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Abstract

Glycocalyx is suggested to play an important role in the pathogenesis of biomaterial-centered infection. Using an accurate and sensitive method to quantify glycocalyx and bacterial adherence, we have demonstrated that the producer of the most glycocalyx also exhibited the highest adherence index, whereas low producers exhibited the least (p less than 0.01). Additionally, at various concentrations the high producer had the greater tendency to adhere and grow on stainless steel wires and tubes (p less than 0.001). The adherence index, referred as the ratio of tritiated thymidine uptake on wires to colony forming units (CFU), was also the highest in high producers. The adherence index increased as the glycocalyx index increased. It was suggested that glycocalyx production enhanced the adherence of Staphylococcus epidermidis to biomaterials and caused persistent and intractable infections. In short, the glycocalyx index and the adherence index can be reliable indices of biomaterial-centered infection.

KEYWORDS: glycocalyx, bacterial adherence, Staphylococcus epidermidis biomaterial infection, colony forming units

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Glycocalyx Production and Adherence of *Staphylococcus* to Biomaterials

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Glycocalyx is suggested to play an important role in the pathogenesis of biomaterial-centered infection. Using an accurate and sensitive method to quantify glycocalyx and bacterial adherence, we have demonstrated that the producer of the most glycocalyx also exhibited the highest adherence index, whereas low producers exhibited the least ($p < 0.01$). Additionally, at various concentrations the high producer had the greater tendency to adhere and grow on stainless steel wires and tubes ($p < 0.001$). The adherence index, referred as the ratio of tritiated thymidine uptake on wires to colony forming units (CFU), was also the highest in high producers. The adherence index increased as the glycocalyx index increased. It was suggested that glycocalyx production enhanced the adherence of *Staphylococcus epidermidis* to biomaterials and caused persistent and intractable infections. In short, the glycocalyx index and the adherence index can be reliable indices of biomaterial-centerted infection.

Key words : glycocalyx, bacterial adherence, *Staphylococcus epidermidis* biomaterial infection, colony forming units

Staphylococcus epidermidis, once considered a harmless microorganism, has been demonstrated as a pathogen of prosthetic cardiac valves (1, 2), cerebrospinal fluid shunts (3-5), and biomaterials (6-9). In *S. epidermidis,* the glycocalyx or slime consists of an extensive, diffuse and polyanionic matrix (10, 11). This matrix or slime layer may serve a protective function and is involved in the development and persistence of biomaterial-centered infections (7, 12-15). Once implaned into hosts, biomaterial implants such as prosthesis and catheters become sites for bacterial colonization and infection (12, 16, 17). The presence of causative bacteria on the biomaterials is associated with increasing resistence of antibiotic therapy and removal of implants (12, 13, 17-19). Nevertheless, the interrelationship of bacterial adherence and glycocalyx production has not been investigated. The intention of this study was to determine the role of glycocalyx in bacterial adherence with an accurate, sensitive and simple assay to quantify glycocalyx on stainless steel wires which simulate the surfaces of biomaterial implants.

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Materials and Methods

Microorganism. Three strains of *Staphylococcus epidermidis* were obtained from eleven patients. The bacterial strains were cultured in agar plates prepared from Mueller Hinton broth (Difco Laboratory, Detroit, MI, USA). All bacteria strains were maintained by transferring them to fresh agar plates every 3 to 4 weeks. The strains of *Staphylococcus epidermidis* were categorized into high (No. 8), medium (No. 4) and low (No. 7) producers according to their glycocalyx productions which were quantified with previously described method (20). Strain No. 8 (high producer), Strain No. 4 (medium producer) and Strain No. 7 (low producer) were chosen for this study.

Quantification of glycocalyx production. Glycocalyx production from the adhered bacteria of each strain was individually quantified on the wall of each culture tube by toluidine blue staining and spectrophotometry as described (20).

Each glass culture tube (12 × 75 mm; American Scientific Products, McGaw Park, IL, USA) contained 1 ml of trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 0.25 % (wt/vol) glucose. A loop of organisms was picked from a single colony on an agar plate, inoculated in the test tube and incubated at 37°C for 24 h.

Each bacterial culture tube was decanted and washed twice, each with 1 ml of water. Bacterial slime was fixed with Carnoy's solution (mixing 10 ml of glacial acetic acid, 30 ml of chloroform with 60 ml of absolute alcohol) and stained with 0.1 % toluidine blue. After 1 h, the dye was decaned and the excessive stain was removed by washing twice, each with 3 ml of water. One ml of 0.2 M NaOH was added to each culture tube to hydrolyze the biofilm by heating for 1h at 85°C. Each sample was then vortexed and cooled to room temperature. The optical density (OD) of glycocalyx was determined by spectrophotometer (Milton Roy, Rochester, NY, USA) at 590 nm.

Tritiated thymidine uptake. Tritiated thymidine uptake of bacteria was measured by quantifying DNA content which is relative to the adhered bacterial numbers. A single colony of *S. epidermidis* was cultured in trypticase soy broth supplemented with 0.25 % (wt/vol) glucose and 0.1 μCi ^{3}H (specific activity 16.7 Ci/mmol; New England Nuclear, Boston, MA, USA) in the presence of a stainless steel wire and incubated at 37°C for 24 h. Each stainless steel wire of uniform size simulated the biomaterial implants. The incubated wire was retrieved and the glass culture tube was decanted. Both the retrieved stainless steel wire and the glass culture tube were washed 3 times, each with 1 ml of water. The washed wire was then transferred to another culture tube and 1 ml of 0.2 M NaOH was added to hydrolyze the biofilm by heating the tube at 85°C for 1 h. The hydrolysis of the biofilm was similar to that on the wall of the tube. After the hydrolyzed biofilm were vortexed and cooled to room temperature, the tritiated thymidine uptake was determined for each sample by placing the hydrolyzed biofilm into a scintillation vial containing 6 ml of scintillation liquid and counted in a scintillation counter (Packard Downers Grove, IL, USA).

Optical density of glycocalyx versus tritiated thymidine uptake on the wall of test tube. A whole colony of the organism was transferred to a glass culture tube to make a homogeneous suspension. Bacterial inoculum of 10, 30, 50 or 80 μl was again transferred to individual glass culture tubes containing 1 ml of trypticase soy broth supplemented with 0.25 % (wt/vol) glucose. All tubes were divided into 2 series, one series of the tubes for quantification of glycocalyx and another series of the tubes for determination of tritiated thymidine uptake.

The glycocalyx production and thymidine uptake on the wall of the test tube was determinded as described above. The glycocalyx index was defined as the glycocalyx production (OD) per fixed amount of bacteria (tritiated thymidine uptake) measurable on the wall of the test tubes (eq. 1).

The equation simply represented the relative glycocalyx production of different strains of *S. epidermidis.*

$$\text{Glycocalyx index} = \frac{\text{OD of glycocalyx on tube}}{\text{tritiated thymidine uptake on tube}} \quad \text{(eq. 1)}$$

Tritiated thymidine uptake on the stainless steel wire versus colony forming units (CFU) in broth culture. Assessment of bacterial adherence to the stainless steel wire of different strains was performed by comparing the tritiated thymidine uptake on the stainless steel wire incubated in the broth culture over the common denominator, CFU in that same broth culture of individual strains of *S. epidermidis.*

A stainless wire was placed in the individual glass test tube containing 10, 30, 50 or 80 μl of bacterial inoculum of *S. epidermidis* No. 8, No. 4 or No. 7. Following the incubation for 24 h, the stainless steel wires were removed and processed for quantification of tritiated thymidine uptake of the adhered bacteria on the stainless

steel wire. Quantification of bacteria in the broth culture was performed by serial dilution and analysis of CFU.

The ratio of tritiated thymidine uptake (relative bacterial numbers) on the stainless steel wire to CFU in the broth culture is defined as the adherence index of *S. epidermidis* (eq. 2). This index may be used to indicate the ability of bacteria to adhere to stainless steel wire or implants.

$$\text{Adherence index} = \frac{\text{tritiated thmydine uptake on wire}}{\text{CFU in broth culture}} \quad \text{(eq. 2)}$$

Statistics. All measurements were performed in quadruplicate and repeated at least 3 times. Statistical analyses were performed with Student's *t*-test.

Results

Tritiated thymidine uptake. Tritiated thymidine uptake of adhered bacteria on each wire and on the wall of each glass culture tube was measured for each strain. For the high producer (No. 8), thymidine uptakes on both the wire and the wall of glass tube were the highest among the 3 strains ($p < 0.01$). A gradation of tritiated thymidine uptake was shown either on the wire or the wall of the glass tube; the high producer (No. 8) had the highest uptake, the low producer (No. 7) had the lowest uptake and the medium producer (No. 4) showed moderate uptate (Fig. 1). The figure also shows that the high producer demonstrated the greatest growth tendency on wire and on the wall of glass tube, whereas the low producer showed the least tendency.

Optical density (OD) of bacterial glycocalyx. For all strains, the OD of bacterial glycocalyx on the wall of the glass tube increased linearly with the tritiated thymidine uptake (Fig. 2).

Bacterial adherence to wires. Viable bacterial cells concentrations (CFU) in bacterial broth culture were calculated and correlated to the tritiated thymidine uptake on wire which had been immersed in the same broth. For each strain, the adherent growth or the tritiated thymidine uptake on wire was a linear function of viable bacterial concentration (CFU/ml) in broth culture (Fig. 3).

Glycocalyx index and adherence index. Calculation of glycocalyx index and adherence index revealed that glycocalyx productions of the

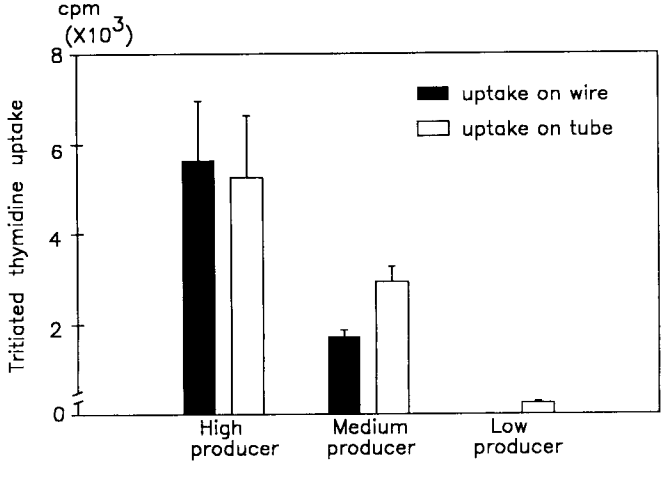

Fig. 1 Tritiated thymidine uptake on the surface of wire and the wall of the glass culture tube for the high, medium, and low producer of *S. epidermidis* (24-h culture). The high producer exhibits the highest uptake both on wires and on the tubes among the 3 strains ($p < 0.01$). The medium producer has higher uptake than the low producer ($p < 0.001$).

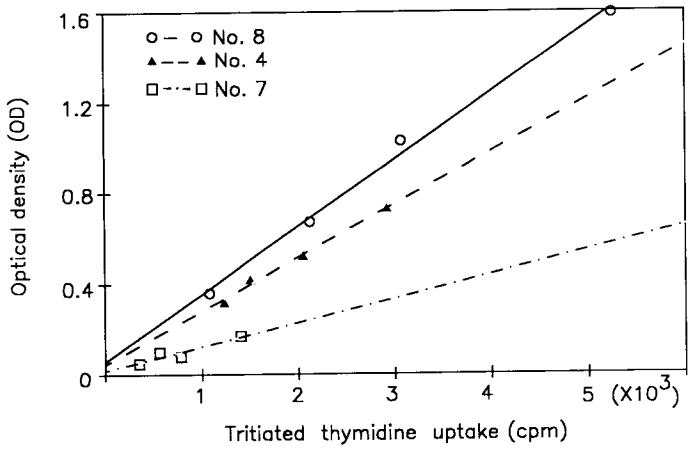

Fig. 2 Glycocalyx production versus tritiated thymidine uptake on the wall of the glass culture tube at various amounts of bacterial inoculum (24-h culture) (○: high producer; ▲: medium producer; □: low producer).

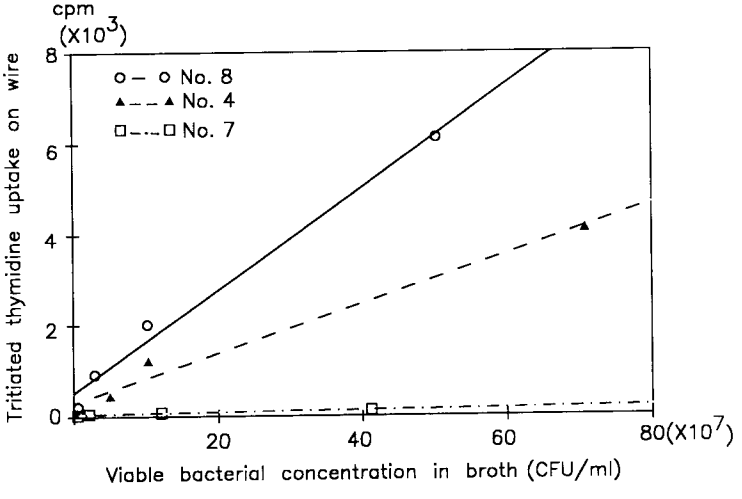

Fig. 3 Tritiated thymidine uptake on wires versus CFU in various amounts of bacterial inoculum (○: high producer; ▲: medium producer; □: low producer).

adhered bacteria and the adherence of individual strains appeared in gradations (Table 1). The high producer had the highest glycocalyx index and adherence index. Fig. 4 shows that the

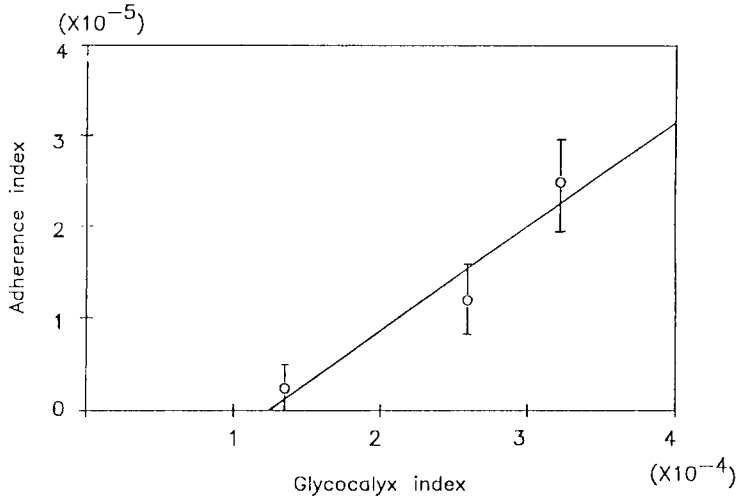

Fig. 4 Glycocalyx index and adherence index of each strain. The adherence index increases as the glycocalyx index increases ($r = 0.9$, $p < 0.001$).

Table 1 Glycocalyx index and adherence index of the high, medium, and low producer strains of *Staphylococcus epidermidis*

Bacterial strain	Glycocalyx index ($\times 10^4$)	Adherence index ($\times 10^5$)
High producer	3.22 ± 0.14	2.48 ± 0.61
Medium producer	2.59 ± 0.09**	1.19 ± 0.34**
Low producer	1.35 ± 0.30**	0.24 ± 0.13**

**indicates $p < 0.01$ compared to the high producer.
Values are given as mean ± SEM.

adherence index is a linear function of glycocalyx index ($r = 0.9$, $p < 0.001$).

Discussion

Recent studies suggest that glycocalyx produced by *Staphylococcus epidermidis* may be involved in the pathogenesis of the biomaterial-centered infection (12, 16, 17). Some strains of *S. epidermidis* have a greater ability for adherent growth than other strains (8). Although scanning electron microscopic observations of others show strain variation of *S. epidermidis* in glycocalyx production, they do not permit quantitative comparison of glycocalyx production (21, 22). Using the glycocalyx index and the adherence index, we were able to demonstrate a strong and positive correlation between glycocalyx production and the adherent growth capacity.

The growth of 3 strains exhibited a gradation of adherence towards the substrata, the stainless steel wire or the glass tube. The high producer strain of *S. epidermidis* showed the greatest adherent growth on the stainless steel wire, whereas the low producer strain had the least growth on the substrate. Our results showed that adherent growth of each strain was strongly correlated to bacterial glycocalyx production. It appeared that bacterial glycocalyx production of individual strains may account for its infectivity and resistance to antibiotic therapy (22). Strains

with higher glycocalyx prodution would form more bacterial colonies which adhere to the surface of its substrata to form a continuous biofilm layer. This initial layer would facilitate further and stronger adherent growth. Ultimately, the bacterial biofilm on biomaterials served as a protection against host defenses (13, 17–19) and antibiotics (14, 15).

In short, both glycocalyx index and adherence index were useful markers for bacterial resistance and infectivity. The importance of glycocalyx production in the pathogenesis of biomaterial-centered infections has been recognized. This implies that future understanding of glycocalyx adherence to biomaterial is needed to reduce biomaterial-centered infections.

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