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Preclinical Evaluation of Group B *Neisseria meningitidis* and *Escherichia coli* K92 Capsular Polysaccharide-Protein Conjugate Vaccines in Juvenile Rhesus Monkeys

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Division of Bacterial Products, Office of Vaccine Research and Review,[1] and Division of Veterinary Medicine, Center for Biologics Evaluation and Research,[3] U.S. Food and Drug Administration, Rockville, Maryland 20852; Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20307[2]; North American Vaccine, Inc., Beltsville, Maryland 20705[4]; and Chiron Biocine, 53100 Siena, Italy[5]

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We reported the first use of group B meningococcal conjugate vaccines in a nonhuman primate model (S. J. N. Devi, C. E. Frasch, W. Zollinger, and P. J. Snoy, p. 427–429, *in* J. S. Evans, S. E. Yost, M. C. J. Maiden, and I. M. Feavers, ed., *Proceedings of the Ninth International Pathogenic Neisseria Conference*, 1994). Three different group B *Neisseria meningitidis* capsular polysaccharide (B PS)-protein conjugate vaccines and an *Escherichia coli* K92 capsular polysaccharide-tetanus toxoid (K92-TT) conjugate vaccine are here evaluated for safety and relative immunogenicities in juvenile rhesus monkeys with or without adjuvants. Monkeys were immunized intramuscularly with either B PS–cross-reactive material 197 conjugate, B PS-outer membrane vesicle (B-OMV) conjugate, or N-propionylated B PS–outer membrane protein 3 (N-pr. B–OMP3) conjugate vaccine with or without adjuvants at weeks 0, 6, and 14. A control group of monkeys received one injection of the purified B PS alone, and another group received three injections of B PS noncovalently complexed with OMV. Antibody responses as measured by enzyme-linked immunosorbent assay varied among individual monkeys. All vaccines except B PS and the K92-TT conjugate elicited a twofold or greater increase in total B PS antibodies after one immunization. All vaccines, including the K92-TT conjugate, elicited a rise in geometric mean B PS antibody levels of ninefold or more over the preimmune levels following the third immunization. Antibodies elicited by N-pr. B–OMP3 and B-OMV conjugates were directed to the N-propionylated or to the spacer-containing B PS antigens as well as to the native B PS complexed with methylated human serum albumin. None of the vaccines caused discernible safety-related symptoms.

Meningitis and septicemia caused by *Neisseriae meningitidis* continue to be major health problems, particularly in children and young adults. Serogroups A, B, and C are responsible for about 90% of meningococcal diseases worldwide. Serogroup B accounts for over 50% of meningococcal infections in the United States and ~80% in some European countries. The mortality rate is ~10%, and survivors may be left with various degrees of permanent neurologic deficiencies.

Licensed capsular polysaccharide vaccines against *N. meningitidis* serogroups A, C, Y, and W135 are available. These polysaccharide vaccines elicit protective antibody responses of diverse isotype compositions in subjects over 2 years of age. In contrast, there are no licensed vaccines against group B meningococcal infections. The capsular polysaccharide of group B *N. meningitidis* (B PS) is a homopolymer of α(2→8)-linked sialic acid identical to that of *Escherichia coli* K1 (K1 PS) (29). *E. coli* K1 is a major cause of neonatal septicemia, meningitis, and upper urinary tract infections in humans (26, 28, 29). For both pathogens, the capsular polysaccharide is considered a virulence factor (26, 29) and anticapsular antibodies are believed to be protective (5, 17, 33, 36). However, the B or the K1 PS is poorly immunogenic in humans and laboratory animals (10, 29, 42); antibody responses to these polysaccharides are mostly T cell independent and are of the immunoglobulin M (IgM) class. The poor immunogenicity of B PS is attributed to its conformational complexity (25, 30), internal esterification (30), and structural homology with a developmental antigen present on mammalian fetal tissues (13, 14, 37).

In recent years, the B or the K1 PS has been rendered immunogenic with or without chemical modification by covalently coupling it to carrier proteins (10, 11, 24). The capsular polysaccharide of *E. coli* K92 (K92 PS) is composed of alternate α(2→8)- and α(2→9)-linked sialic acid residues, and on conjugation to tetanus toxoid (TT) via adipic acid dihydrazide (ADH), it produces antibodies reactive with both group B and group C meningococcal polysaccharides (10). The immunogenicities of B PS and K92 PS conjugate vaccines have been evaluated for mice (4, 10, 24). The B PS conjugates induced both IgG and IgM antibodies with specificities for both the spacer-derivatized B PS and the native B PS (4). The in vitro demonstration of binding of α(2→8)-linked polysialic acid antibodies to certain host antigens in mammals (3, 19–22, 38) has raised concerns about the safety for humans of B PS conjugate vaccines.

The purpose of our study was to evaluate the ability of multiple doses of B PS-protein conjugate vaccines to induce functionally active antibodies in a nonhuman primate model. Since there was no clear evidence to suggest which conjugation

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TABLE 1. B PS and *E. coli* K92 vaccines used in the preclinical trial with juvenile rhesus monkeys

Vaccine	% Substitution[a]	Protein/polysaccharide ratio	Dose (μg of polysaccharide/animal	Source(s)	Lot
B PS[b]			25.0	LBP, CBER	SD-986
B+OMV complex[c]		2.7	30.0	WRAIR[d]	BP2-5-7
B-CRM_{197}	0.4	1.0	5.0	Chiron Biocine	MB-CRM-4
B-OMV	2.4	4.5	5.0	LBP, CBER	SD-22B
B-OMV in MPL plus TDM	2.4	4.5	5.0	LBP, CBER	SD-22Z
N-pr. B-OMP3 in $Al(OH)_3$	4.9	3.1	5.0	NAVA	RD-35-59
N-pr. B-OMP_3 in ST	4.9	3.1	5.0	NAVA	RD-74-76
K92-TT	2.9	3.5	5.0	LBP, CBER	SD-23K

[a] Wt/wt.
[b] Purified unconjugated B PS.
[c] The B+OMV complex was prepared from strain 99M (B:2a:P1.2:L3,7) (44). The OMV used in conjugates was prepared from strain M986 (B:2a:P1.2:L3,7) (11).
[d] WRAIR, Walter Reed Army Institute of Research.

chemistry or which adjuvant system would be effective, we chose to evaluate a diverse spectrum of B PS conjugates and adjuvants in juvenile rhesus monkeys.

MATERIALS AND METHODS

Monkeys. Rhesus monkeys (*Macaca mulatta*) of both sexes, 1 to 1.5 years old, were obtained from the monkey breeding colony of the Food and Drug Administration in Yemassee, N.C.. The cost involved in caring for the animals for more than a year and the limited availability of space dictated the use of five animals per group. The monkeys were housed at the primate facility on the campus of the National Institutes of Health, Bethesda, Md.

Vaccines. Seven different group B meningococcal vaccines were included in the study (Table 1). The B PS was conjugated to a variety of protein carriers, such as group B meningococcal outer membrane vesicles (OMV) from strain M986 (B:2a:P1.2:L3,7) (11, 12), cross-reactive material 197 (CRM_{197}) protein (a nontoxic mutant of diphtheria toxin) (4), and the group B meningococcal recombinant outer membrane protein 3 (OMP3) of serotype 15 (24, 40). The K92 PS was conjugated to TT (11). B PS conjugated to OMV (B-OMV) and K92 PS conjugated to TT (K92-TT) conjugate vaccines contained the corresponding polysaccharide covalently coupled to the protein carrier via the six-carbon spacer ADH (11, 12). The B PS conjugated similarly to CRM_{197} (B-CRM_{197}) via ADH was prepared at Chiron Biocine, Siena, Italy (4). The N-propionylated B PS (N-pr. B) was coupled to OMP3 by reductive amination at the North American Vaccine Inc. (NAVA), Beltsville, Md. (24, 40). The B PS noncovalently complexed with the group B meningococcal OMV (the B+OMV complex) prepared from strain 99M (B:2a:P1.2:L3,7) at the Walter Reed Army Institute of Research, Washington, D.C. (43), and the B PS purified in the Laboratory of Bacterial Polysaccharides (LBP), Center for Biologics Evaluation and Research (CBER), were included as unconjugated B PS control vaccines. The vaccines were used in saline or in adjuvant formulations (Table 1) and are referred to in this report as B-OMV, B-CRM_{197}, N-pr. B–OMP3, K92-TT, the B+OMV complex (noncovalent), and the unconjugated B PS.

Immunizations. Eight groups of five monkeys were immunized intramuscularly in the flank with 5.0 μg of the polysaccharide as a part of each conjugate in 0.125- to 0.5-ml volumes. Three injections were given at weeks 0, 6, and 14 (Table 2). Since the duration between the immunizations is important for the secondary response, second and third immunizations were kept 4 and 8 weeks apart, respectively. One of the control groups received one injection of the purified B PS alone (25 μg), and the other received three injections of the B+OMV complex (30 μg of the PS per dose). The B-CRM_{197} and K92-TT conjugate vaccines were injected as saline formulations. The B-OMV conjugate was used both in saline and in adjuvant formulation admixed in 50 μg of the Ribi adjuvant, i.e., monophosphoryl lipid A plus trehalose dicorynomycolate (MPL+TDM; Ribi Immunochem Research Inc., Hamilton, Mont.). The N-pr. B–OMP3 conjugate was injected with aluminum hydroxide [$Al(OH)_3$; 1 mg/ml] or with stearyl tyrosine (ST; 1 mg/ml) as the adjuvant. Monkeys that received three immunizations of B-CRM_{197}, B-OMV in MPL+TDM, and N-pr. B–OMP3 in $Al(OH)_3$ were reimmunized with 25 μg of the B PS alone in saline at week 50 to study the ability of the unconjugated B PS to elicit a possible anamnestic response which is seen if the initial immunization induced T-cell help and immunological memory.

Collection of blood. Monkeys were bled for automated blood chemistries, complete blood counts, and renal and hepatic function tests at weeks 0, 2, 8, and 16, and for antibody analyses before immunization (week 0) and at weeks 2, 6, 8, 14, 16, 22, and 30 following the first immunization (Table 2). Some conjugate-immunized monkeys that received the B PS alone at week 50 were prebled before this immunization and then 2 weeks later (week 52). Sera were stored frozen at −70°C until tested.

Serology. An enzyme-linked immunosorbent assay (ELISA) was performed to measure antibodies specific for B PS (11, 12). The B PS was noncovalently complexed with methylated human serum albumin (B+mHSA) and used as the coating antigen (2, 11, 12) with all rhesus monkey sera. To measure antibodies directed to the substituted B PS, the B PS covalently coupled to HSA via ADH (B-ADH-HSA) (11, 12) and N-pr. B conjugated to HSA without ADH (N-pr. B–HSA) (11, 24, 40) were prepared and used as additional coating antigens with some rhesus monkey sera. Alkaline phosphatase-labeled goat antihuman total Ig, IgG, and IgM reagents were used (Kirkegaard and Perry Laboratories, Gaithersburg, Md.); the antihuman reagents were chosen, since a preliminary comparison revealed no significant difference between antihuman and antibaboon (a gift from Ronald C. Kennedy, Southwest Foundation for Biomedical Research, San Antonio, Tex.) reagents in their specificities for rhesus antibodies. A pool of high-responder monkey antiserum was assigned an arbitrary total antibody unitage of 1,000 U/ml and was used as the reference standard for measuring B PS antibodies. The ELISA results were calculated with a log-logit computer program and averaged antibody estimates from multiple dilutions for each serum (16). Anticapsular IgG and IgM levels were expressed as A_{405} at 100 min times the dilution factor; multiple dilutions were also done in an attempt to demonstrate parallelism with the reference serum.

In order to differentiate antibodies directed to epitopes formed by the spacer, an ADH-containing conjugate was synthesized with the type 9V capsular polysaccharide (9V PS) of *Streptococcus pneumoniae*. The pneumococcal 9V PS has a repeat unit composed of D-glucuronic acid, 2-acetamido-2-deoxy-D-mannuronic acid, galactose, and glucose and is chemically unrelated to the B PS. The degree of ADH substitution (2.0%) was kept similar to that of the B-ADH-HSA conjugate used in ELISAs as one of the coating antigens.

Statistical analysis. The antibody levels obtained from different rhesus monkey groups were compared by Student's *t* test.

TABLE 2. Immunization and bleeding schedules used in the rhesus monkey trial

Week	Immunization	Blood drawn for:	
		Antibody analysis	Blood counts and chemistries and renal and liver function tests
0	+[a]	+	+
2	−	+	+
6	+	+	−
8	−	+	+
14	+	+	−
16	−	+	+
22	−	+	−
30	−	+	−
50	+[b]	+	−
52	−	+	−

[a] Blood samples were collected before immunization.
[b] A sample of blood was also collected before immunization with the B PS. Monkeys that received three immunizations of B-CRM_{197}, B-OMV in MPL+TDM, and N-pr. B-OMP3 in $Al(OH)_3$ were reimmunized with 25 μg of the B PS in saline.

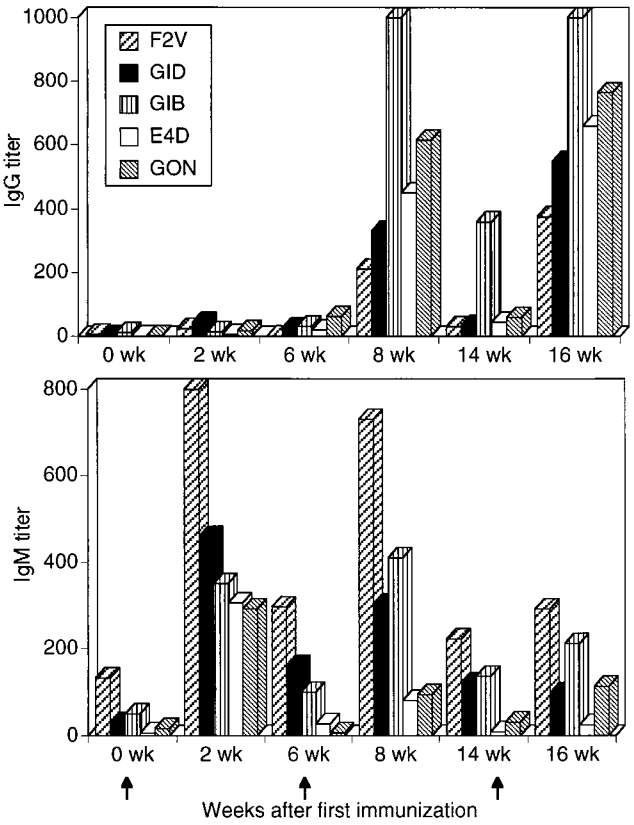

FIG. 1. IgG and IgM titers of individual monkeys (F2V, GID, GIB, E4D, and GON) immunized with the N-pr. B–OMP3 conjugate in $Al(OH)_3$ as measured by ELISA with N-pr. B-HSA as the coating antigen. Arrows indicate the weeks at which monkeys were immunized. Data correspond to A_{405}s normalized to 100 min times the dilution factor.

RESULTS

Vaccines. The compositions of different vaccines used in this study are shown in Table 1. The percent substitution of ADH-containing B and K92 PS conjugates ranged between 0.44 and 2.9% (wt/wt). The protein/polysaccharide ratio of ADH- and *N*-propionyl-substituted conjugates varied between 1.0 and 4.5.

Blood chemistry. The complete blood counts and the results of 24 automated blood chemistries, including a large number of renal and hepatic liver function tests, performed 2 weeks following each immunization remained normal. The postimmunization values obtained were not significantly different from the preimmunization values and fell within the expected normal range, suggesting the absence of abnormal changes due to multiple immunizations with different B PS vaccines, including conjugates. The animals remained outwardly healthy during the entire study.

B PS-specific antibodies. Total antibody and IgG and IgM levels were measured. The B PS-specific preexisting total antibody levels of most of the monkeys ranged between 0.5 and 169.5 U/ml, but two animals showed titers of 1,117.5 and 770.5 U/ml; the geometric mean preimmune level was 12.8 U/ml. Preimmune antibodies were predominantly of the IgM class, as represented in Fig. 1. All monkeys with less than detectable or low preimmunization levels developed higher postimmunization anti-B PS levels. There was a wide variation in capsular antibody responses among animals immunized with the various B PS vaccines or the K92 vaccine; in most cases, the geometric mean and median antibody titers were comparable.

As expected, immunization of juvenile rhesus monkeys with one dose of the purified B PS alone (25 μg) did not elicit a rise in total antibody levels (Table 3). All B PS-containing conjugate vaccines elicited elevated levels of antibodies compared with those of the B PS alone and the K92-TT conjugate after one immunization (Table 3). The antibody titers were not uniform in all five animals after each immunization. The highest level of antibodies after the first immunization was elicited by the N-pr. B–OMP3 administered in $Al(OH)_3$ (468.0 U/ml) followed by the B-OMV administered in MPL+TDM (98.2 U/ml). Except for the N-pr. B–OMP3 conjugate in $Al(OH)_3$, all other vaccines elicited highest B PS antibody levels after the third immunization. Following three immunizations, the B-OMV conjugate administered with the MPL+TDM adjuvant elicited the highest level of antibodies reactive with the B+mHSA antigen (309 U/ml), followed by the N-pr. B–OMP3 conjugate administered in $Al(OH)_3$ (199 U/ml) and the B-CRM_{197} conjugate (191 U/ml). About a two- to fourfold difference existed between anti-B PS levels elicited by saline and adjuvant formulations of the B-OMV conjugate after each immunization. After the first and second immunizations, the N-pr. B–OMP3 conjugate vaccine elicited 11- to 16-fold higher anti-B PS antibody responses when administered with $Al(OH)_3$ than when given with ST. Fourteen weeks following the third immunization, the anti-B PS levels declined yet remained at about two to four times the preimmunization levels.

TABLE 3. Antibody responses to the B PS in juvenile rhesus monkeys vaccinated with B PS and K92 conjugate vaccines

Vaccine	Geometric mean total antibody response[b] (U/ml) to B+mHSA antigen at week:									
	0	2	6	8	14	16	22	30	50[c]	52
B PS	18.0[e]	15.1								
B+OMV complex	8.3[n]	52.7	18.6	61.4	20.5	109.1[o]	44.3	45.3		
B-CRM_{197}[a]	17.0[h]	65.7	41.4	69.0	37.1	190.7[i]	53.6	42.9	29.3	35.9
B-OMV[a]	11.6[j]	25.1	12.2	30.0	15.0	109.8[k]	32.6	24.3		
B-OMV in MPL+TDM[a]	33.1[l]	98.2	68.3	59.7	32.2	309.2[m]	115.9	74.5	70.4	76.8
N-pr. B-OMP3 in $Al(OH)_3$	20.8[d]	468.0[f]	98.1	589.9[g]	58.5	199.2[t]	114.0	53.6	24.7	34.4
N-pr. B-OMP3 in ST	6.4[p]	28.3	12.4	53.0	17.7	157.7[q]	49.7	38.8		
K92-TT[a]	7.7[r]	7.9	10.4	11.8	10.1	89.1[s]	24.3	14.4		

[a] Contained the spacer ADH.

[b] Groups of juvenile rhesus monkeys ($n = 5$) were immunized intramuscularly at weeks 0, 6, and 14 as described in Materials and Methods. Statistical analysis was by Student's *t* test. *g* and *f* versus *d*, $P \leq 0.03$; *i* versus *h*, *k* versus *j*, and *q* versus *p*, $P \leq 0.01$; *m* versus *l*, *t* versus *d*, and *o* versus *n*, $P = 0.02$; *s* versus *r*, $P = 0.05$. The differences in antibody responses observed at weeks 50 and 52 were not statistically significant.

[c] Monkeys that received three immunizations of B-CRM_{197}, B-OMV in MPL+TDM, and N-pr. B-OMP3 in $Al(OH)_3$ were reimmunized with 25 μg of the unconjugated B PS, and antibody levels were measured at week 52.

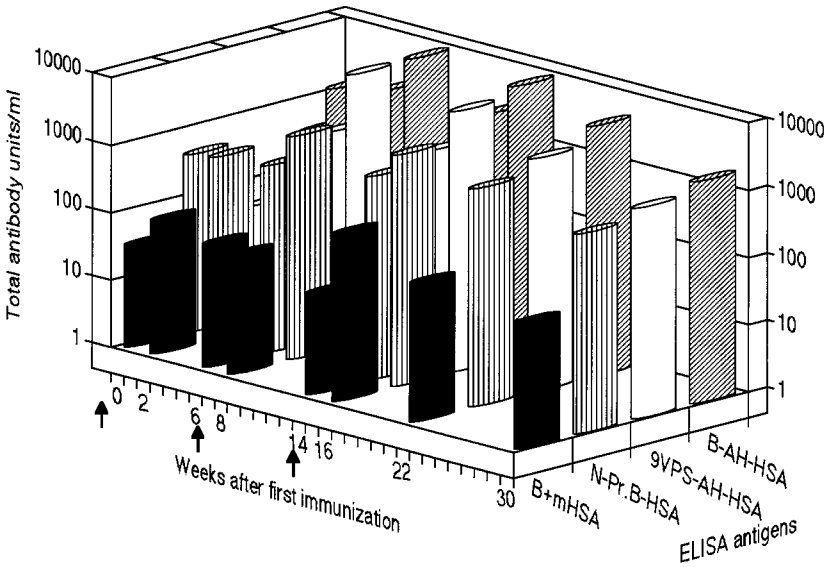

FIG. 2. Geometric mean titers of antibodies in monkeys ($n = 5$) immunized with the ADH-containing B-OMV conjugate in MPL+TDM as measured by ELISA with B PS adhered to microwells by different means. Arrows indicate the weeks at which the monkeys were immunized. The coating antigens were B+mHSA (filled bars), N-pr. B-HSA conjugated without ADH (striped bars), 9V PS-HSA conjugated via ADH (9VPS-AH-HSA; open bars), and B-HSA covalently conjugated via ADH (B-AH-HSA; hatched bars).

The K92-TT conjugate was less immunogenic than the B PS conjugate vaccines. Monkeys immunized with the K92-TT conjugate showed an approximately 11-fold rise in the geometric mean B PS antibody levels only after the third immunization.

Although the N-pr. B–OMP3 conjugate in $Al(OH)_3$ induced the highest levels of total B PS-specific antibodies after the first and second immunizations, the antibody response elicited by the B-OMV conjugate admixed in MPL+TDM was comparatively more durable as measured at week 50 and was about three times higher than the level induced by the N-pr. B–OMP3 conjugate in $Al(OH)_3$ (Table 3). At week 50, monkeys that received three immunizations with B-CRM197 conjugate, B-OMV conjugate in MPL+TDM, and N-pr. B–OMP3 conjugate in $Al(OH)_3$ were reimmunized with the unconjugated B PS in saline. The geometric mean levels of B PS antibodies reactive with the B+mHSA antigen complex, measured at week 52, increased in all three study groups; however, the increase was not statistically significant compared to antibody levels at week 50 (Table 3).

Figure 1 represents the IgG and IgM antibody levels elicited by three immunizations of a representative B PS conjugate, i.e., N-pr. B–OMP3 conjugate given in $Al(OH)_3$, as measured with the N-pr. B–HSA conjugate as the coating antigen. In addition to a class switch from IgM to IgG seen by week 16 in most monkeys, the figure also illustrates preexisting B PS antibody levels and variations in postimmunization antibody levels in individual monkeys. A similar isotype pattern was observed with the rest of the B PS vaccines except the B+OMV complex (noncovalent), which evoked predominantly an anti-B PS IgM response (not shown).

Antibodies to substituted B PS. Figure 2 depicts the geometric mean antibody levels elicited by the B-OMV conjugate administered in MPL+TDM as measured by ELISAs using different forms of B PS coating antigens: the B+mHSA complex, N-pr. B–HSA conjugated by reductive amination (without ADH), and B-ADH-HSA. The antigenically unrelated pneumococcal 9V PS conjugated to HSA via ADH (9V PS-ADH-HSA) was also included as a coating antigen to measure antibodies directed to the ADH spacer. The B-OMV conjugate vaccine elicited substantially higher levels of antibodies to the ADH-containing B PS than to the B+mHSA complex. Even though both the ADH-containing B PS and pneumococcal 9V PS coating antigens used in ELISAs had similar degrees of ADH substitution, antibody levels to the ADH-containing B-HSA conjugate antigen were significantly higher than those reactive with the ADH-containing 9V PS-HSA antigen. Interestingly, antibodies reactive with the B-HSA conjugate antigen were 18- to 246-fold higher than those reactive with the unconjugated B PS antigen, i.e., the B+mHSA complex. Additionally, B-OMV conjugate-induced antibodies bound better to the conjugate form of the N-pr. B (the N-pr. B–HSA antigen), although this antigen lacked ADH, suggesting that the B PS in conjugate form acts as a better binding antigen than the B+mHSA complex.

Figure 3 shows the geometric mean antibody levels elicited by N-pr. B–OMP3 conjugate vaccines administered in $Al(OH)_3$ or in ST as measured by an ELISA with the B+mHSA complex in contrast to N-pr. B–HSA. Both formulations of N-pr. B–OMP3 conjugates elicited substantially higher antibody levels directed to N-pr. B conjugated to HSA than to the native B PS complexed with HSA, similar to the previous observation made by Jennings and coworkers about nonprimates (24).

DISCUSSION

Our results indicated that B PS conjugated to various carrier proteins of medical importance by different synthetic schemes are immunogenic and show T-cell-dependent properties in a nonhuman primate species.

A wide range in levels of antibody to the B PS in prevaccination sera of juvenile rhesus monkeys was observed. The $\alpha(2\rightarrow8)$-linked sialic acid polymer similar to the B PS is found in several divergent commensal gram-negative bacterial species (1, 9). Natural exposure of the monkeys to one or more of these species most likely induced the antibodies reactive with

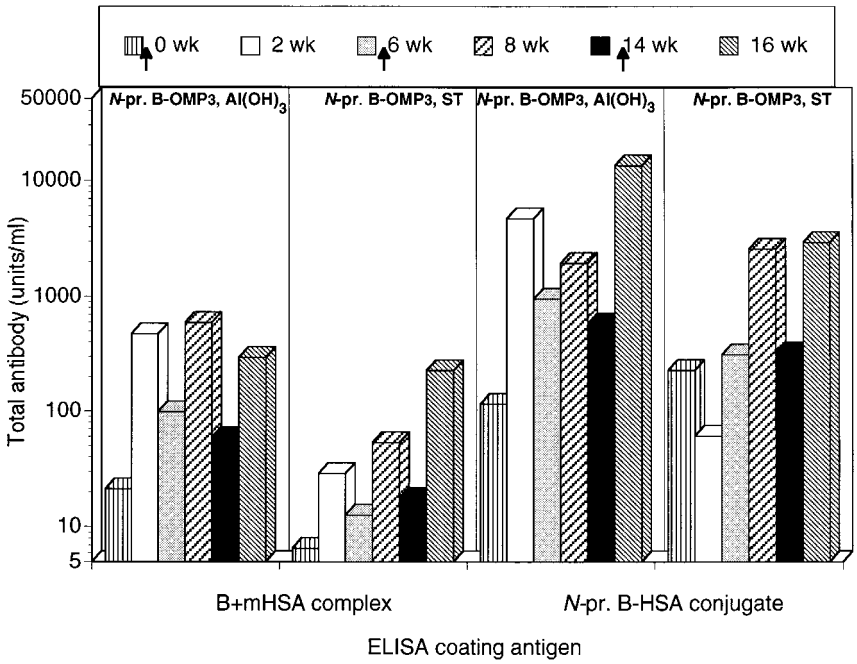

FIG. 3. Geometric mean titers of antibodies in two groups of monkeys ($n = 5$) immunized with the N-pr. B–OMP3 conjugate in $Al(OH)_3$ or in ST as measured by ELISA with the B+mHSA complex and N-pr. B-HSA as coating antigens. Neither the conjugate immunogen nor the coating antigens contained ADH. Arrows indicate the weeks at which monkeys were immunized.

the B PS. About 80 to 90% of apparently healthy humans develop such antibodies (44) by the same mechanism.

Juvenile rhesus monkeys failed to respond with a demonstrable rise in levels of antibody to a single immunization with the unconjugated B PS (25.0 μg). This is consistent with the previous observation made for humans vaccinated with the purified B PS (42). However, the B PS conjugate vaccines were significantly more immunogenic than the unconjugated B PS. Animals that received three injections of various conjugates composed of diverse protein carriers and B PS with different degrees of substitution, in doses lower than those used in humans with licensed *Haemophilus influenzae* type B (Hib) conjugates, did produce substantially elevated B PS-specific antibodies (Table 3). There was variation in B PS antibody responses among individual monkeys immunized with B PS conjugates with or without an adjuvant; the antibody titers were not always uniform (Fig. 1). The geometric mean levels of antibody elicited in monkeys immunized with the B-CRM_{197} conjugate were higher than those induced by the B-OMV conjugate; however, the difference did not reach statistical significance. The difference in immunogenicities may be carrier related or related to the differences in the degree of substitution of B PS and the protein/polysaccharide ratio of the two conjugates.

Adjuvants such as $Al(OH)_3$, ST, and MPL+TDM further augmented the immunogenicities of the B PS conjugate vaccines. The N-pr. B–OMP3 conjugate admixed in ST elicited the highest levels of B PS antibodies after three immunizations (Table 3). In contrast, the N-pr. B–OMP3 conjugate administered in $Al(OH)_3$ produced significantly higher levels of B PS antibodies (468.0 and 589.9 U/ml) after the first and the second immunizations compared to preimmune levels ($P < 0.03$); the third immunization reduced the geometric mean level of antibody (199.2 U/ml). In this group, aluminum ions may have selectively increased titers of antibody to the B PS (34). Induction of a strong primary response in infants receiving a single immunization with the Hib PS conjugated to *N. meningitidis* outer membrane protein has been reported previously (6), but Hib PS conjugated to TT, CRM_{197}, or diphtheria toxoid required repeated doses to elicit an immune response (8, 18) similar to those of the saline formulations of some conjugates used in our study.

The durability of immune responses elicited by the B PS conjugate vaccines was studied up to 30 weeks and in some groups up to 52 weeks. The B PS antibody levels declined 30 weeks after the first immunization but remained higher than the preimmunization levels in each group (Table 3). Reimmunization with the native unconjugated B PS 36 weeks after the third immunization (at week 50) of rhesus monkeys that had previously received three immunizations of B-CRM_{197} conjugate, B-OMV conjugate admixed in MPL+TDM, or N-pr. B–OMP3 conjugate vaccine admixed in $Al(OH)_3$ resulted in a booster injection response that was higher than the level of pre-booster injection antibody measured at week 50 but not significantly so (Table 3). However, the post-booster injection antibody levels detectable with the B+mHSA complex as the coating antigen remained notably higher than the preimmune levels (at week 0). Although immune responses to B PS and the Hib polysaccharide cannot be compared because of the contrasting immunogenic and conformational properties of the two polysaccharides, the effect we observed differs from the effect of the booster injection reported with the Hib PS in infants previously immunized with Hib conjugates (18, 41). The reasons for the lack of a strong memory response in our study are unclear, but this response may be related to the relatively lower doses of conjugate used in the study or due to the labile antigenic nature of the unconjugated B PS. Additional studies at molecular and cellular levels may provide insights into whether the lack of a significant secondary response reflects conformational differences between the conjugated B PS contained in the priming doses and the unconjugated B PS used with the boosting immunization or whether it

is due to the possible inability of the B PS conjugates to induce sufficiently high numbers of memory B cells.

The K92-TT conjugate was comparatively less immunogenic than the B PS conjugates. At the PS dose used in this study, the geometric mean level of B PS antibody elicited by the K92-TT conjugate vaccine was low after the first two immunizations (Table 3). However, all monkeys showed responses to booster injections after the third immunization with significantly elevated levels of antibodies (89.0 U/ml) ($P = 0.05$). It is not uncommon for some bacterial PS conjugates to evoke a low or no antibody response in rhesus monkeys. In one study, none of the seven rhesus monkeys in the group that received three immunizations with doses as high as 50.0 μg of a pneumococcal type 6A-TT conjugate responded (39). It is possible that use of a higher K92 PS dose or addition of an adjuvant could have induced a better primary immune response.

An essential criterion for vaccine immunogenicity is the capacity of the vaccine to elicit a specific immune response to the target antigen. Rabbit and murine antibodies elicited by B-TT and B-CRM_{197} conjugates synthesized via ADH and by N-pr. B-TT conjugates have been shown to elicit antibodies directed both to the substituted and to the native B PSs (4, 24). Similarly, in our study, a portion of the rhesus antibodies induced by the B PS conjugates was directed to the ADH-containing polysaccharides or N-pr. B. However, both types of conjugates also elicited elevated levels of antibodies in rhesus monkeys that were reactive with the B+mHSA complex. These antibodies acquire importance because of their seemingly conflicting functional potentials and possible autoimmune activities. The safety-related significance of ADH-specific antibodies in vivo is not known.

The use of native N-pr. B and ADH-derivatized B PS covalently coupled to HSA or noncovalently complexed with mHSA as coating antigens in ELISAs to study the binding of antibodies elicited by different conjugates revealed interesting results. These findings suggested that the process of conjugation may provide antigenic stability to the B PS or may preserve the helical conformation of the B PS necessary for its antigenicity and immunogenicity. Similar observations have been made with K1 PS-OMV conjugate and B-OMV conjugate-induced monoclonal antibodies, the specific binding of which could be inhibited much more effectively with the conjugated K1 or B PS than with the unconjugated PS (12).

The antibody response to the B PS or to the B+OMV complex is mostly IgM (31, 42, 44). Our results suggest that conjugation of the B PS to protein carriers induces both IgG and IgM responses. A typical class switch in immune response from IgM to IgG was observed in most monkeys following immunizations with the B PS conjugates (Fig. 1). The results are suggestive of the T-cell-dependent nature of the B PS in conjugate formulation.

There has been considerable speculation about possible immunopathologic effects of conjugate-induced B PS antibodies. The cross-reactive polysialoglycoproteins are reported to be present on certain mammalian tissues during early developmental stages (13) but decline rapidly with age after birth. Antibodies to α(2→8)-linked polysialic acid and those to the N-pr. B-TT conjugates have been shown to bind to certain mammalian fetal tissues or to polysialylated human embryonal brain glycopeptides in vitro (3, 14, 19, 21–23). However, to date, there has been no demonstration of the occurrence of such binding in vivo. There is lack of experimental evidence to show that a mere presence of structurally similar antigens in host tissues or a mere binding of antibodies with these structures in vivo results in autoimmune adverse effects (30). For instance, antibodies to the PS of serogroup W135 *N. meningitidis* are shown to bind to fetal brain structures in vitro (19). Yet the W135 PS is an active component of the licensed tetravalent or other commercial meningococcal polysaccharide vaccines, which have been proven to be safe and protective both in children and in adults (7, 20). Saukkonen and coworkers (38) demonstrated positive immunofluorescence in vitro in brain sections of 1- to 13-day-old rats treated with a polyclonal or an IgG monoclonal anti-B PS antibody. However, intraperitoneal injection of the same anti-B PS antibodies into infant rats or into the mother rats 2 days prior to parturition did not result in any binding to brain tissues of infant rats. This absence of binding in vivo is attributed to the existence of an effective blood-brain barrier (38).

The accessibility of antigenically similar fetal host structures for binding with circulating B PS-specific antibodies is another important issue that has not been addressed adequately. Long chains of polysialic acid capable of forming the required unique conformational determinant (21, 24, 25) to produce optimal binding with the specific antibodies are not believed to be present in adults. Indirect evidence for this comes from multiple reports in the literature. Absence of adverse manifestations was reported in a 81-year-old male who carried as much as 23 mg of a B PS-reactive IgM monoclonal antibody/ml in his circulation (27), suggesting the absence of a breakdown of natural tolerance. Naturally occurring anti-B PS antibodies are present in 80 to 90% of healthy adults (44) and in mothers who have given birth to healthy full-term babies (10). Anti-B PS antibodies, including IgG, have been demonstrated in human cord sera of apparently healthy babies (10). Further, Zollinger et al. (44) and Lifely et al. (31) have reaffirmed the safety of OMP-induced anti-B PS antibodies by demonstrating no ill effects or clinically significant hematological or biochemical abnormalities due to B PS antibodies in humans immunized with OMV vaccines noncovalently complexed with the B PS. This absence of harmful immunopathologic effects observed in humans with circulating anti-B PS antibodies may be due to many reasons. Several unique conditions need to be met for efficient binding between α(2→8)-linked polysialic acid and specific antibodies, and there are several difficulties in fulfilling these conditions on the surfaces of host cells in vivo. First is the unavailability of polysialoglycoproteins in sufficiently high amounts on host tissues, particularly those of adults. Second, even when antibodies are present in sufficient amounts in certain tissues (fetal), binding of antibodies to these structures is highly dependent on the accessibility of these antigens in an antigenically functional three-dimensional conformation with little or no internal esterification (30). Availability of host sialic acid in an optimal chain length is needed to meet the conformational requirement and to accomplish efficient binding with specific antibodies (21, 22). The antigen-combining site of anti-B PS antibodies requires, for saturation, an oligomer with 10 or more sugars presenting as a conformational epitope (25, 27). The exact chain length, specific conformation, and extent of internal esterification of polysialic acid present on fetal mammalian tissues are unknown. All these facts combined with the reported decreased avidity of B PS antibodies at human body temperature (10, 32) suggest the difficulties that exist in vivo in a host for accomplishing an effective antigen-antibody interaction capable of inducing harmful autoimmune effects. Rhesus neural cell adhesion molecules are also known to express polysialic acid postnatally (35). In our study, high levels of anti-B PS antibodies elicited by multiple immunizations of juvenile rhesus monkeys with various B PS conjugates did not result in any discernible safety-related adverse effects. We, however, performed no immunohistopathological studies, and a limited number of animals were studied.

This study suggests that the B PS conjugates prepared by different synthetic schemes are immunogenic in a primate species. Further studies are needed to determine whether this model is predictive of the human response to B PS conjugate vaccines. The polysaccharide and OMV antigens of group B *N. meningitidis* are important virulence determinants which satisfy the necessary criteria for use as vaccine components. Several clinical trials have been carried out with OMP vaccines in humans with efficacies of 50 to 80% (15). However, OMP vaccines may be serotype specific and, as used, have not protected young children who are highly susceptible to group B meningococcal diseases. Our results suggest the feasibility and potential efficacy of a new conjugate vaccine composed of two major protective antigens of group B meningococci (B PS and OMV), one enhancing the immunogenicity of the other in the conjugate form (46). Saline formulations of B PS and K92 conjugates may require higher doses and/or multiple immunizations to induce sufficiently high levels of antibodies to the native B PS. The in vitro parameters used to study the functional significance of conjugate-induced rhesus monkey anti-B PS antibodies (45), and of anti-OMV and anti-LPS antibody responses elicited by B-OMV conjugate vaccines used in this preclinical vaccine trial, are reported in a separate paper (46). Together, the results allow us to conclude that multiple immunizations of juvenile rhesus monkeys with various B PS conjugate vaccines appear to be safe and immunogenic. However, the safety parameters used in this study are insufficient to justify clinical trials of B PS conjugates in children and in women of child-bearing age. Nevertheless, our observations made of a primate species with an immune system closely related to that of humans suggest the feasibility of an initial evaluation of various B PS conjugate vaccines in a small number of adult male human volunteers with close and careful monitoring. Human antibodies thus generated by different B PS conjugate vaccines should then be evaluated for functional properties (bactericidal and opsonophagocytic) and protective efficacy in animal models.

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