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Species of Alpha-Hemolytic Streptococci Possessing a C-Polysaccharide Phosphorylcholine-Containing Antigen

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An enzyme-linked immunosorbent assay technique was used to detect and quantify C-polysaccharide-like antigen in strains of alpha-hemolytic streptococci classified into species by following the latest taxonomic recommendations. C-polysaccharide-like antigen is found only in *Streptococcus oralis*, *S. mitis*, and *S. pneumoniae*, which are genetically closely related.

C polysaccharide (PnC), the major cell wall teichoic acid of *Streptococcus pneumoniae*, consists of ribitol-containing repeating pentasaccharide units to which phosphorylcholine is attached (7). The lipoteichoic acid of *S. pneumoniae* also contains phosphorylcholine and has an important role in the regulation of autolysin activity (5). Phosphorylcholine-containing cell wall components have recently been implicated in pneumococcal virulence (13).

Cross-reactions with PnC are well known among alpha-hemolytic oral streptococci (12), but studies have been hampered by the problems of species determination. The classification of oral streptococci has been clarified by recent rRNA cataloging and DNA hybridization studies (2, 4), and routine identification has been improved by using a phenotypic scheme of biochemical identification (1, 3, 4). In the light of these taxonomic developments, we sought to identify the species of oral streptococci which carry phosphorylcholine-containing antigens.

Representative strains of the currently recognized species of alpha-hemolytic streptococci, as determined by DNA-DNA hybridization and phenotypic testing as previously described (1, 3, 15), were studied. These include *S. mutans* KPSK2, 161, and B48; *S. sobrinus* B 542, OMZ 176, SL-1^T, OMZ 65, TH 21, and TH 62; *S. salivarius* NCTC 8618, A385, NCTC 8606, and H53; *S. vestibularis* JW 3, LV 71, and MM1^T; *S. sanguis* NCTC 7863^T and KPE 2; *S. parasanguis* 85-81, UC 4989, MGH 143, SS 897, SS 895, and FW 213; *S. gordonii* NCTC 7868, HF 90A, and M5; *S. crista* CR 311, CR 3, and AK 1; *S. oralis* NCTC 7864, LVG 1, PC 1467, OPA 1, ST 11, ST 12, and ST22; and *S. mitis* K 208, NCTC 10712, ST 20, and ST 31. Ten clinical isolates of *S. pneumoniae* SP1 to SP10 were obtained from sputum samples submitted to the Department of Medical Microbiology.

All strains were grown overnight at 37°C in brain heart infusion broth (Unipath, Basingstoke, England). The viable count of each culture was determined by the method of Miles and Misra (9a). A 1-ml portion of culture was centrifuged in a microcentrifuge, and the supernatant was retained. The cells were washed twice by centrifugation in phosphate-buffered saline (pH 7.5). Lysozyme (50 mg/ml; Sigma Chemical Co., Poole, England) was added in 100-μl volumes to the deposit, and the mixture was incubated for 6 h at 37°C. This method caused complete disruption of the cell wall, as demonstrated by loss of the Gram reaction and by disintegration of cells.

Microtitration plates (M29A; Dynatech, Billingshirst, England) were coated with a 1:2,000 dilution of mouse immunoglobulin M monoclonal anti-phosphorylcholine antibody 5/88 (Universal Biologicals, London, England) in 0.06 M bicarbonate buffer (pH 9.6) by overnight incubation at 4°C. After the plates were washed with 10 mM Tris containing 0.15 M NaCl, 0.05% Tween 20, and 1 mmol of Ca^{2+} (TBSTC), samples were added and incubated for 1 h at room temperature. C-reactive protein was purified as previously described (6) and conjugated with horseradish peroxidase by the periodate method (10). After further washes, 100 μl of C-reactive protein conjugate (1.7 μg/ml) diluted in TBSTC was added and the plates were incubated for 3 h at room temperature. ABTS peroxidase substrate (Kirkegaard-Perry, Gaithersburg, Md.) was added after the plates were washed four times with TBSTC, and the optical density at 405 nm was read with an automated enzyme-linked immunosorbent assay (ELISA) reader (Titertek Multiscan MC; Flow Laboratories, England).

Preparations of streptococci were studied in a screening assay with a limit of detection of 10^{-9} ng of PnC antigen per CFU. Calcium dependence was proven by repeating the assays as above but substituting 10 mmol of EDTA (Sigma Chemical Co.) per liter for calcium in the conjugate buffer. Reactions were shown to be phosphorylcholine inhibitable by repeating the assay with 10 mg of phosphorylcholine (Sigma Chemical Co) per ml in the C-reactive protein conjugate buffer. PnC-like antigen was quantified by comparing the result of dilutions of streptococcal antigens with those obtained from a purified PnC antigen (provided by J. Lui) standard curve on each ELISA plate. The total quantity of PnC-like antigen per milliliter was divided by the viable count to give the quantity of PnC-like antigen per CFU. The PnC-like antigen concentration in culture supernatants were determined by similar methods.

PnC-like antigen was not detected in *S. sobrinus*, *S. salivarius*, *S. vestibularis*, *S. sanguis*, *S. parasanguis*, *S. gordonii*, or *S. crista* in the screening assay. Antigen was

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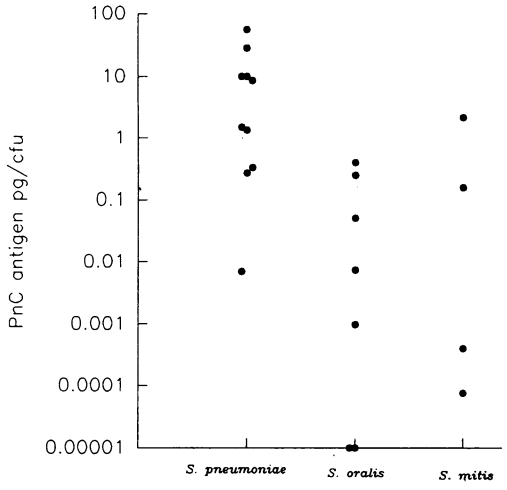

FIG. 1. Results of quantitative assay of phosphorylcholine-containing PnC-like antigen in strains of alpha-hemolytic streptococci of the *S. oralis* group.

found in all strains of *S. pneumoniae*, *S. oralis*, and *S. mitis* and in a single strain of *S. mutans*. Repeated quantitative testing of this strain failed to detect the presence of PnC antigen. Inhibition studies with phosphorylcholine and EDTA in all positive strains resulted in optical densities of less than 0.056 in all wells. PnC-like antigen was found in culture supernatants of *S. pneumoniae* but not in the other species (data not shown). The results of quantification of PnC-like antigen are reported in Fig. 1.

Previous studies have demonstrated the presence of PnC-like antigen among alpha-hemolytic streptococci, although its distribution at the species level cannot be inferred by the identification schemes used. PnC antigen was found in 18 of 52 "*Streptococcus mitior*" strains studied by a coagglutination technique. All other streptococcal "species" examined were negative, but the criteria used to assign isolates to species in this study are not recorded (12). In another study, PnC cross-reacting antigens were found in 80 of 120 alpha-hemolytic streptococci, but the species of these isolates were not identified (11). The binding measured in the latter study was not completely inhibited by phosphorylcholine, which suggests that some of the cross-reaction measured was due to antibody reactivity to the carbohydrate component of PnC (11). The presence of choline in the cell wall was used along with peptidoglycan directly cross-linked with lysine in the definition of *S. oralis* (8). Choline was not found in the single isolate of *S. mitis* studied.

This study demonstrates that PnC-like antigens are confined to *S. oralis* and that the species shown to be most closely related to it by nucleic acid studies are *S. mitis* and *S. pneumoniae* (2, 4).

Pneumococcal virulence has been associated with cell wall turnover in a rabbit meningitis model (13). Organisms with a high spontaneous loss of phosphorylcholine-containing components induced a greater inflammatory response in the rabbit meninges (13). Studies of inflammation in the rabbit model indicate that fractions of killed pneumococci which contain techoic acid polymers induced inflammation when injected into the cisterna magna (14). Bacteremia with *S. oralis* and *S. mitis* but not other oral streptococci has been associated with adult respiratory distress syndome in neutopenic subjects (9).

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