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Novel Aspects of the Z and R3 Antigens of *Streptococcus agalactiae* Revealed by Immunological Testing

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Group B streptococci (GBS) are important human and bovine pathogens which can be classified by a variety of phenotype- and gene-based techniques. The capsular polysaccharide and strain-variable, surface-anchored proteins are particularly important phenotypic markers. In an earlier study, a previously unrecognized protein antigen called Z was described. It was expressed by 27.2% of GBS strains from Zimbabwe, usually in combination with R3 protein expression. In this study, a putative Z-specific antiserum actually contained antibodies against two different antigens named Z1 and Z2; Z1 was >250 kDa in molecular mass. Z1, Z2, and R3 generated multiple stained bands on Western blots and showed similar chromatographic characteristics with respect to molecular mass, aggregate formation, and charge. Of 28 reference and prototype GBS strains examined, 8/28 (28.5%) isolates expressed one, two, or all three of the Z1, Z2, and R3 antigens; 4/28 expressed all three antigens; 2/28 expressed Z2 and R3; 1/28 expressed Z1 only; and 1/28 expressed R3 only. Twenty (71.5%) of the 28 isolates expressed none of the three antigens. Expression of one or more of these antigens was shown by isolates of the capsular polysaccharide types Ia, Ib, V, and IX and NT strains and occurred in combination with expression of various other strain-variable and surface-localized protein antigens. When used as serosubtype markers, Z1, Z2, and R3 affected existing GBS serotype designations for some of the isolates. For instance, the R3 reference strain Prague 10/84 (ATCC 49447) changed serotype markers from V/R3 to V/R3, Z1, and Z2. Other isolates may change correspondingly, implying consequences for GBS serotyping and research.

Subspecies classification of *Streptococcus agalactiae* (group B streptococci [GBS]) is important in epidemiological settings and in efforts to identify highly virulent variants of the bacterium, which is an important pathogen in humans, notably in neonates. A variety of typing techniques have been used for this purpose, such as phenotypic marker-based and/or gene-based techniques. For instance, by use of antibody-based and molecular methods such as multilocus sequence typing (MLST) (1) or restriction digest pattern (RDP) typing (2), GBS clones with particularly high virulence for neonates have been identified (1, 2). The highly virulent variants have been associated with a gene called *srr-2* and expression of a surface-linked marker, the ε antigen, which may contribute to the increased virulence (3). Other gene elements and gene products also characterize the highly virulent GBS isolates (4, 5). In addition to a core genome shared by all GBS, GBS genomes contain a large number of strain-variable genes which may encode virulence factors such as strain-variable and surface-linked proteins which may function as adhesins and/or invasins, as targets of protective antibodies (meaning potential vaccine candidates), as enzymes, and as markers for serosubtyping of GBS already serotyped on the basis of the capsular polysaccharide (CPS) antigen (6, 7). These surface-attached proteins, many of which have been well characterized and the genes of which have been sequenced, include the alpha-like proteins (Alp) Cα (encoded by the *bca* gene), Alp1 (*alp1*), Alp2 (*alp2*), Alp3 (*alp3*), Alp4 (*alp4*), and R4 (*rib*) and the non-Alp proteins Cβ (*bac*) and R3 (6).

The R3 protein has been known for many years (8) and has been immunologically characterized to some extent (9–11) but not sequenced. It was expressed by 6.5% of clinical GBS strains from Norway (9) but was expressed by 24% and 21.5%, respectively, of the isolates of two different carrier GBS strain collections from Zimbabwe, where it occurred with particularly high frequency, about 80%, in the CPS type V isolates (10, 12). These findings suggest a major role(s) of the R3 protein in GBS from certain geographical areas. In earlier experiments by some of us employing antiserum which was considered specific for the R3 protein, the R3 antiserum nevertheless contained antibodies against an additional surface-attached and strain-variable GBS protein which was named the Z antigen (11). This observation was pursued by further antibody-based experiments described in the present study, which, among other findings, showed a greater complexity than previously recognized of strain-variable antigens expressed by the R3 reference strain Prague 10/84 (ATCC 49447; serotype V/R3) and several other isolates.

MATERIALS AND METHODS

Bacterial strains. Reference and prototype strains included in the GBS strain collection of this laboratory were selected for testing. All the isolates are shown in Tables 1 and 2, and many of the isolates have been used in earlier studies (10, 11). The CMFR30 strain was included in a carrier GBS strain collection from Zimbabwe (10, 11). Isolates were cultured on blood agar plates or in Todd-Hewitt broth at 35°C for 20 h or for 2 days when used for preparation of whole-cell suspensions for antigen coating of enzyme-linked immunosorbent assay (ELISA) plates.

Serotyping. All isolates included in this study have been tested for CPS type and serosubtype by molecular serotyping methods as described ear-

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TABLE 1 Reference and prototype GBS strains which expressed one or more of the antigens Z1, Z2, and R3, detected by fluorescent antibody testing

Strain	MLST	Result for FAT with antiserum to antigen:			Proposed serotype[d]
		Z1	Z2	R3	
08-07[a]	88	0	3+	3+	**Ia/Alp3, R3**, Z2
CMFR30[a]	8	3+	0	0	**Ib/Cα, Cβ**, Z1
Prague 10/84 (ATCC 49447)	26	3+	3+	3+	**V/R3**, Z1, Z2
08-17[a]	26	3+	3+	3+	**V/R3**, Z1, Z2
04-534[b]	130	3+	3+	3+	**IX/Cα, Cβ, R3**, Z1, Z2
00-884[b]	130	3+	3+	3+	**IX/Cα, Cβ, R3**, Z1, Z2
Prague 24/60 (NCTC 9829)	ND[c]	0	3+	3+	**NT/R3**, Z2
Prague 25/60 (NCTC 9828)	67	0	0	3+	**NT/Alp4, R3**

[a] Prototype strains of the Department of Medical Microbiology, St. Olav's University Hospital, Trondheim, Norway.
[b] Received from Statens Seruminstitut, Copenhagen, Denmark (13).
[c] ND, not determined (repeated attempts to sequence the *glcK* gene failed to provide a reliable sequence).
[d] Serotype and serosubtype markers known before the present study are in bold.

lier (10); many of the strains were also typed by antibody-based typing tests (14, 15).

MLST. Multilocus sequence typing (MLST) was performed as described elsewhere (1). For strains with MLST results described in the literature, our results agreed with those previously reported.

Bacterial extracts. HCl extraction of GBS whole cells was used to prepare antigen-containing extracts as described earlier (11). Briefly, bacteria were treated with 0.2 M HCl at 50°C for 2 h with frequent stirring, neutralization of the suspension by NaOH, and centrifugation, and the supernatant was precipitated with 5% (wt/vol) trichloroacetic acid and then dissolved in phosphate-buffered saline (PBS) (pH 7.2) and precipitated in 70% saturation of ammonium sulfate. The final precipitate was dissolved in PBS with 0.02% (wt/vol) NaN_3, and 4 ml g^{-1} of bacterial pellet was extracted.

Chromatographic separations. HCl-extracted materials were separated by sieve chromatography as described previously with a Sephacryl S-300 HR column (Amersham Pharmacia Biotech AB, Uppsala Sweden) and elution with PBS-NaN_3. A DEAE Sephacel column (Amersham Pharmacia Biotech AB) was used for ion-exchange chromatography (pH 8.0) with elution by stepwise increments of NaCl from 0 to 0.5 M concentrations (11). An immunoadsorption assay was performed using a CNBr-activated Sepharose 4B column (Amersham Pharmacia) according to the recommendations of the manufacturer. Briefly, antiserum to the GBS strain Prague 25/60 (NCTC 9828; serotype NT/Alp 4, R3) was precipitated by 50% saturation of ammonium sulfate, immunoglobulins were bound to the column material, and GBS materials were applied and eluted with PBS-NaN_3 and then with a glycine-HCl buffer (pH 2.6) containing 0.5 M NaCl. All fractions from the chromatographic separations were diluted 1:5 in coating buffer and then used for antigen coating of ELISA plates for probing with appropriate antisera. We considered that the levels of optical density at 405 nm (OD_{405}) recorded in ELISA reflected the concentrations in the fractions of the antigen targeted by the antibodies.

TABLE 2 Reference and prototype GBS strains without detectable expression of any of the antigens Z1, Z2, and R3

Strain	MLST	Serotype
A909 (NCTC 11078)	7	Ia/Cα, Cβ
515 (BAA-1177)	23	Ia/Alp1
090R (NCTC 9993)	25	Ia/Alp1
70339 (NCTC 12907	7	Ia/Cβ, R4/*rib*
335 (NCTC 12906)	23	Ia/Alp1
H36B (NCTC 8187)	6	Ib/Cα, Cβ
18RS21 (NCTC 11079)	19	II/R4/*rib*
08-60[a]	19	II/R4/*rib*
NEM316 (ATCC 12403)	23	III/Alp2
COH1 (BAA-1176)	17	III/R4/*rib*
65604[a]	19	III/R4/*rib*
BM110[b]	17	III/R4/*rib*
15626/81[a,c]	10	IV/Cα, Cβ
3139 (ATCC 49446)	2	IV/Alp1
2603 (BAA-611)	110	V/R4/*rib*
CJB111 (BAA-23)	1	V/Alp3
NT6 (CCUG 29785)	14	VII/Alp1
7271 (Jelinkova)	1	VII/Alp3
JM9(Jelinkova)	1	VIII/Alp3
161757[a,c]	1	V/Alp3

[a] Prototype strains of the Department of Medical Microbiology, St. Olav's University Hospital, Trondheim, Norway.
[b] Received from G. Lindahl, Lund, Sweden.
[c] Negative-control strains.

Antisera. A murine monoclonal antibody (MAb) raised against the R3 protein of the R3 reference strain Prague 10/84 (ATCC 49447; V/R3, i.e., serotype markers as known before the results from the present study were available) was of the immunoglobulin M (IgM) isotype (9). Polyclonal antibodies (PAbs) against the strains Prague 10/84 and Prague 25/60 (NCTC 9828; NT/Alp4, R3) were raised in rabbits against whole cells of the bacteria (14). Initially, in the present study, experiments were performed with sera here called the original anti-Z and anti-R3 sera, both prepared by exhaustive cross-absorptions of the anti-10/84 whole-cell serum as described previously (11). Briefly, the original anti-R3 PAb was prepared by exhaustive absorption of the anti-10/84 whole-cell serum by strain 161757 (V/Alp3) to remove CPS type V antibodies and other antibodies without interest in the context of this study and then absorption by the Z-antigen-positive, R3-antigen-negative Zimbabwean strain CMFR30 to remove anti-Z antibodies (11). The original anti-Z serum was prepared by cross-absorption of anti-10/84 whole-cell serum by strain 161757 and then by strain 9828 to remove the anti-R3 antibodies. Since we discovered in the present study that strain-variable antigens of the 10/84 isolate were more diverse than hitherto recognized, the preparation of putative protein-specific antisera was modified as described in Results. After the cross-absorptions, antibody levels and specificity were estimated by whole-cell-based and HCl extract-based ELISA, immunofluorescence testing, and Western blotting.

Immunological methods. ELISA was performed as described earlier (16). Alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) or anti-mouse IgM (Sigma-Aldrich, St. Louis, MO) was used for detection of antibody binding by solid-phase-bound antigen. Whole cells of GBS or antigens in bacterial extracts were used for coating of ELISA plates (Nunc, Roskilde, Denmark) after checkerboard titrations to secure optimal coating concentrations. The ELISA titer was defined as described previously (11). Fluorescent antibody testing (FAT) was performed as described earlier (14). The signaling was recorded as 0 to 3+. Antisera in a dilution of 1:50 in PBS-NaN_3 were tested.

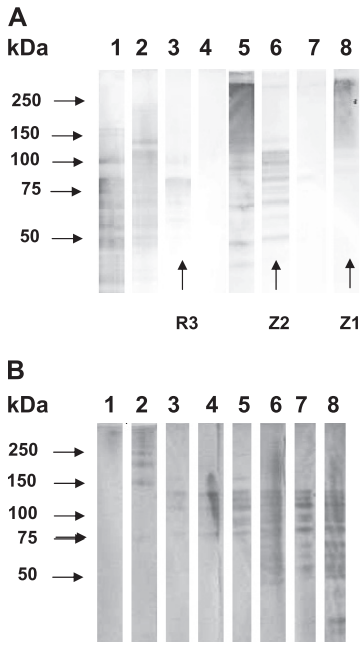

FIG 1 (A) Western blots of SDS lysates of GBS whole cells. Lysates of the strains 08-17 (V) in lanes 1 and 5, 08-7 (Ia/Alp3) in lanes 2 and 6, Prague 25/60 (NT/Alp4, R3) in lanes 3 and 7, and CMFR30 (Ib/Cα, Cβ) in lanes 4 and 8, with lanes 1 to 4 probed with the original anti-R3 serum (1:500) and lanes 5 to 8 probed with the original anti-Z serum (1:500). (B) Western blots of Prague 10/84 (V/R3) extracts. SDS whole-cell extract in lanes 1, 3, 5, and 7 and of partially purified HCl-extracted antigens in lanes 2, 4, 6, and 8, with lanes 1 and 2 probed with the Z1 PAb, lanes 3 and 4 with the Z2 PAb, lanes 5 and 6 with the R3 PAb, and lanes 7 and 8 with the R3 MAb; all antisera were diluted 1:500. The Z1, Z2, and R3 immunoblotting patterns are indicated in panel A.

Western blotting. Western blotting was performed as described previously (17) mostly by testing of materials solubilized from whole cells of GBS by hot SDS or by HCl extraction (17). When HCl-extracted materials which had been partially purified by precipitation and sieve chromatography were tested, 10 μg material per lane was applied. Probing was done with antisera diluted 1:500, and detection of antibody binding used peroxidase-conjugated anti-immunoglobulins (Dako, Roskilde, Denmark).

RESULTS

Anti-Z and anti-R3 antibody targets. The targets for the original anti-Z antibody appeared on Western blotting with very high molecular mass or with much lower molecular mass depending on the GBS isolates (11). Elucidation of the molecular mass variation was initially the goal of the immunoblotting performed. We first tested some Z-antigen-positive clinical isolates and reference and prototype strains by Western blotting and probing with the original anti-Z and anti-R3 PAbs, which we in an earlier study had considered specific for Z and R3, respectively (11). Examples of the testing are shown in Fig. 1A. When probed with either of the two original antisera, strain 08-17 generated very long stained areas (Fig. 1A, lanes 1 and 5). With the original anti-Z PAb, the upper margin of the stained area corresponded to a molecular mass of >250 kDa (Fig. 1A, lane 5). A stained area essentially similar to that generated by the strain 08-17–anti-R3 combination (Fig. 1A, lane 1) was also obtained with the strain 08-07–anti-R3 combination (Fig. 1A, lane 2). However, the 08-17 and 08-07 patterns differed markedly when probed with the original anti-Z PAb (Fig. 1A, lanes 5 and 6). This testing also confirmed previous findings (11) that the reference strain 9828 expressed R3 but no Z antigen (Fig. 1A, lanes 3 and 7). Strain CMFR30, a carrier GBS strain from Zimbabwe, expressed the target which appeared on Western blots with extremely high molecular mass on probing with the original anti-Z PAb (Fig. 1A, lane 8) but without expression of R3 (Fig. 1A, lane 4). Provisionally, we have named the antigen which generated the stained area with extremely high molecular mass of its upper margin (>250 kDa) the Z1 antigen (Fig. 1A, lane 8) and that with lower molecular mass (~135 kDa) the Z2 antigen (Fig. 1A, lane 6), since both of these antigens were detected by using the original anti-Z PAb. It occurred to us that the pattern generated with strain 08-17 and the original anti-Z serum (Fig. 1A, lane 5) could result from expression by 08-17 of both Z1 and Z2 and, if so, that the immunoblotting patterns generated by these two antigens had fused to generate the very long stained area seen in Fig. 1A, lane 5. Our interpretation of these findings became that the original anti-Z serum, prepared from anti-10/84 whole-cell serum, contained antibodies against both Z1 and Z2, meaning that strain 10/84 expressed both of these antigens in addition to R3. On the basis of these findings and the reasoning described above, it should be possible to prepare Z1- and Z2-specific PAbs by further cross-absorption of the original anti-Z serum, for instance, absorption by the Z2-expressing strain 08-07 to obtain anti-Z1 PAb or by the Z1-expressing strain CMFR30 to obtain anti-Z2 PAb. The resulting anti-Z1 and anti-Z2 sera were used in further experiments.

Z antigen was detected because our earlier R3 PAb contained Z antibodies in contrast to the R3 MAb (11). The original anti-R3 PAb used in the present study contained anti-Z2 antibodies due to the fact that for its preparation the antiserum had been cross-absorbed by the Z1-expressing strain CMFR30 but not by a Z2-expressing strain (11). To exclude the possibility that a putative R3-specific PAb should contain Z2 antibodies, another anti-R3 PAb was prepared, namely, by cross-absorption of anti-9828 (NT/Alp4, R3) whole-cell serum, with absorption first by the clinical isolate 08-60 (II/R4) to remove anti-CPS type II antibodies if present (18, 19) and then by strain 65604 (III/R4 [*rib*]) to secure removal of anti-Alp4 antibodies which cross-react with R4 (20). We know from an ongoing study that the resulting 9828 R3 PAb also contained antibodies specific for Alp4 (A. I. Kvam, personal communication). However, we disregarded this since after several hundred clinical GBS strains were tested in our laboratory by an *alp4*-specific PCR (21), no *alp4*-positive isolate had been detected except strain 9828, which is of bovine origin and has been considered a reference strain for both Alp4 and R3. The resulting anti-9828 R3 PAb was used in the experiments described below. Figure 1B depicts immunoblotting results with strain 10/84 SDS whole-cell extract and partially purified HCl-extracted antigens when probed against the Z1 PAb (Fig. 1B, lanes 1 and 2), the Z2 PAb (Fig. 1B, lanes 3 and 4), the 9828 R3 PAb (Fig. 1B, lanes 5 and 6), and the R3 MAb (Fig. 1B, lanes 7 and 8). These results support the interpretation of the results shown in Fig. 1A and, when seen in association with the FAT results shown in Tables 1 and 2, support the inference that the Z1, Z2, and R3 PAbs were specific for the corresponding antigens and that the R3 reference strain 10/84 expressed all three antigens. It is important to note that Z1, Z2, and R3 generated multiple bands on Western blotting. The banding patterns generated by the R3 PAb and R3 MAb were essentially similar (Fig. 1B, lanes 5 and 6 versus lanes 7 and 8), favoring the supposition of R3 protein origin of all the antibody-binding com-

ponents rather than distinct antigens. The anti-Z1, -Z2, and -R3 sera showed titers in whole-cell ELISA of ≥20,000 when 10/84 whole cells or HCl extract was used for coating antigen.

Observations on the Z1, Z2, and R3 antigens. Z1, Z2, and R3 were efficiently released from GBS by HCl extraction at 50°C and were precipitated by 5% trichloroacetic acid and in a 70% saturation of ammonium sulfate. By using whole-cell and HCl extracts from the isolates shown in Table 1 and from several of the isolates shown in Table 2 for antigens, concordant ELISA, FAT, and Western blotting results were obtained in tests using the anti-Z1, -Z2, and -R3 PAbs, results which substantiated the results and interpretations described above. When each of the three antisera was mixed with strain 10/84 HCl extract for antibody neutralization, reactivity of the antibodies in whole-cell-based testing was eliminated. These results accord with the notion that the Z1, Z2, and R3 epitopes which bound antibodies at the bacterial cell surface were present in the HCl extract, which also is consistent with the similarities of Western blotting patterns when SDS-extracted and HCl-extracted antigens were compared (Fig. 1B). However, it can be seen from Fig. 1B, lanes 7 and 8, that exposure of the 10/84 bacteria to HCl may have generated R3 split products of low molecular mass. All three antigens present in the extracts were destroyed by pepsin digestion but resisted degradation by sodium *m*-periodate oxidation, when tested as described previously (11), consistent with the protein nature of the antigens. The antibody-binding capacity of all three antigens was unaffected by heating at 100°C for at least 10 min. When lanes 3 and 4 in Fig. 1B were compared to lanes 5 and 6, it appeared that the immunoblotting patterns of Z2 and R3 looked rather similar. To confirm that the R3 and Z2 antibody-binding sites were located on different molecules rather than both being on the R3 antigen, antibodies raised against strain 9828 (NT/Alp4, R3) were coupled to CNBr-activated Sepharose 4B. Crude 10/84 (V/R3, Z1, Z2) HCl extract was applied to the column, and bound materials were eluted. ELISA testing using materials eluted with PBS, pH 7.2, and materials eluted at pH 2.6 for antigen coating showed that neither of the antigens Z1 and Z2 was bound by the column-attached antibodies while R3 was bound and was eluted at pH 2.6 in considerable amounts, confirming that Z2 and R3 were distinct and immunologically different antigens. On Sephacryl S-300 HR sieve chromatography with 2-ml fractions collected, with voided volume (V_0) corresponding to fraction 25 and apparent volume of distribution during terminal phase (V_t) corresponding to fraction 60, the strain 10/84 R3 was recovered in fractions 26 to 44, Z1 was recovered in fractions 24 to 40, and Z2 was recovered in fractions 26 to 40, all three antigens having peak activity in fraction 34. Thus, as this column has an exclusion limit of 1,500 kDa for globular proteins, molecular aggregate formation and considerable molecular/aggregate heterogeneity of the three antigens are likely. Fractions 40 to 60 contained a large amount of UV-light-absorbing materials without antigenic activity. Upon ion-exchange chromatography (DEAE Sephacel column) and elution with stepwise NaCl concentration increments, all three antigens were eluted in the 0.15 M to 0.3 M NaCl range with peak antigen coating activity at 0.2 M NaCl for R3, 0.15 M NaCl for Z1, and 0.2 M NaCl for Z2. These results accord with the assumption that Z1, Z2, and R3 have rather similar physicochemical properties, meaning that separation of the antigens by conventional chromatographic methods may be difficult while such methods will be efficient in disposing of contaminants.

Z1, Z2, and R3 expression by reference and prototype GBS strains. Tables 1 and 2 show the results of testing isolates of a collection of GBS reference and prototype strains for expression of Z1, Z2, and R3. The testing was performed by FAT, supported for many of the isolates by whole-cell-based or HCl extract-based ELISA or by Western blotting. Of 28 strains examined, including two negative-control isolates, 8 (28.5%) isolates tested positive for one, two, or all three of the antigens Z1, Z2, and R3, most often (4/28) positive for all three antigens, with R3 as the antigen most often (7/28) expressed. One or more of the antigens were expressed by strains of CPS types Ia, Ib, and V; by strains of the recently described CPS type IX (13); and by NT strains. With respect to other surface-anchored proteins, one or more of the three antigens occurred in isolates positive for *bac* (Cβ) or Alp genes. Thus, GBS serotype or serosubtype preferences for Z1, Z2, or R3 expression were not demonstrated by this testing, which, however, was limited to rather few isolates. The majority (71.4%) of the reference and prototype strains, several of which have been important in GBS research around the world, showed no expression of any of the antigens Z1, Z2, and R3 (Table 2). It is important to note that the negative isolates (Table 2) included all eight GBS strains whose genomes had been sequenced up to 2005 (7). If the three antigens are added to already well known antigens in GBS serotyping, the discriminatory power of the typing will certainly be increased, as illustrated by the examples shown in Table 1.

DISCUSSION

In recent studies by some of us, GBS carrier strains from Zimbabwe were tested for a variety of serotype and serosubtype markers, including expression of the GBS protein R3, which was detected by dot blotting of the isolates and probing with a monoclonal anti-R3 antibody (R3 MAb) (10–12). Testing results were checked by probing of an assortment of the isolates with a putative R3-specific polyclonal antibody (R3 PAb), which had been raised against the R3 reference strain Prague 10/84 (V/R3), generally with good agreement between the MAb and PAb testing results. However, two Zimbabwean GBS strains showed positive R3 PAb and negative R3 MAb results (11). By pursuing this observation, we found that the two Zimbabwean strains as well as the homologous 10/84 strain expressed an antigen which was immunologically different from R3. We named this marker the Z antigen, which, to our knowledge, previously has been undetected (11). Obviously, our R3 PAb contained antibodies to the Z antigen in addition to R3 antibodies. These results triggered the experiments described in the present study. Western blotting revealed that, for instance, strain Prague 10/84 expressed an antigen with a molecular mass of >250 kDa named Z1 and an ~135-kDa antigen named Z2, in addition to the R3 antigen. These findings, supported by several other test results, enabled the preparation of putative Z1-, Z2-, and R3-specific antisera, important in further studies of these antigens.

HCl extraction was efficient in releasing Z1, Z2, and R3 antigens from GBS cells. This may be a harsh extraction method, but Western blotting patterns of the HCl-extracted antigens and antigens extracted with hot SDS were similar, and antibody neutralization tests provided evidence that Z1, Z2, and R3 antibody-binding sites exposed on the bacteria were present on the HCl-extracted antigens. Thus, HCl extraction should be a safe method for preparation of these antigens. Our findings agree with the assumption that all three antigens were proteins. When dissolved in

PBS, Z1, Z2, and R3 occurred more or less aggregated, were similarly charged, and appeared with multiple bands on immunoblotting. The similarity in physicochemical characteristics suggests the possibility that Z1, Z2, and R3 may belong to a distinct GBS protein family, which, if so, would add to already-defined antigenic GBS protein families such as the alpha-like proteins (6, 22) or the serine-rich repeat proteins (3, 23, 24). Data on primary structure, not yet available, will be necessary in order to exclude or confirm these suggestions and also may reveal what caused the multiplicity of stained bands on Western blotting—for instance, if the patterns observed can be related to a multiplicity of repeats in the proteins as in several other ladder-forming GBS proteins or to posttranslational modification or were caused by degradation during sample preparation (6, 22, 25).

Z1 showed extremely high molecular mass on Western blotting, a size similar to that of glycosylated streptococcal serine-rich repeat proteins (Srr) (3, 23, 24). Srr-1 and Srr-2 are both known to be surface localized in the bacteria and glycosylated (3, 23, 24). At least, Srr-1 played a role as an adhesin and invasin in human brain microvascular endothelial cells (24). It seems that GBS isolates always possess one of the two Srrs. The majority of strains possess Srr-1, including both the reference strain Prague 10/84 (24) and several of the Z1-, Z2-, and R3-negative strains shown in Table 2 (3, 24). Srr-1 could hardly have been involved in our experiments since we used cross-absorbed antisera from which Srr-1 antibodies must have been removed and isolates known to contain *srr-1* were included among the strains which showed no Z1 antigen expression. Seifert et al. detected *srr-2*-positive strains only among CPS type III, MLST-17/RDP-3 GBS isolates (3). These isolates, which produced the ε antigen, probably encoded by *srr-2*, were highly virulent and associated with serious neonatal GBS disease (3). The strains COH1 and BM 110 (Table 2) were CPS type III, MLST-17 isolates, but both strains were negative in tests with the Z1 antibodies, which also was the case with the δ antigen-expressing strain A909 (3, 26). In considering the very high molecular mass of Z1 in this context, our data suggest the possibility that this antigen may be another previously unrecognized GBS glycoprotein. In 1999, Areschoug et al. described a protein expressed by the 10/84 strain which appeared on SDS-PAGE and immunoblotting with distinct doublet bands of ~100 kDa and ~110 kDa and contained immunoprotective epitopes (27). The relationship of these proteins to the 10/84-expressed Z1, Z2, and R3 antigens remains unclear, but it is a possibility that one of the bands described in reference 27 may represent a truncated version of Z2 and the other may represent a version of R3, although this is hypothetical.

The function of Z1, Z2, and R3, the last described years ago (8), is unknown except for the recognition of these antigens as strain-variable markers which add to a variety of other surface-localized and strain-variable antigens which are useful as serotype/serosubtype markers of GBS. Inclusion of Z1, Z2, and R3 among other markers should provide increased discriminatory power of GBS serotyping, of value in epidemiological settings, although determination of such markers is of little value in evaluation of the relatedness between different isolates on the genomic level (7). The large number of serosubtype markers in GBS makes antibody-based marker detection expensive and cumbersome. However, the immunological uniqueness of the proteins Z1, Z2, and R3, indicated by the data of this and earlier studies (9, 11), suggests that it may become possible to develop molecular typing methods for specific detection of each of the protein-encoding genes, perhaps by including primer sets for the genes in a multiplex PCR. This prediction is based on data which indicate that at least some strain-variable GBS genes which can be identified by gene-specific PCR often encode proteins which possess protein-specific epitopes (20, 21, 28, 29). Knowledge of the usefulness of Z1, Z2, and R3 determination in GBS serotyping is sparse and unknown for GBS from most areas of the world (9–12, 30). In this study, testing of these markers resulted in modification of existing serotype designations in 28.5% of 28 isolates examined, including strains of various CPS types. In a recent study, R3 expression was detected in 21.5% and Z antigen expression was detected in 27.2% of carrier GBS strains from Zimbabwe (11). Thus, data available at this time indicate a potential role of these antigens in GBS serotyping and also suggest that these antigens may have potential as vaccine candidates, for instance, in southern African areas (9–11). Thus, further studies of the antigens focused on in this study, of the genes encoding them, and their immunobiological functions should be worthwhile.

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