IDENTIFICATION OF PROTEIN ANTIGENS OF GROUP B STREPTOCOCCI, WITH SPECIAL REFERENCE TO THE Ibc ANTIGENS

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Group B streptococci can be divided into five types: Ia, Ib, II, III, and IV, on the basis of serological precipitin reactions with their specific capsular polysaccharides (1–4). A sixth type has also been described, the Ic type, which possesses the capsular polysaccharide of type Ia strains and the protein antigens of type Ib strains (1, 2, 5). The shared protein antigens of the Ic and Ib strains have been designated the Ibc protein antigens. Two other protein antigens, the R and the X antigens, which have been found in group A streptococci, are also found in a few group B strains, more specifically, the type III strains (1, 6–8). Antibodies to either the capsular polysaccharides or the Ibc protein antigens are efficacious in mouse protection experiments, but antibodies to the R and X antigens are not (1).

Wilkinson and Eagon (2) found that the acid-extracted Ibc protein was composed of two distinct, separable antigens, the alpha and the beta antigen. The alpha antigen migrates closer to the anode upon immunoelectrophoresis and is susceptible to pepsin digestion but not to trypsin degradation, while the beta antigen is susceptible to both enzymes (2, 5, 9). The two antigens have been found to occur independently of each other in that some isolates can possess both antigens and others only one of the antigens (5, 9).

To more fully understand the nature of the Ibc protein antigens, and also to try to identify previously undetected antigens of group B streptococci, we have analyzed HCl and Triton X-100 extracts of the five prototypic strains: 090, A909, H36B5, 18RS21, and D136C, using a combination of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)[1] and electrophoretic transfer to nitrocellulose sheets. The blots were stained with polyclonal rabbit typing antisera as well as monoclonal mouse sera directed to Ibc proteins.

Materials and Methods

Bacterial Strains and Media. The prototype strains of the five serotypes, Ia, Ic, Ib, II, and III, of group B streptococci, namely, 090, A909, H36B5, 18RS21, and D136C,

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[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HAT, hypoxanthine, aminopterin, thymidine; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

respectively, were used. These were obtained from the Rockefeller University streptococcal collection assembled by the late Dr. Rebecca Lancefield and currently maintained in our laboratory. For the immunochemical studies, culturing of the bacteria was performed in a chemically defined medium (10).

Extraction of Protein Antigens. Late log phase cultures of bacteria were pelleted by centrifugation and extracted with either 0.2 N HCl, at 50°C for 2 h, or with 0.2% Triton X-100, 0.2 M NaCl, 100 mM Na acetate buffer, pH 5.5, at 37°C for 48 h. Extracted protein was precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% wt/vol, and the precipitates prepared for SDS-PAGE (11) as previously described (12). Ibc protein was purified by SDS extraction, TCA precipitation, and gel filtration, as described by Russell-Jones et al. (accompanying paper).

Antisera and Immunological Methods. New Zealand Red or hare brown rabbits were immunized with the following strains: 090R, A909, H36B5, 18RS21, D136C, and the R28 typing strain, T28. Type-specific antiserum was prepared as previously described (1). Enzyme-linked immunosorbent assays (ELISA) (13) were performed in Immulon II microtiter plates (Dynatech Laboratories, Alexandria, VA).

Nitrocellulose Blot Analysis of Antigens. SDS-PAGE in 10% acrylamide gels was performed as described previously by Laemmli (11) using a modification described by Salit and co-workers (12). The proteins were transferred from the gel to nitrocellulose paper (Schleicher and Schuell, Keene, NH) hereafter referred to as the Western blot technique (14). After electrophoresis for 1 h at 1 ampere, the nitrocellulose blot was placed in 0.5% Tween 20 (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS) for 1 h to block the remaining protein-binding sites (15). Antiserum (1:300) or human IgA (4 μg/ml) was added to the PBS-Tween and incubated with the blot for 1 h at room temperature with gentle agitation. Dr. Milan S. Blake kindly provided the serum of a patient with myeloma from which the IgA was purified by the method of Doellgast and Plaut (16). The blot was washed three times for 5 min with a solution of 0.05% Brij 35 (Pierce Chemical Co., Rockford, IL) in 0.9% NaCl and then incubated for 1 h with alkaline phosphatase–conjugated (17) goat anti-rabbit IgG or alkaline phosphatase–conjugated goat anti-human kappa light chain sera diluted 1:500 in PBS plus 0.05% Brij 35. After two further washes in Brij-NaCl and one wash in 0.2% veronal acetate buffer, pH 10.0, the alkaline phosphatase substrate, 3-indoxyl phosphate and nitro blue tetrazolium (Sigma Chemical Co.), was added to the veronal acetate buffer and incubated with the blot at 37°C (18).

Preparation of Monoclonal Antibodies. BALB/c mice were immunized by intraperitoneal injection of 0.2 mg of purified Ibc protein extracted from the Ib typing strain H36B5. Freund's incomplete adjuvant was used. After 28 d the mice received a second intraperitoneal injection of 0.2 mg of the Ibc protein in 0.2 ml of distilled water. On day 32, three mice were sacrificed and their spleens removed for fusion with NS-1 myeloma cells. After fusion, cells were plated in hypoxanthine, aminopterin, thymidine (HAT) medium and maintained on this medium until subcloning was completed, at which time they were grown on Dulbecco's modified Eagle's medium (DME) plus 20% fetal calf serum (FCS). 14 d after fusion the hybridoma supernatants were removed and tested for antibody activity using the ELISA assay. The supernatants from ELISA-positive clones were tested for antigen specificity using the Western blot technique (18). Clones that were reactive on the blots were replated onto a 96 well plate. When cultures were confluent the clones were tested again by ELISA and the most active wells were again replated. Replating and testing were repeated for a total of three subclonings, at which time the clones were assumed to be monoclonal. Subsequent limiting dilution experiments confirmed that these were indeed single clones. The clones were grown to 2 liter volumes in DME plus FCS and concentrated by precipitation with 40% saturated ammonium sulfate. The proteins were redissolved in 0.1 Tris-HCl, 0.1 M NaCl, and, after centrifugation, the supernatants were adjusted to pH 8.0 with 1 M Tris-HCl, pH 8.8 and applied to a protein A–Sepharose column (Pharmacia, Uppsala, Sweden). After washing with buffer, antibodies were eluted with 50 mM citrate buffer, pH 3.0, immediately brought to pH 8.0 with 1 M Tris, pH 8.8, and dialyzed against PBS.

Results

HCl and Triton extracts of prototypic strains of five types of group B streptococci were prepared and the protein antigens analyzed. The Western blots of the extracts of the five typing strains gave rise to a number of stained bands when allowed to react with rabbit typing serum (see Fig. 1). Numerous bands ranging in molecular weight from 130,000 to 20,000 were seen in the Ib and the Ic strains (see Fig. 1 *a*, *b*, *f*, and *g*). These antigens were only "seen" by the Ic and Ib typing sera. According to the operational definition used by the previous primary investigators (1, 2, 5) these antigens are considered to be the Ibc proteins. In contrast, antisera to type II and III serotypes (Fig. 1 *c*, *d*, *h*, and *i*) recognize antigens common to all extracts.

To study the Ibc proteins among a number of different group B strains in more detail, Triton X-100 extracts of a number of strains were applied to five SDS-PAGE gels; one was stained with Coomassie Blue (Fig. 2*A*) and the other four were blotted (Fig. 2*B–E*). Using monoclonal antibodies raised to purified Ibc proteins, it was possible to distinguish two sets of protein antigens, which were also seen by the polyclonal rabbit antisera (Fig. 2). One set of these antigens was seen by the antibody 8A9 (Fig. 2*C*), while another set was seen by a number of monoclonal antibodies and is represented by the pattern shown for antibody 10C6 (Fig. 2*D*). Experiments using differential susceptibility of the antigens to pepsin and trypsin indicated that antibody 8A9 recognized the alpha antigen and antibody 10C6 the beta antigen. The amount of each antigen varied between strains and its presence was not dependent upon the presence of the other antigen. Thus, strain A664 reacted with antibody 8A9 but not with 10C6 or similar antibodies, whereas strain 20RP279 reacted with the monoclonal antibodies represented by antibody 10C6 and not with antibody 8A9. A comparison of Fig. 2*B* with 2*C* and *D* indicates that the majority of the bands produced by the polyclonal rabbit typing serum can be accounted for as the sum of the two patterns produced by the monoclonal antibodies.

When extracts from these strains were also tested for their ability to bind IgA it was found that all the strains which reacted with antibody 10C6 also bound human IgA. In fact it appeared that a similar, but not identical pattern of binding was seen regardless of whether the antibody was that of clone 10C6 or human IgA (compare Fig. 2*D* and *E*). The IgA did not appear to bind to the proteins with which antibody 8A9 reacted (compare Fig. 2*C* and *E*).

A separate group of antigens was found in the Triton extracts of the type III strain, D136C. They varied in molecular weight from 100,000 to 130,000 and reacted with the type III antiserum and the 28R typing serum and, to a lesser degree, with type II antiserum. These antigens appeared to be somewhat acid labile (compare Fig. 1 *d* and *e* with *i* and *j*). Since it is known that the 28R antigen can occur on type III strains and is also acid labile, this group of proteins probably represents the 28R type protein antigens (1, 7, 8).

Two protein antigens were found that appeared to be common to all five strains and reacted with the anti–type II and III sera but not with the anti-Ic or anti-28R sera. The Ib antiserum appeared to weakly react with these antigens only if they were acid extracted. Presumably, antibodies to these determinants were absorbed in the preparation of the Ic typing serum, and were never present

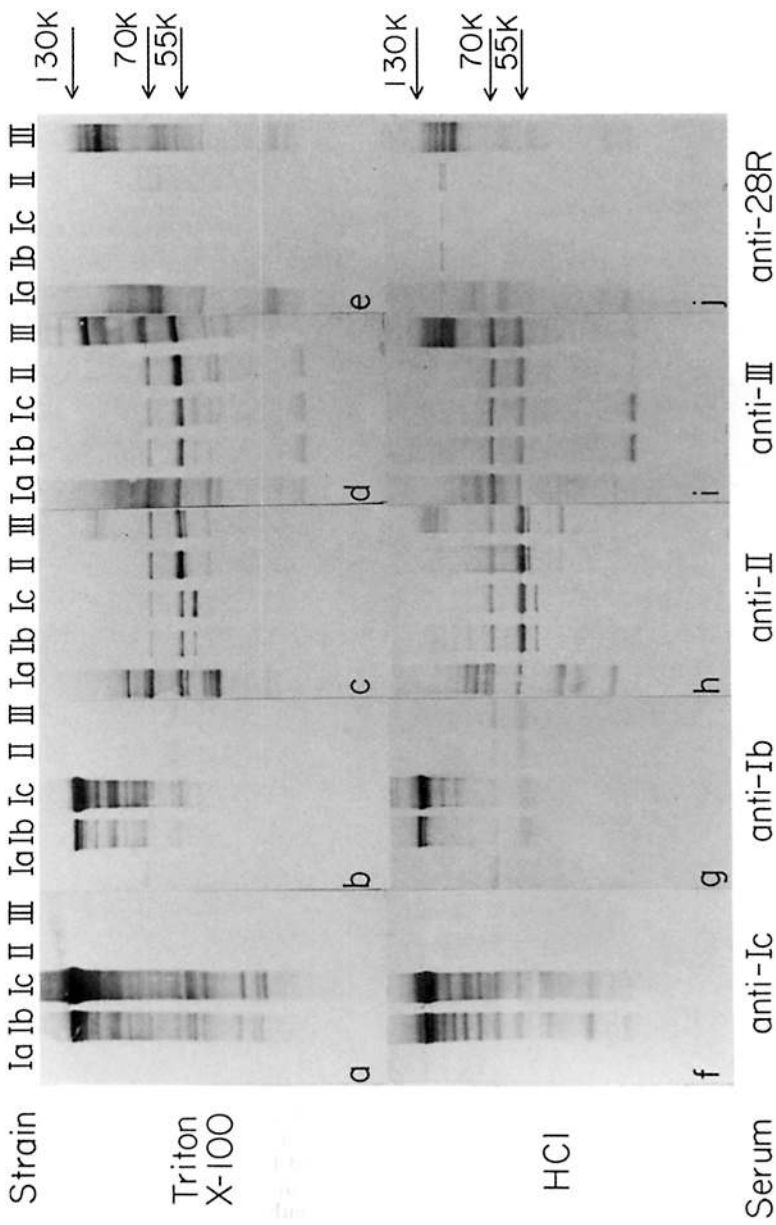

FIGURE 1. Western blot analysis of proteins extracted from the five serotypes of group B streptococci. Strains 090R, A909, H36B/5, 18RS21, and D136C, representing the typing strains of the Ia, Ic, Ib, II, and III serotypes, respectively, were extracted with HCl (*a–e*) or Triton X-100 (*f–j*), subjected to SDS-PAGE and Western blotting, and incubated with the following polyclonal rabbit typing sera: anti-Ic (*a, f*), anti-Ib (*b, g*), anti-II (*c, h*), anti-III (*d, i*), and anti-28R (*e, j*). After washing, the blots were incubated with an alkaline phosphatase–conjugated anti-rabbit IgG and then stained with the alkaline phosphatase substrate, indoxyl phosphate and nitroblue tetrazolium.

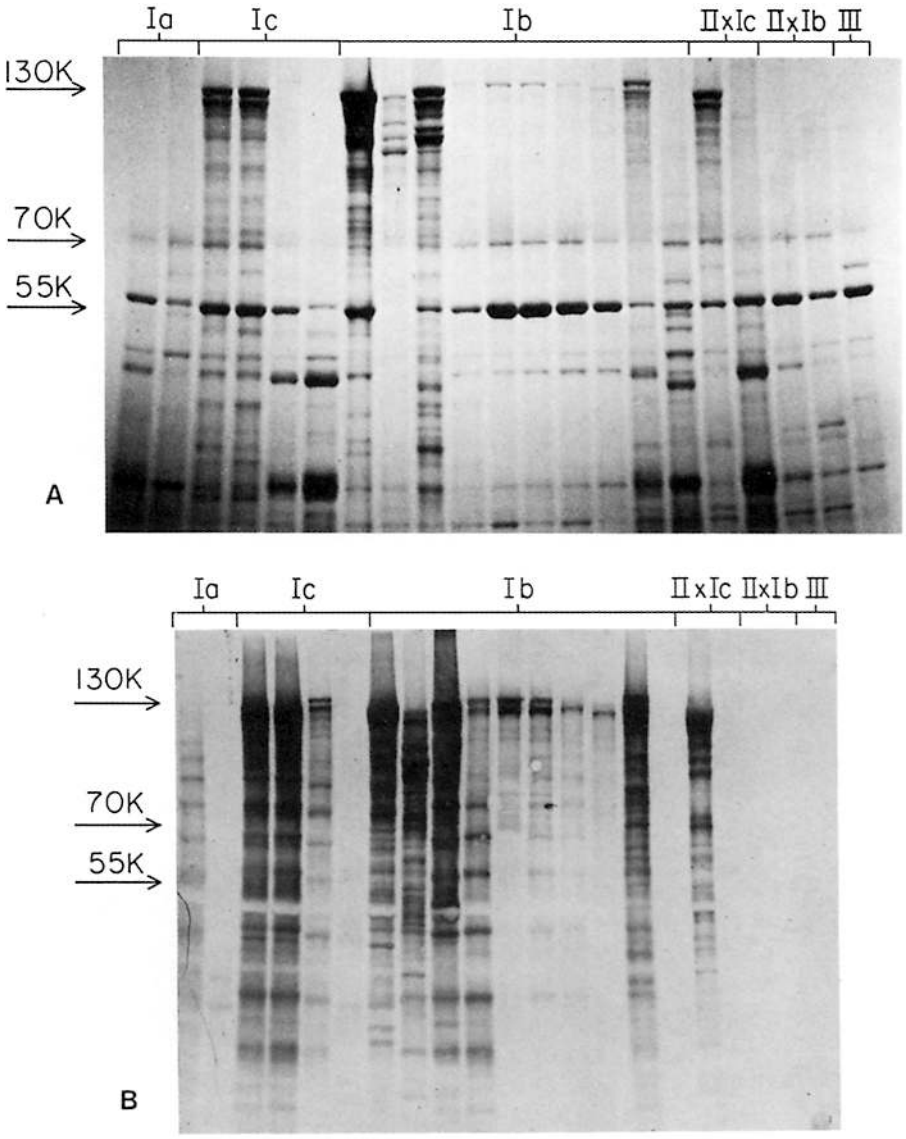

FIGURE 2. Western blot analysis of a number of strains of group B streptococci using monoclonal antibodies raised to the Ibc proteins. A number of strains of various serotypes were extracted with Triton X-100 and the extracts examined by SDS-PAGE using a 10% polyacrylamide gel. One gel was stained with Coomassie Brilliant Blue R (*A*) while duplicate gels were subjected to Western blotting and incubated with either rabbit polyclonal anti-Ic serum (*B*) or different mouse monoclonal antibodies (*C*, 8A9; *D*, 10C6). IgA was incubated with the blot in *E*. After incubation with the appropriate alkaline phosphatase–conjugated antibody, the blots were stained with the histochemical stain for alkaline phosphatase, indoxyl phosphate plus nitroblue tetrazolium. Strains are, from left to right: A664, A665 (type Ia); A909, F345-3, D521, D520 (type Ic); H36B/5, D727, C12B, A943, A707, 7RS69, B390, B631-1, F472, B125 (type Ib); 20RP279, 57RP145 (type II × Ic); F325, F326 (II × Ib); and F471 (type III).

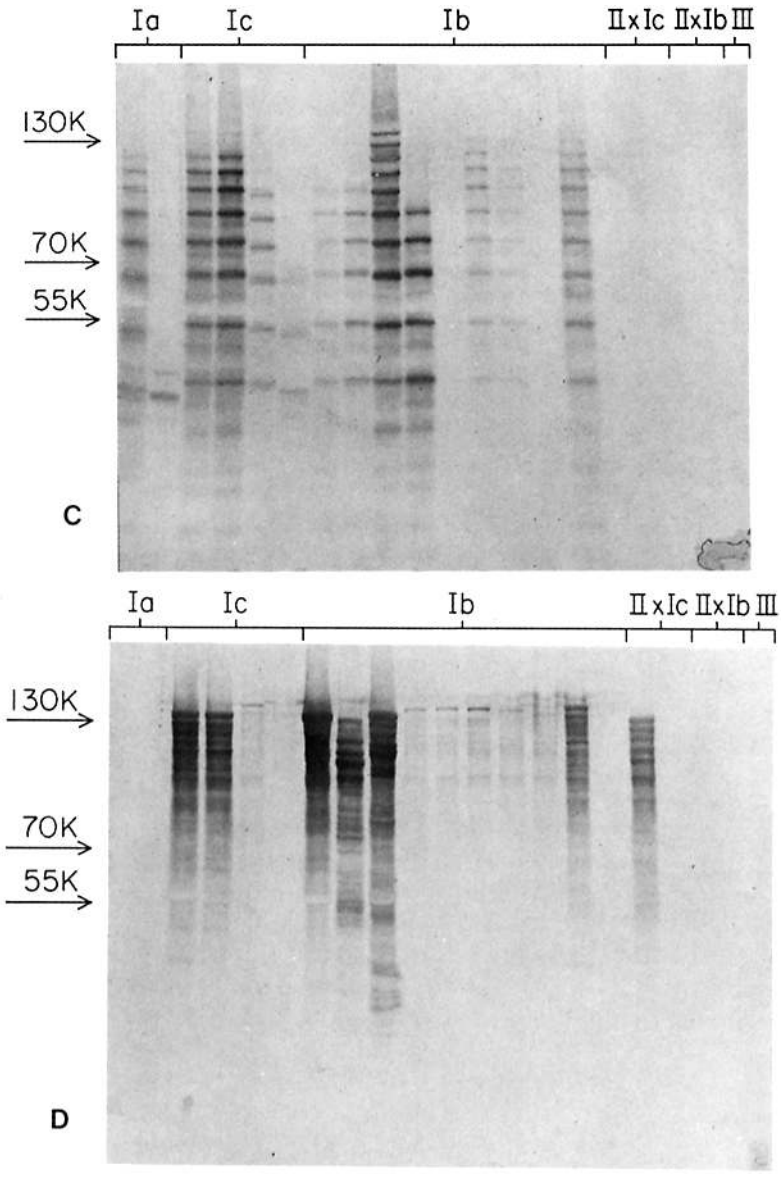
Ia
Ic
Ib
II x Ic
II x Ib
III
130K
70K
55K
C
Ia
Ic
Ib
II x Ic
II x Ib
III
130K
70K
55K
D

FIGURE 2

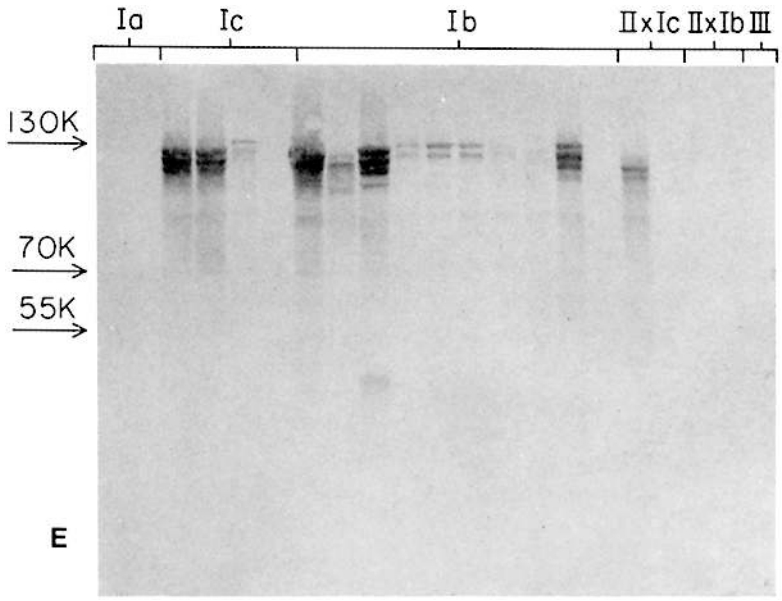

FIGURE 2

in the 28R typing serum. Both of these proteins (55,000 and 70,000 mol wt) were found in both the Triton and acid extracts of all strains.

Discussion

Type Ic and type Ib strains can be differentiated from the other group B streptococcal strains by the presence on the former strains of a number of shared protein antigens, namely, the Ibc proteins, which are not present on the latter strains. The experiments of a number of workers (2, 5, 9) have demonstrated that these protein antigens can be further divided into two antigenic subsets, the alpha and the beta antigens. Determinants of both these antigens were found to occur throughout the length of SDS-polyacrylamide gels (2). The results presented in Fig. 1 *b* and *c* and Fig. 2*B* and *C* are in agreement with the aforementioned findings and show that the Ibc proteins are composed of as many as 30 proteins varying in molecular weight from 130,000 to 20,000, but with a predominant molecular weight of 130,000. It would appear that the larger proteins are readily broken down by acid hydrolysis, or by some endogenous enzyme released by the bacteria during the later stages of log phase culture. Even when log phase bacteria are boiled in SDS to release the large protein, we have found that it is already partially degraded (unpublished observations). Such breakdown of the Ibc proteins was previously suggested by Wilkinson and Eagon (2).

Experiments with monoclonal antibodies have shown that this group of proteins is composed of two antigenically distinct sets of proteins. One set is composed of the breakdown products of a larger 130,000 mol wt "parent" protein and is recognized by antibody 10C6 and the like, as well as the polyclonal

Ib and Ic typing sera. Many of these bands also have the capability of binding human IgA. Another set of proteins was seen by the antibody 8A9 and the Ic typing serum and consisted of at least 12 antigenically similar proteins occurring at regularly spaced intervals throughout the gel, ranging from 10,000 to 120,000 mol wt. It would appear that the antigens seen by the monoclonal antibodies represented by antibody 10C6 occur on a different set of proteins than the antigens seen by antibody 8A9. Furthermore, the amount of each protein varies independently from strain to strain, with some strains having both antigens while others had only one.

Type III, strains of group B streptococci have previously been shown (6–8, 19) to react with an antiserum prepared against group A, type 28 strains. This cross-reactivity is due to the presence of the protein antigen(s), 28R, which has been found in both of these types. This protein appears to be unrelated to the virulence of the streptococci against mice (6–8, 19). A number of proteins were found on the type III strain that reacted strongly with the type III antiserum and the type 28 antiserum. These proteins (100,000–130,000 mol wt; Fig. 1,*i* and *j*) appear to be somewhat acid labile (compare Fig. 1*j* and *e*) in accord with the findings of others (19, 6–8) who found that the anti-28R reactivity is lost upon prolonged heating at pH 2.0.

Two previously undescribed protein antigens (55,000 and 70,000 mol wt) were found to occur in both the Triton and acid extracts of each of the five strains tested (Fig. 1,*b*, *c*, *d* and *g*, *h*, *i*). These two proteins could represent potentially important antigens, especially if antisera to these proteins can be shown to be protective.

Summary

The protein antigens of prototypes of five types of group B streptococcal strains were extracted with HCl or Triton X-100, separated by sodium dodecyl sulfate polyacrylamide electrophoresis, transferred to nitrocellulose, and examined by immunochemical staining. The Ibc proteins are shown to consist of at least two distinct protein antigens and their breakdown products. One antigen, the "beta" antigen, exists primarily as a 130,000 mol wt protein that is also able to bind human IgA. The "alpha" antigen, which has no known function, appears as a number of proteins of various molecular weights from 20,000 to 120,000. Another set of antigens, the R protein antigens of type III strains, has been identified as a group of acid-labile proteins varying in molecular weight from 100,000 to 130,000. In addition, two previously undescribed antigens have been found that are common to all five group B types.

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