Recombinant Group B *Streptococcus* Alpha-Like Protein 3 Is an Effective Immunogen and Carrier Protein

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Conjugate vaccines against pathogens of multiple serotypes are optimized when all components induce functional antibody, resulting in broadened coverage. While most clinical studies of vaccines against group B *Streptococcus* (GBS) have evaluated conjugates composed of capsular polysaccharide (CPS) coupled to tetanus toxoid, conjugates prepared with GBS proteins as carriers have also been efficacious in animals. Here, we report that recombinant GBS alpha-like protein 3 (rAlp3) is both a strong immunogen and a viable carrier protein for type III CPS. The type III CPS-specific immunoglobulin G (IgG) titer rose from <100 to 64,000 among mice that received type III CPS coupled to rAlp3 (III-rAlp3) compared with an absence of a specific response among mice that received an uncoupled mixture. Most (94%) newborn pups born to III-rAlp-vaccinated dams survived challenge with viable type III GBS, compared with 43% survival among those born to dams that received the uncoupled mixture (P < 0.0001). A tricomponent conjugate of type III CPS, rAlp3, and a GBS recombinant beta C protein lacking its IgA binding site (III-rAlp3-rBCP<sub>III</sub>) provided protection against a serotype III strain and a serotype Ia strain bearing beta C protein. High-titered anti-rAlp3 rabbit serum opsonized Alp3-containing strains of two GBS serotypes (types V and VIII) and invasive type III strains bearing the cross-reactive Rib protein for in vitro killing by human peripheral blood leukocytes. Thus, the potential exists for the inclusion of rAlp3 in a GBS vaccine formulated to provide multiserotype coverage.

*Streptococcus agalactiae*, or group B *Streptococcus* (GBS), is an important cause of invasive infection in newborns, pregnant women, the elderly, and immunocompromised adults. The most prevalent GBS serotypes causing neonatal GBS diseases in the world (16, 18, 19), including the United States, Germany, and Malawi, are type Ia (~14% to 30%) and type III (~50% to 68%). Serotype V has emerged as the most common serotype (~30%) isolated from nonpregnant adults (14, 19), and serotypes VI and VIII are prevalent colonizers of pregnant women in Japan (25). Early vaccine trials with purified, uncoupled type Ia, II, and III capsule polysaccharides (CPSs) showed that these antigens evoked low and variable type-specific antibody responses in healthy adults (2, 4). Later vaccine trials of conjugate vaccines created by the covalent attachment of the CPS to an immunogenic carrier protein, such as tetanus toxoid (TT), enhanced the immune response to the CPS and resulted in high levels of type-specific, functionally active antibody predominantly of the immunoglobulin G (IgG) class (1, 40).

Thus far, only a few protein carriers have been used for the preparation of licensed conjugate vaccines (15). Three protein carriers, including diphtheria mutant protein cross-reactive material (CRM<sub>197</sub>), TT, and diphtheria toxoid have been used in *Haemophilus influenzae* type b conjugate vaccines, whereas CRM<sub>197</sub>, TT, and diphtheria toxoid have been used in *Neisseria meningitidis* conjugate vaccines (15, 39). Seven pneumococcal CPSs have each been coupled to CRM<sub>197</sub> to create the multivalent conjugate vaccine against *Streptococcus pneumoniae* in-

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bodies against R28 confer protective immunity against S. pyogenes disease in mice (44). Rabbit antiserum to a GBS purified alpha-like protein, later named Alp2, conferred protection against an alpha-like protein-expressing GBS type V strain in a neonatal mouse model of GBS disease (26). The ability of an alpha-like protein to elicit antibody-mediated protection in experimental animals provided the rationale to investigate whether Alp3 could be used as an immunogen and/or as a protein carrier for CPS antigens in the development of GBS vaccines.

We have recently demonstrated that a recombinant BCP and a recombinant BCP modified to eliminate its human IgA-binding site (rBCP^{Alp3}) were not only effective immunogens but also strong carriers for the type III CPS, creating vaccines with greater serotype coverage than a conjugated vaccine prepared with a non-GBS protein carrier (47). In this report, we describe the expression and purification of a recombinant Alp3 (rAlp3) and demonstrate its immunogenicity in mice and its ability to be a good carrier protein for GBS type III CPS. Type III CPS conjugate vaccines prepared with rAlp3 (III-rAlp3) elicited IgA in adult female mice, which provided protection against lethal GBS challenges to their newborn pups. In addition, rabbit serum to rAlp3 opsonized GBS for in vitro killing by human peripheral blood leukocytes, further confirming the potential of this protein as a viable vaccine antigen.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** GBS strain M781 (type III/Alp3- Rib- BCP^ -), A909 (type Ia/Alp3+ Rib- BCP -), CJB111 (type V/Alp3- Alp- Rib- BCP -), H1A00001 (type V/Alp3- Rib- BCP -), and JM9130013 (type VIII/ Alp3+ Rib- BCP -) were obtained from the Channing Laboratory culture collection. Soluble rBCP^{Alp3} was produced from GBS serotype Ia strain H36B, cloned into pTrcHisA, and expressed by Escherichia coli strain BL21(DE3) (47); soluble rAlp3 was produced from GBS serotype VIII strain H4A0158, cloned into pTrcHisA, and expressed by E. coli strain BL21(DE3) as described previously (7).

Six GBS type III strains (KMP103, KMP105, KMP114, KMP115, KMP120, and KMP121) were isolated from newborns with invasive GBS disease (41). All strains contained rib, with the exception of strain KMP114, which contained alp3 (41).

**Protein expression and purification.** To prepare each recombinant protein, a 1-liter culture of each strain was grown on brain heart infusion agar (BHI, Difco, Sparks, MD) supplemented with 100 μg/ml of ampicillin (Sigma, St. Louis, MO) with shaking at 200 rpm to an optical density at 600 nm of 0.6. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen, Carlsbad, CA) for 3 h at 37°C. Histidine-tagged recombinant proteins were purified by His-bind Fractogel chromatography (Novagen) as described previously (47).

**Generation of GBS conjugate vaccines.** GBS strain type III CPS was purified from GBS strain M781 as previously described (46). To create aldehydes on the sialic acid moieties of type III CPS, 15.2 mg of pure type III CPS was combined with 100 μg/ml of sodium cyanoborohydride (Matreya, Pleasant Gap, PA) to add to each mixture to promote the covalent attachment of the protein(s) to the oxidized CPS. The conjugation procedure, column purification of the conjugates, and biochemical analyses of the vaccines were performed as described previously (47).

**Immunogenicity and efficacy of rAlp3-containing vaccines in mice.** Eight-week-old female outbred CD-1 mice (Charles River Laboratories, Wilmington, MA) were vaccinated intraperitoneally with either rAlp3, III-rAlp3, III-rAlp3-BCP^{Alp3}, or type III CPS plus rAlp3 plus BCP^{Alp3} (III-rAlp3 + BCP^{Alp3}) (10 μg of protein content) mixed with equal amounts of alum (1.3% aluminum hydroxide; Brenntag Biosector, Frederikssund, Denmark) in a total volume of 0.5 ml. Booster doses with alum were administered on days 21 and 42 after the primary dose, and serum was collected before each booster dose. Control dams received saline with alum by the same route and schedule. Mice were then bred on day 56, and their litters were challenged with viable GBS. Neonatal mouse pups (<24 h of age) were challenged with GBS at ~8.5 × 10^6 CFU/pup (~100-fold 50% lethal dose [LD₅₀]) for strain M781, ~5 × 10^6 CFU/pup (~100-fold LD₅₀) for strain A909, ~9 × 10^6 CFU/pup (~200-fold LD₅₀) for strain CJB111, ~7.9 × 10^6 CFU/pup (~7.9-fold LD₅₀) for strain H1A00001, and ~9.0 × 10^6 CFU/ml for strain JM9130013. The challenge dose was administered intraperitoneally in a total volume of 50 μl of Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (Difco). The number of pups that survived the GBS challenge was assessed 48 h after challenge.

**ELISAs.** Specific IgG levels elicited by each vaccine component (type III CPS, rAlp3, and rBCP^{Alp3}) were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (47). Briefly, microtiter plates were coated with the following concentration of primary antigen: 0.2 μg/ml rAlp3, 0.2 μg/ml rBCP^{Alp3}, or 1.0 μg/ml type III CPS-human serum albumin in 0.1 M sodium carbonate buffer (pH 9.8). Mouse sera from each vaccine group were pooled and serially twofold diluted across wells of the coated microtiter plates. Phosphatase-labeled goat anti-mouse IgG (diluted 1:3,000) was used as the secondary antibody. ELISA titers were reported as the reciprocal of the highest dilution corresponding to an optical density at 405 nm of ≥0.2 after 30 min of development at 37°C with 1 mg/ml phosphate substrate (Sigma) dissolved in substrate buffer (1 M Tris, 0.3 mM MgCl₂, pH 9.8).

**Antigenicity of coupled and uncoupled rAlp3.** Competitive inhibition of the binding of mouse antiserum (diluted 1:10,000) to rAlp3-coated microtiter plates was assessed by using rAlp3, II-rAlp3, and III-rAlp3-BCP^{Alp3} as inhibitors at concentrations ranging from 1 to 1,000 ng/ml; competitive inhibition in rBCP^{Alp3}-coated microtiter plates was assessed by using rBCP^{Alp3} and III-rAlp3-BCP^{Alp3} as inhibitors. Percent inhibition was calculated as [(absorbance at 405 nm without inhibitor – absorbance at 405 nm with inhibitor)/absorbance at 405 nm without inhibitor] × 100; the percentage was plotted against the log₁₀ of the inhibitor concentration to generate inhibition curves.

**Rabbit antiserum to rAlp3.** A New Zealand White rabbit was given three 200-μg doses of rAlp3 mixed with incomplete Freund’s adjuvant at 21-day intervals. The rAlp3-specific IgG titer was determined with the use of the rAlp3 ELISA described above and goat anti-rabbit IgG-alkaline phosphatase conjugate as the secondary antibody.

**Whole-cell GBS Western blot analysis.** A 1-nl culture of GBS grown overnight was pelleted by centrifugation, washed with 1 ml PBS (Gibco, Grand Island, NY), and resuspended in 200 μl PBS. A 10-μl aliquot of resuspended cells was mixed with 15 μl loading buffer (NuPAGE LDS sample buffer and NuPAGE sample reducing agent; Invitrogen) and heated at 70°C for 10 min, and proteins were separated on NuPAGE Novex Bis-Tris gels (Invitrogen) according to the manufacturer’s protocol. Western blot analysis of separated GBS proteins was performed with mouse anti-rAlp3 serum followed by phosphatase-labeled goat anti-mouse IgG.

**PCR analysis.** GBS was treated with protoplast buffer (20% sucrose, 10 mM MgCl₂, 10 mM Tris, 0.05% Triton X-100, and 0.5 μM mutanolysin [pH 7.0]) at 37°C for 1 h. Supernatant recovered from the protoplast-treated sample was used directly for PCR amplification. The alp3 gene was amplified with alp3-2F (5'-TGAGGTGATGGCGTGCTGTC-3') and alp3-2R (5’-TGAGGTGATGGCGTGCTGTC-3') and alp3-2R (5’-TCTGTGTCCTCTGAGTGAC-3') at 42°C (annealing temperature of 66°C).

**Functionality of rAlp3 antiserum.** The functionality of rabbit antiserum to rAlp3 was evaluated using an in vitro opsonophagocytosis assay as described previously (5). Briefly, 2.5 μl of rabbit serum collected on day 57 following vaccination with rAlp3 was diluted in 47.5 μl of minimum essential medium (Invitrogen) to yield a final assay dilution of 1:100 when combined with 25 μl of baby rabbit complement (Cedarlane, Burlington, NC), 150 μl of human peripheral blood leukocytes, and 25 μl of GBS. Pooled rabbit antiserum to GBS type V CPS CRM₁₉₀ was used as a positive control. Viable GBS cells were enumerated by quantitative plate counts at time zero and after a 60-min incubation at 37°C with mixing. The difference in the number of GBS CFU was calculated and expressed as the average of two determinations per GBS strain.

**Statistics.** Fisher’s exact test was used to compare the efficacies of GBS vaccines (Instat, version 3.0a; Graphpad Software, San Diego, CA) at a significance level of 0.05.
RESULTS

Composition of the conjugate vaccines. Approximately 34% of sialic acid residues on the type III CPS were oxidized by sodium periodate. Oxidized type III CPS was conjugated to either rAlp3 alone or both rAlp3 and rBCP/H9004 IgA. Purified III-rAlp3 conjugate was composed of 32% (wt/wt) protein and 63% (wt/wt) carbohydrate, while the III-rAlp3-rBCP/H9004 IgA conjugate was composed of 32% (wt/wt) protein and 59% carbohydrate. The presence of rAlp3 in purified conjugate vaccines was confirmed by Western blot analysis (Fig. 1).

Antigenicity of coupled recombinant GBS proteins. Following covalent coupling to oxidized type III CPS, the antigenicity of rAlp3 remained intact, as determined by inhibition ELISA using uncoupled rAlp3 and mouse anti-III-rAlp3-rBCP/H9004 IgA serum (Fig. 2A). Similarly, the antigenicity of rBCP/H9004 IgA was also retained following coupling to type III CPS in the presence of rAlp3 (Fig. 2B). These data imply that the conjugation procedure did not destroy antigenic epitopes of rAlp3 when used alone or in combination with rBCP/H9004 IgA.

Immunogenicity of vaccines in mice. The immunogenicity of uncoupled rAlp3 and two conjugate vaccines containing rAlp3 (III-rAlp3 and III-rAlp3-rBCP/H9004 IgA) in mice was evaluated. An uncoupled mixture (III+rAlp3+rBCP/H9004 IgA) served as a control vaccine. Conjugate vaccines containing rAlp3 either as a single carrier protein or as one of two carrier proteins induced high levels of type III CPS-specific IgG in outbred mice following a single dose (Table 1). A second dose of conjugate vaccines at day 21 served to improve these titers 30- to 60-fold, with no additional increases in titer following the third dose. As expected, uncoupled rAlp3, either alone or admixed with rBCP/H9004 IgA, did not induce type III CPS-specific antibody.

High-titer IgG specific to rBCP/H9004 IgA was also elicited in mice that received conjugates containing this protein either coupled to type III CPS or admixed with type III CPS and rAlp3 (Table 2). Similarly, rAlp3 was highly immunogenic in mice following a single dose, with subsequent increases in specific IgG titers after each of two booster doses (Table 3). The immunogenicity of rAlp3 remained high whether coupled to type III CPS alone or in combination with type III CPS and rBCP/H9004 IgA.

Efficacy of vaccines in neonatal mice. Female mice that received GBS vaccines or saline were mated, and their litters were challenged with viable GBS. Most (94%) pups born to III-rAlp3-vaccinated dams and all 20 pups born to dams that received the combination III-rAlp3-rBCP/H9004 IgA vaccine survived.

### Table 1. GBS type III CPS-specific IgG response in mice elicited by immunization with rAlp3, III-rAlp3, III-rAlp3-rBCP/H9004 IgA, III+rAlp3+rBCP/H9004 IgA, and saline

| Vaccine                              | No. of mice | Titer of type III CPS-specific IgG at day*: |
|--------------------------------------|-------------|--------------------------------------------|
|                                      |             | 0 | 21 | 42 | 56 |
| rAlp3                                | 6           | <100 | 100 | 100 | 100 |
| III-rAlp3                            | 6           | <100 | 2,000 | 64,000 | 64,000 |
| III-rAlp3-rBCP/H9004 IgA             | 9           | <100 | 2,000 | 128,000 | 128,000 |
| III+rAlp3+rBCP/H9004 IgA             | 9           | <100 | 200 | 100 | <100 |
| Saline                               | 10          | <100 | <100 | <100 | <100 |

*Mean of duplicate determinations.
challenge with an ordinarily lethal dose of GBS type III strain M781 (Table 4). Interestingly, 44% survival and 43% survival were observed among pups born to dams that received uncoupled rAlp3 (rAlp3 or III-rAlp3-rBCP<sub>Δ68</sub>, respectively) and challenged with strain M781, a rate significantly greater (P < 0.001) than those for pups born to dams that received saline.

The tricomponent III-rAlp3-BCP<sub>Δ68</sub> vaccine was immunogenic in female mice, and antibody to this vaccine provided protection to 69% (25 survived of 36 challenged) of their pups against challenge with GBS type Ia strain A909 bearing BCP (Table 4). Significant (P < 0.005) reduction in survival was observed among pups born to dams that received an uncoupled tricomponent mixture (III+rAlp3+BCP<sub>Δ68</sub>). None of the vaccines tested were highly efficacious against challenge with GBS strain CJB111, with the highest survival rate of 39% afforded by III-rAlp3 (Table 4). This surprising finding led us to analyze whole GBS cell extracts for the expression of Alp3 among these GBS strains. Despite possessing the alp3 gene, GBS strain CJB111 did not express Alp3 as revealed by a lack of whole-cell reactivity with rAlp3 antiserum (Fig. 3). The lack of Alp3 expression provided a possible explanation for the poor efficacy of the rAlp3-containing vaccines against challenge with CJB111, a type V strain that also does not express the Rib protein or BCP.

Another group of female mice were vaccinated as described above, and their pups were challenged with GBS type V strain H1A00001 and type VIII strain JM9130013, both of which possess the alp3 gene and express Alp3 (Fig. 3). While pooled sera from dams of each of the four vaccine groups (rAlp3, III-rAlp3, III-rAlp3-BCP<sub>Δ68</sub>, and III+rAlp3+BCP<sub>Δ68</sub>) had high rAlp3-specific IgG titers (>1,000,000), the survival among litters was poor: from 0% to 3% for H1A00001 challenge and from 0% to 26% for JM9130013 challenge. These sera were also not functionally active against these GBS strains in the opsonophagocytosis assay (data not shown).

**Rabbit antiserum to rAlp3.** To explore the functional activity of rAlp3 antiserum further, a rabbit was vaccinated with rAlp3 admixed with incomplete Freund’s adjuvant. The titer of rAlp3-specific IgG rose from <100 before vaccination to 656,100 on days 42 and 50 and to 1,968,300 on day 57.

**Opsonophagocytosis of GBS strains by rabbit serum raised to rAlp3.** The in vitro opsonophagocytosis assay was used to further explore the number of GBS serotypes that would be susceptible to the antibody-mediated killing of GBS in vivo. GBS type V strain H1A00001, which possesses alp3 and expresses Alp3 (Fig. 3), was opsonized for killing by human peripheral blood leukocytes in the presence of complement (Table 5). GBS type VIII strain JM9130013, which bears Alp3, was also killed in this assay, in sharp contrast to the lack of opsonization and killing of type V strain CJB111 and type Ia strain A909, both which lack Alp3 (Fig. 3 and Table 5). Interestingly, cells of GBS type III strain M781, which lacks alp3 but possesses the anti-rAlp3 cross-reactive Rib protein (Fig. 3), were opsonized and killed in this assay (Table 5).

The latter findings led us to explore if rAlp3 antiserum would be functionally active against other GBS type III strains. Rabbit antiserum to rAlp3 promoted the killing of six (86%) of seven type III strains isolated from newborns with invasive GBS disease by ≥0.6 log<sub>10</sub> CFU (Fig. 4). Western blot analysis of whole-cell proteins from these strains showed that all but strain KMP114, which was not opsonized and killed, expressed cross-reactive proteins, presumably Rib (Fig. 4); the nonopsonized strain possessed alp3 but did not express Alp3. Rabbit serum to rAlp3 (diluted 1:10,000) reacted with colony blots of all GBS strains used in this study, with the exception of CJB111, A909, and KMP114.

**DISCUSSION**

Conjugate vaccines against pathogens of multiple serotypes are optimized when all components induce functional antibody, resulting in broadened coverage. The search for a protein that would serve as a serotype-independent vaccine against GBS infections has been ongoing since Lancefield and colleagues reported protection of mice against GBS infection by antibody specific to both polysaccharide and protein antigens (27). Several GBS surface proteins, including ACP, BCP, Caspase, Rib, and Sip, have been tested as independent vaccines or as carrier proteins for GBS CPSs (31). As stand-alone vaccines, GBS proteins have not progressed beyond animal testing. Preclinical studies with Sip demonstrated that this antigen would be an ideal vaccine, as it was conserved among serotypes, was highly immunogenic, and protected mice against challenge with most GBS serotypes (9, 38). Unfortunately, however, sera from most women and newborns tested including newborns with invasive GBS disease had naturally occurring antibody to Sip (37), a result that raised concerns about the use of this protein as a vaccine. The presence of naturally occurring human maternal antibody to GBS BCP also failed to correlate with neonatal protection (23) despite promising studies of this protein as a vaccine in animals (33).
Recently, a combination of Sip and three newly described GBS pilus proteins has demonstrated efficacy against several GBS serotypes in mice (36), results that provided a renewed interest in a GBS protein-based vaccine.

Because of the preclinical and clinical successes with GBS CPS-protein conjugate vaccines (20), we chose to examine the role of the Alp3 protein not as a stand-alone vaccine per se but as a carrier protein for CPSs. As it was shown to be a strong immunogen in animals, rAlp3, like other GBS proteins (31), was a good carrier for type III CPS, as revealed by efficacy in mice. Surprisingly, in this animal model, uncoupled rAlp3 conferred partial protection against a GBS type III strain lacking Alp3. GBS Alp3 possessed at least two antigenic determinants: Alp2/Alp3 and Alp3/Rib common sites (34). Repeat regions of Alp3 and of Rib match by 99% and are likely sites for the Alp3/Rib cross-reactivity (24). This cross-reactivity was also reflected in the ability of rAlp3 rabbit serum to opsonize Rib protein-expressing type III strain M781 for killing in vitro, a result that explains the >40% protection against challenge with strain M781 in newborn mouse pups born to dams that received rAlp3. Indeed, the degree of opsonically mediated killing of GBS strains with this antiserum correlated with the expression of Alp3-cross-reactive proteins, as measured among a panel of invasive GBS isolates.

Active immunization of mice with rAlp3 or rAlp3-containing vaccines did not provide protection against challenge with Alp3-expressing GBS strains H1A00001 and JM9130013, nor did antiseraa to these vaccines mediate opsonic killing. However, high-titered rabbit rAlp3 antiserum mediated the opsonization and killing of these strains, a finding that raises the possibility that the Alp3/Rib common site may, under certain conditions, be partially concealed by other surface antigens such as the CPS.

As both Alp3 and Rib proteins occur with high frequency in GBS, usually in strains of different capsular antigen types (8, 22), the Alp3/Rib common site will be expressed more often

![FIG. 3. Reactivity of rAlp3 antiserum with GBS. Whole-cell extracts of GBS type III strain M781 (lane 1), type V strain HA1/0001 (lane 2), and type VIII strain JM9130013 (lane 3) but not type V strain CJ111 (lane 4) and type Ia strain A909 (lane 5) reacted with rAlp3 antiserum. The alp3 gene was PCR amplified with use of specific primers (42). As expected, strain M781, which contains rib but not alp3, expressed Rib that cross-reacted with rAlp3 antiserum. Surprisingly, strain CJ111 did not express Alp3 despite possessing the gene for this protein, and as expected, strain A909 neither possessed alp3 nor expressed the protein.](image-url)

### TABLE 4. Efficacy of rAlp3, III-rAlp3, III-rAlp3-BCP<sup>Alp3</sup>A, and III+rAlp3+BCP<sup>Alp3</sup>A vaccines in neonatal mouse pups born to vaccinated dams

| GBS challenge strain | Dam immunization | No. of dams | No. of surviving pups/no. of pups challenged (% survival) |
|----------------------|------------------|-------------|----------------------------------------------------------|
| M781 (type III Alp3<sup>−</sup> Rib<sup>−</sup> BCP<sup>−</sup>) | rAlp3 | 3 | 18/41 (44)% |
|                      | III-rAlp3 | 3 | 34/36 (94)% |
|                      | III+rAlp3+BCP<sup>Alp3</sup>A | 3 | 20/20 (100)% |
|                      | III+rAlp3+BCP<sup>Alp3</sup>A | 2 | 10/23 (43)% |
|                      | Saline | 4 | 0/43 (0)% |
| A909 (type Ia Alp3<sup>−</sup> Rib<sup>−</sup> BCP<sup>−</sup>) | III-rAlp3| 3 | 25/36 (69)% |
|                      | III+rAlp3+BCP<sup>Alp3</sup>A | 3 | 13/37 (35)% |
|                      | Saline | 2 | 0/28 (0)% |
| CJB111 (type V Alp3<sup>−</sup> Rib<sup>−</sup> BCP<sup>−</sup>) | rAlp3 | 3 | 3/23 (13)% |
|                      | III-rAlp3 | 3 | 14/36 (39)% |
|                      | III+rAlp3+BCP<sup>Alp3</sup>A | 2 | 0/26 (0)% |
|                      | III+rAlp3+BCP<sup>Alp3</sup>A | 3 | 7/43 (16)% |
|                      | Saline | 4 | 6/46 (13)% |

<sup>a</sup> P < 0.001 compared with saline.
<sup>b</sup> P < 0.005 compared with III+rAlp3+BCP<sup>Alp3</sup>A.
<sup>c</sup> P < 0.005 compared with III+rAlp3+BCP<sup>Alp3</sup>A.
<sup>d</sup> P = 0.001 compared with saline and P = 0.04 compared with III+rAlp3+BCP<sup>Alp3</sup>A.

### TABLE 5. Functional activity of rabbit serum to group B streptococcal rAlp3 as measured by an in vitro opsonophagocytosis assay<sup>a</sup>

| GBS strain (serotype) | Complement | Log<sub>10</sub> CFU at: | Log<sub>10</sub> CFU decrease (0–1 h) |
|-----------------------|------------|--------------------------|-----------------------------------|
|                       |            | 0 h                      | 1 h                               |
| H1A00001 (V)          | +          | 5.22                      | 4.64                              | 0.58 |
| H1A00001 (V)          | −          | 5.24                      | 5.90                              | −0.66 |
| CJ111 (V)             | +          | 5.12                      | 5.53                              | −0.41 |
| A909 (Ia)             | +          | 4.86                      | 4.86                              | 0.00 |
| JM9130013 (VIII)      | +          | 5.15                      | 4.82                              | 0.33 |
| M781 (III)            | +          | 5.07                      | 4.07                              | 1.00 |

<sup>a</sup> Rabbit antiserum to anti-type V-CRM<sub>197</sub> (3) with complement, a positive control for type V, promoted a 1.21-log<sub>10</sub> reduction of GBS type V strain CJ111 in this assay.
than either of the two proteins alone. Indeed, expression of the Alp3/Rib common site occurs frequently in GBS, notably in pathogenic serotype III, V, and VIII strains (22, 35), and in group A streptococci that express the Alp3-like protein R28 (43, 44), suggesting that the inclusion of the Alp3/Rib common region in a vaccine might provide at least partial protection against a broader range of streptococci. The ability of rAlp3 antisera to opsonize invasive GBS type III strains bearing the cross-reactive proteins, presumably Rib, is of particular importance given the correlation of low maternal levels of Rib antibody with neonatal GBS disease (28).

BCP, encoded by the \textit{bac} gene, is found on nearly all strains of GBS serotype Ib as well as on some isolates of types Ia, II, and V (31), and approximately 23% of GBS strains contained the \textit{bac} gene according to \textit{bac}-specific PCR analysis (21). Our recently developed rBCP\textsubscript{Ia}/H9004 IgA demonstrated a higher degree of efficacy against a BCP-positive GBS type Ia strain when coupled to type III CPS and rAlp3 than as a component of an uncoupled mixture, despite evoking similar levels of maternal IgG. Mechanisms for this enhanced efficacy are currently unknown but may include an effect of size, given the generation of large polymers due to conjugation or the exposure of protective epitopes by conjugation.

In summary, our data suggest that rAlp3 is a good carrier for GBS CPS, and antisera to this protein is functionally active against GBS strains bearing Alp3 or the cross-reactive Rib protein. In theory, a conjugate vaccine containing type Ia CPS, rBCP\textsubscript{Ib/A}, and rAlp3 could provide coverage against multiple GBS serotypes, serotypes Ia (from the CPS), Ib (from BCP), III (from the cross-reactivity of rAlp3 with Rib), and V and VIII (from rAlp3) or most serotypes causing early- and late-onset neonatal GBS disease in the United States (14, 19).

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