SEROLOGICAL AND BIOLOGICAL CHARACTERISTICS AND PENICILLIN RESISTANCE OF NONHEMOLYTIC STREPTOCOCCI ISOLATED FROM SUBACUTE BACTERIAL ENDOCARDITIS

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The finding of a new variety of nonhemolytic streptococcus, designated as *Streptococcus* s.b.e., has been recently reported (Loewe, Plummer, Niven, and Sherman, 1946). The organism was found 41 times in 113 cultures from 100 cases of subacute bacterial endocarditis. Certain significant cultural and biochemical characteristics were attributed to this new variety as well as a refractoriness to penicillin therapy. However, no data on the *in vitro* sensitivity to penicillin were included in the report. The organism was subsequently studied in great detail (Niven and White, 1946; Washburn, White, and Niven, 1946; White and Niven, 1946; Niven, Kiziuta, and White, 1946), and a considerable degree of homogeneity was attributed to it. Of particular significance was the report (Washburn, White, and Niven, 1946) that all 42 strains of *Streptococcus* s.b.e. studied fell into two distinct serological types as determined by the precipitin method. Five of these strains gave positive tests with both type I and type II antisera. An antiserum prepared by immunizing rabbits with one of the five strains reacted with extracts of all the strains of *Streptococcus* s.b.e. of types I and II. This was believed to be due to the presence of specific components of both types in the cells of the polyvalent strain, rather than to the presence of a common antigenic component.

The grouping of nonhemolytic streptococci on a serological basis, similar perhaps to the Lancefield grouping of the hemolytic streptococci, would be a logical goal since classification based upon cultural and biochemical characteristics has many deficiencies, for example, lack of specificity and the inconstancy of some of the reactions. Solowey (1942) attempted to group serologically the streptococci isolated from cases of subacute bacterial endocarditis and from the human throat and extracted teeth. She found that about two-thirds of all the strains could be differentiated into 14 serological types of which about 50 per cent of the reacting strains from each source fell into two types. No serological differentiation could be shown between the streptococci from subacute bacterial endocarditis and those from the throat and teeth. No correlation of the serological with the biochemical characteristics of the various strains was found.

Dawson, Hobby, and Lipman (1944) investigated the sensitivity to penicillin of 50 strains of nonhemolytic streptococci isolated from subacute bacterial endocarditis cases. The organisms were described grossly as "viridans," "indifferent," and "resembling enterococci." Their sensitivities were expressed in relation to a standard hemolytic streptococcus strain. No findings characteristic

for any particular group could be demonstrated. They concluded that with occasional exceptions, strains of nonhemolytic streptococci isolated from cases of subacute bacterial endocarditis are sensitive to penicillin *in vitro* and that the degree of sensitivity of any particular strain does not correspond with its serological or cultural properties.

The present study on all strains of nonhemolytic streptococci isolated from subacute bacterial endocarditis in this hospital for a period of the last 20 months, concerns itself with the correlation of the serological and biological characteristics of the bacteria with their resistance to penicillin *in vitro*. The results are embodied in the following report.

CRITERIA USED FOR CLASSIFICATION OF ORGANISMS ISOLATED

Soon after original isolation, all organisms were subjected to serological and biological tests and their resistance to penicillin was determined *in vitro*. Thirty-four strains isolated from 32 cases were the subject of this investigation. Four strains were isolated from two recurrent infections (strains 17 and 18, and strains 19 and 20). It may be noted from table 1 that in both patients with recurrences, neither was infected with a *Streptococcus* s.b.e. strain.

It has long been recognized that the classification of the nonhemolytic streptococci is far from satisfactory. Prior to the identification of the *Streptococcus* s.b.e. group, only a few other groups had been more or less clearly differentiated, such as the *Streptococcus salivarius*, the enterococcus groups, and a few others. In order to facilitate the analysis of the significant characteristics of the various strains isolated, the organisms were classified by the following properties:

Streptococcus s.b.e. group. Precipitation with *Streptococcus* s.b.e. antiserum, fermentation of inulin with acid production, hydrolysis of arginine, growth on 40 per cent bile blood agar, absence of mucoid colonies on 5 per cent sucrose agar, and the synthesis of slime in 5 per cent sucrose broth.

Streptococcus salivarius group. Formation of large mucoid colonies on 5 per cent sucrose agar, no change in color on blood agar, failure to hydrolyze arginine, and the ability to ferment inulin and raffinose with the production of acid (Sherman, Niven, and Smiley, 1943).

Enterococcus group. Precipitation with Lancefield group D streptococcus antiserum, marked resistance to penicillin, growth at 45 C, and growth in media containing 6.5 per cent NaCl, and in 40 per cent bile (Evans and Chinn, 1947).

Unclassified Streptococcus group. The organisms that did not possess the specific characteristics described above.

Accordingly, in this study, of the 34 strains isolated, 9 were *Streptococcus* s.b.e., 2 each were *Streptococcus salivarius* and enterococci, and the remaining 21 were considered to be in the heterogeneous group, unclassified streptococci.

SEROLOGICAL CHARACTERISTICS

All strains were studied serologically. The methods employed for the preparation of the precipitating antisera and the carbohydrate extract from the organisms as well as the technique for the precipitin test were those described

TABLE 1

Characteristics of nonhemolytic streptococci isolated from subacute bacterial endocarditis

STRAIN	PRECIPITATION WITH ANTISERUM			BIOLOGICAL CHARACTERISTICS									PENICILLIN RESISTANCE (X standard)
	Str. s.b.e.	Str. 15	Str. Lan. D	Greening—24 hr	Fermentation— inulin	Fermentation— raffinose	Growth—45 C	Growth—6.5 % NaCl	Arginine hydrolysis	Growth—40% bile blood agar	5% Sucrose agar— mucoid colonies	5% Sucrose broth— slime synthesis	
Streptococcus s.b.e.													
1	+	-	-	+	+	-	+	-	+	+	-	+	2
2	+	±	±	+	-	-	+	-	+	+	-	+	5
3	+	-	-	+	+	-	+	-	+	-	-	+	2
4	+	-	-	+	-	+	+	-	+	+	-	+	10
5	+	-	-	+	+	-	+	-	+	+	-	+	10
6	+	-	-	+	+	-	-	-	+	+	-	±	10
7	+	-	-	+	-	-	-	-	+	+	-	±	2
8	+	-	-	+	+	-	+	-	+	+	-	+	15
9	+	-	±	+	-	-	+	-	-	+	-	+	10
Streptococcus salivarius													
10	-	-	-	±	+	+	+	-	-	+	+	+	5
11	-	±	+	-	+	-	+	-	-	+	+	+	15
Enterococcus													
12	-	-	+	+	-	-	+	+	+	+	+	+	150
13	-	-	+	+	-	-	+	+	+	+	+	-	250
Unclassified streptococci													
14	-	-	-	+	-	-	-	-	+	-	+	+	2
15	-	+	-	+	-	+	+	-	-	-	-	-	5
16	-	+	±	-	-	-	+	-	-	-	+	-	5
17	-	+	±	-	-	-	+	-	-	-	-	-	1
18	-	±	±	+	-	-	+	-	-	-	-	-	5
19	-	-	-	-	+	-	±	-	+	+	-	-	2
20	-	-	-	-	-	-	+	-	-	±	-	-	1.5
21	-	-	-	+	-	+	+	-	+	-	-	-	1
22	-	-	-	-	-	-	-	-	-	-	-	-	10
23	-	-	-	+	-	-	-	-	-	-	-	-	2.5
24	-	±	-	-	+	+	-	-	-	-	-	-	5
25	-	-	-	-	-	+	+	-	+	-	-	±	5
26	-	-	-	+	-	-	-	-	+	-	-	+	5
27	-	-	-	+	-	-	-	-	+	-	-	-	5
28	-	-	-	+	-	-	+	-	+	-	-	-	10
29	-	±	±	+	-	-	+	-	-	+	-	-	2
30	-	-	-	+	-	-	+	-	-	-	-	+	10
31	-	-	-	-	-	-	-	-	-	+	-	-	15
32	-	-	±	+	+	-	-	-	+	+	-	+	5
33	-	+	-	+	-	-	+	-	+	-	-	-	1.5
34	-	±	±	+	-	-	+	-	-	-	+	+	5

+ = Strongly or definitely positive; ± = weakly or doubtfully positive; - = negative.

by Lancefield (1933). Antisera were prepared for three strains: (1) *Streptococcus sanguis* (*Streptococcus* s.b.e.), a strain obtained from Washburn that contains both types I and II antigen, (2) a nonhemolytic streptococcus strain in the unclassified group (strain 15), which was isolated from an early case and which did not possess any of the significant biological characteristics ascribed to *Streptococcus sanguis*, and (3) a Lancefield group D hemolytic streptococcus (strain C1). Heat-killed organisms, as recommended by Washburn, were used to prepare the first two antisera, whereas formalin-killed organisms were employed to produce the Lancefield group D antiserum. The precipitin test was conducted with varying amounts of antigen and a constant amount of serum. All tests were accompanied by controls using normal rabbit serum and extract, and the various antisera without any extract, for comparison.

As may be seen from tables 1 and 2, the *Streptococcus* s.b.e. is a distinct serological entity. All *Streptococcus* s.b.e. strains reacted strongly only with the antiserum specific for the group. Extracts prepared from streptococci in the

TABLE 2
Precipitin reactions of extracts of nonhemolytic streptococci from subacute bacterial endocarditis

EXTRACTS TESTED	ANTISERUM					
	<i>Streptococcus</i> s.b.e.		<i>Streptococcus</i> 15		Lancefield D <i>Streptococcus</i>	
	pos.	neg.	pos.	neg.	pos.	neg.
<i>Streptococcus</i> s.b.e.....	9	0	1±	8	2±	7
<i>Streptococcus salivarius</i>	0	2	1±	1	1	1
<i>Enterococcus</i>	0	2	0	2	2	0
Unclassified streptococci.....	0	21	{ 4 4±	13	6±	15

other groups failed to precipitate with *Streptococcus* s.b.e. antiserum. These results confirm the observations of Washburn *et al.* on the serological homogeneity of the *Streptococcus* s.b.e. cultures.

BIOLOGICAL CHARACTERISTICS

All organisms were subjected to a number of cultural and biochemical tests. The media and methods employed were those described by Sherman (1937) plus a few others developed and reported from his laboratory since then. The bacteria were grown under optimum oxygen tensions. The three anaerobic strains (22, 25, and 28) were cultured in a tightly sealed jar from which the air had been thoroughly evacuated and replaced with nitrogen gas. Two of the strains were microaerophilic (27 and 29) and were carried in jars in which carbon dioxide was provided by a lighted candle.

One strain, 33, was typical in all respects of the diphtheroid streptococcus described by Lamanna (1944) but, unlike the Lamanna strain studied by White and Niven (1946), the diphtheroid streptococcus isolated in this series did not prove to be a *Streptococcus* s.b.e. either serologically or biologically.

No single biological characteristic specific for any one variety could be demonstrated, with one exception. The organisms of the enterococcus group were the only ones found able to grow in 6.5 per cent NaCl broth. However, the presence of a combination of concomitant characteristics was highly suggestive for the *Streptococcus* s.b.e. group, namely, growth on 40 per cent bile blood agar, hydrolysis of arginine, fermentation of inulin with the production of acid, absence of mucoid colonies on 5 per cent sucrose agar, and the synthesis of slime in 5 per cent sucrose broth. One unclassified streptococcus, strain 32, possessed all these properties although an extract prepared from it failed to precipitate with specific *Streptococcus* s.b.e. antiserum.

Good correlation between the biological and serological characteristics were

TABLE 3

Biological characteristics of nonhemolytic streptococci from subacute bacterial endocarditis

CHARACTERISTICS	WHITE & NIVEN	STREPTOCOCCUS S.B.E. (9)	STREPTOCOCCUS SALIVARIUS (2)	ENTEROCOCCUS (2)	UNCLASSIFIED STREPTOCOCCI (21)
	<i>per cent</i>	<i>no.</i>	<i>no.</i>	<i>no.</i>	<i>no.</i>
Greening—24 hr.....	98.0	9	1±	2	13
Fermentation inulin.....	81.0	5	2	0	3
Fermentation raffinose.....	33.0	1	1	0	4
Growth—45 C.....	45.0	7	2	2	12
					1±
Growth—6.5% NaCl.....	0.0	0	0	2	0
Arginine hydrolysis.....	100.0	8	0	2	9
Growth—40% bile blood agar.....	72.0	8	2	2	4
					1±
5% Sucrose agar—mucoid colonies.....	0.0	0	2	2	3
5% Sucrose broth—slime synthesis.....	95.0 {	7	2	1	5
		2±			1±

demonstrable for two groups, *Streptococcus* s.b.e. and the enterococci. Table 3 summarizes the significant biological characteristics of the strains of each group isolated in this series.

PENICILLIN RESISTANCE

Resistance to penicillin *in vitro* was determined for all streptococci isolated. A tube dilution method using fresh meat extract broth as the medium and the standard *Staphylococcus aureus* H strain for comparison was employed. The tests were run in 2-ml amounts. Two series of penicillin dilutions varying from 0.5 to 0.005 Oxford units per ml were set up. Tubes were inoculated with suitably diluted 6-hour cultures of the streptococcus and the staphylococcus to give final concentrations of 600 to 800 and 200 to 300 organisms per ml, respectively, at zero hours. If no inhibition proved to be present in this range, a greater amount of penicillin was used for the streptococcus series. The tubes were incubated at 37 C overnight and the end points of complete inhibition were noted the following morning. The amount of penicillin required to inhibit

the streptococci divided by the amount necessary to inhibit the standard organism was the penicillin resistance expressed as the coefficient of resistance as compared to the *Staphylococcus aureus* H standard. The standard organism was inhibited by 0.02 Oxford units per ml.

The resistance to penicillin of the strains under investigation is recorded in tables 1 and 4. It may be seen that, in general, the resistance of organisms of the *Streptococcus* s.b.e. group to penicillin *in vitro* was found to be somewhat higher than that of the organisms of the unclassified group. However, highly resistant strains were not encountered, and all appeared within practical therapeutic range. Only the enterococci showed marked resistance to penicillin.

TABLE 4
Penicillin resistance of nonhemolytic streptococci from subacute bacterial endocarditis

PENICILLIN RESISTANCE (X STANDARD)	STREPTOCOCCUS S.B.E. (9)	STREPTOCOCCUS SALIVARIUS (2)	ENTEROCOCCUS (2)	UNCLASSIFIED STREPTOCOCCI (21)
1	—	—	—	2
1.5	—	—	—	2
2	3	—	—	3
2.5	—	—	—	1
5	1	1	—	9
10	4	—	—	3
15	1	1	—	1
150	—	—	1	—
250	—	—	1	—
1-5	4	1	0	17
6-15	5	1	0	4
Above 16	0	0	2	0

It may also be noted from the tables that identification of *Streptococcus* s.b.e. by this characteristic alone is not feasible.

SUMMARY

The serological and biological characteristics and the *in vitro* penicillin resistance of 34 strains of nonhemolytic streptococci isolated from 32 cases of subacute bacterial endocarditis were investigated.

The report of the existence of a new variety of nonhemolytic streptococcus, based upon serological and biological characteristics and designated as *Streptococcus* s.b.e., is confirmed.

The resistance of the *Streptococcus* s.b.e. (*Streptococcus sanguis* White) group to penicillin was found to be slightly higher than the unclassified streptococci isolated from this disease, but none of the strains were markedly resistant to the antibiotic *in vitro*. The streptococci of the enterococcus group were found to be markedly resistant to penicillin.

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