Serological relationships between serotype-III *Streptococcus sanguis* and Lancefield group-H Streptococci

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Summary. Certain strains of *Streptococcus sanguis* and group-H streptococci have been shown to have similar physiological properties and serological specificities. Serological studies revealed that serotype-III *S. sanguis* shared a common antigen with the so-called “British” group-H streptococci, but not with the “American” group-H streptococci. Serotype-III antigen was extracted in cold 5% trichloroacetic acid from isolated cell walls of *S. sanguis* ATCC 10558, and purified chromatographically. The purified serotype-III antigen consisted of neutral and amino sugars and some phosphorus, and was negatively charged. Hapten inhibition of the quantitative precipitin reaction between serotype-III antigen and antiserum indicated the strong possibility of α -glucosidic linkages being an immunodeterminant of the antigen.

Introduction

Viridans streptococci comprise the largest portion of the microbial flora of teeth, tongue, cheeks and saliva. These include several species such as *Streptococcus sanguis*, *S. mitior* (*mitis*), *S. salivarius*, *S. milleri* and *S. mutans*. *S. sanguis* is one of the first organisms to colonise newly erupted teeth (Carlsson *et al.*, 1970) and is prominent in dental plaque. It was originally isolated from the blood of patients with subacute bacterial endocarditis (s.b.e.), and was designated as *Streptococcus* s.b.e. (Loewe *et al.*, 1946), and later *S. sanguis* (White and Niven, 1946). Strains produced greenish discolouration of blood agar, i.e., α -haemolysis, synthesised dextran from sucrose, hydrolysed arginine to form ammonia, and fermented salicin, lactose and sucrose. Hare (1935) isolated a group of streptococci from the normal human throat and because these strains did not belong to Lancefield's groups A–G, he proposed a new group, H, for them. Taxonomic properties of *S. sanguis* from the blood of patients with endocarditis and group-H streptococci from throats were found to be very similar (Hehre and Neill, 1946) and other workers also reported antigenic similarities between certain strains of *S. sanguis* and the group-H streptococci (Dodd, 1949; Porterfield, 1950; Farmer, 1954; Cole *et al.*, 1976). Meanwhile,

different strains of group-H streptococci were used as vaccines for production of commercial group-H antisera by different companies in the USA and UK. Unfortunately, Cole *et al.* (1976) described later that the serological specificity of these strains was not identical, resulting in confusion about the group-H specificity.

We have previously classified strains of *S. sanguis* isolated from tooth surfaces into at least four serotypes, I–IV, and biotypes A and B (Hamada *et al.*, 1980a) and also noted that serotype-III strains were serologically related to some reference strains of group-H streptococci. In this communication we report that strains of serotype-III *S. sanguis* possess the identical serological specificity as those of group-H streptococci used for production of group-H antisera in England.

Materials and methods

Bacterial strains

A total of 16 reference strains of *S. sanguis* or group-H streptococci was used, the sources of which have been described previously (Hamada *et al.*, 1980a). Some additional strains of group-H *Streptococcus* were kindly provided by Dr M. Hughes (Wellcome Research Laboratories, Kent) and Dr R. M. Cole (National Institute of Health, Bethesda, MD, USA).

The physiological properties of the streptococcal strains used are listed in table I. Streptococcal strains of other species or Lancefield groups were selected from the culture collection in the Department of Dental Research,

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the National Institute of Health, Tokyo. The serotype distribution was made up of 165 strains of *S. sanguis* isolated from tooth surfaces of 26 adults and children and characterised as described previously (Hamada *et al.*, 1980a).

Antisera

The group-H antisera were kindly supplied by Dr M. Hughes (Wellcome Research Laboratories, Kent). Antisera against *S. sanguis* were produced in rabbits by immunisation with whole cells of *S. sanguis* ST3 (serotype I), ATCC 10557 (serotype II), ATCC 10558 (serotype III) or ST7 (serotype IV).

These antisera were used as standard serotype-specific antisera after the application of appropriate absorption procedures (Hamada *et al.*, 1980a). Antisera raised against "American" group-H streptococcus strain F90A, a strain mainly used for production of anti-group H sera in the USA were also used for comparison.

Preparation of antigens from strains of *S. sanguis* or group-H streptococci

Crude antigens for serotyping or grouping were extracted by autoclaving lyophilised whole cells of *S. sanguis* and group-H streptococci in saline (Rantz and Randall, 1955). The type-specific antigen of serotype-III *S. sanguis* was isolated from purified cell walls of *S. sanguis* ATCC 10558. Cell walls (4 g dry weight) were extracted in 200 ml of 5% trichloroacetic acid (TCA) at 0°C for 6 h with stirring. The extract was separated from the cell walls by centrifugation at 20 000 *g* for 30 min. This extraction procedure was repeated twice, and the supernates were combined, neutralised with NaOH solution, dialysed against water, and concentrated. The concentrated antigen extract was applied to a DEAE-Sephadex A-25 column (1.5 × 40 cm) and eluted with 0.05 M (NH₄)₂CO₃, followed by a linear gradient of 0–1.0 M NaCl in 0.05 M (NH₄)₂CO₃. Fractions that reacted

with antiserum to serotype-III *S. sanguis* were combined, concentrated, and applied to a column (1.0 × 45 cm) of Sephadex G-100. Fractions containing serotype-III antigen were combined and lyophilised.

Serological and chemical analyses

Immunodiffusion tests were performed as described previously (Hamada and Slade, 1976). Immunoelectrophoresis in 1.2% Noble agar gel in 0.05 M barbital-HCl buffer (pH 8.6) was performed at 10 mA (2 mA/cm) on a glass slide for 90 min in a cold chamber, followed by overnight incubation at 4°C. Precipitin reactions and their inhibition by various haptenic sugars and sugar derivatives were performed according to the method of Hamada and Slade (1976) and assay for total hexose, hexosamine, phosphorus, rhamnose and protein was done spectrophotometrically (Okahashi *et al.*, 1983). Quantitative determination of individual sugars in the antigen was performed with a Shimadzu GC-7AG gas-liquid chromatograph (GLC) after samples were hydrolysed in 2 N HCl at 100°C for 2 h followed by trimethylsilylation (Hamada *et al.*, 1983).

Results

Physiology and immunological properties of S. sanguis and group-H streptococci

Strains of *S. sanguis* biotype A and group-H streptococci from various sources showed similar physiological properties, such as glucan synthesis from sucrose, α-haemolysis on sheep-blood agar plates, elaboration of H₂O₂, production of ammonia from arginine, and hydrolysis of salicin and inulin. To determine the serological specificities of these strains, antisera were prepared against strains of *S. sanguis* serotypes I–IV. We found that crude RR antigens from serotype III, ATCC 10558, and

Table I. Physiological properties of *S. sanguis* and group-H streptococci

Strain	Results of biochemical tests with						
	salicin	inulin	sorbitol	arginine	glucan	H ₂ O ₂	Haemolysis
<i>S. sanguis</i> (serotype/biotype)							
ATCC 10556 (I/A)	+	+	—	+	+	+	α
ATCC 10557 (II/B)	—	—	—	—	+	+	α
ATCC 10558 (III/A)	+	+	—	+	+	+	α
ST6 (IV/A)	+	+	—	+	+	+	α
ST7 (IV/A)	+	+	—	+	+	+	α
Group H streptococci							
CN474 (UK)	+	+	—	+	+	+	α
CN2814 (UK)	+	+	—	+	+	+	α
F90A (USA)	+	+	—	+	+	+	α

+ = Positive reaction, — = negative reaction.

group-H streptococcal strains CN474 and CN2814 reacted with antisera elaborated against whole cells of serotype III, as well as with commercial group-H antisera from Wellcome Research Laboratories (fig. 1). No precipitin band was observed between the RR antigen of "American" group-H streptococcal strain F90A and antisera to *S. sanguis* ATCC 10558 and group-H streptococcal strains CN474 and CN2814. Antiserum to strain F90A reacted with RR antigens from the homologous strain and with *S. sanguis* ST6. The latter was formerly designated to be serotype IV (fig. 2).

Antiserum to *S. sanguis* ATCC 10558 and "British" group-H antiserum produced a fused precipitin band with antigens from reference strains Challis, Wicky, Hockley and Blackburn and clinically isolated *S. sanguis* strains ST111, ST134 and ST140. These antigens did not react with antisera specific for types I, II and IV, indicating that the serological specificity of *S. sanguis* serotype III is identical with that of "British" group-H streptococci.

Isolation and characterisation of *S. sanguis* serotype-III antigen

Antigens were extracted in cold 5% TCA from isolated cell walls of *S. sanguis* ATCC 10558. Fractionation of the TCA extract through a DEAE-Sephadex A-25 column yielded two hexose peaks (fig. 3). Only the second peak eluted in 0.2–0.3 M NaCl reacted with antiserum to *S. sanguis* ATCC 10558. The fractions that gave this peak were dialysed against water, concentrated and applied to a Sephadex G-100 column. The column was eluted with distilled water, yielding (1) a small,

Fig. 1. Immunodiffusion showing identity of "British" group-H and serotype-III *S. sanguis* strains. Upper wells: RR extracts from strains ATCC 10558, CN474 and CN2814. Lower wells: antisera to "British" group-H streptococcus strain (right and left wells) and *S. sanguis* ATCC 10558 (centre well).

Fig. 2. Immunodiffusion showing identity of serotype-IV *S. sanguis* ST6 and an "American" group-H strain. A = anti-F90A serum; peripheral wells are RR extracts of *S. sanguis* strains ST6, ATCC 10558 and ST7 and group-H streptococcal strains F90A, CN474 and CN2814.

higher mol. wt hexose peak A and (2) a large, lower mol. wt hexose peak B (fig. 4).

Chemical compositions of the peaks A and B were similar (table II). They mainly contained glucose, galactose, rhamnose and glucosamine at an approximate molar ratio of 3:2:3:3, together with small amounts of protein and phosphorus.

Fig. 3. DEAE Sephadex A-25 ion-exchange chromatography of TCA extract of *S. sanguis* ATCC 10558. The column (1.5 × 40 cm) was eluted first with 0.05 M (NH₄)₂CO₃, followed by a linear gradient of 0–1.0 M NaCl in 0.05 M (NH₄)₂CO₃. Total hexose (●—●), phosphorus (○—○), and absorbance at 280 nm (· · ·) are shown. The bar indicates the range in which the fraction gave a precipitin band against anti-10558 serum by the immunodiffusion test.

Fig. 4. Sephadex G-100 gel filtration of partially purified antigen of *S. sanguis* ATCC 10558. Total hexose (●—●), phosphorus (○—○), and absorbance at 280 nm (·····) are shown. The bar indicates the range in which the fraction gave a precipitin band against anti-10558 serum by the immunodiffusion test.

Furthermore, the immunological specificity of these two antigen peaks was found to be identical; the latter peak was used as purified serotype-III antigen because of its better recovery. The serotype-III antigen and RR antigens from whole cells and cell walls of *S. sanguis* ATCC 10558 gave a fused precipitin line against antisera to homologous *S. sanguis* ATCC 10558 and group-H streptococci (fig. 5a).

Immunoelectrophoretic analysis showed that antigens from *S. sanguis* ATCC 10558, including purified serotype-III antigen, were negatively

Table II. Percentage of chemical composition of isolated cell-wall antigen (peaks A and B) of *S. sanguis* ATCC 10558

Component	Percentage composition of peak	
	A	B
Glucose	19.7	18.5
Galactose	12.7	12.7
Rhamnose	17.8	19.9
Hexosamine	19.7	19.7
Phosphorus	3.1	3.3
Protein	2.7	4.0

Fig. 5. Immunodiffusion (a) and immunoelectrophoresis (b) of purified type-III antigen and RR extracts of whole cells (WC) and crude cell walls (CW) of *S. sanguis* ATCC 10558. Immunoelectrophoresis was performed on 1.2% Noble agar gel in 0.05 M barbital buffer (pH 8.6) at 10 mA for 90 min.

charged and migrated towards the anode at pH 8.6 (fig. 5b).

A quantitative precipitin reaction determined the equivalence point of antiserum and antigen for subsequent hapten-inhibition tests. The equivalence zone was reached at an antigen concentration of 0.7 µg when 3 µl of anti-ATCC 10558 serum was used (fig. 6). Hapten-inhibition studies of the quantitative precipitin reaction were performed (table III). Inhibition rates were greatest with panose, a trisaccharide and maltose, a disaccharide, composed of α-linked glucose residues. The common structure in these sugars is α(4 glucosidic linkage. Lactose and α-methylglucoside were moderate inhibitors. These results suggest that the α-glucosidic linkage is a major immunodominant part of the serotype-III antigen.

Discussion

Confusion has arisen from the use in the UK and USA of ill-defined strains of so-called "group-H" streptococci that differ in their serological specificity (Cole *et al.*, 1976; Facklam, 1977).

Fig. 6. Quantitative precipitin curve of purified type-III antigen with anti-ATCC 10558 serum (3 μ l). Antiserum was mixed with different amounts of the antigen and the reaction mixture was incubated for 1 h at 37°C, and for 18 h at 4°C.

Hamada *et al.* (1980a) reported that isolates of *S. sanguis* from human tooth surfaces could be classified into at least four serotypes, I, II, III, IV and in this study we found that purified serotype-III antigen of *S. sanguis* is identical with group-H antigen obtained from "British" but not "American" group-H strains. This may clarify the confusion in the serological classification of group-H streptococci. The purified serotype-III antigen is similar to serotype-II antigen (Koga *et al.*, 1983) in its chemical composition; both antigens contain neutral and amino sugars as well as significant amounts of phosphorus, and are negatively charged. Neither glycerol nor ribitol was detected in either of the antigens. The immunodeterminant of serotype-III antigen appeared to be the α -linked glucosidic linkages, whereas that of serotype-II antigen is N-acetylgalactosamine. It should be noted that widely used laboratory strains of group-H streptococci (i.e., strains Challis, Wicky, Hockley, M5 and Blackburn) have been classified as the serotype-III *S. sanguis*/“British” group H.

Rosan and Argenbright (1982) reported that the phenol-water extract of *S. mitis* ATCC 903 whole

cells contained group-H antigen, and this antigen was found to be glycerol teichoic acid linked with the α -glucosidic residue. Our results, however, showed that this strain possessed no antigenic substance that would react with anti-serotype-III or “British” group-H sera. Furthermore, *S. mitis* ATCC 903 did not produce glucan from sucrose, nor ferment inulin and salicin, although this strain hydrolysed arginine to produce ammonia. Such properties are not consistent with those of serotype-III *S. sanguis* and group-H streptococci. Also, the phenol extracts from most gram-positive bacteria including *S. mitis* ATCC 903, strains of serotypes I, III, IV *S. sanguis*, many Lancefield groups of streptococci and staphylococci contain polyglycerophosphate as a heterophile antigen (McCarty, 1959; Hamada *et al.*, 1980b).

Recently we isolated more than 600 strains of *S. sanguis* from human tooth surfaces for epidemiological studies and found that c. 17% of the total isolates belonged to serotype III. This indicated that serotype-III *S. sanguis*/“British” group-H streptococci were widely distributed in the human oral cavity. On the other hand, we found that very few clinical isolates of *S. sanguis* reacted with antisera elaborated against strain F90A, an “American” group-H strain.

Table III. Hapten inhibition of the quantitative precipitin reaction between type-III antigen and anti-ATCC 10558 serum*

Hapten	Percentage inhibition
D-Glucose	22
D-Galactose	29
L-Rhamnose	19
α -Methylglucoside	36
β -Methylglucoside	23
α -Methylgalactoside	15
β -Methylgalactoside	22
Panose	56
Maltose	52
Lactose	44
Isomaltose	27
Cellobiose	19
Gentiobiose	20
Trehalose	25
Melibiose	19
Raffinose	26
N-acetylglucosamine	25
N-acetylgalactosamine	21

* Anti-ATCC 10558 serum (3 μ l) was incubated with 20 μ mol of haptens for 1 h at 37°C, and then purified type-III antigen (0.7 μ g) was added.

We propose that the antigen from "British" group-H streptococci rather than from the American variety should be considered to be the group-H

antigen of the Lancefield classification. Group-H streptococci appear to be synonymous with serotype-III *S. sanguis* (Hamada *et al.*, 1980a).

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