NOTES

Separation of Antigens of *Streptococcus faecalis* by Gel Filtration

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Previous serological studies of hyaluronidase-positive (HP) and hyaluronidase-negative (HN) strains of *Streptococcus faecalis* suggested that the HP strains contained specific antigens as well as antigens held in common with *S. faecalis* type 3 (B. Rosan and N. B. Williams, Nature, 1966, *in press*). To define the antigens responsible for these relationships, soluble extracts of an HP strain (B39-5) and an HN strain (S161) were compared by use of Ouchterlony agar-gel diffusion techniques. Since the location of the precipitin bands between the wells was partially dependent upon the size and shape of the antigens, separation of the antigens on a Sephadex column (Pharmacia, New Market, N.J.), which acts as a molecular sieve, appeared feasible.

Soluble extracts were obtained from cells grown in Todd-Hewitt broth (Difco) for 24 hr at 37°C aerobically, washed three times in distilled water, and disrupted in a Servall-Ribi cell fractionator (Ivan Sorvall, Norwalk, Conn.) at 37,000 to 40,000 psi at 4 to 15°C. The particulate fraction was removed by centrifugation, and the supernatant fluid was examined in double diffusion plates prepared with 1.5% Noble Agar (Difco) in a borate buffer (pH 8.4). A diagrammatic summary of the observations of the Ouchterlony plates is shown in Fig. 1. The lower wells contained the extracts, S161 and 39-5, and the upper wells contained S161 and 39-5 antisera obtained from rabbits inoculated intravenously with whole cells. Three precipitation bands with lines of identity were noted when S161 antiserum was used against the S161 and 39-5 extracts. Band A represents precipitation of the slowest moving antigen(s) in this system, the B antigen(s) precipitates midway between the antiserum well and the antigen well, and the D antigen(s) precipitates closest to the antiserum well. The reaction with 39-5 antiserum is quite different; the only distinct band of precipitation noted was that formed by the B antigen in the homologous extract. The refractoriness of 39-5 antiserum towards the B antigen of strain S161 and its lack of antibodies against antigens C and D did not seem to be explicable solely in terms of the concentration of antigens in the bacteria. It appeared more likely that these observations reflected subtle chemical and structural differences in the antigens themselves or the architectural arrangements of the antigens within the cells. These suggestions were reinforced when it was found that, in contrast to 39-5 cells, S161 cells were sensitive to lysozyme and 35% more susceptible to *Streptomyces albus* enzymes (McCarty, J. Expil. Med. 96:555, 1952).

Although the three antigens were released upon disruption of cells of both strains, no residual concentration of antigen A and only relatively small amounts of antigen D were detectable in the particulate fraction ("cell wall"), suggesting that these antigens were more closely associated with "cytoplasmic" components. This anatomical localization is supported by the identification of antigen D as the group antigen by use of Ouchterlony and immunoelectrophoretic methods with commercial group D antigen and antiserum (Difco). The group D antigen has been shown to be closely associated with the cytoplasmic membrane (G. Shockman and H. D. Slade, J. Gen. Microbiol. 37:297, 1964). The B antigen was obtained from both strains by leaching the particulate fraction with distilled water, and extraction with cold 5% trichloroacetic acid and with *Streptomyces* enzymes, suggesting that antigen B is more closely associated with the "cell wall." However, later immunoelectrophoretic studies of antigen B extracted from the particulate fractions by trichloroacetic acid or *Streptomyces* enzymes revealed that it contained two components joined by a line of identity. One component behaved identically to the B antigen found in the supernatant fluids of disrupted cells in that it did not migrate in the electric field; the other antigen migrated toward the anode.

To separate antigens A, B, and D from the
supernatant fluid of disrupted cells for further immunochromatography procedures, including ethyl alcohol, propanol, acetone, ammonium sulfate, cetylpyridinium chloride, and differential precipitation of borate complexes, were used (R. L. Whistler and M. L. Wolfrom [ed.], Methods in Carbohydrate Chemistry, vol. 5, Academic Press, Inc., New York, 1965). None was successful in separating the mixture. The differences in the rate of diffusion in the agar suggested that some separation might be achieved by chromatography through Sephadex. Analyses of several combinations of solvent, buffer, and type of Sephadex indicated that the best separation was achieved by use of 0.15 M NaCl solvent and eluent in a G-200 column. Therefore, a column (1.5 by 90 cm) having a bed volume of 123 ml was used in further batch separations. Fractions were collected with a Warner-Chilcot time drop fractionator set at 10 drops per tube (1 ml). Every fifth tube was sampled directly in Ouchterlony plates for presence of antigens with S161 antiserum. Figure 2 shows a typical Ouchterlony plate showing separation of antigens of strain 39-5. Antigen A was faintly detected in fractions 35 and 40, and antigen B was detected in fractions 45 to 85; in fractions 90 to 95, it was mixed with antigen D. The latter antigen is separated in the remaining fractions, 100 to 115.

Further substantiation of the separation was determined by combining and concentrating the fractions which appeared to be immunologically homogeneous and subjecting them to immunoelectrophoresis at 250 v for 2 hr in borate-buffered agar (pH 8.4, \( \mu = 0.01 \)). The antigens were

![Diagrammatic summary of antigenic relationships of strains S161 and 39-5.](image)

**Fig. 1.** Diagrammatic summary of antigenic relationships of strains S161 and 39-5. Upper wells contain antisera; lower wells contain supernatant fluids of disrupted cells (band A takes several days to develop, at which time B and D tend to become diffuse, thus blurring the relationship).
placed in outer wells to be electrophoresed, after which S161 antiserum was added to the central trough and incubated overnight at 4 C. The results of these studies are shown in Fig. 3. Fractions 35 to 40 contained two negatively charged bands showing an identity reaction. These presumably constitute "subunits" of antigen A. In contrast to results obtained in the Ouchterlony plates, the concentrate of fractions 41 to 49 represented a mixture of antigens A and B. The latter appears as a single nonmigrating band in the concentrate of fractions 51 to 75. Not shown in Fig. 3 was the mixture of antigen B and the negatively charged D antigen which are found in concentrates of fractions 76 to 104. Finally, antigen D appeared as a single entity in concentrates of fractions 105 to 125.

To increase the final yield of each antigen, the concentrated mixtures of A and B and B and D were recycled through the Sephadex. The results with a mixture of antigens B and D were satisfactory, but the amounts of antigen A which could be separated from B by recycling were disappointing. However, it was found that 5% trichloroacetic acid precipitated antigen A, and antigen B could be recovered from the supernatant fluid.

In addition to the separation of precipitinogens of S. faecalis by gel filtration, these preliminary investigations suggest that the parent serotype to subtype relationship, which exists between strains S161 and 39-5, respectively, derives from at least two factors. One of these factors is a broader spectrum of precipitins induced by parent serotype, as shown by the appearance of at least three lines of identity against S161 and 39-5 extracts in double diffusion plates. Second, the high degree of specificity of antibody against the B antigen induced by strain 39-5 suggests a slight difference in antigenic determinants of this antigen in relation to the antigen obtained from S161, despite the identity reaction in double diffusion against S161 antiserum. The results confirm and offer an explanation for the observations of M. E. Sharpe and P. M. F. Shattock (J. Gen. Microbiol. 6:150, 1952) that among the group D streptococci subtype cells completely absorb the antibodies against the parent type, but that subtype antibodies are not completely absorbed by the parent type cells. Further studies of the isolated antigens should establish the chemical and structural basis for these interesting relationships.

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