Antibodies against *Streptococcus agalactiae* Proteins cα and R4 in Sera from Pregnant Women from Norway and Zimbabwe

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Group B streptococci (GBS) express strain-variable and surface-localized proteins, which are important serotype markers and targets of protective antibodies. These include the cα and R4 proteins, one or the other of which is expressed by approximately 75% of clinical GBS isolates. These proteins have been considered vaccine candidates. In this study, the cα and R4 proteins were extracted by trypsin digestion of GBS and purified by sequential precipitation with trichloroacetic acid and ammonium sulfate followed by gel filtration chromatography. The proteins were used as antigens in an indirect enzyme-linked immunosorbent assay (ELISA) to measure the levels of cα- and R4-reactive antibodies in sera from pregnant women from Norway (n = 100) and from Zimbabwe (n = 124). Antibody levels in the Norwegian group of women were significantly higher than in the Zimbabwean group, and a higher proportion of the Norwegian women contained appreciable levels of antibodies against both proteins. The antibodies traversed the placental barrier. With individual sera, a significant correlation between the anti-cα and anti-R4 antibody levels was observed and each of the two protein antigens effectively competed for human serum antibodies both against itself and against the other antigen. Inhibition ELISA results demonstrated specificity for each of the proteins of immune antibodies raised in rabbits. These results demonstrate that (i) the majority of women of childbearing age have antibodies against cα and R4, (ii) the levels of these antibodies differ among pregnant women in different parts of the world, and (iii) the normal human serum antibodies may target a common cα and R4 protein site, whereas immune antibodies targeted a different site(s) specific for each protein.

Group B streptococci (GBS) remain an important cause of morbidity and sometimes mortality in neonates and also a cause of maternal infections and infections in nonpregnant adults, in spite of antibiotic and supportive therapy (2, 30).

Both host factors and microbial factors determine the outcome in infected individuals, including colonized newborns (30). The level of serum antibodies to capsular polysaccharide GBS antigens is one important host factor (3, 4). Up to now, nine different capsular antigen types have been defined. Among these, types Ia, Ib, II, and III predominate in many parts of the world (19, 26), but serotype V GBS has emerged as an increasingly important pathogen in some areas (11) and serotype VI and VIII strains are important pathogens in Japan (21).

Many GBS strains also produce one or more proteins characterized by, among other things, resistance to trypsin digestion and formation of ladder-like banding patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. These proteins include the cα protein of the c protein fraction of GBS (7, 10), the R1 to R4 proteins (8, 13, 18, 23), protein Rib (31), a Rib-like protein (1), and cα-like proteins (20, 22). Large tandem repeats are additional characteristic features of these proteins (20, 27, 32), which harbor epitopes targeted by protective antibodies, according to experimental models (1, 17, 22, 31). For this reason the proteins or protein fragments have been considered vaccine candidates (17). In this context the cα and R4 proteins should be of particular interest, since some 75% of GBS isolates harbor one or the other of these proteins (19). On this basis we figured that maternal antibodies against ladder-forming proteins could be one of the host factors which determine the outcome in newborns colonized by GBS, as has been suggested by others (25). For this reason we have measured the levels of serum antibodies against cα and R4, the most prevalent of the ladder GBS proteins, in two groups of pregnant women, one from Norway and one from Zimbabwe.

MATERIALS AND METHODS

**Human sera.** A total of 100 sera from pregnant women in the Trondheim area of Norway and 124 sera from pregnant women in the Chimihoyi area of western Zimbabwe were examined. Sera were collected during checkup visits to physicians or maternity clinics by women between the fourth and eighth month of pregnancy and were handed to us blinded after routine testing in hospital laboratories. Only sera which tested negative for hepatitis B surface antigen and human immunodeficiency virus were included in the study. The women ranged in age from 17 to 38 years. For five Norwegian women giving birth at full term, sera from both the mothers and the babies were collected and analyzed. Sera were kept at −20°C until tested.

A commercial human gamma globulin preparation (165 mg/ml; Pharmacia, Uppsala, Sweden) was used throughout the study.

**Bacterial strains and culture.** GBS strains NCTC 12906 (strain 335; serotype Ia/cα) and 65604 (serotype III/R4), our prototype strains for the cα and R4 proteins, respectively, were used for the preparation of the cα and R4 proteins. The bacteria were cultured in Todd-Hewitt broth (10), harvested by centrifugation (10,000 × g; 15 min), and washed with phosphate-buffered saline (PBS), pH 7.2.

**Antigen preparation.** The bacteria were extracted with trypsin (1 mg/ml) in 50 mM Tris buffer (pH 8.0); there was 5 ml of the solution per g of wet bacterial pellet.

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The suspension was incubated at 37°C for 4 h and centrifuged (10,000 × g; 15 min).

Proteins in the supernatant were precipitated with 5% (wt/vol) trichloroacetic acid (TCA) at 4°C for 20 h. The precipitate, which was collected by centrifugation, was dissolved in PBS and dialyzed against PBS, and proteins were precipitated with ammonium sulfate (pH 7.0; 72% saturation) at 4°C for 20 h. The final precipitate, collected by centrifugation, was dissolved in a small volume of PBS and applied to a Sephacryl S-200 HR (Pharmacia) column (60 by 1.5 cm). The column was equilibrated and eluted with PBS at an elution rate of 3 ml/h. Fractions of 1.6 ml were collected and were used in a dilution of 1:10 for coating microtiter plates to detect the presence of the cα or R4 antigen by probing with the appropriate antisera. The fractions, which contained the cα or R4 protein, were pooled and kept at −20°C in small aliquots. The fractions were also tested using the Streptex kit (Murex Biotech. Ltd., Dartford, England).

**Antisera.** Anti-cα and anti-R4 monoclonal antibodies (MAbs) used in this study were those described previously (7, 8). Polyclonal antibodies (PAbs) included antibodies raised in rabbits against whole cells of strains 335 and 65604, as described previously (8), and antisera raised against the purified cα (7) and R4 (8) proteins. In addition, rabbit antisera against whole cells of GBS strains ATCC 12400 (090) and NCTC 11079, reference strains for capsular antigen types Ia and II, respectively, were used.

**Immunological techniques.** Immunoblotting was performed as described previously (28). Briefly, fractions positive for the cα or R4 protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, Calif.), and probed against PAbs (1:500) or MAbs (1:500). Antibody binding was detected using the appropriate peroxidase-conjugated anti-immunoglobulin preparation (1:1,000).

For direct enzyme-linked immunosorbent assay (ELISA), the coating capacity of the cα and R4 preparations was tested by checkerboard titration using the appropriate PAbs (1:500), MAbs (1:500), and gamma globulin preparation (1:2,000). The ELISA was performed as described previously (28). Briefly, coating (50 μl/well) was performed at 4°C for 20 h. Incubation with human serum, antisera, or alkaline phosphatase-conjugated antibodies to immunoglobulins (1:1,000; Sigma) of the appropriate species proceeded at 20°C for 1 h. Incubation with substrate (p-nitrophenyl phosphate) was at 37°C for 30 min and was followed by reading the signal at 405 nm. Negative controls included testing without antigen and testing without human or animal antibodies. Washings were performed using PBS with 0.05% (vol/vol) Tween 20 (PBST), which also served as the diluent. All tests were performed in duplicate. Background signal was recorded but was not subtracted from the recordings obtained with the various sera. The results are presented as ELISA ratio obtained by dividing the mean of the optical density at 405 nm (OD405) signaled by the human serum when tested in a dilution of 1:200 by that recorded with gamma globulin when tested in a dilution of 1:2,000.

Inhibition ELISA was performed by mixing equal volumes of the antigen solution tested for inhibition or PBST (positive control) and appropriately diluted gamma globulin, MAbs, or PAbs and by incubating the mixture at 20°C for 1 h. The mixture was then tested as in the indirect ELISA. The gamma globulin and antisera were used in dilutions which resulted in OD405 recordings for the positive controls in the range of 1.000 to 1.200. Inhibition was expressed as a percentage reduction of the OD405 recorded with the competing antigen from the OD405 signals by the positive control, tested on the same microtiter plate.

**Statistical methods.** The Mann-Whitney U test was used for comparison of differences between population groups. The Stat-Med statistical program was used for linear regression and Pearson’s correlation calculations. Values of P <0.05 were considered statistically significant.

**RESULTS**

The cα and R4 antigens. GBS strains 335 (Ia/cα) and 65604 (III/R4) were digested by trypsin, and extracted material was isolated by TCA precipitation and then by ammonium sulfate precipitation. The cα or R4 proteins, which were contained in the precipitate were further purified by sieving chromatography. Both proteins were eluted from the Sephacryl S-200 HR column as sharp and symmetrical peaks corresponding to the void volume of the column. Fractions close to the total volume of the column contained UV light-absorbing material, but coats prepared with this material showed no MAbs or PAb binding activity. The pooled void volume fractions gave rise to ladder-like patterns in Western blotting, as previously described for cα (7) and R4 (8); the patterns seemed identical when probed with the anti-whole-cell PAbs and MAbs, respectively. The material showed no reactivity when tested by the Streptex kit, and coats prepared with it were negative when tested by ELISA with rabbit antibodies used to detect capsular antigens Ia and III, respectively, in a fluorescent antibody test (5, 9). For both of the protein antigens, a dilution of 1:10 was optimal for coating in an ELISA to detect human or animal anti-protein antibodies. In the ELISA, the anti-cα MAb and PAb recognized only cα and the corresponding anti-R4 antibodies recognized only the R4 protein. Antisera against whole cells of our reference strains for capsular antigens Ia and II (which do not express the cα and/or the R4 protein), both of which were sera with high levels of antibodies against the homologous strain, showed no antibody activity against cα and R4 when dilutions from 1:100 were tested.

**Levels of anti-cα and anti-R4 antibodies in sera from pregnant women.** Figure 1 shows the titration curves for cα and R4-reactive antibodies in a commercial human gamma globulin preparation. An increase or decrease in the ELISA ratio of 0.200 to 0.300 corresponded to approximately a doubling or halving, respectively, of the antibody concentration. Repeated testing of the gamma globulin (1:2,000) showed OD405 readings (means ± standard deviations) of 0.906 ± 0.234 against cα and 0.984 ± 0.249 against R4 with background signaling values of 0.112 ± 0.049 and 0.096 ± 0.030, respectively, similar to the background signaling recorded with a 1:200 dilution of individual human sera.

The results recorded with sera from pregnant women are shown in Table 1 and Fig. 2. For both population groups, immunoglobulin G (IgG) antibody activity in the sera from pregnant women from Norway and Zimbabwe are shown in Table 1 and Fig. 2. For both population groups, immunoglobulin G (IgG) antibody activity in the sera from pregnant women from Norway and Zimbabwe.

**TABLE 1. Levels of antibodies to the GBS proteins cα and R4 in sera from pregnant women from Norway and Zimbabwe**

<table>
<thead>
<tr>
<th>Serum source</th>
<th>No.</th>
<th>Median (range) ELISA ratio* with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cα coat</td>
</tr>
<tr>
<td>Norway</td>
<td>100</td>
<td>0.675 (0.150–2.559)</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>124</td>
<td>0.350 (0.140–1.400)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R4 coat</td>
</tr>
<tr>
<td>Norway</td>
<td>100</td>
<td>0.735 (0.120–1.830)</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>124</td>
<td>0.370 (0.100–1.470)</td>
</tr>
</tbody>
</table>

* P < 0.05 for both the cα coat and the R4 coat.
levels varied greatly among individuals. However, both anti-c⁰ and anti-R4 antibody levels were significantly higher in the Norwegian population than in the Zimbabwean population. On the basis of an arbitrary selection of ELISA ratios of >0.250 as an indication of the presence of these antibodies, 91% of the Norwegian sera and 69% of the Zimbabwean sera contained anti-c⁰ IgG antibodies and 92 and 73% of the sera, respectively, contained anti-R4 antibodies.

When paired sera from five mothers and their newborns were examined, the babies showed levels of antibodies against both protein antigens almost equal to the levels of their mothers. On average, 10%-lower ELISA ratios were recorded with sera from the offspring.

Specificity of antibodies. It was observed that individual sera showed the same or nearly the same ELISA ratios for the anti-c⁰ and anti-R4 antibodies, as if the two antigens measured the same antibody. This accordance was evaluated for the first 20 sera tested in each of the two population groups examined. For both the Norwegian sera (Fig. 3) and the Zimbabwean sera, the correlation between the c⁰ and R4 ELISA ratios was highly significant (r = 0.977 and 0.915, respectively; P < 0.01), supporting the suspicion that the human serum antibodies recognized a target(s) which was common to the c⁰ and R4 proteins. This supposition was substantiated by the finding that c⁰ and R4 neutralized the R4-reactive human serum antibodies to the same extent (84 and 82% inhibition for the c⁰ and R4 proteins, respectively) but not the vaccination-induced R4-reactive rabbit antibodies (1 and 100% inhibition for the c⁰ and R4 proteins, respectively). Analogous results were obtained in the inhibition ELISA when the c⁰ protein was used for coating and human or immune anti-c⁰ antibodies were used for probing, i.e., both c⁰ and R4 neutralized the c⁰-reactive human antibodies but only c⁰ neutralized the anti-c⁰ antibodies raised in animals (data not shown).

DISCUSSION

In this study we measured the levels of serum IgG antibodies against GBS proteins c⁰ and R4 in groups of pregnant women from Norway and Zimbabwe. One of these proteins (rarely both) is expressed by at least 75% of GBS strains (19).

Since both c⁰ and R4 are ladder-forming GBS proteins and since these proteins are targets of protective antibodies in experimental models (1, 17, 22, 24, 31), these antigens have been considered vaccine candidates either alone or as the protein component in a capsular polysaccharide-protein conjugate vaccine (17). On this basis we hypothesized that the testing described in the present study could be the measurement of serum antibodies which are important in protection against GBS disease, particularly neonatal disease.

After trypsin extraction and sequential precipitation with TCA and ammonium sulfate and gel filtration, the proteins appeared immunologically homogenous, as evidenced by the failure to detect serogroup or capsular polysaccharides, by Western blotting findings, and by the results of testing with antisera raised against whole cells of GBS strains which do not express the c⁰ or R4 protein. We anticipated that trypsin, efficient for extraction of these proteins (13), would cleave a variety of contaminating GBS proteins and thereby would facilitate separation of the contaminants from the high-molecular-weight c⁰ and R4 proteins by the fractionation procedure.

We chose to match antibody levels in the population groups tested against the levels in a human gamma globulin preparation, although it has been established that different commercial gamma globulin preparations vary considerably in GBS antibody levels, including opsonic activity (14, 33), mostly depending on the donor pool (33). The latter observation is consistent with our findings that the Norwegian group of pregnant women had significantly higher levels of both c⁰- and R4-reactive antibodies than a corresponding group of Zimbabwean women. This difference cannot be attributed to GBS carrier rate in pregnancy, which was higher in a Zimbabwean (29) than in a Norwegian (15) group of pregnant women. Genetic factors and/or factors related to socioeconomic standards and nutritional status may account for this difference. The design of the
study did not permit adequate evaluation of the impact of such factors. Although antibody levels varied up to manyfold between individuals, at least 70% of the Zimbabwean women and 90% of the Norwegian women had c- and R4-reactive antibodies. For c- this is considerably higher than the value found previously by testing blood donors (6). However, the results of the present study compare favorably with the results of testing of anti-R4 antibodies by Western immunoblotting (12). In that study 92.5% of colonized mothers and 54% of noncolonized mothers had detectable levels of these antibodies. Some investigators have noticed an increase in anti-GBS antibodies in urogenital secretions, but less of an increase in serum antibodies, in GBS carriers compared to noncarriers (16). We did not discriminate between carriers and noncarriers among the individuals tested but considered it likely that the anti-c- and -R4 antibodies detected in the human sera were induced by GBS carriage.

We noticed that the c- and R4-reactive antibodies showed identical or similar levels of signaling in ELISA for both individual human sera and the gamma globulin preparation and that this accordence was statistically significant. This prompted inhibition experiments which showed that c- and R4 were equally effective as competing antigens for antibodies in the gamma globulin preparation, irrespective of which of the two proteins was used for coating, indicating a common c- and R4 site targeted by the human antibodies. To our knowledge this is the first presentation of such specificity of human antibodies recognizing the c- and R4 antigens. This was unlike what was found for the antibodies induced in animals, which demonstrated specificity for the homologous antigen. The c- protein, the most extensively characterized of the ladder-forming GBS proteins, has epitopes in the repeats and in the N-terminal region, both of which are targeted by protective antibodies (17). Our results support the notion that the natural human serum antibodies target c- and R4 sites which are distinct from the sites targeted by the immune antibodies induced in animals. In that case, the functions of the human antibodies, such as immunoprotection in neonates and adults, may differ from the sites targeted by immune antibodies induced in animals (17, 24, 31). This remains a challenge for future studies.

In conclusion, our data show that serum from the majority of adult women from an African and a Scandinavian country contained serum antibodies which recognized the GBS proteins c- and R4. These antibodies probably target sites different from the sites targeted by immune antibodies raised in animals. The human c- and R4-reactive antibodies require further studies, including studies to clarify their immunobiological function.

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