Demonstration of the Capsular Antigens of Bovine Group B Streptococci by the Serum-Soft Agar Method

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The elaboration of type-specific capsular antigens by group B streptococci can be demonstrated by the serum-soft agar technique. Group B streptococci isolated from bovine mastitis, namely, strains 9F, 14Mi, 8Mo, 44B, and 4S, were shown to form diffuse and compact types of colony morphology in serum-soft agar. Immunochemical and chemical analyses of antigens isolated from diffuse and compact colonies of strain 9F indicated that the diffuse-type growth of this strain was due to the elaboration of a galactose-rich surface antigen, whereas the compact 9F strain was devoid of this antigen. Specific 9F antiserum was effective in converting the diffuse 9F colonies of the compact type, indicating the presence of capsular material. Preliminary evidence suggests that the serum-soft agar technique could also be used to determine the antigenic diversity of the surface antigens of group B streptococci, thus providing an effective means of typing those organisms.

The antigenic classification of group B hemolytic streptococci is based on the occurrence of distinct type-specific cell surface antigens (2). Epidemiological studies on the transmission of these organisms have depended largely upon recognizing these antigens on the cell surface of the isolated streptococcal strains. Group B streptococci derived from human infections can, in most instances, be classified into one of the five known major serotypes on the basis of these antigens (2). However, nontypable strains, although isolated from human sources, appear to be more common among the strains isolated from bovine mastitis (4). The relatively high incidence of non typable strains among the bovine group B streptococci may be due to the fact that these organisms possess a greater diversity of type-specific surface antigens. In addition to this diversity, the lack of sufficiently potent antiserum that can unequivocally detect low levels of capsular antigen or the lack of monospecific antiserum that can discriminate between cross-reactive antigens present in crude bacterial antigen preparation may also contribute to the difficulties in typing many of the group B streptococci of bovine origin.

Pike, using the modified soft agar method of Ward and Rudd (9), demonstrated the elaboration of capsular hyaluronic acid by group A streptococci (5). In addition, Yoshida has shown that the elaboration of capsular material by *Staphylococcus aureus* can be increased by the incorporation of normal serum into the soft

agar medium (10). This report attempts to determine the feasibility of using the serum-soft agar (SSA) method to demonstrate the elaboration of capsular material by group B streptococci isolated from bovine mastitis. In addition, a specific antiserum-soft agar method was used in an attempt to determine the antigenic diversity among bovine strains of group B streptococci.

MATERIALS AND METHODS

Strains of streptococci. Types Ia (090/14), Ib (H36B/60/1), Ic (A909/14), II (18RS21/45/1), and III (D136C) were kindly supplied by R. C. Lancefield, Rockefeller University. "Compton" strains SS 970 (X protein) and SS 971 (R protein) were obtained from H. W. Wilkinson, Center for Disease Control, Atlanta, Ga. The bovine strains were obtained from R. Eberhardt, Veterinary Science Department, Pennsylvania State University.

Antisera. Standard type-specific antisera were obtained from R. C. Lancefield. The remaining antisera were obtained from rabbits immunized with the bovine strains according to the procedures described by McCarty and Lancefield (3).

Media and growth conditions. The organisms were grown in 10 ml of Todd-Hewitt broth containing 0.15% agar and 0.01% normal serum according to the procedure described by Pike (5) and incubated under 10% CO₂ for 18 h at 37 C.

Serological methods. Procedures for the qualitative precipitin test have been previously described (3). The double-diffusion analysis in agar was performed according to the method described by Tan and Kunkel (8). Immunoelectrophoretic analysis was performed by the method Kane et al. (1).

Isolation of surface antigens. Capsular antigens were isolated from whole cells by boiling at pH 7.0 and were purified by a combination of diethylaminoethylcellulose chromatography and Bio-Gel filtration as previously described (1).

RESULTS

In an attempt to determine the feasibility of using the SSA method as a means of detecting capsular materials elaborated by group B streptococci, the five known group B serotypes and the two Compton strains, which lack type-specific polysaccharide antigens, were tested. The Compton strains, X and R, formed compact-type growth, whereas the encapsulated strains, D136C, 18RS21, A909, H36B, and 090, formed the diffuse type of growth (Fig. 1). These results suggest that the diffuse-type colonial morphology formed in SSA may be due to the elaboration of capsular material by these strains similar to that observed for group A streptococci by Pike (5). This notion is supported by the fact that the formation of the diffuse colonial morphology can be selectively converted to the compact type by homologous antiserum, which is rich in type-specific capsular antibodies, but not by heterologous antisera. In addition Quellung or capsular swelling reactions confirmed the results of the SSA test in that capsular material was detected on strains D136C, 18RS21, A909, H36B, and 090, whereas strains X and R revealed the absence of capsular material.

The presence of capsular material in group B streptococci isolated from local dairy herds was determined by the SSA technique. Both compact and diffuse types of growth were exhibited by the bovine-derived organisms (Fig. 2). Strains 14Mi and 9F showed the diffuse-type growth, whereas strains 8Mo and 44B showed the compact-type growth and strain 4S showed a mixture of both growth types. To support the view that the diffuse-type growth was due to

the elaboration of capsular material into the soft agar, several experiments were performed. In the first study, encapsulated strain 9F was inoculated into soft agar containing either normal, homologous, or heterologous antiserum. Diffuse-type growth was obtained when strain 9F was grown in the presence of normal serum or heterologous antiserum, whereas cells grown in the presence of homologous antiserum formed the compact-type growth (Fig. 3). These results suggest that strain 9F has a surface structure that is immunologically distinct from strain 55J and that antibodies directed against the 9F surface antigen can prevent the formation of the characteristic diffuse-type growth of the encapsulated 9F strain.

In another experiment, individual colonies of a 9F variant strain, which formed only the compact-type growth, were isolated from specific SSA cultures and subsequently subcultured into additional specific SSA. This transfer procedure was repeated until the culture consisted of a predominance of only the colonies with compact-type growth. Figure 4 shows the results of the comparative study of colony morphology between the parent strain of encapsulated 9F and its selected variant strain. Note that the variant strain (left) formed the typical compact-type growth, whereas the parent strain formed the diffuse-type growth. In addition, Quellung reactions, using the diffuse and compact 9F strains and specific 9F antiserum, demonstrated the presence of capsular material on the diffuse 9F cells, whereas the compact 9F cells exhibited no capsular material.

The cell surface antigens of both the parent and variant 9F strains were extracted from whole cells by mechanical mixing at 95 C in pH 7.0 buffer. The cells were removed by centrifugation, and the supernatant was dialyzed and lyophilized. Depicted in Fig. 5 are the results of the immunoelectrophoretic analysis of the supernatant preparations. The antigen prepara-

FIG. 1. Serum-soft agar analyses of the diffuse-type growth of the conventional encapsulated group B streptococcal serotypes Ia (090), Ib (H36B), Ic (A909), II (18RS21), and III (D136C) and the compact-type growth of non-encapsulated strains X and R in the presence of normal rabbit serum.

FIG. 2. Serum-soft agar analyses of selected strains of group B streptococci isolated from bovine mastitis cases in local dairy herds in the presence of normal rabbit serum.

tion from the parent strain (Fig. 5, well 1) consisted of two acidic antigens. In contrast, the preparation derived from the variant strain (well 2) consisted of only a single acidic antigen, which appeared to be similar to the less electrophoretically mobile antigens observed in the parent strain preparation. Additional absorption studies indicated that the compact variant was devoid of the more acidic polymer. These results suggest that the acidic polymer, which is present in the encapsulated strain and not in the variant strain, may be responsible for the diffuse-type growth of the parent 9F strain in SSA.

The acidic antigen that is unique to the parent 9F strain was purified by Bio-Gel P-100 filtration and diethylaminoethylcellulose chromatography (1). Figure 6 shows the results of the double-diffusion analysis in agar of the acidic antigen. The purified acidic antigen (Fig. 6, well 1) formed only a single precipitin band with anti-9F serum. In contrast, crude extracts from strain 9F (well 2) as well as from heterologous strains (wells 3–5) formed a multiplicity of precipitin bands with anti-9F serum. The large inner band was shown to be due to the group-specific carbohydrate. Subsequent chemical

analysis indicated that the acidic antigen of strain 9F consisted of a predominance of galactose with small amounts of an acidic compound and an unknown compound with an *Rf* value in the range of fucose. Attempts are now in progress to determine the chemical features of this acidic antigen and its biological role in the infectious process.

Serotyping of strain 9F. Attempts were made to serotype strain 9F by the SSA method. In this procedure, 0.1 ml of anti-9F serum was incorporated into 10 ml of soft agar and the culture tubes were inoculated with encapsulated group B streptococcal serotype Ia, Ib, Ic, II, or III. With the exception of strain D136C (type III), all of the serotypes formed their typical diffuse-type growth in the presence of anti-9F serum. In the case of type III, the colonies that formed in the SSA appeared to be only moderately diffuse. These results suggest that bovine strain 9F may have a surface antigen that is related to the type III group B streptococci. However, anti-9F serum absorbed with type III capsular antigen had no appreciable effect on converting the encapsulated 9F strain into the compact-type growth in SSA. Also, diffuse-type growth was obtained when 9F was

FIG. 3. Colonial morphology of encapsulated bovine strain 9F in serum-soft agar containing normal rabbit serum, anti-9F-serum, and anti-55J serum.

grown in the presence of anti-9F serum absorbed with the 9F acidic antigen, whereas compact-type growth was obtained with unabsorbed 9F serum. These results indicate that the acidic antigen of encapsulated strain 9F is immunologically distinct from the five known type-specific capsular antigens of group B streptococci. However, in view of the fact that anti-9F serum had a slight effect on the type III cells, the 9F surface antigen may have an antigen that is immunologically similar, but not identical, to an antigen carried by the type III streptococci (strain D136C).

DISCUSSION

This report suggests that elaboration of capsular-like polysaccharide antigens by group B hemolytic streptococci can be detected by the SSA method as described by Pike (5). This conclusion is supported by three types of evidence. First, encapsulated prototypes, representing the five known group B streptococcal serotypes, formed capsular-rich diffuse colonies in SSA, whereas the standard Compton strains, which are devoid of capsules, formed capsular-defi-

cient compact colonies. Second, encapsulated strains can readily be converted to the capsular-deficient compact strains in soft agar by specific capsular antibodies. Third, a polysaccharide surface antigen was isolated from culture filtrates of diffuse-type strain 9F but not from cultures of its compact-type variant.

In recent years, the immunological role of the capsular antigens of group B streptococci has been carefully investigated. Interest in this area has been stimulated by the observations that group B streptococci, a common bovine pathogen, can also be an important etiological agent of neonatal meningitis. Significantly, the prognosis of this disease depends upon adequate and prompt treatment, and therefore rapid identification of the etiological agent is vital in neonatal meningitis therapy. Lancefield has previously shown that a successful scheme for the typing of group B streptococci depends upon the availability of type-specific capsular antigens on the cell surface of the isolated organisms. For example, group B streptococci lacking these surface antigens have been difficult to classify by conventional serolog-

FIG. 4. Serum-soft agar analyses of the diffuse-type growth of encapsulated strain 9F and the compact-type growth of the non-encapsulated variant derived from the parent strain 9F in the presence of normal rabbit serum.

FIG. 5. Immunoelectrophoretic analysis of surface antigens isolated from encapsulated strain 9F (9F, diffuse) and the non-encapsulated variant (9F, compact) depicted in Fig. 4. Well 1, Diffuse colony preparation; well 2, compact colony preparation; trough, anti-9F serum.

FIG. 6. Double-diffusion analysis in agar between the purified acidic antigen of encapsulated strain 9F and crude extracts of encapsulated strain 9F and other heterologous diffuse strains and anti-9F serum. Well 1, Purified 9F antigen; well 2, crude extract of strain 9F; wells 3–5, crude extracts of encapsulated strains 14Mi, 60R, and 79-0, respectively; well 6, crude extract of strain 44B; well 7, anti-9F serum.

ical methods. Stableforth, using specific precipitin and agglutination procedures, demonstrated at least 16 types of bovine group B streptococci (7). In an extension of this observation, Pattison et al. (4), recognizing the cross-reactive X and R proteins, grouped these bovine strains into 6 types, rather than 16, on the basis of surface polysaccharides. The results presented in this study suggest that encapsulated strains of group B streptococci can be serotyped by the SSA method in spite of the fact that these organisms may also carry a multiplicity of cross-reactive cell wall-associated antigens.

Preliminary evidence suggests that the SSA method, using appropriate anticapsular sera, can be used to determine the antigenic diversity of the surface antigens of a variety of group B streptococci. For example, SSA analyses of over 80 group streptococcal strains isolated from bovine mastitis cases in Pennsylvania and possessing cross-reactive cell wall antigens revealed that 77.6% were immunologically related to strain 9F. Double-diffusion analyses in agar indicated that all of the related strains had the galactose-rich surface antigen that is,

in part, responsible for the formation of the diffuse-type growth in SSA by strain 9F streptococci.

Recently, Romero and Wilkinson (6) presented evidence indicating that encapsulated group B streptococci can be rapidly identified by the fluorescent antibody technique. Although this procedure was suggested to be effective in the identification of clinical isolates rich in capsular material, its widespread usage was in question in view of the inadequate availability of commercial, polyvalent antisera that are rich in the appropriate capsular antibodies. The importance of the capsular type-specific antigens of group B streptococci can not be overlooked; therefore, more effort should be directed toward recognizing the possible diverse capsular substances that may be present in group B streptococci.

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