Antibodies raised in animals against the *Streptococcus agalactiae* proteins c α and R4 and normal human serum antibodies target distinct epitopes

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The targets for normal human serum antibodies that react with proteins c α and R4 isolated from group B streptococci (GBS; *Streptococcus agalactiae*) have been studied and compared with the targets for murine monoclonal and rabbit polyclonal antibodies raised against these proteins. The proteins were extracted by trypsin digestion and purified by precipitations and gel filtration and testing was based on enzyme immunoassays. The immune antibodies showed specificity for the corresponding protein, targeted that protein in Western blotting and recognized their targets after heat treatment (100 °C) of the proteins. Human antibodies in a commercial gammaglobulin preparation targeted a site(s) common to c α and R4. This target failed to bind the antibodies in Western blotting and was destroyed by heating. c α - and R4-reactive antibodies in sera from healthy pregnant women recognized the common, heat-labile determinant(s), but contained little or no antibodies against the heat-stable c α - or R4-specific determinants. These results are consistent with the notions that (i) the normal human antibodies and the immunization-induced animal antibodies targeted different sites on the c α and R4 proteins and that (ii) the natural human antibodies targeted conformational epitopes and the immune antibodies targeted linear epitopes. These findings are important for further clarification of GBS immunology and immunoprotection in humans.

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INTRODUCTION

Group B streptococci (GBS; *Streptococcus agalactiae*) are important pathogens in humans, particularly in neonates, who frequently become colonized during delivery as a result of urogenital GBS carriage in the mother. Studies from different parts of the world have demonstrated variable GBS carriage rates in pregnant women of up to more than 30 % (Moyo *et al.*, 2000). Among factors that determine the outcome when neonates become colonized by GBS (Schuchat, 1998), the level of protective serum antibodies transferred from the mother to the fetus seems to be important, notably antibodies against the capsular polysaccharide antigens (Baker & Kasper, 1976; Baker *et al.*, 1980). Nine different GBS capsular antigen types are known and have been designated Ia, Ib and II–VIII. In addition to the type-specific anti-capsular antibodies, antibodies against surface-localized and strain-variable protein antigens may be important in immunoprotection according to results

obtained in animal models and opsonophagocytic assays (Lachenauer & Madoff, 1996; Larsson *et al.*, 1996; Madoff *et al.*, 1991; Stålhammar-Carlemalm *et al.*, 1993). These antigens include the c α and c β proteins of the c protein fraction of GBS (Bevanger & Maeland, 1979). c α and a number of other proteins belong to a family of ladder-forming GBS proteins (Wästfelt *et al.*, 1996), so designated on the basis of the banding patterns observed in immunoblotting. This group of proteins includes the R proteins R1–R4 (Flores & Ferrieri, 1996; Lancefield & Perlmann, 1952) and protein Rib (Stålhammar-Carlemalm *et al.*, 1993), which is probably identical to R4 (Bevanger *et al.*, 1995; Smith *et al.*, 2002). Sequence analysis of some of the proteins has demonstrated similarity between them: up to 100 % identity for some stretches of the proteins (Lachenauer *et al.*, 2000; Wästfelt *et al.*, 1996).

Since antibodies raised in animals against ladder-forming GBS proteins are protective in animal models (Lachenauer & Madoff, 1996; Larsson *et al.*, 1996; Madoff *et al.*, 1991; Stålhammar-Carlemalm *et al.*, 1993), we speculated that corresponding human serum antibodies might be important

Abbreviation: GBS, group B streptococcus.

in defence against GBS disease in humans. For that reason, we recently measured antibodies against the c^{α} and R4 proteins of GBS (Moyo *et al.*, 2001), the ladder-forming proteins that occur at the highest frequency (Kvam *et al.*, 1995). We found that 70 % or more of pregnant women tested from Norway and Zimbabwe had appreciable levels of antibodies against these proteins, with highest levels in the group of Norwegian women (Moyo *et al.*, 2001). Our previous data also suggested that the specificity of the c^{α} - and R4-reactive human antibodies might be different from that of immune antibodies raised in animals against these proteins (Moyo *et al.*, 2001). The present study describes the results of further work on the two categories of antibodies, notably their specificity, since this might be important in the context of functional capability of the antibodies and means to discriminate between the natural and immune antibodies in serological testing.

METHODS

Human sera. Sera from 30 pregnant Norwegian women and 30 pregnant Zimbabwean women were selected arbitrarily from larger collections of sera from pregnant women described in a previous report (Moyo *et al.*, 2001). The sera were collected during antenatal check-up visits from women without known GBS or any other infectious disease. Only sera that tested negative for hepatitis B virus and human immunodeficiency virus were used (Moyo *et al.*, 2001). A commercial preparation of human gammaglobulin (165 mg ml⁻¹; Pharmacia) was used in a number of experiments.

Bacterial strains. Strains 335 (Ia/ c^{α}) and 65604 (III/R4), our prototype strains for the c^{α} and R4 proteins, were respectively used for preparation of the c^{α} and R4 antigens. The bacteria were preserved at 4 °C on sealed blood-agar plates and cultured at 37 °C on blood-agar plates or in Todd-Hewitt broth as described previously (Bevanger & Maeland, 1979). Harvested bacteria were washed with PBS (pH 7.2).

Antigens. The procedures for extraction and purification of the c^{α} and R4 proteins synthesized by the GBS strains 335 and 65604 have been described previously (Moyo *et al.*, 2001). Briefly, the proteins, both of which are resistant to degradation by trypsin (Bevanger *et al.*, 1992, 1995), were extracted by trypsin digestion of whole bacterial cells, precipitated with 5 % (w/v) trichloroacetic acid and then with ammonium sulphate (72 % saturation). The final precipitate was chromatographed by gel filtration on a Sephacryl S-200 HR column (Pharmacia), which was equilibrated and eluted with PBS containing 0.02 % sodium azide. The proteins were eluted at a position corresponding to the void volume fractions. The fractions with peak activity as antigen in ELISA were combined and kept at -20 °C. The proteins appeared immunologically homogeneous, as evidenced by immunoblotting and other antibody-based tests (Moyo *et al.*, 2001).

Antisera. The preparation and properties of rabbit antisera against whole cells of the GBS strains 335 and 65604 and of antisera against purified c^{α} and R4 have been described previously (Bevanger & Maeland, 1977; Bevanger *et al.*, 1992, 1995). Briefly, rabbits were given several intravenous injections with formalin-killed bacteria over a period of 3 weeks (Bevanger & Maeland, 1979) or were immunized intradermally with the c^{α} or R4 protein, which had been purified by immunosorbent chromatography (Bevanger *et al.*, 1992, 1995). Animals were bled 1–2 weeks after the last injection. For the generation of c^{α} -specific mAb, BALB/c mice were immunized with killed A909 (Ia/ c^{α} / c^{β}) bacteria (Bevanger *et al.*, 1992) and, for anti-R4 mAb, with

immunosorbent-purified R4 protein (Bevanger *et al.*, 1995). Clones that synthesized c^{α} - or R4-specific monoclonal IgG were used for production of the ascites used in this study.

Indirect ELISA. The test was performed as described previously (Moyo *et al.*, 1999, 2001). Briefly, c^{α} or R4 protein recovered from the gel-filtration column was used to coat microtitre plates (Sero-Wel; Bibby Sterilin) after determining optimal antigen dilution for coating by checkerboard titration (Moyo *et al.*, 1999). The coating was performed at 4 °C for 20 h and incubation with antibodies at 20 °C for 1 h. Alkaline phosphatase-conjugated anti-immunoglobulins (Sigma) were used and *p*-nitrophenyl phosphate was used as substrate. PBS containing 0.05 % (v/v) Tween 20 (PBST) was used as diluent and for washings. A_{405} was recorded. When twofold dilutions of sera were tested, the ELISA titre was defined as the reciprocal of the highest serum dilution that showed an A_{405} of at least 0.200 above the background signal, which was determined by testing serum in wells that contained no antigen. For measuring antibody levels in sera from pregnant women, sera were tested at a dilution of 1 : 200 (Moyo *et al.*, 2001). Antibody levels were expressed as ELISA ratios obtained by dividing the mean A_{405} recorded for individual human sera tested in duplicates by that recorded for the gammaglobulin preparation tested in duplicate at a dilution of 1 : 2000 on the same microtitre plate.

ELISA inhibition. The test was performed in order to examine whether antigens in a bacterial extract would neutralize the anti- c^{α} and/or anti-R4 antibodies. Undiluted or diluted bacterial extract, or PBST in the positive control, was mixed with an equal volume of antiserum or the gammaglobulin preparation, which had been diluted so as to give an A_{405} of approximately 1.000 for the positive control. The mixture was incubated at 20 °C for 1 h and then tested in the indirect ELISA as described above. The antibody-neutralizing capacity of an extract is expressed as percentage reduction caused by the extract of the A_{405} recorded for the uninhibited positive control.

Western blotting. Western blotting was performed according to Laemmli (1970) and a previous report from our laboratory (Moyo *et al.*, 1999). Briefly, undiluted material from the gel-filtration column which contained the c^{α} or R4 protein was subjected to SDS-PAGE, transferred to PVDF membranes (Bio-Rad) and probed with gammaglobulin (1 : 2000), individual human sera (1 : 200), polyclonal rabbit antibodies (pAb; 1 : 500) or mAb (1 : 1000). Probing with the appropriate horseradish peroxidase-conjugated anti-Ig and colour development were performed as described previously (Moyo *et al.*, 1999).

RESULTS AND DISCUSSION

Both the murine monoclonal and rabbit polyclonal anti- c^{α} and anti-R4 antibodies showed strong reactivity in ELISA against the homologous protein, but no or very weak binding activity against the heterologous protein (Table 1). Normal rabbit serum showed no reactivity against the c^{α} and R4 proteins (not shown). In contrast to the immune antibodies, antibodies in a human gammaglobulin preparation recognized both antigens (Table 1).

In order to compare further the human and immune antibodies, ELISA inhibition was performed. The R4 protein strongly inhibited the rabbit anti-R4 antibodies, whereas c^{α} did not, confirming the R4-specificity of the pAb (Fig. 1). By contrast, both c^{α} and R4 inhibited the human antibodies that recognized the R4 protein, although not completely within the range of concentrations of the inhibiting proteins. Fig. 1 shows the results of a representative experiment, which was

Table 1. ELISA titres of c^α- and R4-reactive antibodies in rabbit antisera, murine mAbs in ascitic fluid and a human gammaglobulin preparation

Immunizing antigen	Species	Antigen used in ELISA	
		c ^α	R4
Polyclonal antisera			
Strain 335 whole cells (Ia/c ^α)	Rabbit	25600	200
Strain 65604 whole cells (III/R4)	Rabbit	400	51200
Purified c ^α	Rabbit	25600	200
Purified R4	Rabbit	200	3200
Monoclonal antibodies			
Strain 335 whole cells (Ia/c ^α)	Mouse	25600	< 200
Strain 65604 whole cells (III/R4)	Mouse	< 200	6400
Gammaglobulin preparation			
None	Human	3200	6400

Fig. 1. ELISA competition using R4 protein-coated microtitre plates and rabbit anti-R4 antibodies inhibited by R4 (○) or c^α (●) extract or human gammaglobulin inhibited by the R4 (□) or c^α (■) extract.

confirmed on repetition. Concordant results were obtained in analogous inhibition tests using anti-c^α antibodies and solid-phase-bound c^α, i.e. neutralization of the anti-c^α immune antibodies only by c^α and neutralization of the human antibodies recognizing c^α by both c^α and R4 (not shown). Taken together, these results support the notion that the immune antibodies recognized a protein-specific epitope(s), whereas the normal human serum antibodies recognized a different epitope(s) that is shared by c^α and R4.

In Western blotting, we observed no antibody binding when the gammaglobulin preparation or individual human sera were probed against c^α and R4. In these tests, all of the sera examined were used at dilutions that showed an A₄₀₅ of > 1.000 in ELISA. The antibodies raised in animals resulted in ladder-like banding patterns, which are known features of these proteins (Bevanger *et al.*, 1992, 1995; Madoff *et al.*, 1991; data not shown). Other investigators have used Western blotting in order to measure human serum anti-

bodies against R4, but with electrophoresis performed in non-denaturing polyacrylamide minigels (Fasola *et al.*, 1996), which may explain the discrepancy. To test further the possibility that the failure to detect binding of the human serum antibodies by c^α and R4 in Western blotting was due to denaturation of the proteins in hot SDS, we tested whether heating itself would affect antibody binding. As seen from Fig. 2, heating of c^α in PBS at 100 °C for 15 min nearly eliminated its ability to bind the human antibodies but not its ability to bind the murine or rabbit immune antibodies, not even when the heating was prolonged for up to 60 min. Analogous results were obtained when the R4 protein was exposed to heating and then tested for binding of the corresponding immune or natural human antibodies. The antigen preparations were exposed to heating for 30 min in a step-wise fashion in the range 40–100 °C. Binding of the human antibodies was unaffected by heating of c^α and R4 up to 60 °C but, on the basis of A₄₀₅ recordings, was reduced by

Fig. 2. ELISA results (A₄₀₅) for rabbit anti-c^α antibodies (open columns), monoclonal anti-c^α antibodies (hatched columns) and a human gammaglobulin preparation (filled columns) probed against microtitre plates coated with c^α that had been untreated or heat-treated (100 °C) for the times indicated.

25 % by heating at 70 °C and by 60 % at 80 °C and was maximally affected at temperatures ≥ 90 °C. These results were interpreted to mean that the c^{α} and R4 epitopes that were recognized by the human antibodies were destroyed by the denaturing effect of the heating of the proteins, whereas the epitopes targeted by the immune antibodies resisted the denaturation. The results further support the above notion that the natural human and immune antibodies target different epitopes. Similar discrepancies between natural and induced antibodies have been described before (Guilbert *et al.*, 1982).

Experiments were performed to test the extent to which heat-denaturation of c^{α} and R4 would affect antibody binding by the proteins when individual human sera were tested, altogether using 60 sera from pregnant women from Norway and Zimbabwe. Individuals showed great variation of antibody levels (Table 2; Fig. 3), with generally higher levels in the Norwegian population than in the Zimbabwean population, as observed earlier (Moyo *et al.*, 2001). Compared with the

levels of signal in tests with the untreated antigens, the levels were reduced significantly with the heat-treated antigens, down to background levels for the majority of sera. The results indicate that the major component of the natural c^{α} - and R4-reactive human antibodies was directed against the heat-sensitive determinant(s) common to c^{α} and R4.

This study has shown that immune anti- c^{α} and anti-R4 antibodies recognize protein-specific epitopes that resist denaturation by SDS and/or heating, consistent with targets determined by amino acid sequences. Natural human c^{α} - and R4-reactive antibodies recognize targets that are shared by the two proteins and are destroyed by the denaturing procedures, consistent with binding sites determined by conformation. It has been shown that conformation also plays an important role in the binding by c^{α} of immune antibodies, in this case conformational effects of variations in the number of c^{α} repeat units (Madoff *et al.*, 1996). In the present study, we can exclude this type of protein structure variation, as all the final experiments were performed using the same c^{α} and R4 protein preparations. Rather, our results focus on the route of immunization as an important factor, parenteral for the immune antibodies and, presumably, mucosal presentation for the normal human antibodies. Mucosal immunization with GBS induced immune responses in both mice (Hordnes *et al.*, 1995) and humans (Hordnes *et al.*, 1996), with a major contribution by broadly cross-reacting antibodies (Hordnes *et al.*, 1996), which could have included c^{α} -/R4-reactive antibodies corresponding to the normal human antibodies measured in the present study. It will be important to test whether humans immunized during the course of invasive GBS disease or by parenteral vaccination (Paoletti *et al.*, 2000) generate antibodies against the heat-stable or heat-labile epitopes. Our observation that the putative conformation-determined epitope was heat-labile should facilitate the design of tests to discriminate between the two categories of antibodies.

Both c^{α} and R4 belong to a GBS protein family that includes members with large, tandemly arranged repeats (Michel *et al.*, 1992; Wästfelt *et al.*, 1996) and with considerable sequence similarity between different proteins (Lachenauer *et al.*, 2000; Michel *et al.*, 1992; Wästfelt *et al.*, 1996). This similarity may have resulted in conformational structures in different proteins that can be recognized by the same natural human serum antibodies as a result of antigenic mimicry. Findings described previously (Moyo *et al.*, 2001) as well as the data shown in Table 1 argue against a contaminating non- c^{α} /R4 antigen shared by the GBS strains 335 and 65604 as a target for the normal human serum antibodies.

Although the results described in the present study and in a previous study should broaden our knowledge of GBS immunology, they leave several challenges for further work, including investigations on the levels and specificity of c^{α} -/R4-reactive antibodies in mucosal secretions, factors that determine the generation of cross-reacting versus protein-specific antibodies and the biological functions of the cross-reacting versus the protein-specific antibodies.

Table 2. Binding of antibodies in sera from healthy pregnant women by untreated and heat-treated c^{α} and R4 proteins

Proteins were heat-treated at 100 °C. Binding was measured as the ELISA ratio as described in Methods. In all cases, the difference between antibody levels with untreated and heat-treated antigen was significant ($P < 0.05$; Mann–Whitney U test).

Antigen	Heat-treatment	Median (range) ELISA ratio with sera from:	
		Norway ($n = 30$)	Zimbabwe ($n = 30$)
c^{α}	—	0.414 (0.092–1.367)	0.248 (0.092–1.001)
c^{α}	+	0.107 (0.067–0.272)	0.105 (0.081–0.550)
R4	—	0.532 (0.097–1.143)	0.273 (0.107–1.077)
R4	+	0.131 (0.067–0.278)	0.120 (0.085–0.298)

Fig. 3. Distribution of ELISA ratios for antibodies in sera from 60 pregnant women, 30 from Norway and 30 from Zimbabwe, tested against R4 protein that had been untreated (open columns) or heat-treated (100 °C, 30 min) (hatched columns).

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