Antigenic Determinants of Alpha-Like Proteins of *Streptococcus agalactiae*

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The majority of group B streptococcus (GBS) isolates express one or more of a family of surface-anchored proteins that vary by strain and that form ladder-like patterns on Western blotting due to large repeat units. These proteins, which are important as GBS serotype markers and as inducers of protective antibodies, include the alpha C (Cα) and R4 proteins and the recently described alpha-like protein 2 (Alp2), encoded by *alp2*, and Alp3, encoded by *alp3*. In this study, we examined antigenic determinants possessed by Alp2 and Alp3 by testing of antibodies raised in rabbits, mainly by using enzyme-linked immunosorbent assays (ELISA) and an ELISA absorption test. The results showed that Alp2 and Alp3 shared an antigenic determinant, which may be a unique immunological marker of the Alp variants of GBS proteins. Alp2, in addition, possessed an antigenic determinant which showed specificity for Alp2 and a third determinant which showed serological cross-reactivity with Cα. Alp3, in addition to the determinant common to Alp2 and Alp3, harbored an antigenic site which also was present in the R4 protein, whereas no Alp3-specific antigenic site was detected. These ELISA-based results were confirmed by Western blotting and a fluorescent-antibody test. The results are consistent with highly complex antigenic structures of the alpha-like proteins in a fashion which is in agreement with the recently described structural mosaicism of the *alp2* and *alp3* genes. The results are expected to influence GBS serotyping, immunoprotection studies, and GBS vaccine developments.

*Bacterial strains.* GBS strains used in this study were reference, prototype, and clinical isolates described in previous reports (10, 20). For the preparation of the protein antigens Cα, Alp2, Alp3, and R4, the following GBS strains were used: NCTC 12906 (strain 335, serotype Ia/Cα), which is negative for the genes *alp* and *rib*; strain ATCC 12403 (D136C) (serotype III/R1 *alp2*), which has been subjected to complete genome sequencing and is negative for *bca*, *alp3*, and *rib* (7); the clinical strain 64/95 (serotype V/R1 *alp3*), which is *alp2* and *rib* negative; and strain 65604 (serotype IV/R4 *rib*), which is *alp* negative. Strain 64/95, not strain JM9, was initially chosen for the preparation of Alp3, since it was reported that JM9 was targeted by anti-R4 serum (13). The isolates were cultured on Stretches of the ladder-forming proteins show sequence homology to a considerable extent (12, 25), consistent with the structural mosaicism of these proteins (12). This could explain the serological cross-reactivity between these proteins, such as that between Cα and Alp2 (12, 13), and the reason that an anti-R1 protein serum recognized both GBS strains which possessed *alp2* encoding Alp2 and strains which possessed *alp3* encoding Alp3 (Maeland and Lyng, Abstr. 13th Eur. Cong. Clin. Microbiol. Infect. Dis.). As these proteins (i) play a role as serotype markers, (ii) may be important in the pathogenesis of GBS disease, and (iii) may be considered vaccine candidates, there is a need for further clarification of their immunological features.

The aim of the present study was to clarify antigenic determinants harbored by Alp2 and Alp3. The results show (i) that Alp2 possesses at least three sites for antibody binding and Alp3 possesses at least two sites, all of which have distinct immunological specificities, and (ii) that some of these sites probably are protein specific, whereas other sites caused serological cross-reactivity between some of the ladder-forming GBS proteins.

*Materials and Methods.*

*Bacterial strains.* GBS strains used in this study were reference, prototype, and clinical isolates described in previous reports (10, 20). For the preparation of the protein antigens Cα, Alp2, Alp3, and R4, the following GBS strains were used: NCTC 12906 (strain 335, serotype Ia/Cα), which is negative for the genes *alp* and *rib*; strain ATCC 12403 (D136C) (serotype III/R1 *alp2*), which has been subjected to complete genome sequencing and is negative for *bca*, *alp3*, and *rib* (7); the clinical strain 64/95 (serotype V/R1 *alp3*), which is *alp2* and *rib* negative; and strain 65604 (serotype IV/R4 *rib*), which is *alp* negative. Strain 64/95, not strain JM9, was initially chosen for the preparation of Alp3, since it was reported that JM9 was targeted by anti-R4 serum (13). The isolates were cultured on
blood agar plates or in Todd-Hewitt broth (4). All isolates included in this study were tested by PCR for possession of the \( \text{alp} \) and \( \text{rib} \) genes.

**Antiserum.** Rabbit antiseria against GBS whole cells or against the purified proteins \( \text{Ca} \) and \( \text{R4} \) were raised as described in previous reports (2, 3). Murine monoclonal antibodies against \( \text{Ca} \) (1) and \( \text{R4} \) (2) were used in enzyme-linked immunosorbent assays (ELISA) to identify these proteins in gel filtration fractions. A rabbit anti-\( \text{R1} \) protein serum raised against the \( \text{alp} \)-positive strain ATCC 12403 (20) was used for identification of \( \text{Alp} \).

**Immunological techniques.** Absorption of antiseria was performed either with an excess of whole cells of GBS, i.e., with a volume of bacterial pellet at least four times the volume of undiluted antiserum, or with graded densities of bacteria. In the latter experiments, fivefold dilutions of a GBS suspension of \( \sim 10^{10} \) bacteria ml\(^{-1} \) were prepared, and an equal volume of appropriately diluted antiserum was added. Absorption was carried out at 20°C for 60 min and followed by centrifugation at \( 10^3 \times g \) for 15 min. All dilutions were made with phosphate-buffered saline, pH 7.2 (PBS), which contained 0.02% Na\(_2\)N\(_3\) or 0.05% Tween 20.

ELISA was performed as described previously (17) using reagents in 50-µl volumes for testing in duplicate and alkaline phosphatase-conjugated anti-immunoglobulin G antibodies (Sigma). The ELISA titer was defined as the reciprocal of the highest serum dilution resulting in an optical density at 405 nm (OD\(_{405}\)) of at least 0.200 above the background, which was determined by testing without antigen. In the ELISA absorption test with graded doses of bacteria, we used antiserum in a final dilution which signaled an OD\(_{405}\) in the 1.000-to-1.500 range when it was tested unabsorbed. In these experiments, percent OD\(_{405}\) reduction was calculated. A reduction of \( \geq 20\% \) from the OD\(_{405}\) signaled by the positive control was considered evidence of antibody binding by the bacteria used for absorption. This limit was based on the OD reductions which were recorded when strains without the appropriate protein and gene were tested.

**Fluorescent-antibody testing.** Fluorescent-antibody testing was performed and signaling was recorded as described previously (3). Western blotting of sodium dodecyl sulfate-solubilized whole cells of GBS strains and probing with antibodies were performed according to a previously reported method (19).

**Purification of antigens.** The protein antigens \( \text{Ca} \), \( \text{Alp2} \), \( \text{Alp3} \), and \( \text{R4} \) were prepared by acid extraction of whole cells of GBS. Bacteria washed with PBS-Na\(_2\) were extracted with 5% (wt/vol) trichloroacetic acid at 4°C for 20 h. After centrifugation, the sediment with the protein which had been released and precipitated with the acid was suspended in PBS and solubilized by neutralization followed by centrifugation at \( 10^3 \times g \) for 15 min. Constituents in the supernatant were again precipitated with trichloroacetic acid (5%), taken up in PBS-Na\(_2\), and applied to a Sephacryl S-200 HR column (Amersham Pharmacia Biotech AB), which was eluted with PBS-Na\(_2\) as described previously (21). Fractions were diluted 1:10 and used as coating antigens in ELISA for antigen-based detection of the protein. Fractions which contained the protein of interest were pooled, dialyzed against 10 mM Tris buffer, pH 8.0 (basic buffer), and applied to a DEAE Sephacel column (Amersham Pharmacia Biotech AB) equilibrated with the basic buffer. Stepwise elution with increasing concentrations of NaCl up to 0.5 M NaCl in the basic buffer was performed. Nearly all of the various proteins were eluted in the range of 0.1 to 0.25 M NaCl, and this material was used as the antigen in ELISA after testing for the optimal concentration for coating by checkerboard titration. The immunological homogeneity of the protein was assessed as described previously (21).

**Oligonucleotide primers.** Primer pairs for the \( \text{alp} \) and \( \text{rib} \) genes were constructed (Eurogentech S.A., Liege, Belgium) according to recommended specifications (9) and were as follows: for \( \text{alp2} \) (GenBank no. AF208158), the pair was ba23S1-bal2A2, and the estimated length of the amplicon was 426 bp; for \( \text{alp3} \) (GenBank no. AF245663), the pair was ba23S1-bal2A3, and the length of the amplicon was 321 bp; for \( \text{alp} \) (alp2 plus alp3), the pair was bcaS1-balA, and the length of the amplicon was 446 bp; and for rib (GenBank no. US8333), the pair was ribS2-ribA2, and the length of the amplicon was 225 bp.

**PCR.** For all primer sets, PCR was performed in every detail as described previously (15), including detection of the PCR products by electrophoresis in 2% (wt/vol) agarose gels. Testing of reference and prototype GBS strains confirmed the previously described specificity of the PCRs for the \( \text{alp} \) and \( \text{rib} \) genes (9).

**RESULTS AND DISCUSSION**

**Purification of antigens.** Harvested bacteria of the GBS strains ATCC 12403 (III/R1 \( \text{alp2} \)) and 64/95 (V/R1 \( \text{alp3} \)), both of which were \( \text{rib} \) negative by PCR, and the \( \text{alp} \)-negative strains 335 (Ia/Ca) and 65604 (III/R4 \( \text{rib} \)) were extracted with trichloroacetic acid, and the material was applied to a Sephacryl S-200 HR column. The fractions were tested for the presence of \( \text{Alp2} \) or \( \text{Alp3} \) by probing with an anti-\( \text{R1} \) protein serum (20), which could be done due to the cross-reactivity of \( \text{R1} \) antibodies with \( \text{Alp} \) (12, 13), and for the presence of \( \text{Ca} \) or \( \text{R4} \) by probing with the appropriate monoclonal or polyclonal antibodies (1, 2). All four proteins were separated from vast amounts of constituent material that absorbed UV light as shown in Fig. 1 for \( \text{Alp2} \) and appeared at or shortly after the void volume fraction of the column. When we tested fractions with antiserum against whole cells of the bacteria, signaling in ELISA differed little from that recorded with the \( \text{R1} \)-specific antiserum. The recognition of \( \text{Alp3} \) by anti-\( \text{R1} \) antibodies was similar to the recognition of \( \text{Alp2} \) shown in Fig. 1. Materials in the pooled fractions which contained the protein were then subjected to ion-exchange chromatography. The eluted material was used as the antigen in ELISA.

**Reactivity and cross-reactivity of antibodies.** Table 1 shows...
TABLE 2. Results of absorption of antibodies against antigenic Alp2 and Alp3 determinants with reference and prototype GBS strains

<table>
<thead>
<tr>
<th>GBS strain used for absorption</th>
<th>OD&lt;sub&gt;405&lt;/sub&gt; reduction (%) in test system&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 12403 (III/R1 alp2)</td>
<td>97 89 37 3</td>
</tr>
<tr>
<td>64/95 (V/R1 alp3)</td>
<td>86 0 8 97</td>
</tr>
<tr>
<td>ATCC 49447 (V/R3)</td>
<td>6 0 3 0</td>
</tr>
<tr>
<td>65604 (III/R4 rib)</td>
<td>0 5 0 98</td>
</tr>
<tr>
<td>NCTC 12906 (Ia/Cα)</td>
<td>0 0 95 0</td>
</tr>
<tr>
<td>15626/81 (IV/Cβ)</td>
<td>10 4 0 0</td>
</tr>
</tbody>
</table>

<sup>a</sup> For definition of test systems, see Table 1, which shows that Alp2 was used as the antigen in test systems 1, 2, and 3, and that Alp3 was used in test system 4.

Results obtained with antisera tested against all four protein antigens. In addition to the expected homologous reactivity, this testing revealed cross-reactivity between Alp2 and Alp3, between Alp2 and Cα, and between Alp3 and R4. These results confirmed those of earlier reports on cross-reactivity between laddering streptococcal proteins (13, 23), observations which prompted the inclusion of Cα and R4 in the present study on the alpha-like proteins. Results in accordance with those shown in Table 1 were obtained with additional antisera. Another anti-Alp2 serum tested also showed low or no antibody activity against Cα, whereas different anti-Cα sera targeted Alp2, although with comparatively low antibody activity. Antisera against whole cells of GBS 335 (Ia/Cα) or 65604 (III/R4 rib) showed results similar to those obtained with the antisera against purified Cα or R4, as shown in Table 1. Monoclonal or polyclonal antibodies against Cβ (22) or R3 (10) showed no binding by any of these antigens (not shown).

Normal rabbit sera showed titers of ≤400. The results indicate that the alpha-like proteins harbor several distinct antigenic determinants. These determinants were further explored by using the antigen-antibody combinations called test systems 1 to 4 in Table 1 and by recording signaling in ELISA after further cross-absorption of the antisera used in the four test systems. In this setting, Alp2 was the coating antigen in systems 1, 2, and 3, and Alp3 was the coating antigen in system 4.

Alp2-associated antigenic determinants. Table 2 shows the results of one of several experiments with concordant results. The results obtained for test system 1 confirmed the existence of an antigenic determinant shared by Alp2 and Alp3, here named Alp2/Alp3 common. Testing of three other alp2-positive strains, the only alp2 strains detected among 48 clinical GBS strains examined, and of five alp3-positive clinical strains by the absorption test also revealed expression of Alp2/Alp3 common by these isolates, whereas all five alp-negative strains showed negative results. No cross-reactivity of Alp2/Alp3 common with R3, R4, Cα, or Cβ was observed (Table 2). These results accord with the notion that Alp2/Alp3 common may be a unique marker of the Alp variants of GBS proteins, although only a few strains have been tested so far. Since antibodies in antisera specific for R1 targeted both Alp2 and Alp3, the Alp2/Alp3 common site may be the target for antibodies in anti-R1 sera used for serotyping (5, 20), supporting the previous suggestion that Alp2 and Alp3 may be variants of the R1 protein (Maeland and Lyng, Abstr. 13th Eur. Cong. Clin. Microbiol. Infect. Dis.). The sequences of the 172-amino-acid-long N-terminal region of Alp2 and Alp3 matched completely (12), and this region may harbor the Alp2/Alp3 common site, although this possibility is conjectural at present.

Only the alp2-positive strain 12403, among those shown in Table 2, recognized the antibodies in test system 2. Three additional alp2 strains performed similarly to 12403 in test system 2, but none of five alp3 strains or five alp-negative strains did. Since the determinant involved in test system 2 was not present in Cα, Cβ, R3, or R4, it may be unique to the Alp2 proteins. This antigenic determinant, to our knowledge not recognized previously, we have provisionally called Alp2 specific. Alp2 specific should be useful in the identification of Alp2 by antibody-based methods. Its location in the Alp2 molecule remains enigmatic.

The serological cross-reactivity between Alp2 and Cα shown in Table 1 and previously noted by other investigators (12, 13) was confirmed by ELISA absorption in test system 3 (Table 2) and was caused by an antigenic determinant (Alp2/Cα common) which was harbored by only the Alp2- or Cα-expressing strains. It seems that Alp2/Cα common was only weakly immunogenic when it was located in Alp2, and Alp2 strain 12403 ranged far below Cα strain 335 in its capacity to neutralize the anti-Alp2/Cα common antibodies which had been generated by immunization with Cα (Fig. 2a). Also, this antibody caused only weak binding with an atypical pattern in Western blot analyses against Alp2 (Fig. 3B, lane 2), compared to the pat-
tern seen with anti-Alp2-specific antibodies (Fig. 3B, lane 1). These findings accord with the notion that the serological cross-reactivity between the Alp2 and Co common sites was only partial, presumably due to imperfect structural matching of these sites. A possible location for Alp2/Co common in Alp2 could be an 82-amino-acid stretch towards the C terminus which showed an 88% match with Co (lane 2), and normal rabbit serum (lane 3); lanes 4 and 5, blots of strain 64/95 (V/R1 alp3) probed with anti-Alp3/R4 common antibodies raised against Alp3 (lane 4) and R4 (lane 5). All sera were used at a dilution of 1:400.

Alp3-associated antigenic determinants. Experiments using test system 4 (Table 2) with Alp3 as the coating antigen confirmed the cross-reactivity found between Alp3 and R4 shown in Table 1. The results were the same whether the antibodies used in system 4 had been raised against Alp3 or against R4. We have attributed this cross-reactivity to a shared antigenic determinant (Alp3/R4 common) which was not present in Alp2, Co, Cβ, or R3. This means that Alp3 possessed at least two antigenic determinants: Alp2/Alp3 common and Alp3/R4 common. An Alp3-positive and an R4-positive strain showed equally strong capacities to bind to the Alp3/R4 common antigenic antibodies (Table 2 and Fig. 2b), consistent with the identity or near-identity in immunological specificity of the Alp3- and R4-associated Alp3/R4 common sites. Repeats of Alp3 and of protein Rib (24), which probably is identical to R4 (Smith et al., Abstr. 15th Lancefield Int. Symp. Streptococci Streptococcal Dis.), matched by 99% (12) and are likely sites for the harboring of the Alp3/R4 common site. Testing of five alp3-positive but rib-negative strains showed results similar to that recorded for strain 64/95, and testing of five clinical rib-positive but alp3-negative strains showed results similar to that recorded for strain 65604 (Table 2). These data are consistent with earlier findings that protein Rib showed cross-reactivity with the Streptococcus pyogenes protein R28 (23), which later was shown to have 98% sequence homology with Alp3 (12, 23) and thus probably can be considered an S. pyogenes variant of the GBS Alp3 protein. As both alp3/Alp3 and rib/R4 occur with high frequency in GBS, usually in strains of different capsular antigen types (9), the Alp3/R4 common site will be expressed more often than either of the two proteins. It is important to be aware of this and of other shared antigenic determinants when preparing antisera for GBS serotyping or for other purposes. For instance, antisera raised against R4 will target Alp3-positive but R4-negative GBS strains, resulting in false typing results, unless the antibodies targeting the Alp3/R4 common site have been removed. None of our data indicated the existence of an Alp3-specific determinant analogous to the Alp2-specific determinant. This fact will hamper antibody-based detection of Alp3, since signaling in tests with both anti-Alp2/Alp3 common and anti-Alp3/R4 common antibodies will be required for its identification by serological means. On the other hand, we cannot exclude the possibility that an Alp3-specific immunological marker may exist but has escaped detection by us.

Immunofluorescence testing and Western blotting. The antisera used in the ELISA absorption tests were further cross-absorbed by one or two additional reference or prototype GBS strains as required in order to prepare sera specific for each of the antigenic Alp determinants. The absorbing strains were selected on the basis of serotype, cross-reactivity in the fluorescent-antibody test, and possession of the alp and rib genes. The final sera were tested by immunofluorescence against all of our 15 reference and prototype GBS strains and by Western blotting against whole-cell lysates of GBS. This testing confirmed the results obtained in ELISA and ELISA absorption tests with respect to antigenic Alp determinants. Examples of the Western blotting results are shown in Fig. 3. The Alp2/Alp3 common antibodies (Fig. 3A), Alp2-specific antibodies (Fig. 3B, lane 1), and Alp3/R4 common antibodies (Fig. 3B, lanes 4 and 5) generated ladder-like banding patterns and showed cross-reactivity as expected on the basis of the ELISA-based testing. The Alp2/Co common antibodies diverged from the other antibodies in that they generated only a single band (Fig. 3B, lane 2).

Comments. This study has shown that the alpha-like proteins are highly complex immunologically, with antigenic sites which may be protein specific and sites which are identical or nearly identical in immunological specificity to antigenic sites in other laddering GBS proteins. These findings are consistent with the structural mosaicism and sequence homologies of the genes encoding these proteins (12). Also, we cannot exclude the possibility of the existence of antigenic Alp sites not unveiled in this study, such as an Alp3-specific site analogous to the Alp2-specific site.

The results obtained by the immunofluorescence and ELISA absorption tests have confirmed that the antigenic Alp sites described here are available for antibody binding at the bacterial cell surface, at least in some GBS strains. This finding is consistent with earlier observations that antisera prepared against purified Alp induced protection in experimental infection models (13, 23), as did an anti-R1 serum prepared from anti-whole-cell serum (20). However, it may be that antisera used in the protection studies contained antibodies against more than one of the antigenic determinants harbored by that Alp, meaning that the contribution to the protective activity of antibodies against each antigenic determinant is not known. This is a challenge that should be addressed by further immunoprotection studies, as it should be important to identify the
antigenic domain with the highest protective potential, notably in the context of GBS vaccine development. The results of the present study have substantiated the idea that it is possible to prepare site-specific anti-Alp sera for use in experiments along these lines. Of particular interest would be the protective potential of antibodies against the determinant Alp3/R4 common, as this site is prevalent in GBS, notably in serotype III and serotype V isolates (9, 11, 18). Clarification of other immunobiological functions of site-specific anti-Alp antibodies, such as their role in GBS-epithelial cell (23) and GBS-phagocyte interactions, is desirable. Not least, site-specific anti-Alp-specific antibodies have potential in GBS serotyping. Such studies would benefit from more-detailed knowledge of the molecular structures of the antigenic Alp sites, like the information which could be gained by epitope mapping.

REFERENCES