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Dextran Production as a Possible Virulence Factor in Streptococcal Endocarditis[1] (40216)

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Coleman and Williams (1) have subdivided the viridans streptococci into species based on biochemical tests and the capacity of the bacteria to synthesize extracellular polysaccharides from sucrose. *S. mutans* species of viridans streptococci produce at least two different types of extracellular polysacchride: a water soluble dextran with mainly α-(1 $\rightarrow$ 6) glucan linkages, and a branched water-insoluble, cell-surface associated dextran with both α-(1 $\rightarrow$ 3) and α-(1 $\rightarrow$ 6) glucan linkages (2). Cell-surface associated dextrans are essential for the adherence of *S. mutans* to hard surfaces such as glass or tooth enamel (3–4) and play an important role in the development of dental caries (5). *S. sanguis* species also adhere to teeth (5) and produce several types of dextrans, including a cell-surface associated insoluble form (6–9).

Dextran forming *S. sanguis* and *S. mutans* species were isolated in 28–45% of patients with streptococcal endocarditis (6, 10–13) whereas only 11–12% of the streptococci isolated from blood cultures after dental extraction were dextran producers (10, 12). Elliott (12, 14) has suggested that the higher incidence of dextran producing organisms in endocarditis patients may be due to the increased stickiness of dextran coated organisms on endocardial surfaces.

In these studies, an experimental rabbit model of streptococcal endocarditis developed by Garrison and Freedman (15) and modified by Durack, Beeson, and Petersdorf (16, 17) was used to test Elliott's dextran virulence hypothesis by contrasting the *in vivo* infectivity of dextran producing and dextran negative strains of streptococci. In addition, the infectivity of a stable ultraviolet-induced dextran negative mutant of *S. sanguis* was compared to its dextran producing parent strain. The dextran virulence hypothesis was further tested by comparing the infectivity of streptococci in broth media with and without the sucrose substrate necessary for dextran production.

Methods. The model of streptococcal endocarditis used in these studies has been described by us previously (18). Briefly, sterile platelet and fibrin thrombi were induced on the aortic valve and endocardium of New Zealand white rabbits by passing a polyethylene catheter down the right carotid artery into the left heart. Twenty-four hours after placement of the catheter, the animals were divided into groups and were inoculated intravenously through a marginal ear vein with 1.0 ml portions of an 18 hour tryptic soy yeast (TSY) extract (Difco) broth culture of the test strain. Five percent sucrose (Difco) tryptic soy yeast broth was used in specific experiments. Inoculum size was varied by serial dilution of an 18 hour culture in TSY broth. All inocula were quantitated by serial 10-fold dilutions in saline and culture of portions in blood agar pour plates.

Normal rabbits without catheter induced endocardial lesions rapidly clear streptococci from the blood without colonizing the endocardium (19), whereas animals with endocardial lesions are regularly infected at high inoculum sizes (16). The presence or absence of endocardial infection was determined 24–48 hr after the intravenous injection of organisms with the catheter left in place. Endocardial vegetations weighing 10–280 mg were excised, weighed and homogenized in TSY broth, and serial 10-fold dilutions of the homogenate were incorporated into blood agar pour plates to permit quantitation of the

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number of streptococcal colony forming units (CFU) per milligram of vegetation. Vegetations were considered sterile when less than 10 colony forming units were seen in pour plates containing 0.5 ml of undiluted tissue homogenate after incubation at 35° centigrade for 48 to 72 hr. The inoculum at which 50% of the animals were infected (ID_{50}) was calculated by the method of Litchfield and Wilcoxon (20).

Strains of streptococci originally isolated from endocarditis patients were obtained from S. D. Elliott (Oxford), D. T. Durack (Seattle), C. Thornsberry (Atlanta) and C. Watanakunakorn (Cincinnati). Dextran production from sucrose by streptococci was determined using the ethanol-precipitation method of Hehre and Neill (6). By this method *S. sanguis* and *S. mutans* strains were dextran producers, while *S. mitis* and *S. salivarius* were dextran negative.

S. sanguis, serotype II (NCTC 7864) was subjected to ultraviolet irradiation using the procedures of Johnson *et al.* (4). Serial dilutions of irradiated organisms were plated on Mitis Salivarius (MS) agar (Difco). Alterations in polysaccharide synthesis are expressed as colonial variants on MS agar which contains 5% sucrose. Small, flat, nonadherent, creamy colonial mutants were selected after sequential incubation in anaerobic jars and then 5% CO_2 for 18–24 hr at 35° centigrade. Although several mutants produced less ethanol-precipitable dextrans than the parent strain, none were completely deficient in dextran production.

Since it seemed likely that the different types of dextran produced by *S. sanguis* were produced by separate enzymes (7–9), a mutant strain from the first series of irradiation experiments was irradiated again to inactivate a second enzyme system. A number of second stage small, flat, nonadherent colonial mutants differed from the appearance of first stage mutants and were found to be dextran negative when tested for ethanol-precipitable polysaccharides.

These mutants were screened with a more sensitive assay which measured the uptake of a ^{14}C label from [^{14}C]sucrose into methanol-precipitable or cell-associated polysaccharides using the method of Robrish *et al.* (21). Incorporation of the label through glycolysis was inhibited by the addition of 29 m*M* NaF (9, 22). Replicate tubes of 0.5 ml broth cultures containing 0.3 μCi of Sucrose-U-[^{14}C] (specific activity, 0.1 μCi/mole of sucrose) were incubated at 37° for up to 3 hr. Incorporation was stopped by addition of 4.5 ml of methanol and transfer to an ice bath. The contents of each tube were collected on glass filters and washed 20 times with 5 ml of methanol. Washing reduced the residue of [^{14}C]sucrose on the filter from uninoculated control broth solutions to less than 150 cpm. Filters were dried and counted in a Packard liquid scintillation spectrometer with a scintillation fluid containing 15.2 g of Omnifluor (NEN) per gallon of toluene.

Only one of the second stage dextran negative mutants was used in this study. Comparison of the mutant *S. sanguis* to the parent strain and dextran negative control (*S. mitis*) showed the absence of dextran synthesis (Fig. 1) by the failure to incorporate a ^{14}C label from sucrose. Both the mutant and the *S. mitis* culture did incorporate small amounts of label barely discernible in Fig. 1. This labeled material is believed to be due to synthesis of cell wall polysaccharides. The colonial morphology of the dextran negative mutant of *S. sanguis* (NCTC 7864) on blood agar and response to biochemical testing with arabinose, sorbitol, mannitol, enulin, raffinose, lactose, and esculin were the same as the parent organism.

Results. The ability of dextran-producing streptococci and dextran-negative strepto-

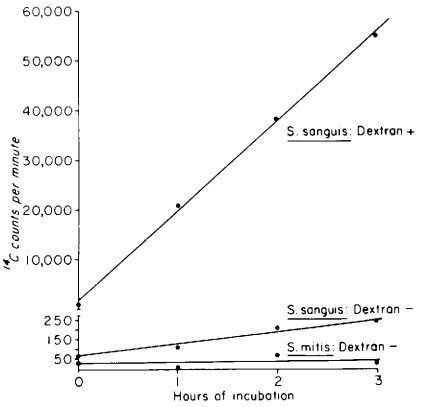

FIG. 1. ^{14}C Incorporation from sucrose, u-[^{14}C] by the *S. sanguis* parent strain, dextran mutant, and *S. mitis* strain.

cocci to induce endocarditis in groups of rabbits injected with varying inoculum sizes was determined, and the results are presented in Tables I–III. The infectivity of an organism was measured by the proportion of animals infected in each group after the intravenous injection of different inoculum sizes. The most infective organisms produced a higher proportion of infected animals at lower inoculum sizes. The number of organisms at which 50% of the animals were infected was calculated for each strain by the method of Litchfield and Wilcoxon (20) and its presented in the tables with an estimate of the 95% confidence limits of the observation.

Two strains of dextran-producing streptococci did not appear significantly more infective than two dextran-negative organisms when grown in TSY broth (Table I).

The ID_{50} of the dextran producing parent *S. sanguis* of $\log_{10}$ 4.8 cfu (4.3–5.2: 95% confidence limits) was not significantly different from the dextran negative mutant ID_{50} of $\log_{10}$ 4.4 cfu (3.6–5.1: 95% confidence limits) when grown in TSY broth. In order to verify the *in vivo* stability of the dextran negative mutant, mutant organisms isolated from endocardial vegetations from three infected rabbits were shown not to incorporate [^{14}C] label from [^{14}C]sucrose in broth cultures after passage through the rabbits.

TSY broth not supplemented with sucrose may permit the development of small amounts of cell surface associated dextrans in

TABLE I. RESULTS OF INFECTIVITY EXPERIMENTS COMPARING DEXTRAN PRODUCING TO DEXTRAN NEGATIVE STRAINS OF VIRIDANS STREPTOCOCCI USING ORGANISMS GROWN IN TRYPTIC SOY YEAST BROTH.

Organism	Inoculum size ($\log_{10}$ cfu)	# Rabbits infected/# Rabbits total (%)	Infectious doses 50% ($\log_{10}$ cfu) (95% confidence limits)
Dextran positive:			
1. *S. sanguis*, I	7.0	8/10 (80)	5.1 (4.1–6.2)
	5.2	6/10 (60)	
	3.2	1/9 (11)	
2. *S. mutans*	6.1	5/8 (63)	5.1 (3.8–6.4)
	5.0	4/8 (50)	
	4.5	3/8 (37)	
Dextran negative:			
1. *S. mitis*	7.5	6/9 (67)	6.4 (5.1–7.7)
	5.3	4/12 (33)	
	4.4	2/9 (22)	
	2.6	0/9 (0)	
2. *S. mitis*	7.0	11/11 (100)	6.3 (5.7–6.9)
	6.3	6/12 (50)	
	5.6	3/12 (25)	

TABLE II. RESULTS OF INFECTIVITY EXPERIMENTS COMPARING DEXTRAN POSITIVE S. SANGUIS, SEROTYPE # (NCTC 7864) AND A DEXTRAN NEGATIVE MUTANT GROWN IN TRYPTIC SOY YEAST BROTH.

Organism	Inoculum size ($\log_{10}$ cfu)	# Rabbits infected/# rabbits total (%)	Infectious dose 50% ($\log_{10}$ cfu) (95% confidence limits)
S. sanguis, II	8.0	42/43 (98)	4.8 (4.3–5.2)
Dextran positive	6.6	9/9 (100)	
	5.3	37/45 (82)	
	4.2	3/16 (19)	
	3.4	1/10 (10)	(117 Rabbits)
	3.0	1/10 (10)	
S. sanguis, II	8.3	7/7 (100)	4.4 (3.6–5.1)
Dextran negative mutant	7.3	8/8 (100)	
	6.2	7/7 (100)	
	5.2	6/8 (75)	
	4.1	4/9 (44)	(46 Rabbits)
	3.2	1/7 (14)	

TABLE III. RESULTS OF INFECTIVITY EXPERIMENTS COMPARING DEXTRAN POSITIVE S. SANGUIS, SEROTYPE II (NCTC 7864) AND A DEXTRAN NEGATIVE MUTANT GROWN IN 5% SUCROSE TRYPTIC SOY YEAST BROTH.

Organism	Inoculum size (log_{10} cfu)	# Rabbits infected/# rabbits total (%)	Infectious dose 50% (log_{10} cfu) (95% confidence limits)
S. sanguis, II	8.8	8/8 (100)	3.6 (2.8–4.1)
Dextran positive	8.5	8/8 (100)	
	7.7	21/21 (100)	
	5.6	6/7 (86)	
	5.5	15/19 (79)	
	4.7	6/9 (67)	
	4.5	8/11 (73)	(83 Rabbits)
	3.6	0/10 (0)	
S. sanguis, II	7.6	8/8 (100)	4.7 (4.1–5.3)
Dextran negative mutant	6.7	7/9 (78)	
	5.3	7/8 (88)	
	4.8	5/8 (63)	
	4.3	7/11 (64)	
	3.8	1/9 (11)	(60 Rabbits)
	2.8	0.7 (0)	

S. sanguis and *S. mutans* species due to the presence of trace amounts of sucrose in the media (23, 24). To amplify the difference between the dextran mutant and its dextran producing parent strain, both parent and mutant strains were grown in TSY broth supplemented with 5% sucrose (Table III). The sucrose grown *S. sanguis* II (NCTC 7864) dextran producing parent strain had a lower ID_{50} (log_{10}, 3.6 cfu) than when grown in TSY broth alone (ID_{50}; log_{10}, 4.8 cfu), suggesting that dextran production may have increased its virulence by approximately 16-fold. In contrast, the sucrose grown dextran negative mutant ID_{50} was unchanged (TSY: ID_{50} log_{10} 4.4 cfu vs. sucrose TSY: ID_{50} log_{10} 4.7 cfu) when grown in sucrose TSY broth.

These results show that *S. sanguis* II organisms grown in sucrose supplemented broth were more virulent than the same organisms grown in the absence of sucrose ($P < 0.05$, Litchfield and Wilcoxon method (20)). A mutant unable to produce cell surface associated dextrans did not demonstrate increased virulence when grown in sucrose supplemented broth. Comparison of the dextran negative mutant grown in sucrose to the dextran producing parent strain grown in sucrose suggested decreased infectivity of the dextran negative mutant, but the difference was not significant at the $p \leq 0.05$ level (Litchfield-Wilcoxon method (20)). In conclusion, dextran production appears to be a virulence factor for the production of experimental endocarditis in rabbits with traumatized endocardium after exposure to *S. sanguis* bacteremia.

Discussion. Local factors that influence the adherence of bacteria to endocardial tissues and initiate the development of bacterial endocarditis in man are unknown. Kerr (25) and Angrist *et al.* (26) have suggested that bacteria are deposited on the endocardium from the blood stream. In addition, Angrist *et al.* (26) have proposed that the actual nidus for endocardial colonization is not the endocardial cell, but sterile fibrin and platelet vegetations that form at the site of damage to the endothelium.

In the rabbit model of experimental streptococcal endocarditis used in these studies, Durack, Beeson, and Petersdorf (16, 17) have demonstrated that intravenously injected streptococci colonize previously induced sterile endocardial vegetations with organisms found both free on the surface of the vegetation and within neutrophils and monocytes adherent to the surface of the vegetation. These observations suggested that the model might be employed to investigate organism and host parameters promoting the localization of bacteria in endocardial vegetations. tations.

The current investigations into the role of extracellular dextran production by streptococci upon endocardial colonization were prompted by evidence that dextrans coating the surface of certain species of streptococci allow these organisms to adhere to teeth and to produce dental caries (5). The species *S.*

mutans has a unique property, not shared with other dextran-producing streptococci, of causing extensive dental caries on smooth enamel surfaces in sucrose fed experimental animals (5). In the presence of sucrose, *S. mutans* will synthesize dextrans and adhere to hard surfaces such as glass, tooth enamel, or wire. *S. mutans* organisms grown without sucrose do not synthesize dextran, do not adhere to hard surfaces, or produce dental caries on smooth surfaces (5). The attachment of sucrose grown *S. mutans* organisms is blocked by treating the cells with dextranase or anti-dextran immunoglobulin (27). Mutants of *S. mutans* have been produced that are unable to synthesize insoluble cell-associated dextrans. Those mutants were unable to adhere to hard surfaces (4) or produce smooth surface dental caries in rats (3).

S. sanguis has been less extensively studied, but adheres to teeth (5, 28) and appears to produce several types of dextran from sucrose, including an insoluble cell-surface associated form that mediates adhesion to hard surfaces. Although the role of cell-surface associated dextrans in the adherence of *S. sanguis* has not definitely been established, this species was selected in the current studies because it causes endocarditis more frequently than *S. mutans* (11).

Valone and Sande (29) have used the rabbit model to examine the hypothesis that dextran production by *S. sanguis* is a virulence factor. They have reported that a sucrose grown dextran producing strain of *S. sanguis* treated with dextranase to remove surface associated dextrans possessed a higher ID_{50} ($\log_{10}$ 7.26 cfu) than untreated sucrose grown organisms ($\log_{10}$ 6.58 cfu). Further studies by the same group (30) have shown increased adherence by dextran coated *S. sanguis* organisms to *in vitro* fibrin and fibrin-platelet preparations compared to the same organism treated with dextranase.

Other investigators have rejected the hypothesis of Angrist *et al.* (26) that endocardial colonization results from seeding sterile thrombotic vegetations and have examined the adherence of bacteria directly to untraumatized endothelial cells. Gould *et al.* (31) studying the adherence of different bacterial species to portions of human or canine aortic valve leaflets have shown differences in adherence that correlate with the incidence of endocarditis in man produced by each of the species. In this model, dextran-producing streptococci did not appear to be more adherent to untraumatized canine and human endothelium than dextran-negative streptococci (32). However, *Streptococcus mutans* strains grown in sucrose were shown to adhere *in vitro* more readily than strains not grown in sucrose broth to traumatized canine aortic valve sections (33).

This study supports previous observations (29, 30) that dextran production by *Streptococcus sanguis* contributes to the ability of these organisms to produce endocarditis *in vivo* in rabbits. The dextran negative mutant used in these studies was identical to the parent strain in biochemical reactions and appearance on blood agar and was stable during passage through animals. The experimental model required large numbers of animals to determine the ID_{50} within reasonable statistical limits and did not demonstrate the smooth dose response relationships necessary for ID_{50} calculations using smaller numbers of animals. This makes determination of 10-fold differences in infectivity tedious with this model. The use of a dextran negative mutant to examine dextran mediated *S. sanguis* virulence in producing endocarditis has not been reported previously. Dextran mediated virulence may occur by direct surface adhesion as in the *in vitro* fibrin-platelet model of Scheld and Sande (30), indirect adherence by other components of the growth media or bacterial byproducts absorbed to the dextrans, or may be more complicated involving organism antibody interrelationships. Further *in vitro* studies using serum with high and low antibody titers to *S. sanguis* should be conducted in the fibrin-platelet model to clarify these relationships.

Summary. The hypothesis that dextran production from sucrose by *Streptococcus sanguis* contributes to the infectivity of this organism in the development of bacterial endocarditis was tested in a rabbit model of experimental endocarditis using a dextran producing strain and an ultraviolet induced dextran-negative mutant. Sucrose grown dextran producing organisms were more infective than organisms grown in broth solutions without sucrose, whereas sucrose did not in-

crease the infectivity of the dextran negative mutant. Dextran production appears to be a virulence factor in the production of streptococcal endocarditis.

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