Novel Interactions of Glycosaminoglycans and Bacterial Glycolipids Mediate Binding of Enterococci to Human Cells*\(^\d\)

Received for publication, March 4, 2009 Published, JBC Papers in Press, April 23, 2009, DOI 10.1074/jbc.M901460200

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**Enterococcus faecalis** is among the most important nosocomial pathogens. The intestinal mucosa is considered to be the main site used by these bacteria for entrance and dissemination. A better understanding of the mechanisms involved in colonization and invasion of enterococci may help to devise methods to prevent infections in hospitalized patients. Glycosaminoglycans, which are present on the surface of all eukaryotic cells, were investigated with regard to their role as host receptors for adhesion of **E. faecalis**. Competitive binding assays, enzymatic digestion, and reduction of the sulfation of the glycosaminoglycan chains indicated that heparin and heparan sulfate, but not chondroitin sulfate B, played important roles in adhesion of **E. faecalis** 12030 to Caco2 cells. By using proteinases and carbohydrate oxidation by sodium meta-periodate to modify the bacterial surface, it could be demonstrated that a sugar-containing molecule rather than a protein is the bacterial ligand mediating adhesion to eukaryotic cells. Preincubation of Caco2 cells with the enterococcal glycolipid diglucosyldiacylglycerol but not other carbohydrate cell wall components inhibited bacterial binding. These results may indicate that heparin and/or heparan sulfate on host epithelial cells and diglucosyldiacylglycerol, either itself or as a partial structure of lipoteichoic acid, are involved in enterococcal adhesion to colonic epithelia, the first step in translocation from the intestinal tract.

Enterococci are currently the third most common pathogen isolated from bloodstream infections and the second most common nosocomial pathogen in the United States (1, 2). Enterococci are responsible for three to four cases of nosocomial bloodstream infection per 10,000 hospital discharges (3). These bacteria contribute significantly to additional hospitalization and patient mortality (4). Enterococci are endogenously resistant to a variety of antibiotics and have increasingly acquired more resistance mechanisms to antimicrobials, allowing them to survive in hospital and nursing home settings (5). The difficulty in treating serious enterococcal infections emphasizes the importance of understanding virulence factors that may be targeted by alternative therapeutics. The rapid increase in vancomycin-resistant enterococcal strains (6) and their general ability to pass this trait on to other pathogens, *i.e.* *Staphylococcus aureus* (7), indicates we are confronting an urgent and expanding problem on multiple fronts.

Enterococci seem to have a propensity to colonize and infect certain tissues (such as the gastrointestinal tract, the urogenital tract, and the cardiac endothelia), although they are never found to cause disease in other tissues (e.g. lower respiratory tract). A main route of invasive infection by enterococci is likely initiated by adherence of the microbe to host cells followed by translocation to the gastrointestinal submucosa (8). Glycosaminoglycans (GAGs)\(^{\d}\) expressed on the surface of mammalian epithelial cells often represent prominent receptors for pathogens (9, 10). It has been shown previously that heparan sulfate proteoglycans on professional and nonprofessional phagocytes mediate invasion of *Enterococcus faecalis* into cells, where they can resist specific killing mechanisms (11). Because of their complex nature, heparan sulfate proteoglycans are made up of a variety of different structures that have a well-defined distribution in different tissues, which is likely an important factor in binding of bacteria to cells (12). In this study we evaluated the role of host glycosaminoglycans and bacterial surface structures in mediating binding of **E. faecalis** to mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A human cell line, Caco2, derived from colon carcinoma was used in this study. Cells were cultivated in DMEM supplemented with 10% fetal bovine serum and 1% nonessential amino acids in a 5% CO\(_2\) atmosphere. All the experiments were performed on cells between the 15th and 25th passage.

**Bacterial Strains**—The following strains were used in this study: *E. faecalis* 12030 (13); FA2-2 (14); type 5/type 9 (15);

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* This work was supported, in whole or in part, by National Institutes of Health Grants AI050667 (to J. H.) and GM-38060 (to R. J. L.).

\(^{\d}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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2 The abbreviations used are: GAG, glycosaminoglycan; HS, heparan sulfate; CS, chondroitin sulfate; DGlcDAG, diglucosyldiacylglycerol; LTA, lipoteichoic acid; WTA, wall teichoic acid; DMEM, Dulbecco’s modified Eagle’s medium; ANOVA, analysis of variance; MGlcDAG, monoglucosyldiacylglycerol; SPR, surfactant plasmon resonance.
V583 (16); OG1RF (17); and E. faecalis 12030ΔbgsA, a deletion mutant in a glycosyltransferase (18).

Bacterial Culture and Isolation of Cell Wall Glycoconjugates—E. faecalis strains were grown to mid log phase at 37 °C without agitation in tryptic soy broth. Prior to infection of cells, bacteria were washed and resuspended in DMEM. The number of bacteria in the inoculum was initially estimated from previously derived growth curve determinations and confirmed by viable counts for each experiment. LTA was extracted and purified from E. faecalis 12030 as described elsewhere (19). Wall teichoic acid (WTA) was extracted using trichloroacetic acid, as described previously (20), and further purified by size exclusion chromatography using a Sephacryl S-200 column (1 × 90 cm; GE Healthcare) followed by anion-exchange chromatography using a Sephadex Q-Sepharose (1× 10 cm; Q-Sepharose) using a gradient of 0 to 1 M NaCl. Purity was assessed by 1H NMR analysis. Total membrane lipids were prepared from the organic phase after butanol extraction and purified according to the method of Bligh and Dyer (21) and others (22, 23). The preparations were analyzed by TLC as described elsewhere using α-naphthol staining and appropriate standards (22). Glycolipids were isolated by preparative layer chromatography. Silica gel plates were loaded with Bligh/Dyer extracts, and the lipid mixture was separated by CHCl₃/MeOH/H₂O (65:25:4, v/v). Spots staining with α-naphthol were scraped off the plate and eluted by CHCl₃. Purity of the glycolipids was evaluated by TLC as described above.

Chemicals—Reagents used in this study were purchased from Sigma, except for the following: DMEM (PAN Technologies); trypsin/EDTA, nonessential amino acids, and gentamicin (Invitrogen); tryptic soy broth (Merck); fetal bovine serum (PAA Clone); porcine intestinal heparin and HS (Celsus Laboratories); 2-O-desulfated heparin, 6-O-desulfated heparin, and de-N-sulfated heparin (Neoparin Inc.). Sensor streptavidin chip. To prepare the heparin chip, biotinylated heparin was immobilized with an streptavidin sensor chip. The successful immobilization of GAGs was confirmed by the observation of an ~300-resonance unit increase in the sensor chip. To prepare the heparin chip, biotinylated heparin was immobilized with an ~70-resonance unit increase.

Inhibition of Adhesion by Heparin, 2-O-Desulfated Heparin, 6-O-Desulfated Heparin, De-N-Sulfated Heparin, HS, and CSB—Glycosaminoglycans were diluted in DMEM and added in various concentrations to the epithelial cells 30 min prior to addition of the bacterial inoculum. The adhesion assay was then performed as described above.

Heparin Lyase and Chondroitin Lyase ABC Treatment of the Cells—To test the ability of E. faecalis 12030 to adhere to the epithelial cells after depletion of some of the surface glycosaminoglycan chains, heparin lyase and chondroitin lyase ABC were added to the cells 10 min prior to infection in concentrations between 0.125 and 2 units/ml (25). Additionally, 50 min after infection, the medium was supplemented with the same amount of enzyme, and the incubation was continued for another hour.

Sodium Meta-periodate Treatment—Treatment of bacteria with various amounts of sodium meta-periodate (0.125–1 mM) for 60 min at 4 °C in the dark was used to study the contribution of surface carbohydrates in bacterial adhesion to Caco2 cells. Sodium meta-periodate was neutralized using ethylene glycol. Purified glycolipids were treated with sodium meta-periodate at 1 mM at 4 °C on a rotor rack in the dark followed by neutralization with ethylene glycol.

Inhibition of Enterococcal Binding to Caco2 Cells Using Cell Wall Carbohydrates—Glycolipids (67.5–250 μg/ml), LTA (50–500 μg/ml), or WTA (50–500 μg/ml) isolated from enterococcal cell walls were added to the cells 30 min before infection. The assay was then performed as described above.

Sodium Chlorate Treatment of the Cells—Sodium chlorate (an inhibitor of GAG chain sulfation) (26) was added to the cells 48 h prior to the experiments in a concentration between 12.5 and 50 mM as described elsewhere (27). For screening of different enterococcal strains a concentration of 50 mM was used.

Measurement of Glycolipid Interaction with GAGs Using Surface Plasmon Resonance (SPR)—Surface plasmon resonance measurements were performed on a BIAcore 3000 operated using the version software. Buffers were filtered and degassed for SPR assay. The biotinylated GAGs were immobilized to a streptavidin chip based on the manufacturer’s protocol. The biotinylated heparin was prepared by reaction of sulfo-N-hydroxysuccinimide long-chain biotin (Pierce) with free amino groups of unsubstituted glucosamine residues in the polysaccharide chain. To prepare other biotinylated GAGs (HS, chondroitin sulfate (CS) A, CSB, and CSC), GAGs (5 mg) and amine-PEO3-biotin (5 mg) were dissolved in 200 μl of H₂O and 10 mg of NaCNBH₃ was added. The reaction mixture was heated at 70 °C for 24 h, after that a further 10 mg of NaCNBH₃ was added, and the reaction was heated at 70 °C for another 24 h. After cooling, the mixture was desalted with the spin column (3000 Da). The biotinylated GAGs were collected and freeze-dried. To prepare the GAGs chip, biotinylated HS, CSA, CSB, and CSC were immobilized on different flow cells in a streptavidin sensor chip (CSA immobilized flow cell served as a control). The successful immobilization of GAGs was confirmed by the observation of an ~300-resonance unit increase in the sensor chip. To prepare the heparin chip, biotinylated heparin was immobilized with an ~70-resonance unit increase.

For the measurement of glycolipid interaction with GAGs using BIAcore, the glycolipid suspension was prepared in HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4) by heating to 65 °C for 15 min in a water bath and then sonicated for another 15 min. Glycolipid samples diluted in HBS running buffer (0.01 M HEPES, 0.15 M NaCl, pH 7.4) were injected at a flow rate of 30
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μl/min. At the end of the sample injection (90 s), the same HBS running buffer (0.01 M HEPES, 0.15 M NaCl, pH 7.4) was flowed over the sensor surface to facilitate dissociation for 180 s. After a 3-min dissociation time, the sensor surface was regenerated with injections of 30 μl of 2 M NaCl. The response was monitored as a function of time (sensogram) at 25 °C. The SPR data were processed by using BIAevaluation software (version 4.0.1, GE Healthcare).

Statistical Methods—Multigroup comparisons were done using ANOVA (PRISM4, GraphPad software).

RESULTS

Inhibition of the Bacterial Binding to the Colonic Cell Line Caco2 Using Polysulfated Polysaccharides—When confluent monolayers of Caco2 cells were incubated for 2–4 h at 37 °C with ~100 bacteria/cell, adherence of bacteria correlated with the time of incubation (i.e. more bacteria adhered after longer incubation periods). For the invasion assay a higher multiplicity of infection (1000:1) was used because at a multiplicity of infection of 100:1 only few bacteria could be detected inside the cells. The number of internalized bacteria was ~4 logs smaller than the number of adherent bacteria (data not shown). The involvement of GAGs in the adherence of bacteria to the host cells was investigated using different concentrations of heparins, HS and CSB, to preincubate Caco2 cells monolayers. These polysulfated polysaccharides reduced (up to 56%) the adherence of E. faecalis 12030 to the epithelial cell cultures (Fig. 1, A and B). The highest inhibition was obtained by using the glycosaminoglycan heparin (56% at a concentration of 500 μg/ml); HS also significantly decreased the level of adherence of the bacteria by 50% (concentration of 1000 μg/ml). Binding of bacteria to Caco2 cells was not significantly affected when using CSB (13% at 1000 μg/ml). The inhibition of the bacterial binding was dose-dependent when HS was used (Fig. 1B). For heparin, a dose-dependent inhibition was observed for all but the highest concentration used (Fig. 1A). This latter observation may be explained by the unphysiologically high inhibitor concentration. The structural complexity of heparin is determined by a series of modifications during its biosynthesis, regarding mainly sulfation of the aminosugars and/or uronic acids. To identify the importance of these modifications for bacterial binding, inhibition assays were performed using chemically modified heparins as inhibitors (28). As shown in Fig. 2, preincubation of cell monolayers with 500 μg/ml normal heparin and 6-O-desulfated heparin decreased bacterial binding by 58 and 56%, respectively. In contrast, both 2-O-desulfated heparin and de-N-sulfated heparin partially lost their inhibitory ability (21 and 23% inhibition, respectively).

Enzymatic Digestion of Polysulfated Polysaccharide Chains Decreases Adherence of E. faecalis 12030 to Caco2 Cells—The role of GAGs in bacterial adhesion was further investigated by treating the target cells with heparin lyase I (which cleaves heparin and HS chains) and chondroitin lyase ABC (which degrades chondroitin sulfate chains). The strongest reduction in adherence occurred after treatment of the cells with heparin lyase I, which reduced enterococcal adhesion by 25–52.5% in a dose-dependent manner, whereas chondroitin lyase ABC had little effect on enterococcal adherence (Fig. 3).

Reduction of Enterococcal Binding to Epithelial Cells by Decreasing Sulfation of GAG Chains—Caco2 cells were grown for 48 h in the presence of sodium chlorate (12.5–50 mM) to decrease sulfation of GAG chains. Under these conditions,
adherence of the bacteria was reduced by up to 42% (Fig. 4). To
determine whether GAGs also mediate adhesion to other
enterococcal strains, we tested five more strains in addition to
E. faecalis 12030. Two of the strains (FA2-2 and type 9) showed
a significant reduction in adhesion to cells treated with sodium
chlorate compared with untreated Caco2 cells (Fig. 5). This was
comparable with the inhibition obtained when the reference
strain E. faecalis 12030 was used (41% inhibition for FA2-2 and
68% inhibition for type 9). No significant difference between
the treated and untreated cells was detected for the other
strains tested (type 5, OG1-RF, and V583).

Modifications of the Bacterial Cell Surface
—To study the
specific interactions between bacteria and GAGs, bacterial cells
were treated with proteinase K, Pronase E, or sodium meta-
periodate. Using bacteria treated with proteinase K or Pronase
E, adherence of enterococci to epithelial cells was not impaired,
making a protein unlikely to mediate bacterial binding (data not
shown). The role of bacterial polysaccharides in adhesion to
Caco2 cells was tested after incubation of bacteria with sodium
meta-periodate (0.125–1 mM). This treatment resulted in a dose-
dependent reduction of enterococcal adhesion to the epithelial
cells of 2–100% (Fig. 6). Meta-periodate oxidation and protein-
ase digestion did not affect bacterial viability, as demonstrated
by viable counts (data not shown).

Inhibition of Enterococcal Binding to Caco2 Cells Using LTA,
WTA, and Glycolipids
—Incubation of the epithelial monolay-
ers with LTA (50–500 μg/ml) and WTA (50–500 μg/ml) did
not have any effect on bacterial adhesion (data not shown).
Using extracts of total membrane lipids, which include glyco-

FIGURE 2. Inhibition of bacterial binding to the epithelial cells using
chemically modified heparins. Normal heparin, 2-O-desulfated heparin,
6-O-desulfated heparin, and de-N-sulfated heparin (all at 500 μg/ml concent-
tration) were used as inhibitors of bacterial binding. Heparin and 6-O-desul-
fated heparin significantly reduced bacterial binding to cells (*, p > 0.05,
against heparin using ANOVA with Dunnett’s multiple comparison test),
whereas 2-O-desulfated heparin and de-N-sulfated heparin partially lost their
inhibitory activity (#, p < 0.05 against heparin). Bars represent average ± S.E.

FIGURE 3. Reduction of bacterial attachment to the epithelial cells using
GAG lyases. Heparin lyase I (1 and 2 units/ml) and chondroitin lyase ABC (1
and 2 units/ml) were used to digest the GAG chains for 10 min prior to the
infection and for 110 min after bacterial infection (*, p < 0.001, against the
control containing no enzymes using ANOVA with Bonferroni’s multiple com-
parison test). Bars represent average ± S.E.

FIGURE 4. Decreasing bacterial binding using sodium chlorate as an
inhibitor of sulfation for the GAG chains. Sodium chlorate in various con-
centrations (12.5–50 mM) was added to the adhesion assay 48 h prior to the
experiment. Untreated cells were used as a negative control. All concentra-
tions reduced binding significantly (p < 0.001 using ANOVA with Bonferroni’s
multiple comparison test) as compared with the untreated cells. Data points
represent individual replicates, and lines represent the mean.

FIGURE 5. Inhibition of different E. faecalis strains binding to Caco2 cells
using sodium chlorate treatment. Reduction of GAG chain sulfation by
sodium chlorate treatment (50 mM) decreased the ability of E. faecalis 12030,
FA2-2, and type 9 strains to adhere (using ANOVA with Bonferroni’s multiple
comparison test). Meanwhile for the other three strains tested, there was no
significant difference in binding to Caco2 cells (data not shown). Bars repre-
sent average ± S.E. +, with sodium chlorate; –, without sodium chlorate.

FIGURE 6. Increasing bacterial binding using sodium chlorate as an
inhibitor of sulfation for the GAG chains. Sodium chlorate in various con-
centrations (12.5–50 mM) was added to the adhesion assay 48 h prior to the
experiment. Untreated cells were used as a negative control. All concentra-
tions reduced binding significantly (p < 0.001 using ANOVA with Bonferroni’s
multiple comparison test) as compared with the untreated cells. Data points
represent individual replicates, and lines represent the mean.
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**FIGURE 6.** Effect of sodium meta-periodate treatment of *E. faecalis* 12030 on attachment to Caco2 cells. Bacterial cells were treated with sodium meta-periodate (0.125–1 mM). Bacteria grown in the same conditions, but not treated with sodium meta-periodate, were used as a control (*, p < 0.001 ANOVA with Bonferroni’s multiple comparison test using sodium meta-periodate and untreated bacteria). Bars represent the average ± S.E.

**FIGURE 7.** Glycolipids isolated from enterococci inhibit bacterial attachment to Caco2 epithelial cells. Confluent monolayers of Caco2 cells were incubated with glycolipids (67.5–125 µg/ml) from *E. faecalis* 12030 for 30 min before addition of bacteria to measure the adherence. Cells incubated only with DMEM were used as comparison. Both concentrations of glycolipids (i.e., 62.5 µg/ml, *, p < 0.01, and 125 µg/ml, #, p < 0.001) significantly reduced bacterial binding using ANOVA with Bonferroni’s multiple comparison test. Data points represent individual replicates, and lines represent the mean.

**FIGURE 8.** Inactivation of glycolipids with sodium meta-periodate eliminates inhibitory activity. Treatment of glycolipids with 1 mM sodium meta-periodate resulted in a complete loss of the inhibitory activity of enterococcal glycolipids on adherence to Caco2 cells. (p < 0.01, ANOVA with Dunn’s multiple comparison test). GL, glycolipids. Data points represent individual replicates, and lines represent the mean.

Glycolipids (concentrations between 67.5 and 250 µg/ml), adhesion to the cells was inhibited by up to 59%, and maximum inhibition was observed at a concentration of 125 µg/ml (Fig. 7). To assess if inhibitory effects of the extracts were because of glycolipids, total membrane lipids were treated with 1 mM sodium meta-periodate. Compared with native material, which reduced adhesion significantly (43%), oxidized glycolipids were not able to inhibit bacterial binding (Fig. 8).

**Inhibition of Enterococcal Binding to Caco2 Cells by Purified Glycolipids**—To further corroborate their role in bacterial binding and exclude nonspecific effects mediated by other membrane lipids, the two major glycolipids of *E. faecalis* (MGlcDAG and DGlcDAG) were purified (Fig. 9). Whereas MGlCAG did not significantly affect bacterial binding, DGlcDAG inhibited adhesion up to 47% (Fig. 9).

**Surface Plasmon Resonance Measurement of Glycolipid-GAGs Interactions**—The interaction between glycolipids and GAGs was examined by SPR. Biotinylated GAGs were immobilized on a streptavidin sensor chip, and the glycolipid in the running buffer was injected at different concentrations. Sensorgrams of DGlcDAG-heparin, DGlcDAG-HS, DGlcDAG-CSB, and DGlcDAG-CSC interactions are shown in Fig. 10. Surface plasmon resonance demonstrated a stronger binding signal (resonance units) of HS to DGlcDAG, in comparison with CSB or CSC (Fig. 10, B and D). The binding of heparin and HS to DGlcDAG was concentration-dependent (Fig. 10, A and D), suggesting the interaction is specific. The *K_d* value for DGlcDAG binding to heparin can be estimated at ~60 µM. The binding of CSB or CSC to DGlcDAG was not concentration-dependent, and the interaction was low. No binding was observed (negative signal) for MGlcDAG-GAGs interactions (Fig. 10C).

**DISCUSSION**

Many pathogenic bacteria establish infections after adhering to host surfaces by means of specific surface structures or motifs. Adhesion is usually the first step leading to colonization and subsequent infection. The intestinal tract is a source of many nosocomial pathogens, including *E. faecalis*, which can cause invasive infections such as bacteremia or endocarditis in otherwise healthy individuals. Other bacteria that are phylogenetically related, such as group A or group B streptococci, colonize different host epithelia and infect distinct target organs. Various structures on eukaryotic cells and tissues have been identified as targets for adhesion by microorganisms (29). Many of these molecules have been characterized as belonging to the family of proteoglycans (9). Several investigators have used purified GAGs as competitive inhibitors of adhesion to study
the role of these molecules in the attachment of various pathogens to host surfaces (9). Different glycosaminoglycans are distinguished based on the charge of the carbohydrate chains. Chondroitin sulfate usually contains one sulfate group per disaccharide unit, although in HS the amount of sulfate residues varies between 0.8 and 1.4. Heparin is the most sulfated glycosaminoglycan, with an average of 2.4 sulfate groups per disaccharide (9). Differences in binding to specific glycosaminoglycans and tissue-specific expression profiles of proteoglycans may account for tropism of pathogens in invasive infection and colonization (12).

It has been shown recently that binding of Escherichia coli O157:H7 to colonic epithelial cells is blocked by heparin and HS in a dose-dependent fashion (30). Adhesion to GAGs has also been demonstrated in a variety of Gram-positive organisms as follows: Streptococcus pneumoniae binds to heparin, HS, and chondroitin sulfate (31); staphylococci bind to CSA (32) and HS (33); Streptococcus pyogenes recognizes dermatan sulfate, HS, and heparin (34); and Streptococcus mutans binds mainly heparin (35). The binding partners for glycosaminoglycans on the bacterial surface have been characterized for some bacterial species, whereas for others there is still controversy. All the known binding ligands of proteoglycans to date are surface proteins. Baron et al. (36) and Auperin et al. (37) found that H9251 protein of Streptococcus agalactiae binds to proteoglycans on the ME180 cervical epithelial cell line. In S. pyogenes Frick et al. (34) showed that there is a strong correlation between M protein expression and the ability of the bacteria to bind to glycosaminoglycans, especially dermatan sulfate. A different protein, ActA, has been shown to be responsible for the attachment of Listeria monocytogenes to host cell heparin and HS (38).

Baldassarri et al. (11) showed that heparin, HS, and to a lesser extent CSA act as the host receptors for enterococci on phagocytes. Our data corroborate most of the findings of Baldassarri et al. (11). In contrast to their results, however, we did not see any inhibition by CSB in our experiments. These differences may be explained by the fact that we used a different cell line (i.e. Caco2) derived from colon cancer, whereas Baldassarri et al. (11) used professional and non-professional phagocytes.

The data presented here confirm that in the early stages of infection enterococci use heparan sulfate-containing proteoglycans as receptors on the host cells. Inhibition of bacterial binding was observed in the presence of soluble heparin and HS. Pretreatment of the cells with heparin lyase I and sodium chlorate also reduced bacterial binding to Caco2 cells. Binding of ligands to heparan sulfate depends

**FIGURE 9.** DGlcDAG but not MGlcDAG inhibits enterococci binding to Caco2 cells. Monolayers of Caco2 cells were incubated with MGlcDAG or DGlcDAG (250 μg/ml) for 30 min before the adherence assay. Cells incubated only with DMEM were used as comparison. Data points represent individual replicates, and lines represent the mean. For the two major membrane glycolipids of *E. faecalis* 12030 strain, DGlcDAG is the main glycolipid present in the wild type, whereas the mutant deficient in a glycosyltransferase produces only MGlcDAG (see also supplemental Fig. 1). ns means not significant.

**FIGURE 10.** SPR sensograms of the interaction of DGlcDAG and MGlcDAG with host GAGs. A, sensograms of interaction of DGlcDAG with heparin. Concentrations of DGlcDAG (from top to bottom) are as follows: 100, 75, 50, and 25 μM, respectively. B, sensograms of interaction of DGlcDAG (100 μM) with GAGs. HS, red; CSB, green; CSC, blue. C, sensogram interactions of MGlcDAG (100 μM) with GAGs. Heparin, red; HS, blue; CSB, green; CSC, pink. D, binding response of DGlcDAG at 50 μM (white), 75 μM (gray), and 100 μM (black) to GAGs.
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on defined patterns and orientations of the sulfo and carboxyl groups along the polysaccharide chain (39). Heparin is distinguished from heparin sulfate by much higher levels of N- and O-sulfo groups. In recent years, numerous heparan sulfate biosynthetic enzymes have been identified that are involved in the modification of the heparan sulfate chain (40). These modifications include 2-O-sulfation of iduronic and glucuronic acid, N-sulfation of glucosamine, as well as 6-O-sulfation and 3-O-sulfation of glucosamine (40). Here we provide evidence that N-sulfation and 2-O- but not 6-O-sulfation are required for DGlcDAG-heparan sulfate interaction. This conclusion is supported by the observations that 6-O-desulfated heparin was able to inhibit bacterial binding, whereas 2-O-desulfated heparin as well as de-N-sulfated heparin partially lost their inhibitory activity. These findings support the conclusion that specific sulfate groups on cellular heparin sulfate rather than the total level of sulfation may be important for mediating DGlcDAG-host cell interaction. Interestingly, the sulfation pattern required for inhibition of DGlcDAG binding was different from the sulfation pattern mediating hepatitis C virus envelope glycoprotein-heparan sulfate interaction (28). These findings further confirm the relevance and specificity of these modifications for DGlcDAG-heparan sulfate interactions.

Inhibition of bacterial binding to epithelia using sodium chlorate was used to investigate whether utilization of GAGs is strain-specific. Because adhesion of three of six strains tested could be inhibited by sodium chlorate treatment, we conclude that, although proteoglycans may not act as receptor for all the enterococcal strains, this mechanism is not a unique feature of E. faecalis 12030.

Our data indicate that a carbohydrate cell wall structure, and not a protein, is involved in adhesion to Caco2 cells because treatment with sodium meta-periodate abrogated binding of the bacteria, whereas treatment with proteinases did not impair it. There are only a few examples of glycoconjugates mediating adhesion to eukaryotic cells. Guzman et al. (41) treated E. faecalis isolates with sodium meta-periodate showing that adherence to the human cell line Girardi heart is mediated by carboxylate residues on the bacterial surface. By using d-galactose and l-fucose, they were able to inhibit binding of the bacteria to the heart cells. Beachey and co-workers (42) studied adhesion of S. pyogenes to Hep-2 cells and found that LTA is part of a two-step adhesion process to the host cells. The role of LTA in invasion of brain microvascular endothelial cells by group B streptococci was studied by Doran et al. (43) in a deletion mutant of the iagA gene. IagA is responsible for the synthesis of DGlcDAG, the cell surface anchor of LTA, and its inactivation resulted in attenuated virulence and reduced translocation from the bloodstream into the cerebrospinal fluid (43). The authors concluded that LTA is the bacterial cell surface structure that interacts with the blood-brain barrier endothelium.

Using a mutant in a glycosyltransferase gene with high similarity to iagA, we were able to demonstrate that this mutant is also deficient in DGlcDAG. The mutant strain adhered significantly less to Caco2 cells (18). Here we show that purified MGlcDAG efficiently inhibited adhesion, although DGlcDAG had no effect.

Because we demonstrated that a large proportion of enterococcal strains bind to Caco2 cells through highly sulfated heparan sulfates and that bacterial glycolipids are able to specifically inhibit this attachment, we were interested in measuring the interaction between these molecules. Using surface plasmon resonance experiments, a high affinity interaction of heparin was only shown for DGlcDAG, whereas for MGlcDAG, which differs only by the lack of a terminal α-D-glucose (Fig. 9), no detectable binding was observed. As for the binding of DGlcDAG to different GAGs, high affinity binding was observed for heparin and HS, which represent the most sulfated GAGs present on eukaryotic cells. DGlcDAG is found in the enterococcal cell wall as a membrane glycolipid and also as a partial structure of LTA (44). Because of the spatial arrangement of the cell wall, a direct interaction of membrane-bound glycolipids with GAGs is difficult to envision. Both glycolipids and LTA, however, are also shed from the cells during growth and turnover of the bacterial cell wall (45). LTA is also known to be able to insert its glycolipid anchor into eukaryotic membranes (46, 47). We therefore hypothesized that LTA shed from planktonic bacteria is inserted into the eukaryotic membrane and interacts with GAGs by its kojibiose residue of the glycolipid anchor. By its polyglycerol phosphate moiety, which now extends toward the interface between host cells and pathogens, it binds to a bacterial cell surface receptor, which yet needs to be defined. A similar model was suggested for S. pyogenes (32). This model is supported by our experimental findings as follows: (i) because saturating the LTA-binding sites on the eukaryotic cell by preincubation with DGlcDAG, a bridging by reoriented LTA would be blocked; and (ii) by using the LTA molecule for preincubation no such inhibition would be achieved due to the additional presence of polyglycerol phosphate in the inhibitor, the putative binding moiety to the bacterial cell surface receptor. Further experiments will need to clarify the exact mechanism of the adhesion. To our knowledge, however, this is the first time that bacterial adhesion mediated by the interaction of two carbohydrate structures has been demonstrated.

Although several mechanisms, receptors, and putative virulence factors have been proposed for the interaction of enterococci with mammalian cells, there is still no good understanding of the occasional transition from usually benign commensal to deadly pathogen. Future studies of the role of surface proteoglycans and carbohydrate containing surface components may help to develop new strategies to prevent and treat enterococcal infections.

Acknowledgments—We thank Dr. G. Pier and Dr. O. Prucker for helpful discussions and to D. Lawrie for editorial help.

REFERENCES

GAGs and Bacterial Glycolipids Mediate Binding of Enterococci