}H-] salivary mucin oligosaccharides for 3 h at 37°C. The mixture was then fractionated at 4°C on columns (1.5 x 110 cm) of Sephadex G-200 using 0.1 M Tris/HCl, pH 7.2. Eluates were monitored by liquid scintillation spectrometry, absorbance at 280 nm, or the Lowry protein assay (12).

Neuraminidase Assay: Bacteria or extracts were incubated at a concentration of 2 x 10^9 cells in 0.01 M PBS, pH 7.0, with [^{3}H-] sialyllactitol (~ 100,000 cpm) for 1-24 h at 37°C. The reaction was terminated by heating at 100°C for 2 min. Following centrifugation, digests were applied to columns of Dowex 1-X2, H^+ (bed volume 1.5 ml) and eluted first with 5 bed volumes of distilled water then 5 bed volumes of 2 N formic acid. Aliquots were then assayed by liquid scintillation spectrometry. Clostridium perfringens neuraminidase (Sigma Chem. Co., Type VI) was used as a positive control ([^{3}H-] lactitol in the water wash) while heat treated bacteria (80°C, 45 min) provided the negative control ([^{3}H-] sialyllactitol in the formic acid eluate).

Reagents: All simple sugars, colominic acid (poly-NeuAcα2,8NeuAc), and N-Acetylneuramin-lactose (Grade 1) were obtained from Sigma Chem. Co. NeuAcα2,3Galβ1,3GalNAcol was prepared from fetuin as described by Bhoyroo and Spiro (13). [^{3}H-] sialyllactitol (~ 0.7 mCi/mMol) was prepared as described by Frisch and Neufeld (14). NeuAcα2,3Galβ1,3[^{3}H-]GalNAcol (9.1 mCi/mMol) and Galβ1,3[^{3}H-]GalNAcol (7.3 mCi/mMol) were prepared by alkaline-borotritide cleavage of the lower molecular weight human salivary mucin (7).

RESULTS AND DISCUSSION

Previous studies have demonstrated that human salivary mucin can agglutinate selected strains of Streptococcus sanguis (2). Due to the limited availability of human mucin, bacterial mediated hemagglutination was used as a substitute assay. Initial studies were carried out to screen reference strains and clinical isolates of S. sanguis for their ability to agglutinate RBC of various species. The general pattern of hemagglutination was guinea pig (4^+) > rat > rabbit, human (A,B,O) (2^+) >> sheep > chicken. Accordingly, guinea pig RBC were employed in all further studies. Four strains of S. sanguis (ATCC 10556, ATCC 10557, G9B, and clinical isolate KS32AR) which strongly agglutinated guinea pig RBC were used. Several simple sugars were tested for their ability to inhibit hemagglutination of guinea pig RBC with S. sanguis strains G9B and KS32AR (Table 1). N-Acetylneuraminic acid was the best inhibitor, reversing hemagglutination by 50% at concentrations of 0.5 to 1.0 mM. The only other simple sugar tested which gave inhibition was galactose. Hemag-

TABLE 1

HEMAGGLUTINATION INHIBITION[a)]

Inhibitor	50% Inhibition [mM]	
	G9B	KS32AR
1. N-Acetylneuraminic acid	0.5	1.0
2. Galactose	4	8
3. Potassium sulfate	25	33
4. Fucose	30	33
5. N-Acetylgalactosamine	>100	50
6. N-Acetylglucosamine	>100	>100
7. Galactosamine-HCl	>100	>100
8. Glucose	>100	>100
9. Mannose	>100	>100

a) Bacterial cells were not heat treated.

glutination was not inhibited by glucuronic acid or galacturonic acid (Table 2) suggesting that the inhibition seen with N-Acetylneuraminic was specific and not due to charge. Support for the specific interaction of N-Acetylneuraminic with S. sanguis was shown by hemagglutination inhibition with colominic acid (poly-NeuAcα2,8NeuAc) (Table 2). Studies by Jaques et al. (15) have demonstrated that the glycerol side chain (C-7→C-9) of N-Acetylneuraminic acid binds calcium ions. Since colominic acid, a polymer of NeuAc whose linkages involve the glycerol group is unable to effectively bind calcium ions (15), it is unlikely that interaction of colominic acid with S. sanguis involves calcium bridging between the bacterial surface and N-Acetylneuraminic acid.

The inhibition data suggest two specific mechanisms of interaction between S. sanguis and carbohydrate ligands. First, the bacteria contains separate carbohydrate binding components; one with specificity for N-Acetylneuraminic acid and the other with specificity for galactose. Second, the bacteria contains a component having specificity towards an oligosaccharide structure containing both N-Acetylneuraminic acid and galactose. Several lines of evidence support the latter mechanism. For example, the concentrations of galactose required to give 50% inhibition were lower (0.5-1.0 mM)

TABLE 2

HEMAGGLUTINATION INHIBITION[a)]

Inhibitor	50% Inhibition [mM]			
	G9B	10556	KS32AR	10557
1. NeuAc	0.5	1.0	1.0	1.0
2. NeuAcα2,8NeuAc[b)]	8	8	4	4
3. D-Glucuronic Acid	>33	>33	>33	>33
4. D-Galacturonic Acid	>33	>33	>33	>33
5. Gal	4	2	8	8
6. Galβ1,4Glc[c)]	8	2	8	4
7. NeuAcα2,3Galβ1,4Glc[d)]	0.04	0.08	0.04	0.08
8. NeuAcα2,3Galβ1,3GalNAcol[e)]	0.005	0.01	0.02	0.01

a) Similar results were obtained with untreated or heat treated bacteria.
b) Colominic acid.
c) Lactose.
d) Sialyllactose (85% NeuAcα2,3Galβ1,4Glc and 15% NeuAcα2,6Galβ1,4Glc).
e) Prepared from fetuin.

for neuraminidase treated RBC than untreated cells. More conclusive evidence was obtained by inhibition studies with oligosaccharides containing both N-Acetylneuraminic acid and galactose (Table 2). The most potent inhibitor tested was the trisaccharide: NeuAcα2,3Galβ1,3GalNAcol. Parallel studies demonstrated that these reactions were not heat sensitive nor dependent upon cell associated neuraminidase activity (strains ATCC 10557 and KS32AR); and this suggested that the carbohydrate binding protein is a lectin.

To begin characterizing the carbohydrate binding protein from Streptococcus sanguis, clinical isolate KS32AR was extracted with lithium 3,5-diiodosalicylate using conditions which did not appreciably disrupt cell walls. Approximately 20 mg of lyophilized extract (~ 7 mg protein) were obtained from 6.5×10^{13} cells. The extract was devoid of neuraminidase activity. To determine for the presence of the carbohydrate binding component, the LIS extract was incubated with [^{3}H-] mucin oligosaccharides and binding was demonstrated by chromatography of the incubation mixture on Sephadex G-200. As shown in Figure 1, 70% of the acidic trisaccharide was bound to the LIS extract in a protein peak eluting just after the void volume. In comparison,

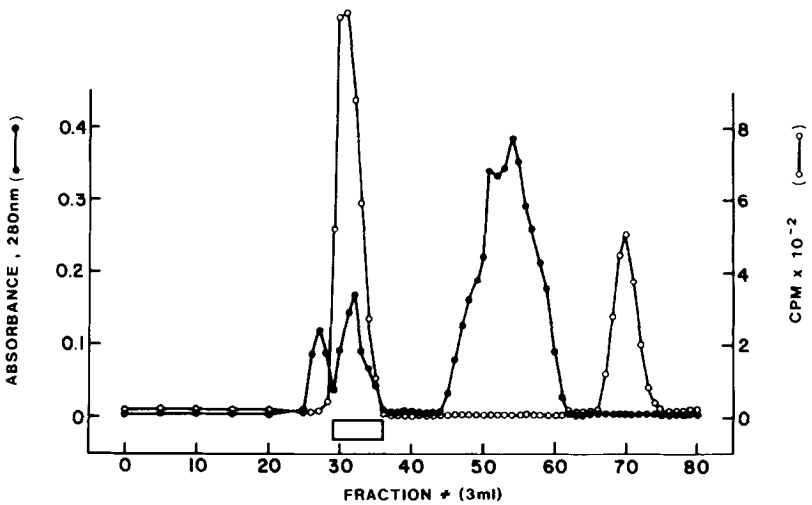

Figure 1 Sephadex G-200 gel filtration of the LIS extract from S. sanguis KS32AR and NeuAcα2,3Galβ1,3[^{3}H-]GalNAcol. The incubation mixture contained 11 mg (3 mg protein) of bacterial extract plus 12 nmol (~ 120,000 cpm) of [^{3}H-] mucin trisaccharide. Greater than 90% of the cpm were recovered from the column fractions.

only 23% of the neutral disaccharide (Galβ1,3GalNAcol) was bound and localized in this protein peak. These results parallel the specificity demonstrated by the hemagglutination inhibition studies.

The mechanisms whereby oral bacteria interact with saliva and salivary coated oral surfaces are complex. The elegant studies by Gibbons and colleagues (see references 1 and 6 for review) have demonstrated the selective nature of bacterial adherence to oral surfaces. Studies such as these and others (2-4) have suggested that oral bacteria may contain surface lectins and/or enzymes which serve as receptors for the oligosaccharides of salivary glycoproteins which are free in saliva or coating oral surfaces. In contrast, others have suggested that the interactions described are non-specific and involve ionic and hydrophobic interactions between the bacterial surface components and salivary macromolecules (5,16,17). We postulate that non-specific forces may initially function to align salivary glycoproteins with the bacterial surface to maximize specific interactions such as those described in this study.

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