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Quantitative Assay of Glycocalyx Produced by Viridans Group Streptococci That Cause Endocarditis

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A quantitative method to determine glycocalyx production by strains of viridans group streptococci from patients with endocarditis is presented. There is good correlation between this new tryptophan quantitative assay and qualitative assays employing polysaccharide stains (ruthenium red, periodic acid-Schiff, and Cellufluor) or the Molisch test. The quantification of the glycocalyx production in glucose substrate in vitro by viridans group streptococci correlates with the size of cardiac vegetation and ease of antimicrobial sterilization in experimental endocarditis. The relationship of in vitro quantification of glycocalyx to maintenance of infection, morbidity of infection, and antimicrobial treatment is discussed.

Viridans group streptococci, the most common cause of bacterial endocarditis, produce an exopolysaccharide (glycocalyx) which correlates with adherence to damaged heart valves (5). The amount of glycocalyx is correlated with the size of infected cardiac vegetations (9) and resistance to antimicrobial therapy (2). These relationships have commonly been measured by qualitative light microscopy assays, using the polysaccharide stains ruthenium red, periodic acid-Schiff, and Cellufluor (9).

To further study the role of glycocalyx in endocarditis pathogenesis, a quantitative spectrophotometric assay has been developed which measures cell-adherent glycocalyx produced by viridans group streptococci grown in glucose substrate. The assay is based on the tryptophan reaction of Shetlar et al. (13) and is chemically similar to standard colorimetric methods used in carbohydrate liquid chromatography detection (14). We report the use of this assay in the evaluation of endocarditis-producing clinical isolates of viridans group streptococci.

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MATERIALS AND METHODS

Test strains. Viridans group streptococci from 28 patients with endocarditis at San Francisco General Hospital and Truman Medical Center (Kansas City, Mo.) were identified to the species level by the method of Pulliam et al. (12) and stored at −70°C in tryptic soy broth with 20% glycerol (Remel Laboratories, Lenexa, Kans.). For in vitro qualitative and quantitative assays of glycocalyx production, organisms were grown for 48 h in pooled normal rabbit serum (tested to contain 75 to 76 mg of glucose per dl) as the source of carbohydrate.

Qualitative assays. Smears of in vitro-grown bacteria on glass slides were fixed and stained with periodic acid-Schiff, ruthenium red, and Cellufluor M2R. For Cellufluor staining, slides were incubated in a 0.1% solution of the fluorochrome and examined with a fluorescence microscope with exciter, dichroic, and barrier filters of 490, 510, and 530 nm, respectively. For ruthenium red staining, 0.15% ruthenium red–0.5% glutaraldehyde in 100 mM cacodylate buffer was applied for 1 h; then 0.05% ruthenium red–5.0% glutaraldehyde was applied for 2 h, followed by exhaustive buffer washes. Cardiac vegetations were fixed and 4-μm-thick sections were stained in periodic acid-Schiff with Evans blue counterstain. All slides were read blinded by one investigator and unblinded by a second reader, using a scale of 0 to 4 for a qualitative estimate of glycocalyx production.

The Molisch test was performed on the material produced by viridans group streptococci in rabbit serum after centrifugation at 950 × *g* for 10 min, suspension in saline, and sonication as in the tryptophan assay. Two drops of 10% alpha-naphthol in absolute alcohol was added to 0.5 ml of the resultant supernatant. The mixture was then underlaid with 0.5 ml of sulfuric acid. The carbohydrates bordering the concentrated acid were dehydrated to the furfural derivative condensing with the alpha-naphthol to a reddish violet color at the junction of the two liquids. A positive reaction is evidence for the presence of polysaccharide (8).

Animal model of endocarditis. Endocarditis was induced in rabbits by the procedure described by Garrison and Freedman (4) and modified by Durack and Beeson (3) and Perlman and Freedman (10). Streptococcal isolates incubated in Todd-Hewitt broth for 7 h to an inoculum of 10^8 to 10^9 CFU/ml were injected into a marginal ear vein. Therapy was started with 300,000 U of procaine penicillin intramuscularly every 12 h, starting 24 h after the infection was initiated. All animals with negative blood cultures were excluded from analysis. The catheter was left in place across the aortic valve during therapy. At necropsy, cardiac vegetations were removed and prepared for quantitative culture and microscopic examination.

Quantitative (tryptophan) assay. For tryptophan assay, clinical isolates were streaked on blood agar plates and a uniform inoculum was made in two 4-ml tubes of pooled normal rabbit serum and grown at 37°C for 48 h. The duplicate samples were centrifuged (950 × *g*, 10 min). The organisms were suspended in 4 ml of 0.9% saline and sonicated at 60% power for 30 s (Artek model 300). The stripped organisms were packed by centrifugation (950 × *g*, 10 min). One milliliter of the supernatant which contained the polysaccharide was precipitated by adding it in drops to 10 ml of absolute alcohol and packed by centrifugation (2,400 × *g*, 15 min). The packed precipitate, dissolved in 1 ml of distilled water, was digested in 7 ml of 77% sulfuric acid and

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TABLE 1. Tryptophan quantification of glycocalyx production by 10 clinical isolates of viridans group streptococci

Streptococcal species	No. of isolates assayed	A_{500} (mean ± SD)
S. morbillorum	9	0.255 ± 0.024
S. salivarius	9	0.183 ± 0.034
S. salivarius	10	0.100 ± 0.021
S. salivarius	5	0.181 ± 0.020
S. sanguis II	12	0.147 ± 0.013
S. mutans	5	0.153 ± 0.029
S. mitis	8	0.268 ± 0.027
S. mutans	5	0.146 ± 0.006
S. sanguis II	5	0.149 ± 0.012
S. anginosus	5	0.169 ± 0.022

cooled for 10 min in an ice bath. One milliliter of cold 1% tryptophan was added to each tube and mixed. After exposure to a boiling water bath for 20 min, the tubes were cooled in ice, and sample A_{500} was read on a spectrophotometer (model 640; Varian, Palo Alto, Calif.) against a distilled water blank which was run through the reaction. The carbohydrate in the glycocalyx dehydrates in the presence of the concentrated acid to a furfural. The brownish violet color that develops is the condensation product of furfural with the aromatic amine tryptophan.

To measure the contribution of contaminants (protein) to the assay, a standard curve of bovine serum albumin was compared with dextran over a range of 60 μg to 1 mg/ml. A tryptophan assay was also run on 0.1-mg/ml solutions of 10 monosaccharides, reading both A_{500} and scanning between 400 and 600 nm.

In daily assays, either a standard curve of 520,000-M_w dextran or a single 0.1-mg/ml sample was included as a check on run-to-run variability.

RESULTS

Multiple tryptophan assays were performed on 28 clinical isolates of endocarditis-producing viridans group streptococci.

The 10 strains representing the most commonly isolated organisms are shown in Table 1. The quantitative assay was found to be quite reproducible and correlated with the qualitative assays (Table 2). Five strains with low, moderate, and high glycocalyx production were selected for initiation of endocarditis in the rabbit model. The size of the vegetations (weights) produced in the experimental model of endocarditis both in control animals (untreated) and in penicillin-treated animals correlated with the tryptophan quantitative assay (Table 3). The glycocalyx quantification also correlated with the rapidity of sterilization of infected vegetations by penicillin (Table 4).

TABLE 2. Qualitative and quantitative glycocalyx production by viridans group streptococci[a]

Streptococcal species	In vitro qualitative assay[b]			Tryptophan quantification (A_{500} [mean ± SD])
	Ruthenium red	Periodic acid-Schiff	Cellufluor	
S. salivarius	0	0	1	0.100 ± 0.021
S. sanguis II	0–1	0–1	0–1	0.147 ± 0.013
S. anginosus	2	3	2–3	0.169 ± 0.022
S. mitis	4	4	3–4	0.268 ± 0.027
S. morbillorum	4	4	4	0.255 ± 0.024

[a] All the species tested gave positive results by the Molisch test.
[b] Qualitative estimates ranged from 0 to 4+.

TABLE 3. Correlation between vegetation weights in the rabbit endocarditis model and tryptophan quantification of glycocalyx[a]

Streptococcal species	Tryptophan quantification (A_{500} [mean ± SD])	Mean wt in mg of vegetation at day 5 (no. of animals)	
		Untreated controls	Treated with penicillin
S. salivarius	0.100 ± 0.021	47 (12)	24 (12)
S. sanguis II	0.147 ± 0.013	57 (10)	46 (5)
S. anginosus	0.169 ± 0.022	71 (6)	51 (4)
S. mitis	0.268 ± 0.027	133 (4)	90 (10)
S. morbillorum	0.255 ± 0.024	186 (8)	101 (7)

[a] The correlation coefficients for the methods were 0.905 for A_{500} value from tryptophan assay and mean vegetation weight of control animals and 0.981 for A_{500} value and mean vegetation weight of treated animals.

Protein contributed negligibly to the assay result; bovine serum albumin produced only 0.2% of the absorbance value shown by dextran when both were assayed at 1.0 mg/ml. Spectrophotometric scanning of tryptophan-assayed monosaccharides, glycocalyx, and dextran showed similar peaks for α-D-glucose, dextran, and glycocalyx. Maximum A_{500}s of 100-μg samples were 0.614 to 0.617 for glucose and 0.644 for dextran.

DISCUSSION

This quantitative assay is reproducible with in vitro-grown viridans group streptococci. The data suggest that it is predictive of glycocalyx production in vivo. The glucose substrate used for in vitro quantification was normal, unenriched rabbit serum, containing 75 mg of glucose per dl, which was totally utilized by the viridans group streptococci.

The glycocalyx appears to protect bacteria from human defense mechanisms (leukocytes, antibody, and complement) (1, 7). It may protect bacteria from the action of antibiotics (6). The measurement of the cell-adherent glycocalyx, which we report in this study, is important in that this is the growth that is probably contributing to the physical carbohydrate barrier that limits antimicrobial penetration and activity in endocarditis. A large fraction of the glycocalyx from high-glycocalyx-producing strains is lost from the bacterial surface into the serum growth medium. The adherent glycocalyx appears to be tightly bound, in that doubling the sonication time to 1 min does not greatly improve yield. Electron microscopy on 30-s-stripped organisms shows that some glycocalyx remains. This glycocalyx may also form a physical barrier in the microcolony of infection in the cardiac vegetation.

TABLE 4. Rate of killing of streptococci in vegetations during penicillin therapy

Streptococcal species	MIC/MBC (U/ml) of penicillin	Tryptophan quantification (A_{500} [mean ± SD])	No. (%) of vegetations sterile on day:	
			2	5
S. salivarius	0.312/0.312	0.100 ± 0.021	6/6 (100)	12/12 (100)
S. sanguis II	0.156/0.312	0.147 ± 0.013	5/7 (71)	5/5 (100)
S. anginosus	0.312/0.312	0.169 ± 0.022	2/4 (50)	4/4 (100)
S. mitis	0.312/0.312	0.268 ± 0.027	0/8 (0)	0/10 (0)
S. morbillorum	0.156/0.156	0.255 ± 0.024	0/6 (0)	0/7 (0)

The quantitative assay may have many applications. By correlating with sizes of vegetations, it may be a predictor of embolic complications. Viridans group streptococci with high tryptophan assay values are less easily sterilized when these strains are used to produce animal model endocarditis; the assay value may be a guide to antibiotic therapy in endocarditis, particularly prosthetic valve endocarditis. Other potential applications include the ability to predict the pathogenicity of blood culture isolates and to further characterize the biochemical makeup of the glycocalyx.

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