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Immunogenicity and Protective Efficacy of the Alpha C Protein of Group B Streptococci Are Inversely Related to the Number of Repeats

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Infection by group B streptococci (GBS) is an important cause of bacterial disease in neonates. Alpha C protein is a protective cell surface-associated protein of GBS. This protein contains a repeat region flanked by N and C termini. Variable expression of tandem repeating units of alpha C proteins had been found among clinical isolates of GBS. We examined the effect of the number of repeats on the immunogenicity of the alpha C protein and its ability to elicit protection from GBS infection in a neonatal mouse model. Mice were immunized with purified alpha C proteins of constructs containing various numbers of repeats ($n = 1, 2, 9$, and 16) and the N- and C-terminal regions. Both the N-terminal and the repeat regions contain protective and opsonic epitopes. Antibody responses to the alpha C protein constructs with various numbers of repeats were tested with enzyme-linked immunosorbent assay plates coated with either native, nine-repeat alpha C protein or "repeatless" N-terminal antigen. An inverse relationship was found between the number of repeats and the immunogenicity of the alpha C protein; this effect was most pronounced on titers of antibody to the N-terminal region. An inverse relationship was also observed between the number of repeats and protective efficacy, i.e., mouse dams immunized with 5 μg of one- or nine-repeat alpha C protein transferred protective immunity to 65 or 11% of their pups, respectively ($P < 0.0001$). Thus, the presence of multiple repeats appears to lessen the antibody response to the complete alpha C protein, and especially the antibody response to its N-terminal region, and suggests a mechanism whereby repeat elements contribute to the evasion of host immunity.

Group B streptococci (GBS) are the leading cause of meningitis, pneumonia, and sepsis in neonates (1). The alpha C protein is a protective cell surface antigen present in approximately 50% of all clinical isolates and in 90% of the non-type III GBS isolates (10). The cloned alpha C protein gene (*bca*) contains a series of nine identical tandem repeats, flanked by an N- and a C-terminal region (Fig. 1) (20). The repeat region contains a protective epitope, as has been shown by passive immunization with a repeat-region-specific monoclonal antibody, 4G8 (15, 18). The N-terminal region of the alpha C protein also contains protective epitopes (15). The C-terminal region appears to be anchored in the cell wall (23) and has not been shown to have a role in protective immunity.

The alpha C protein in clinical isolates of GBS strains varies in molecular size (62.5 to 167 kDa), as has been shown by Western blotting with monoclonal antibody 4G8 (18). These variations in molecular size correspond to the number of tandem repeats within the *bca* gene, which encodes the alpha C protein (19, 20). Variation in repeat number occurs by intragenic deletions within the *bca* gene, presumably by homologous recombination. GBS expressing alpha C protein with a diminished number of repeats (one or two repeats) can escape antibody-mediated immunity, because loss of repeating units results in loss of conformational epitopes, thus rendering the protein unrecognizable by antibodies elicited to the native, nine-repeat protein (7, 19). If a decreased number of repeats confers a selective advantage, this raises the question of why multiple tandem repeats occur within the gene and protein.

Using passive immunization with rabbit antiserum elicited to repeat number-variant constructs of the alpha C protein, we previously demonstrated that antibodies raised to low-number-repeat alpha C protein had greater protective efficacies than those elicited to the wild-type, nine-repeat protein (7). In the present study, the effects of the number of repeats on the immunogenicities and protective efficacies of the complete alpha C protein and of its N-terminal region were examined. Active immunization of mouse dams was followed by challenge of neonatal mice with an otherwise lethal inoculum of GBS (22). The mouse model allows us to directly examine the effects of the number of repeats on the magnitude and functionality of the immune response to these GBS antigens. In addition, the individual contributions of repeat and N-terminal regions to protective immunity when both were presented in a one-repeat construct were examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* HMS174(DE3) (5) and BL21(DE3) (25), containing the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase gene (26), were used as recipients for the plasmids (pT7-7) that contained the various repeat regions flanked by an N-terminal and a C-terminal region (Fig. 1). The plasmids (pT7LM2, pT7LM7, pT7LM16, and pT7LM39) have been described previously (7), and the N-terminal region has been cloned in pET24a (pDEK14) (15).

Expression and purification of alpha C proteins. *E. coli* HMS174(DE3) and BL21(DE3), containing plasmids with the different repeat and N-terminal constructs, were grown to an A_{650} of 0.6 in tryptone medium (Difco, Detroit, Mich.) plus ampicillin (100 μg/ml). Expression of the repeat and N-terminal proteins was induced by IPTG (0.4 mM) for 3 h and stopped by chilling on ice. Alpha C proteins were purified as described previously (7). For the purification of the "repeatless" N-terminal region of the alpha C protein, plasmid pDEK14 (15),

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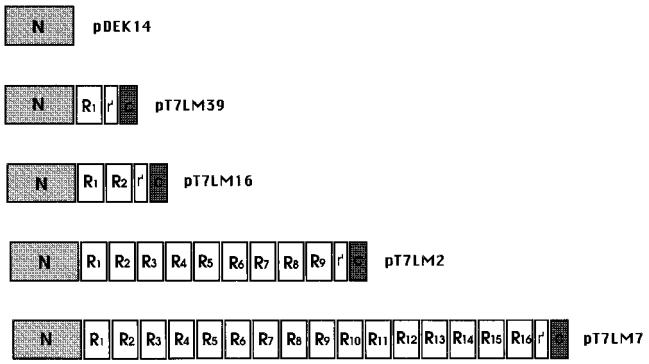

FIG. 1. Schematic view of the multimeric repeat constructs and the N-terminal region of the alpha C protein gene. The repeatless N-terminal construct (top) contains only the N-terminal region of the protein. Each of the other constructs contains a repeat region flanked by N- and C-terminal domains. The pT7LM2 construct with nine repeats is the native alpha C protein. C, C terminus; N, N terminus; R, repeat; r′, partial repeat.

containing the N-terminal sequence fused with a poly-histidine tag upstream of the T7 promoter, was used. Two to 5 mg of purified N-terminal protein was obtained from 100 ml of culture of *E. coli* BL21(DE3) containing plasmid pDEK14. An affinity column charged with Ni^{2+} (Novagen, Madison, Wis.) with high affinity for histidine was used for the purification of repeatless N-terminal protein.

ELISA and ELISA inhibition. Antibodies in mouse antiserum elicited to 1-, 2-, 9-, and 16-repeat proteins and to the N-terminal alpha C protein were measured by enzyme-linked immunosorbent assay (ELISA) (7). ELISA inhibition was measured with the formula percent inhibition = $\{[A_{405(\text{uninhibited control})} - A_{405(\text{sample})}]/A_{405(\text{uninhibited control})}\} \times 100\%$.

Immunogenicity of alpha C proteins in mice. To determine the immunological responses of the mice to the constructs of the alpha C protein of GBS with various numbers of repeats, female mice (CD-1; Charles River Labs, Wilmington, Mass.) that were 8 weeks of age were immunized intraperitoneally with 5, 10, or 25 μg of purified alpha C proteins containing 1 repeat, 2 repeats, 9 repeats, or 16 repeats or with the purified repeatless N-terminal region of the alpha C protein of GBS. Concentrations of the purified proteins were determined by the bicinchoninic acid method (Pierce, Rockford, Ill.). Immunizing doses were prepared by dilution of a single stock solution for each antigen. The purified proteins were adsorbed to 0.5 ml of aluminum hydroxide (alhydrogel) as the adjuvant. Groups of four mice were used for each immunization. Primary immunization was given on day 0, and a booster injection was given on day 21. Sera were collected each day before immunization and 3 weeks after the last dose and tested for antibody levels by ELISA.

Mouse protection studies. A neonatal mouse model of GBS infection was used to assess the protective efficacies of active immunization with one- and nine-repeat and N-terminal alpha C proteins (22). Mice were immunized intraperitoneally with 5, 10, or 25 μg of the purified proteins plus aluminum hydroxide on day 0 and boosted on days 21 and 42. Mice were bred 1 week after the last booster, and neonatal mouse pups (born 4 weeks after the last booster) were challenged intraperitoneally (within 48 h after birth) with 3×10^4 to 5×10^4 CFU of GBS strain A909 (10^3 times the 50% lethal dose). Strain A909 expresses polysaccharide Ia and cell surface proteins α (nine repeats) and β. Survival was assessed 48 h after challenge, and survival data for groups whose dams had been immunized with different proteins were compared by Fisher's exact test.

Immunoprecipitation of N-terminal antibodies. Antibodies to N-terminal antigen were removed by immunoprecipitation according to published methods (2, 8) with minor modification. Lipids in the rabbit antisera were removed by centrifugation in an Eppendorf centrifuge for 15 min at 14,000 × *g* at room temperature. Subsequently, rabbit antisera were incubated at 37°C for 2 h with purified N-terminal antigen (1.5 mg of N-terminal antigen/ml of antiserum). A second incubation of 300 μg of N-terminal antigen/ml of serum was required to remove small quantities of residual antibodies to the N-terminal region. The concentration of antigen required to remove N-terminal antibodies was estimated by ELISA inhibition. Antisera were incubated overnight at 4°C, and precipitated antigen-antibody complex was removed by centrifugation in an Eppendorf centrifuge for 15 min at 14,000 × *g* at 4°C. Antibodies to N-terminal and native alpha (nine-repeat construct) antigens, before and after absorption with N-terminal antigen, were quantified by ELISA.

Opsonophagocytic killing after removal of N-terminal antibodies. Opsonophagocytic assays were performed to determine the functionalities of antibodies to the repeat and N-terminal regions of the alpha C protein of GBS (3). Rabbit antiserum of different dilutions (range of final dilutions, 1/50 to 1/5,000), human polymorphonuclear leukocytes ($\sim 3 \times 10^6$), and human complement were used for killing of GBS strain A909 ($\sim 1.5 \times 10^6$ CFU). The amount of opsonophagocytic killing (log kill) was determined by subtracting the log of the number of colonies surviving the 1-h assay from the log of the number of CFU at the zero time point. Opsonophagocytic killing was compared by the two-tailed *t* test.

Protection from GBS after removal of N-terminal antibodies. The mouse protection assay was adapted from Rodewald et al. (22). Groups of four CD-1 outbred pregnant mice were injected intraperitoneally (3 to 4 days before delivery) with 0.5 ml of undiluted rabbit serum elicited to one-repeat alpha C protein (7) (i) containing antibodies to the repeat and N-terminal regions, (ii) containing antibodies to the repeat region only (N-terminal antibodies were removed by immunoprecipitation), or (iii) containing antibodies to the repeat and N-terminal regions (incubated with an irrelevant protein, bovine serum albumin [BSA], as a control). Antiserum elicited to polysaccharide Ia coupled to tetanus toxoid (Ia-TT) and preimmune serum were used as positive and negative controls. Pups were challenged intraperitoneally with 3×10^4 to 5×10^4 CFU of GBS strain A909 (10^3 times the 50% lethal dose) within 48 h of birth. Survival was assessed 48 h after challenge, and survival data for groups whose dams had been immunized with different antisera were compared by Fisher's exact test.

RESULTS

Immunogenicity of the alpha C protein. To determine the effect of the number of repeats on the immunogenicity of the alpha C protein, antisera from mice immunized with different repeat constructs were compared for antibody titers to the native alpha C protein (nine-repeat construct) by ELISA. One-repeat alpha C protein was the most and 16-repeat alpha C protein was the least immunogenic (Fig. 2A). There was a significant inverse relationship between the number of repeats and the immunogenicity of the alpha C protein ($P = 0.015$; Jonckheere nonparametric k-sample test) (11).

Immunogenicity of the N-terminal region. To determine the effect of the number of repeats on the immunogenicity of the N-terminal domain within the alpha C protein, antisera from mice immunized with the different repeat constructs and with the repeatless N-terminal construct of the alpha C protein were compared for antibodies to N-terminal protein by ELISA. Figure 2B shows a strong inverse relationship between the number of repeats and the immunogenicity of the N-terminal region. The highest titers were shown by antisera elicited to repeatless N-terminal protein, and the lowest titers were shown by antisera elicited to 16-repeat alpha C protein. The trend of inverse relationship was highly significant ($P < 0.0001$, Jonckheere nonparametric k-sample test). Antibody levels elicited by the different antigens were compared by the Wilcoxon test: for the N-terminal construct versus the 1-repeat construct, $P = 0.028$; for the N-terminal construct versus the 16-repeat construct, $P = 0.026$; for the 1-repeat construct versus the 16-repeat construct, $P = 0.0266$; and for the 1-repeat construct versus the 9-repeat construct, $P = 0.15170$. A similar inverse relationship was found with rabbit antisera (data not shown).

Mouse protection study. The protective efficacies of one- and nine-repeat alpha C proteins and the N-terminal protein were compared. Mice were immunized with 5, 10, or 25 μg of the purified proteins. A dose-response effect was observed on the level of antibody but not on the level of protection of mice immunized with the nine-repeat construct (Table 1). However, no effect on the level of antibody or the level of protection was observed for mice immunized with the one-repeat construct (Table 1). When results of immunizations with the one- and nine-repeat alpha C protein constructs were compared to determine protective efficacies, differences were most pronounced at the 5-μg dose. At this dose, a considerably higher level of protection was obtained by the one- than by the nine-repeat alpha C protein construct (Table 1). However, at the 10- and 25-μg doses, differences in levels of protection diminished (Table 1). Immunization with N-terminal protein produced a

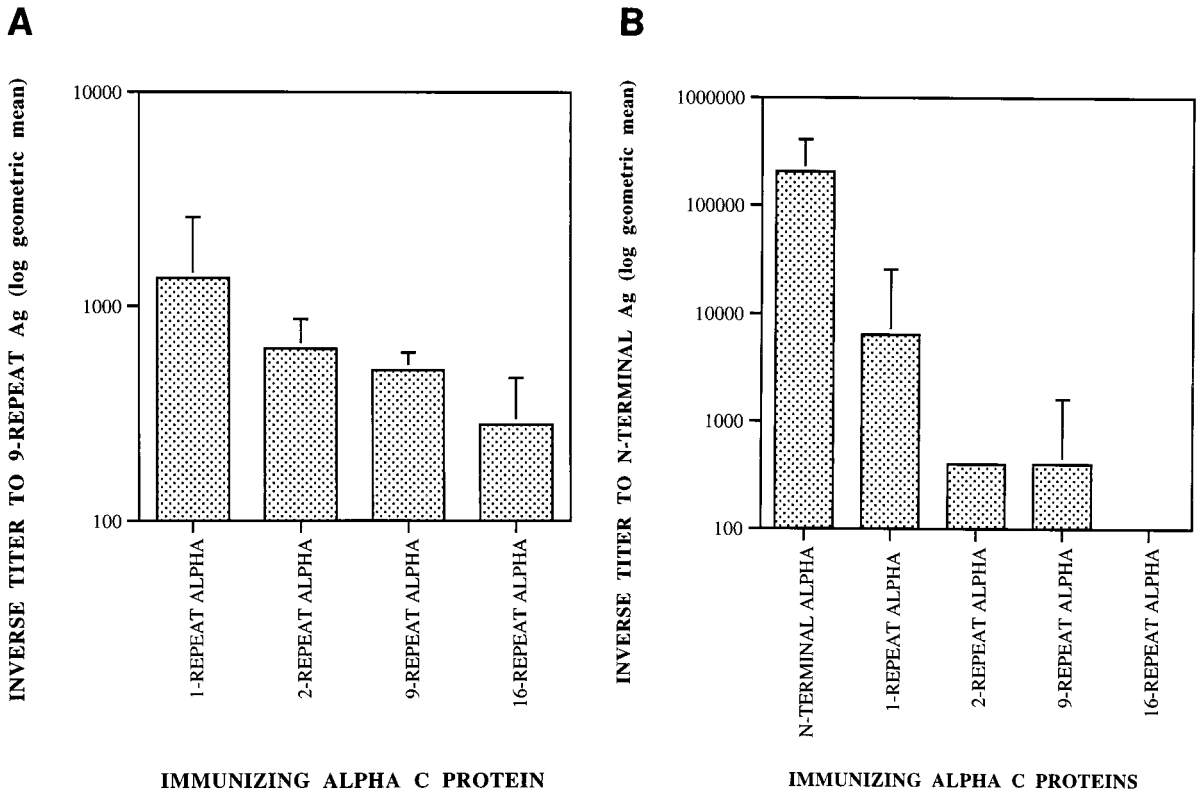

FIG. 2. ELISA showing the effect of the number of repeats on the immunogenicity of mice to native (nine repeats) alpha C protein (A) or to the N-terminal region of alpha C protein (B). The mouse antisera used here were raised to 1-, 2-, 9-, and 16-repeat alpha C protein and to N-terminal alpha C protein (25 μg/mouse). Microtiter plates were coated with purified nine-repeat alpha C protein (A) or with N-terminal alpha C protein (B) at 1 μg/ml. Twofold serial dilutions were made of the antisera (starting dilution, 1:100). The *x* axis represents the number of repeats; the *y* axis represents the log geometric mean of inverse titers of antisera from four mice per immunization. Ag, antigen. The error bars represent the standard deviation of three independent experiments.

dose-response effect in levels of antibody and protection (Table 2).

Relative contributions of repeat and N-terminal regions to GBS protection by one-repeat alpha C protein. Since both repeat and N-terminal regions are protective, and since both are present in the one-repeat construct, the relative contribution to GBS protection of each region of that protein was studied. ELISA inhibition with antisera elicited to one-repeat alpha C protein, with purified one-repeat and N-terminal proteins as inhibiting antigens, and with the one-repeat alpha C protein as the coating antigen suggested that the antibodies were predominantly directed to the repeat region and that they

TABLE 1. Protection of neonatal mice from GBS by active immunization with one- and nine-repeat alpha C protein

Immunizing antigen	Dose of immunization (μg/mouse)	Titer to native alpha C protein	No. of pups alive/total no. of pups	% Survival[a]
One-repeat alpha C protein	5	10,763	15/23	65
	10	51,200	21/33	64
	25	12,800	8/15	54
Nine-repeat alpha C protein	5	504	4/38	11
	10	3,805	18/33	55
	25	6,400	15/40	37
Ia-TT (positive control)	2	Negative	12/12	100
Saline (negative control)	0	Negative	4/22	18

[a] Significant differences in levels of protection of the various antigens were determined by Fisher's exact test. The results were as follows: for the one- versus the nine-repeat antigen at 5 μg/mouse, $P < 0.0001$; for the one- versus the nine-repeat antigen at 10 μg/mouse, $P = 0.6170$; for the one- versus the nine-repeat antigen at 25 μg/mouse, $P = 0.3629$; and for the Ia-TT conjugate versus the negative control, $P < 0.0001$.

TABLE 2. Protection of neonatal mice from GBS by active immunization with repeatless N-terminal antigen of the alpha C protein

Immunizing antigen	Dose of immunization (μg/mouse)	Titer to N-terminal antigen	No. of pups alive/total no. of pups	% Survival[a]
N-terminal protein	5	5,382	12/46	26
	10	30,444	20/32	63
	25	86,108	14/22	64
Ia-TT (positive control)	2	Negative	28/29	97
Saline (negative control)	0	Negative	3/29	10

[a] Significant differences in levels of protection of the different antigens were determined by Fisher's exact test as follows: for the N-terminal antigen at 5 μg/mouse versus the negative control, $P = 0.1398$; for the N-terminal antigen at 10 μg/mouse versus the negative control, $P < 0.0001$; for the N-terminal antigen at 25 μg/mouse versus the negative control, $P < 0.0001$; for the N-terminal antigen at 5 μg/mouse versus the positive control, $P < 0.0001$; for the N-terminal antigen at 10 μg/mouse versus the positive control, $P = 0.0013$; for the N-terminal antigen at 25 μg/mouse versus the positive control, $P = 0.0032$; and for the positive control versus the negative control, $P < 0.0001$.

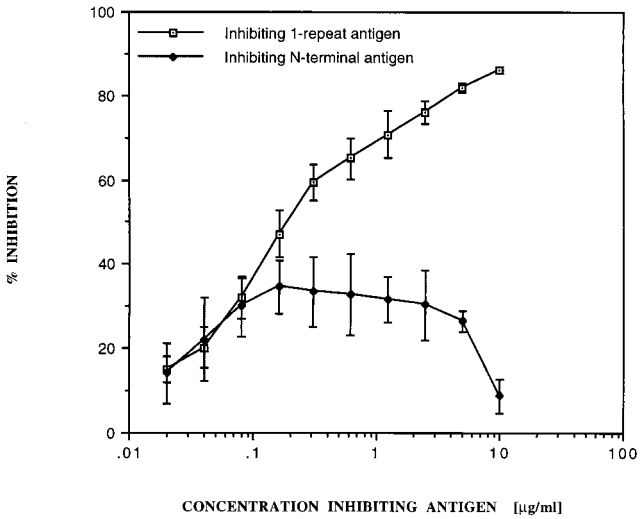

FIG. 3. ELISA inhibition showing the presence of antibodies to the repeat region and to the N-terminal region of alpha C protein in antiserum elicited by the one-repeat construct that protected 65% of the pups from GBS challenge. Antisera from four mice immunized with 25 μg of one-repeat alpha C protein were pooled. Plates were coated with purified one-repeat alpha C protein; one-repeat alpha C protein and N-terminal alpha C protein were used as inhibiting antigens (starting concentration, 10 μg/ml). Error bars represent the standard deviation of three independent experiments.

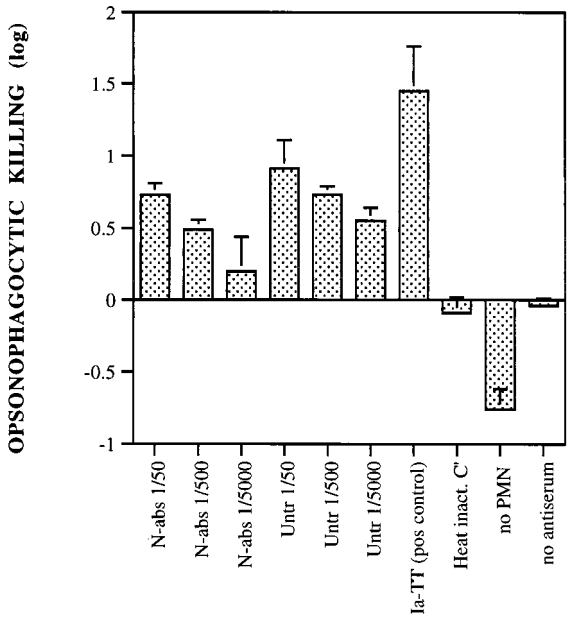

FIG. 4. Opsonophagocytic killing of GBS strain A909 by rabbit antiserum elicited to the one-repeat alpha C protein construct before (untreated [Untr]) and after (N terminus absorbed [N-abs]) removal of antibodies to the N terminus. Results of three independent experiments were averaged and are shown in this figure. Error bars represent the standard deviation of three independent experiments. Negative controls included the use of heat-inactivated complement (heat inact. C′), the absence of polymorphonuclear leukocytes (PMN), and the absence of antiserum. As a positive control (pos control), rabbit antiserum elicited to protective Ia-TT was used. The two-tailed *t* test showed no significant differences between opsonophagocytic killing by one-repeat construct-elicited antiserum with (Untr) and without (N-abs) antibodies to the N terminus: N-abs versus untreated 1:50 dilution ($P = 0.0696$), versus untreated 1:500 dilution ($P = 0.1237$), or versus untreated 1:5,000 dilution ($P = 0.2780$).

were minimally directed to the N-terminal region (Fig. 3). However, when the same ELISA inhibition was performed with the repeatless N-terminal construct as the coating antigen, a strong inhibition of antibody binding to the coating antigen was found (data not shown). This result suggests that the N-terminal region in the repeat-containing construct is less available for antibody binding than the N-terminal region in the repeatless construct. Antisera elicited in rabbits to one-repeat alpha C protein gave similar results (data not shown).

To determine the relative contributions to opsonophagocytic killing and neonatal mouse protection of antibodies to the N-terminal and repeat-containing regions within the protective one-repeat construct, we performed absorption experiments to remove N-terminus-specific antibodies from antiserum raised to the one-repeat construct. For these experiments, rabbit instead of mouse antisera were used, since insufficient volumes of mouse antisera were available. Mouse and rabbit antisera elicited to one-repeat alpha C protein gave similar results in both levels of protection and levels of antibody (reference 7 and this study). Antibodies to the N terminus were removed by two successive immunoprecipitations with purified N-terminal antigen. ELISA using microtiter plates coated with repeatless N-terminal antigen and native alpha C protein (which contains nine repeats and the N-terminal region) showed that nearly all of the antibodies to the N terminus were removed by immunoprecipitation (titer declined from 102,400 to 3,200) and that the ELISA titer to the wild-type nine-repeat protein declined by only a single dilution (from 204,800 to 102,400). Opsonophagocytic killing of A909 by one-repeat construct-elicited antibodies showed no significant decrease after removal of antibodies to the N terminus (Fig. 4). Similarly, neonatal protection from GBS declined from 74 to 61% after removal of antibodies to the N terminus (Table 3), which was not significant. Sham immunoprecipitation with BSA did not reduce the ELISA titers and, as expected, did not result in a significant decrease in opsonophagocytic killing.

DISCUSSION

This study has shown an inverse relationship between the number of repeats and the immunogenicity of the alpha C protein of GBS. Our data demonstrate that increasing the number of tandem repeats lowers the immunogenicity of the purified antigen, a finding that has implications for the design of vaccines based on repeat-containing proteins. Moreover, elicitation of a weaker antibody response by infection with

TABLE 3. Protection of neonatal mice from GBS by one-repeat construct-elicited antibodies before and after removal of antibodies to the N terminus

Rabbit antiserum elicited to:	No. of pups alive/total no. of pups	% Survival[a]
One-repeat construct, untreated	28/38	74
One-repeat construct, N terminus absorbed	20/33	61
One-repeat construct, BSA absorbed	23/39	59
Ia-TT (positive control)	33/37	90
Preimmune serum (negative control)	7/48	15

[a] Significant differences in levels of protection for different antisera were identified by Fisher's exact test as follows: for the untreated one-repeat construct versus preimmune serum, $P < 0.0001$, and for Ia-TT versus preimmune serum, $P < 0.0001$. Not significantly different were the levels of protection for the untreated one-repeat construct versus the N-terminal absorbed one-repeat construct ($P = 0.3113$) and the untreated one-repeat construct versus the BSA-absorbed one-repeat construct ($P = 0.2295$).

GBS containing alpha C protein with a high number of repeats than by infection with GBS expressing alpha C proteins with a low number of repeats may enhance the ability of the high-number repeat GBS to escape protective immunity. The inverse relationship between the number of repeats and immunogenicity was most pronounced for the N-terminal region of the alpha C protein. The highest levels of antibody to the N-terminal region were found in the repeatless N-terminal construct, the next highest levels were found in the antisera elicited to the 1-repeat construct, and the lowest levels were found in the antisera elicited to the 16-repeat construct. The N-terminal region of the nine-repeat protein is an effective target for antibody-mediated killing of GBS and protection from GBS infection (reference 15 and this study). These results imply that the domain can be bound by antibody but that it does not evoke an antibody response. A similar phenomenon was found for the N-terminal region of the parasitophorus antigen P126 of *Plasmodium falciparum* (4). Banic et al. found that the N-terminal region was poorly immunogenic when it was exposed to the immune system as part of the P126 protein in mice, but that it elicited antibodies that could also bind the N-terminal region in the native molecule in P126 when it was exposed as a synthetic peptide corresponding to the N-terminal region of the P126 antigen. Therefore, the N-terminal region of the P126 antigen was antigenic but not immunogenic when it was presented as part of the P126 antigen. The explanation for the diminished immunogenicity of the N-terminal region of the alpha C protein, within the context of the repeat-containing protein, is not known. Although the relative mass of the N-terminal region decreases as the number of repeats increases, this does not explain the observed differences. For example, 10 μg of repeatless N-terminal protein contains approximately the same mass of N-terminal peptide as 25 μg of the two-repeat construct. The titer for the repeatless N-terminal construct was 1:30,444 (Table 2), and that for the two-repeat construct was 1:400 (Fig. 2B).

The effect of the number of repeats on protective efficacy of active immunization with alpha C protein of GBS confirmed results of passive-immunization studies (7). Differences in levels of protection were thought to be due to differences in antigen binding; i.e., antibodies raised to the antigens with higher numbers of repeats failed to recognize the antigens with smaller numbers of repeats present in escape mutants. In this study, mice were actively immunized with the alpha C protein constructs and alum rather than Freund's adjuvant was used, more closely modelling potential human immunization.

In this study, mouse dams immunized with 5 μg of one- or nine-repeat alpha C protein transferred protective immunity to 65 or 11% of their pups, respectively, following challenge with alpha-positive GBS. These findings were similar to those obtained by passive immunization with rabbit antisera. However, differences in the protective efficacies in this model may have been due both to differences in the immunogenicities of the antigens and to the different populations of antibodies elicited. When the immunization dose was increased to 10 or 25 μg, levels of protection increased for nine-repeat protein but never exceeded the level of protection by one-repeat protein. Thus, the maximum level of protection was reached for one-repeat alpha C protein at a dose of 5 μg or lower but to obtain higher levels of protection for nine-repeat alpha C protein, a higher immunizing dose was required.

One-repeat alpha C protein is more immunogenic than nine-repeat alpha C protein (Table 1). A possible explanation for the difference in levels of immunogenicity is that the one-repeat construct contains both T- and B-cell epitopes but that the nine-repeat construct acts mostly as a T-cell-independent antigen due to the presence of the repeated epitopes. Multivalent antigens with repetitive epitopes, such as polysaccharides, activate B cells without the assistance of T-helper cells, as do polymeric proteins with repeating epitopes (e.g., bacterial flagellin) (16). T-cell-independent antibody responses tend to be weaker and are not boosted with repeated immunization. Thus, the repeat-containing alpha C protein may be more prone to activate B cells in a classic T-cell-independent manner, which might account for the immunologic properties of this antigen.

Since both the repeat and N-terminal regions have protective properties and since both regions, when presented in the one-repeat construct, induce antibodies, questions arose as to the relative contribution of each domain to protection. Opsonophagocytic killing of GBS strain A909 in the presence of antiserum elicited to the one-repeat construct was unaffected by removal antibodies to the N terminus (Fig. 3), which suggests that the repeat region in the one-repeat construct was predominantly responsible for protection of the pups challenged by A909. To test this hypothesis, the same one-repeat construct-elicited antiserum was studied, with and without antibodies to the N terminus, for protection of neonates from GBS challenge. Although 97% of the antibodies to the N terminus were removed by immunoprecipitation, no significant differences in protection of neonatal mice from GBS were found before and after removal of antibodies to the N terminus. These results support the hypothesis that, in a construct with repeat and N-terminal regions, the repeat region is predominantly responsible for protection. We concluded from these results that the N-terminal region contributed substantial protection only when it was presented in a repeatless construct.

Results of our present and earlier studies (7, 19) suggest that one function of variation in the number of repeats is to escape antibody-mediated immunity. Intragenic recombination within the repeats of the alpha C protein gene (*bca*) may result in GBS with lower-number repeat proteins and, presumably, with higher-number repeat proteins (18, 19). We demonstrated that loss of repeating units by intragenic recombination in the repeat region of the alpha C protein is an important mechanism of escape from antibody-mediated immunity (19). The present study suggests that GBS with higher-number repeat alpha C proteins may elicit less antibody to the protein, thus conferring a selective advantage to isolates containing higher-number repeat alpha C proteins and helping to explain why these strains occur among human isolates. The fact that the number of repeats within the alpha C protein appears to be normally distributed, with a mean of 9 or 10 repeats among clinical isolates (18), may imply selection for both greater and lesser numbers of repeats.

Most GBS express surface proteins with multiple tandem repeats (17, 24, 27). Repeat-containing antigens are found in many other pathogens as well, with diverse examples including *Plasmodium* sp., group A streptococci, pneumococci, and *Blastomyces dermatitidis* (9, 12–14, 21, 27, 28). The role of repeating elements within these proteins has never been completely elucidated, although functions attributed to them have included binding to host structures (6) and antigenic variation (12, 18). An additional attribute may be the ability of the repeat content of a protein antigen to modulate the immune response to the antigen. With the alpha C protein, repeat content affects not just the overall immunogenicity of the protein but the epitope specificities of the antibodies elicited to the protein. This factor will need to be considered in the design of vaccines based on repeat-containing proteins. If this phenomenon occurs during infection, the presence of multiple repeats may contribute to the pathogenicity of the organism by diminishing the ability of

the host to mount an effective antibody response to the organism.

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