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Bactericidal Antibody Responses of Juvenile Rhesus Monkeys Immunized with Group B *Neisseria meningitidis* Capsular Polysaccharide-Protein Conjugate Vaccines

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Reports on the bactericidal activities of antibodies to group B *Neisseria meningitidis* capsular polysaccharide (B PS) are conflicting. Using three different complement sources, we analyzed the bactericidal activities of sera of juvenile rhesus monkeys immunized with five conjugate vaccines of B PS synthesized by different schemes, an *Escherichia coli* K92 conjugate, and a noncovalent complex of B PS with group B meningococcal outer membrane vesicles (B+OMV) (S. J. N. Devi, W. D. Zollinger, P. J. Snoy, J. Y. Tai, P. Costantini, F. Norelli, R. Rappuoli, and C. E. Frasch, Infect. Immun. 65:1045–1052, 1997). With rabbit complement, nearly all preimmune sera showed relatively high bactericidal titers, and all vaccines, except the K92 conjugate, induced a fourfold or greater increase in bactericidal titers in most of the monkeys vaccinated. In contrast, with human complement, most prevaccination sera showed no bactericidal activity and in most of the vaccine groups, little or no increase in bactericidal titer was observed. However, the covalent conjugation of P BS and OMV (B-OMV) administered with and without the Ribi adjuvant induced relatively high bactericidal titers which persisted up to 30 weeks. An analysis of the specificities of bactericidal antibodies revealed that absorption with *E. coli* K1 cells did not change the bactericidal titer with human complement but reduced the titers observed with the rabbit and monkey complements. A significant increase in anti-lipopolysaccharide (LPS) antibodies was elicited by the B-OMV conjugates, and nearly all of the bactericidal activity with human complement could be inhibited with the purified group B meningococcal L3,7,8 LPS. B-OMV covalently coupled via adipic acid dihydrazide elicited significantly elevated levels ($P \leq 0.02$) of anti-OMV antibodies compared to those of the noncovalently complexed B+OMV. An initial small-scale evaluation of B PS conjugates in adult human males appears feasible, with careful monitoring, to settle the inconsistent reports of the importance of source of complement in eliciting bacteriolysis. Subsequent analysis of resultant human antibodies for bacteriolysis, opsonophagocytosis, and protective efficacy in animal models may be the first step toward answering safety- and efficacy-related concerns about B PS conjugate vaccines.

Meningitis and septicemia due to group B *Neisseria meningitidis* continue to cause high morbidity and mortality in children and adults worldwide. There are no vaccines against group B meningococcal infections licensed in this country. Outer membrane vesicle proteins (OMV) have been used as investigational vaccines which are serotype specific and induce a significant, though often transient, response (19, 35). The capsular polysaccharides (PSs) of serogroup A, B, and C *N. meningitidis* are T cell independent in nature, but unlike group A and C meningococcal PSs, the serogroup B PS (hereafter called B PS) is poorly immunogenic. However, the B PS has been successfully rendered immunogenic by covalently conjugating or noncovalently combining it with protein carriers (5, 6, 11, 16, 20, 34).

The bactericidal assay, in general, is accepted as a surrogate for evaluating the functional properties of bacterial PS-specific antibodies, although the assay results may not always reflect or predict protection in vivo against every disease. Complement-dependent bactericidal activity is one of the mechanisms by which meningococcal anti-PS antibodies are believed to exert biological effects (12). The historic study in the late 1960s by Goldschneider, Gotschlich, and Artenstein (12) showed that serum antibodies directed to the capsular PSs of group A and C *N. meningitidis* are bactericidal and protective. These workers also established an association between age-related susceptibility to meningococcal disease (including group B) and lack of bactericidal antibody.

In the early 1980s, Zollinger and Mandrell (36) showed that anti-B PS antibodies elicited by natural infections or by vaccination with a noncovalent complex of B PS with OMV (the B+OMV complex) are bactericidal only with heterologous (rabbit), not with homologous, complement. Whether the same is true for antibodies elicited by the B PS covalently conjugated to protein carriers by diverse synthetic schemes has not been well studied. A recent report by Mandrell et al. (22) of B PS-specific human monoclonal antibodies and a preliminary abstract by Tai et al. (29) on polyclonal primate antibodies elicited by a chemically modified B PS conjugate reexamined the role of complement sources in bacteriolysis. Both studies showed that under appropriate conditions, B PS antibodies are in fact bactericidal in vitro in the presence of homologous complement and are more so in the presence of heterologous rabbit complement.

In an accompanying paper, we report the characteristics, safety, and relative immunogenicities of various B PSs and related conjugate vaccines in juvenile rhesus monkeys (9). The

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purpose of that 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 chemistry or which adjuvant system would be effective, we chose to evaluate a diverse spectrum of group B meningococcal conjugates and adjuvants in juvenile rhesus monkeys. The bactericidal activities of sera of immunized rhesus monkeys tested with three different complement sources, the specificities of functional antibodies elicited by the conjugate vaccines, and anti-OMV and anti-lipopolysaccharide (LPS) antibody levels elicited by OMV-containing B PS vaccines are reported.

MATERIALS AND METHODS

Reagents. Alkaline phosphatase-labeled goat anti-human total immunoglobulin (Ig) (IgG plus IgM plus IgA), purchased from Kirkegaard and Perry Laboratories, Gaithersburg, Md., was used in all enzyme-linked immunosorbent assays (ELISA). GC agar with defined supplement (23) and tryptic soy agar were from Difco Laboratories, Detroit, Mich. Gey's balanced salt solution containing 0.2% gelatin, Ca^{++}, and Mg^{++} (GBSSG) and Dulbecco's phosphate-buffered saline (PBS) were obtained from Life Technologies, Grand Island, N.Y.

Vaccines and immunization. The different B PS vaccines and the *Escherichia coli* K92 PS-tetanus toxoid (K92-TT) conjugate vaccine, their sources, their doses, and the schedule used in immunizing rhesus monkeys are described in detail in the accompanying paper (9). Briefly, the B PS conjugates were a combination of B PS and cross-reactive material 197 (CRM_{197}) protein (B-CRM_{197}), a covalent conjugation of B PS and OMV (B-OMV), B-OMV in monophosphoryl lipid A plus trehalose dicorynomycolate (MPL+TDM), N-propionylated B PS (N-pr. B) conjugated to outer membrane protein 3 (OMP3) in stearyl tyrosine (ST), and N-pr. B-OMP3 in $A1(OH)_3$, and the B+OMV complex was used as a nonconjugate control vaccine. In addition, the *E. coli* K92 PS [composed of alternate $\alpha(2\rightarrow8)$ and $\alpha(2\rightarrow9)$ polysialic acids] conjugated to TT was also used. Juvenile rhesus monkeys of both sexes, five per group, were immunized three times intramuscularly at weeks 0, 6, and 14 with one of the conjugates (5.0 μg of a PS as a part of a conjugate per animal); the B+OMV complex (noncovalent) was given in 30.0-μg doses. The sera were collected before immunization and at 2, 6, 8, 14, 16, 22, and 30 weeks after the first immunization and were stored at −70°C until use.

Bacterial strains and antigens. Group B *N. meningitidis* 8765 (B:15:P1.3:L3,7) was used in the bactericidal assay. Strain 8765 expresses LPS that is partially sialylated, but the effect of the degree of sialylation on the sensitivity of the strain to complement-mediated killing by antibodies of different specificities was not determined. *E. coli* K1 strain BORT (O18:K1:H7, *O*-acetyl$^-$) was used for absorption of B PS-specific antibodies. Strain M986 (B:2a:P1.2:L3,7) was used for the preparation of the OMV used in conjugation to the B PS. Strain M986-NCV1, a noncapsular variant of group B *N. meningitidis* (B:2a:P1.2:L3,7), was used for the preparation of the OMV for use in ELISA (9). B PS was purified by the standard method (13, 32). L3,7,8 LPS was purified from strain 8532 (B:15:P1.3:L3,7,8), L1 LPS was purified from strain 6940 (B:19:P1.6:L1), L4 LPS was purified from strain NE (B:4:?:L4), and L12 LPS was purified from strain 7897 (A:?:?:L12) of *N. meningitidis*. LPSs used in ELISA and inhibition experiments were purified by the hot phenol-water method of Westphal and Jann (31) and were pelleted twice in an ultracentrifuge to remove capsular PSs and nucleic acids. Purified OMP with less than 1% LPS was prepared from strain 8529 (B:15:P1.3:L3,7) by extraction with 3% Empigen BB–0.5 M $CaCl_2$–0.1 M sodium acetate (pH 5.0) followed by ethanol fractionation, solubilization of the OMP fraction in 1% Empigen BB–0.05 M Tris-HCl–0.15 M NaCl–0.01 M EDTA (pH 8.0), and precipitation three times with ammonium sulfate from the 1% Empigen BB solution.

Complement sources. Three sources of complement were used. Rabbit complement was obtained from 3- to 4-week-old animals. Human and monkey complements were from adult volunteers and nonimmunized juvenile monkeys, respectively. Each source of complement was prescreened for the absence of activity against the test strain, and serum pools made from those lacking activity were used in the assays. All complement sera were stored at −70°C and were thawed on ice just prior to use.

Anti-OMV antibodies. An ELISA was performed to measure total antibodies specific for OMV, i.e., OMP plus LPS (6). The ELISA results were calculated with a log-logit computer program and averaged antibody estimates from multiple dilutions for each serum. A human serum with a known concentration of OMV antibodies was used as the reference standard, and the results were expressed in micrograms per milliliter.

Anti-LPS antibodies. LPS antibody titers in sera of rhesus monkeys immunized with various vaccines were measured by an ELISA with the purified LPS of immunotype L3,7,8 noncovalently complexed to bovine serum albumin as the coating antigen (25 μg/ml) (37). The plates were coated with the antigen in carbonate buffer, pH 9.6, at 37°C for 2 h. The sera were tested in doubling dilutions. Dulbecco's PBS containing 0.5% bovine serum albumin, 0.5% casein, 0.2% sodium azide, and 10 mg of phenol red/liter was used as the dilution buffer and for blocking. Alkaline phosphatase-labeled goat anti-human total Ig (IgG, IgM, and IgA) was used as the second antibody (1:500), and incubations with primary and secondary antibodies were carried out overnight at room temperature. Results were expressed as arbitrary antibody units per milliliter (optical density at 410 nm [OD_{410}] × reciprocal dilution at that point/50,000) and were based on the average of three points from the linear portion of each curve (approximate OD range, 0.15 to 1.0).

Bactericidal assay. Human and rabbit complement sources were used with all serum samples; the assays performed with rabbit complement were done with a threefold dilution series (38, 39). Some serum samples were reassayed with normal rhesus monkey serum as the complement source, but there was not enough normal monkey serum with no bactericidal activity to test all the samples. The assays were done in 96-well microplates with a total volume of 0.1 ml (23). About 2,000 organisms per well were incubated at 37°C for 1 h in a candle extinction box. Viable CFU were enumerated after plating by the tilt method on GC agar with defined supplement and overnight incubation at 37°C.

Controls in duplicate included (i) active complement (50 μl of assay buffer, 25 μl of active complement, 25 μl of bacterial suspension), (ii) inactive complement (50 μl of assay buffer, 25 μl of heat-inactivated complement, 25 μl of bacterial suspension), and (iii) a positive control consisting of serial twofold dilutions of a known positive serum (50 μl ml per well), active complement (25 μl), and bacterial suspension (25 μl). The control wells were plated before and after the 1-h incubation period to monitor the viability of the bacteria during the incubation period. Reciprocal serum dilutions resulting in 50% killing were chosen as the endpoints.

Analysis of specificities of bactericidal antibodies. The specificities of bactericidal antibodies elicited by the B-OMV conjugate administered with and without MPL+TDM were studied by absorption and inhibition.

(i) Absorption with *E. coli* K1 cells. Pooled sera taken at 8 weeks from five monkeys that had been vaccinated with the B-OMV vaccine in MPL+TDM were diluted 1:10 with GBSSG and absorbed four times with *E. coli* K1 cells. The cells were grown overnight on tryptic soy agar, washed once, suspended in GBSSG, and adjusted to an OD_{600} of 2.0. The cell suspension (1.25 ml) was dispensed into each of several 1.5 ml-centrifuge tubes, and the cells were pelleted with a model 12 Microfuge (Beckman Instruments, Inc., Palo Alto, Calif.) at 12,000 × *g* for 15 min at 4°C. The supernatant was discarded, the cells were suspended in 1.25 ml of serum, and the suspension was rotated at 4°C for 1 h. The suspension was centrifuged at 12,000 × *g* for 15 min at 4°C, and the supernatant was transferred to a new tube containing pelleted cells. After the last absorption, the serum was sterile filtered with a 0.22-μm-pore-size membrane filter and was tested along with unabsorbed serum for bactericidal activity with human, rabbit, or monkey sera as complement sources.

The serum collected at 8 weeks, before and after absorption with *E. coli* K1 cells, was assayed by ELISA for antibody levels to purified B PS bound to the microwell plate via poly-L-lysine and to M986 outer membrane complex and purified OMPs of strain 8529 (B:15:P1.3:L3,7), the same type and subtype as the strain used in the bactericidal assay. Assays were done as described above for anti-LPS antibodies. In the anti-B PS assay, 25 μg of poly-L-lysine in carbonate buffer/ml was used to coat the wells, followed by two washes with Dulbecco's PBS without calcium and incubation with 25 μg of B PS in carbonate buffer/ml for 1 h at 37°C. The plates were then blocked, and the test was completed as described above.

(ii) Bactericidal inhibition study with different LPSs and with B PS. Pooled sera collected at week 16 from monkeys in the two groups that had been vaccinated with the B-OMV vaccine with and without MPL+TDM were pooled, and the bactericidal titers of the two pools against strain 8765 (B:15:P1.3:L3,7,8) were determined. Dilutions of serum that gave about 90% killing (1:100 and 1:200) were used in the inhibition test. A constant amount of diluted serum (25 μl) was added to serial twofold dilutions (25 μl) of the inhibiting antigen, i.e., LPS (L3,7,8; L1; L4; and L12) or B PS, in a 96-well microplate and incubated at 37°C for 1 h. Complement and bacteria were then added to the wells, and the assay was completed as for the regular bactericidal assay.

As a control for nonspecific inhibition by the LPS preparations, a rhesus monkey serum containing bactericidal antibodies with specificity mainly against PorA (P1.3) was included in the assay. This serum was from a monkey vaccinated with a group B meningococcal OMP-detoxified LPS vaccine in a different study (33) and was identified as having mostly anti-PorA activity by comparative testing for activity against a PorA deletion mutant (38) and its isogenic parent. None of the antigens inhibited this serum under the same conditions.

RESULTS AND DISCUSSION

In an accompanying article, we report the B PS-specific antibody levels elicited in juvenile rhesus monkeys by seven different formulations of B PS vaccines (9). Here we report the anti-OMV and anti-LPS antibody levels in sera of rhesus monkeys immunized with B PS covalently conjugated to and noncovalently complexed with group B meningococcal OMV and the functional activities of resultant antibodies.

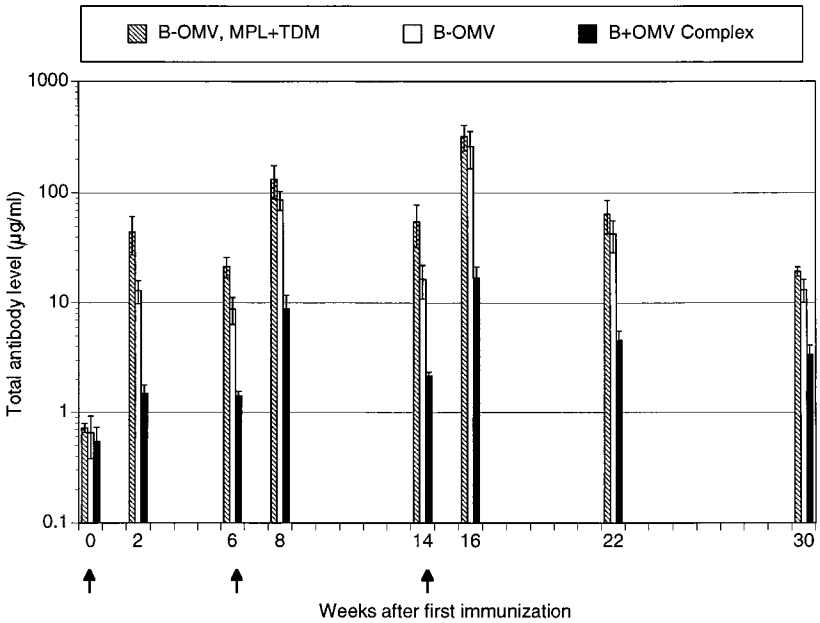

FIG. 1. Total anti-OMV antibody titers (means ± standard errors) versus M986 OMV of three rhesus monkey groups as measured by ELISA. Study groups included monkeys ($n = 5$) immunized three times with the B+OMV complex or the B-OMV conjugate vaccine administered in saline or in MPL+TDM. Arrows indicate the weeks at which monkeys were immunized.

OMV-specific antibodies. The PS and OMV antigens of group B *N. meningitidis* are important virulence determinants which satisfy the necessary criteria for use as vaccine components. Several efficacy trials with humans have been carried out with OMP vaccines with efficacies of 50 to 80% (11). However, OMP vaccines may not be effective against all group B meningococcal strains and, as used, have not protected young children who are highly susceptible to group B meningococcal diseases. Interestingly, covalent coupling of the B PS to meningococcal OMV enhanced the immunogenicities of both components of the conjugate. The total levels of antibody to the OMV component (a mixture of OMP and LPS) elicited by the B-OMV conjugate in saline or in MPL+TDM are depicted in Fig. 1. Antibody levels obtained with the noncovalently complexed B+OMV are also shown for comparison. It should be noted that the OMV preparation used in the B-OMV conjugate was prepared from a different strain by a method different from the one used in the noncovalent vaccine formulation complexed with the B PS. The highest levels of anti-OMV antibodies were observed in monkeys that received the B-OMV conjugate in MPL+TDM following each of three immunizations. The OMV antibody levels remained higher than the preimmunization levels by week 30. The anti-OMV levels elicited by the B-OMV conjugated via adipic acid dihydrazide and administered in saline were 8- to 13-fold higher than those elicited by the noncovalently complexed B+OMV ($P \leq 0.02$). The reason for this enhanced effect is not known. However, a similar increase in immunogenicity to the protein carriers cytolysin and elastase of *Vibrio vulnificus* has been reported after covalent attachment to the *V. vulnificus* PS (7). From our study, it is not clear whether the process of conjugation rendered OMV more immunogenic or whether the enhanced immunogenicity to OMV was due to the adjuvant or immunomodulating effect of the associated LPS or simply a result of the differences in the OMV preparations used.

LPS antibodies. The clinical studies with OMV vaccines have consistently shown poor response to the LPS. In one study, the group B meningococcal LPS-derived oligosaccharide-OMP conjugate hardly induced an immune response to LPS without an adjuvant (30). Figure 2 shows the geometric mean levels of anti-LPS antibody elicited in our study by three LPS-containing B PS conjugates: B-OMV, B-OMV in MPL+TDM, and the B+OMV complex (noncovalent). The increase in anti-LPS antibodies observed in rhesus monkeys immunized with the saline formulation of the B-OMV conjugate was four- to eightfold greater than in those vaccinated with the B+OMV complex.

Bactericidal antibodies. Literature on the bactericidal activities of B PS-specific antibodies in the presence of homologous complement is inconsistent. While some researchers have demonstrated complement-mediated bacteriolysis with homologous complement (22, 29), others, using different conditions, have not (36). One of the main objectives of our study was to evaluate whether rhesus monkey antibodies elicited by the B PS and related conjugate vaccines behave differently from naturally occurring or infection-induced B PS antibodies described in published studies with reference to bacteriolysis in vitro.

We used rabbit and human complements with sera from all immunized groups and the monkey complement with some rhesus monkey sera. When rabbit complement was used, nearly all of the juvenile rhesus monkeys had relatively high prevaccination titers of bactericidal antibodies (range, 1:3 to 1:7,290, and geometric mean titer, 1:90). With the exception of K92-TT conjugate, all other vaccines induced a fourfold or greater increase in titer (mean ± standard error) in most of the animals vaccinated (Table 1). The increased titers, however, did not persist through week 30. By week 30, the serum bactericidal titers of most monkeys had returned to prevaccination levels. Our results obtained with rabbit complement are consistent with those of published reports (22, 36) in that rabbit complement markedly enhanced the bactericidal activities of B PS-specific antibodies in monkeys immunized with various B PS vaccines. Similar augmentation by rabbit complement has

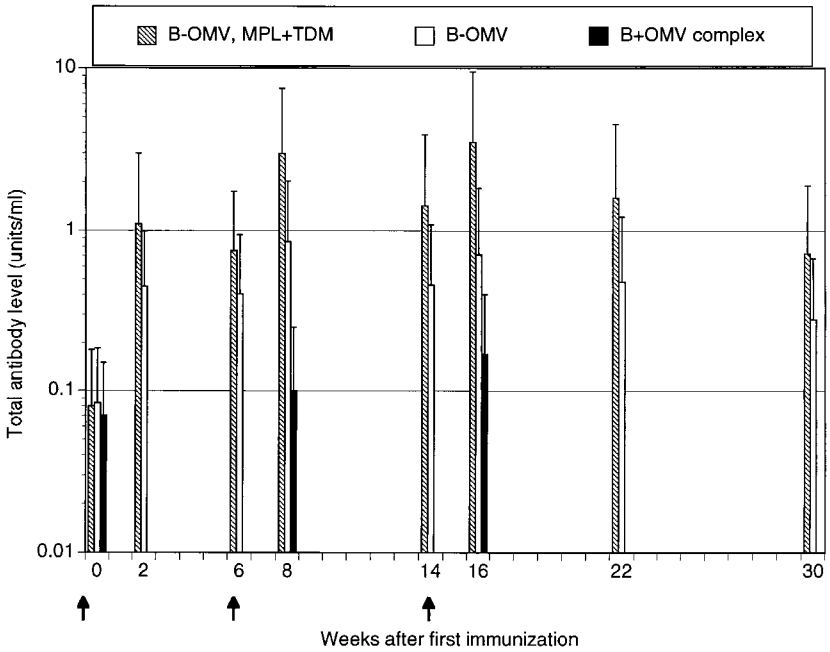

FIG. 2. Total anti-L3,7,8 LPS antibody titers (means + standard errors) of three rhesus monkey groups as measured by ELISA. Study groups included monkeys ($n = 5$) immunized three times with the B+OMV complex or the B-OMV conjugate vaccine administered in saline or in MPL+TDM. Arrows indicate the weeks at which monkeys were immunized.

also been reported with human IgM antibodies to the capsular PSs of group C *N. meningitidis* and *Haemophilus influenzae* type b (14, 22).

When human complement was used, most prevaccination rhesus monkey sera showed no bactericidal activity and in most of the vaccine groups, little or no increase in bactericidal activity was observed after immunization. However, in contrast to the reported absence of bactericidal activity of anti-B PS antibodies elicited by the noncovalently complexed B+OMV vaccine (36), the B-OMV conjugate administered in saline and in MPL+TDM induced relatively high titers of bactericidal antibodies; these antibodies persisted through week 30 (Table 2). These results, by and large, are consistent with a recent report that used human complement (22). As discussed below, however, these antibodies do not appear to be specific for the B PS. Because only a small amount of normal rhesus monkey serum that was suitable for use as complement was available, we sought to validate the use of human complement as a substitute for homologous monkey complement in comparing the bactericidal activities of the sera with homologous and heterologous complements. The pre- and postvaccination sera from a total of nine monkeys selected from the different vaccine groups were tested together with monkey, human, and rabbit complements. The results with the monkey complement (not shown) were similar to the results obtained with human complement except that both the pre- and postvaccination titers were somewhat higher with monkey complement. Monkeys vaccinated with conjugate vaccines not containing meningococcal OMV showed no significant rises in bactericidal titers when the homologous monkey complement was used. When rabbit complement was used, however, these same animals showed substantial rises in bactericidal titers.

The B-CRM_{197} conjugate-induced murine antibodies reactive with the unmodified B PS have been shown to possess

TABLE 1. Bactericidal titers of sera from monkeys immunized with various B PS vaccines and the K92-TT conjugate obtained with rabbit complement

Wk after first immunization	Vaccine used[a]						
	N-pr. B-OMP_3 in $A1(OH)_3$	B-OMV in MPL+TDM	B-OMV	B-CRM_{197}	N-pr. B-OMP3 in ST	B+OMV complex	K92-TT
0[b]	8.1 ± 1.9	7.4 ± 1.1	7.1 ± 1.4	4.9 ± 0.9	6.8 ± 0.6	4.5 ± 1.5	6.5 ± 1.0
2	14.7 ± 0.8	10.6 ± 0.4	10.0 ± 1.4	9.7 ± 0.9	8.4 ± 0.6	7.8 ± 1.6	7.1 ± 0.8
6	10.9 ± 1.8	9.0 ± 0.6	8.4 ± 1.1	7.8 ± 0.6	7.8 ± 0.3	5.2 ± 1.1	6.8 ± 0.9
8	13.5 ± 1.1	10.0 ± 0.3	10.3 ± 1.1	9.0 ± 0.8	8.4 ± 0.6	7.1 ± 1.2	6.8 ± 0.9
14	9.7 ± 1.7	8.7 ± 0.8	8.4 ± 1.3	7.1 ± 0.4	6.8 ± 0.6	5.8 ± 1.4	6.8 ± 0.9
16	11.6 ± 0.6	12.8 ± 0	9.0 ± 1.2	7.8 ± 0.6	7.8 ± 0.6	8.4 ± 1.5	6.5 ± 1.0
22	9.3 ± 1.5	9.7 ± 0.9	7.4 ± 1.3	5.8 ± 1.3	7.1 ± 0.4	4.9 ± 0.9	6.5 ± 1.0
30	8.7 ± 1.7	7.8 ± 1.3	7.1 ± 1.4	5.2 ± 1.1	6.8 ± 0.6	3.6 ± 0.8	6.5 ± 1.0

[a] Mean $\log_2$ reciprocal titers ± standard errors of the mean ($n = 5$).
[b] Preimmunization sample.

TABLE 2. Bactericidal titers of sera from monkeys immunized with the B PS vaccines and the K92-TT conjugate with human complement

Wk after first immunization	Vaccine used[a]						
	N-pr. B-OMP3 in $A1(OH)_3$	B-OMV in MPL+TDM	B-OMV	B-CRM_{197}	N-pr. B-OMP3 in ST	B+OMV complex	K92-TT
0[b]	0.0	0.0	0.0	0.4 ± 0.4	1.4 ± 1.4	0.0	0.2 ± 0.2
2	0.2 ± 0.2	8.4 ± 0.4	5.4 ± 1.2	1.8 ± 1.0	2.2 ± 1.3	0.0	1.0 ± 0.2
6	0.0	6.8 ± 0.4	3.2 ± 1.4	1.0 ± 1.0	1.4 ± 1.4	0.0	0.2 ± 0.2
8	0.8 ± 0.8	7.4 ± 0.8	4.8 ± 1.4	1.0 ± 1.0	1.6 ± 1.4	0.0	3.0 ± 1.0
14	0.0	6.2 ± 0.4	2.4 ± 1.1	0.4 ± 0.4	1.4 ± 1.4	0.0	0.4 ± 0.2
16	0.8 ± 0.6	8.6 ± 0.2	6.4 ± 1.2	0.4 ± 0.4	2.2 ± 1.4	0.0	0.4 ± 0.4
22	0.0	6.8 ± 0.5	4.6 ± 0.7	0.4 ± 0.4	1.4 ± 1.4	0.0	0.2 ± 0.2
30	0.0	5.8 ± 0.5	3.2 ± 0.6	0.4 ± 0.4	1.4 ± 1.4	0.0	0.2 ± 0.2

[a] Mean $\log_2$ reciprocal titers ± standard errors of the mean ($n = 5$).
[b] Preimmunization sample.

biological activities against group B *N. meningitidis* (4), whereas only that population of antibodies (mouse) produced by the N-pr. B-TT conjugates and reactive with cell-associated B PS or B PS linked to a column matrix with a long spacer arm were reported to be bactericidal (17). In our study, except for occasional samples showing low titers, sera of rhesus monkeys immunized with B-CRM_{197} and N-pr. B-OMP3 conjugates did not exhibit bacteriolysis of group B *N. meningitidis* 8765 in presence of human complement. However, in another preliminary study, the N-pr. B-OMP3 conjugates were reported to induce antibodies that were bactericidal with human complement (29). The exact reasons for these varied observations are not known. Several differences in experimental conditions used in our study and those used by the other group (29) might be responsible for the discrepant results.

Specificities of bactericidal antibodies. Since the B-OMV conjugate was composed of B PS, OMV, and some LPS, the specificities of bactericidal antibodies were determined by absorption and inhibition studies. *E. coli* K1 cells express a capsule consisting of $\alpha(2\rightarrow8)$-linked sialic acid similar to the one expressed on the surfaces of group B *N. meningitidis* cells (18). In our study, absorption with *E. coli* K1 (*O*-acetyl$^-$) cells of a pool of the sera collected at 8 weeks from the monkeys that received the B-OMV conjugate in MPL+TDM did not change the bactericidal titer with human complement. However, notably, absorption with *E. coli* K1 cells resulted in eight- and twofold reductions in the bactericidal titers observed with rabbit and rhesus complements, respectively (Table 3). More interestingly, the absorption with *E. coli* K1 cells did decrease the ELISA titer to the B PS antibody from 5.39 U (preabsorption) to 0.63 U. The biologic significance of these observations or the reasons for the diverse effects of complement from diverse sources are unknown. The limited studies we have performed thus far do not provide one with an explanation for these intriguing observations.

Since the B-OMV conjugate vaccine formulations also contained ~5 to 10% of meningococcal LPS, the specificities of bactericidal antibodies active with human complement were further assessed by inhibition with a purified group B meningococcal LPS. Interestingly, nearly all of the bactericidal activities with human complement of two pools of the sera collected at week 16 from monkeys vaccinated with the B-OMV conjugate in MPL+TDM could be inhibited with the purified L3,7,8 LPS, with 50% inhibition seen at 1 μg of LPS/ml (Fig. 3A); the inhibition was reproducible. The antibody responses of the immunized groups of monkeys to the L3,7,8 LPS were verified by an ELISA (Fig. 2). L1, L4, and L12 LPSs and the B PS did not inhibit bacteriolysis (Fig. 3A). The control serum of a rhesus monkey vaccinated with a group B meningococcal OMP-detoxified LPS vaccine in a different study (38) was not inhibited by the L3,7,8 LPS or by the L1, L4, and L12 LPSs (Fig. 3B). Our observation is contrary to the results of published reports. The clinical studies with OMV vaccines have consistently shown that most of the bactericidal antibodies were directed to the class 1 and class 5 proteins (26). In this study, however, the target strain used in the bactericidal assay did not share class 1, 2, or 5 OMPs with the vaccine strain. Studies of candidate meningococcal LPS-containing vaccines, including LPS-derived oligosaccharide-protein conjugates, have also reported the inability of anti-LPS antibodies to be bactericidal to group B meningococci (15, 25, 30).

It is well known that antibodies to group B meningococcal OMV are bactericidal (11, 34, 38). Since the serotype used for the OMV component of the B-OMV conjugate (B:2a:P1.2:

TABLE 3. Bactericidal and ELISA titers of a pooled serum collected at week 8 from monkeys vaccinated with B-OMV conjugate in MPL+TDM before and after absorption with *E. coli* K1 cells

Type of pooled serum collected at wk 8	Bactericidal assay result[a] with complement from:			ELISA result[b] with coating antigen:		
	Humans	Rabbits	Monkeys	B PS	Outer membrane complex[d]	OMP[e]
Unabsorbed	160	2,560	640	5.39 ± 0.52	655 ± 40	75 ± 6.2
Absorbed[c]	160	320	320	0.63 ± 0.04	583 ± 56	66.8 ± 3.6

[a] Reciprocal antibody titers from duplicate experiments (reciprocal dilution of serum).
[b] ELISA units ± standard errors of the mean; n = four determinations.
[c] Uncorrected for slight dilution that occurred during absorption.
[d] From *N. meningitidis* M986 (B:2a:P1.2:L3,7).
[e] From *N. meningitidis* 8529 (B:15:P1.3:L3,7).

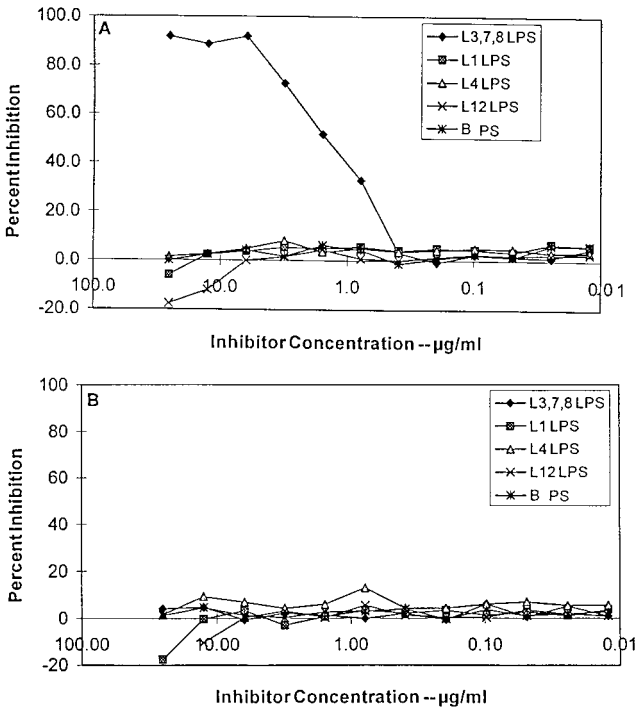

FIG. 3. Inhibition of bacteriolysis active with human complement in a pooled serum collected at week 16 from monkeys ($n = 5$) immunized with the B-OMV conjugate in MPL+TDM (A) and in the serum collected 8 weeks postvaccination of a rhesus monkey immunized with a purified OMP vaccine (B). The LPSs (L1; L4; L12; and L3,7,8) and B PS antigens were used as inhibitors at the different concentrations indicated. Group B *N. meningitidis* 8765 (B:15:P1.3:L3,7) was used in the bactericidal assay.

L3,7) and the one used in the bactericidal assay (B:15:P1.3: L3,7,8) were not the same, no comments can be made as to the contribution of antibodies elicited by the OMP component of the B-OMV conjugate vaccine to bactericidal activity.

Bacteriolysis results from a complex interaction between specific antibodies, the pathogenic bacterium, and the complement. Several factors influence the outcome of a bactericidal assay, including the strains of pathogens used and their growth conditions, the ratio of antibodies to bacterial cells used in the assay, and the buffer system (1, 2, 22, 30). Some strains, though fully encapsulated, are completely resistant to killing by B PS antibodies and require 5- to 10-fold higher concentrations of antibodies to bring about killing (22). We used the GBSSG buffer and a single strain of group B *N. meningitidis* (B:15:P1.3: L3,7,8) in bactericidal assays throughout. A target strain heterologous to the vaccine strains was used to increase the probability of detecting bactericidal antibodies to the B PS.

The biological functions of an antiserum are determined by the epitope specificities, amounts, and isotype compositions of the antibodies present. Some (sub)classes of antibodies are better complement activators than others, while some can even inhibit lysis (10). Temperature is another factor that affects the efficiencies of binding of antibodies to the B PS in an assay (3, 36). We performed absorption assays at 4°C, a temperature at which binding of B PS antibodies is known to be more effective than at the human body temperature of 37°C (36). Relative avidity of antibodies is also an important factor (28). Use in our study, as a complement source, of a human serum that was not agammaglobulinemic but might have contained B PS antibodies at less than the detectable levels may possibly have contributed to the results obtained. A low-avidity human chimeric IgG antibody with specificity to B PS (5E1) showed no bactericidal activity with either rabbit or human complement (22, 24). Similar results were obtained with some B-OMV conjugate-induced murine (low-avidity) IgG monoclonal antibodies (MAbs) which were also not bactericidal with rabbit complement even at high concentrations (1.5 mg/ml) when tested in an assay that used Dulbecco's buffer system (8). Many rhesus monkey sera used in bactericidal assays in our study had various levels of antibodies of both the IgG and the IgM class.

Our study has certain limitations. First, we confined absorption studies (with *E. coli* K1 cells) to the sera taken after the first immunization (week 8) only. Had we also tested sera (with shifted isotype composition) taken after the second and third immunizations for bactericidal inhibition following absorption, it is possible that we would have obtained different results. Second, we limited our absorption studies to *E. coli* K1 cells and to the B PS attached to ELISA plates via poly-L-lysine. Inhibition with soluble B PS (Fig. 3A) failed to affect the bactericidal titer of a pooled serum taken at week 16 from monkeys vaccinated with the B-OMV conjugate vaccine. Mandrell et al. (22) have successfully used purified soluble B PS to inhibit the bactericidal activities of B PS-specific human MAbs active with human complement. It is known from a previous study that the B PS-specific MAbs generated by B- or *E. coli* K1 PS-OMV conjugate vaccines show binding and functional diversities (8). The B PS expresses microheterogeneous epitopes, and the expression of epitopes on the B PS is dependent on the configuration in which B PS is presented to the antibodies (8). Some MAbs which failed to agglutinate with group B *N. meningitidis* or *E. coli* K1 cells were able to react specifically with the purified B PS by precipitation or by immunodot analysis. More importantly, the B PS proved to be a better inhibitor when used as a covalent conjugate or a noncovalent complex with a protein than when attached via poly-L-lysine (8). In the present study, we did not use a B PS-human serum albumin conjugate or a noncovalent B PS and methylated human serum albumin complex for absorption and inhibition in addition to *E. coli* K1 cells. Irrespective of the varied and inconclusive reports of the bactericidal functions of B PS conjugate-induced antibodies, the potential possibility that B PS-specific antibodies may also function by an opsonophagocytic mechanism in monkeys or in humans cannot be ignored, since phagocytosis is known to be an efficient mechanism of protection against group B *N. meningitidis* in vitro (24, 27).

In conclusion, under the conditions used, the bactericidal activities of antibodies elicited by various B PS conjugate vaccines appeared to be highly diverse. The B-OMV conjugate elicited levels of bactericidal antibodies active with the human complement higher than those of other B PS or K92 conjugate vaccines. Thus, the B-OMV conjugates appear to have the potential to improve the efficacies of the group B meningococcal vaccines currently being evaluated by producing antibodies to two potential protective antigens, i.e., the PS and OMV components. The observation that the bactericidal antibodies (active with human complement) induced by the B-OMV conjugate in MPL+TDM could be absorbed by the purified LPS but not by absorption with *E. coli* K1 cells is intriguing and warrants further evaluation. The significance of the 50% (one well) decrease in the bactericidal activity of B-OMV conjugate-immunized rhesus monkey sera (active with the rhesus complement) that was absorbed by *E. coli* K1 is unclear, since there was a small uncorrected dilution effect resulting from multiple (four) absorptions. Nevertheless, there may have been some anti-B PS-specific bactericidal activity expressed with monkey

complement. This suggests the need to use homologous rhesus complement rather than the human complement.

The biologic relevance of augmented lysis in the presence of rabbit complement and the absence of lysis by rhesus antibodies elicited by B PS conjugates other than the B-OMV conjugate with human complement is uncertain. The inconsistent published reports about the ability of B PS antibodies to produce lysis with human complement suggest that the phenomenon may be unrelated to the source of complements used but may potentially be related to the relative avidity. The varied reports and observations on one in vitro parameter of possible biologic activity should not deter further functional evaluation of B PS conjugates. An equine anti-B PS antibody lacking bactericidal activity in vitro has been shown to be functionally effective and to confer protection in experimentally infected animals (21). This suggests that mechanisms other than complement-mediated bacteriolysis also play a role in protection against group B meningococcal infections. The observation that the antibodies evoked by some B PS conjugate vaccines were bactericidal with the homologous rhesus complement and could be absorbed with *E. coli* K1 cells supports an initial evaluation of these conjugates (with careful monitoring) in a small number of adult male human volunteers. The conflicting reports on the importance of source of complement for bacteriolysis of group B *N. meningitidis* may then be settled by evaluating the resultant human antibodies for bactericidal activity with homologous complement, for opsonophagocytosis, and for protective efficacy in appropriate animal models.

REFERENCES

1. **Amir, J., M. G. Scott, M. H. Nahm, and D. M. Granoff.** 1990. Bactericidal and opsonic activity of IgG1 and IgG2 anticapsular antibodies to *Haemophilus influenzae* type b. J. Infect. Dis. **162:**163–171.
2. **Amir, J., X. Liang, and D. M. Granoff.** 1990. Variability in the functional activity of vaccine-induced antibody to *Haemophilus influenzae* type b. Pediatr. Res. **27:**358–364.
3. **Azmi, F. H., A. H. Lucas, H. L. Spiegelberg, and D. M. Granoff.** 1995. Human immunoglobulin M paraproteins cross-reactive with *Neisseria meningitidis* group B polysaccharide and fetal brain. Infect. Immun. **63:**1906–1913.
4. **Bartoloni, A., F. Norelli, C. Ceccarini, R. Rappuoli, and P. Costantino.** 1995. Immunogenicity of meningococcal B polysaccharide conjugated to tetanus toxoid or CRM_{197} via adipic acid dihydrazide. Vaccine **1:**463–470.
5. **Devi, S. J. N., J. B. Robbins, and R. Schneerson.** 1991. Antibodies to poly[(2→8)-α-*N*-acetylneuraminic acid] and poly[(2→9)-α-*N*-acetylneuraminic acid] are elicited by immunization of mice with *Escherichia coli* K92 conjugates: potential vaccines for groups B and C meningococci and *E. coli* K1. Proc. Natl. Acad. Sci. USA **88:**7175–7179.
6. **Devi, S. J. N., C. E. Frasch, W. Zollinger, and P. J. Snoy.** 1994. Immunization of juvenile rhesus monkeys with group B *Neisseria meningitidis* capsular polysaccharide-protein conjugate vaccines, 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. SSC, Reading, England.
7. **Devi, S. J. N., U. Hayat, C. E. Frasch, A. S. Kreger, and J. G. Morris, Jr.** 1995. Capsular polysaccharide-protein conjugate vaccines of carbotype 1 *Vibrio vulnificus*: construction, immunogenicity, and protective efficacy in a murine model. Infect. Immun. **63:**2906–2911.
8. **Devi, S. J. N., A. Karpas, and C. E. Frasch.** 1996. Binding diversity of monoclonal antibodies to α(2→8) polysialic acid conjugated to outer membrane vesicle via adipic acid dihydrazide. FEMS Immunol. Med. Microbiol. **14:**211–220.
9. **Devi, S. J. N., W. D. Zollinger, P. J. Snoy, J. Y. Tai, P. Costantini, F. Norelli, R. Rappuoli, and C. E. Frasch.** 1997. Preclinical evaluation of group B *Neisseria meningitidis* and *Escherichia coli* K92 capsular polysaccharide-protein conjugate vaccines in juvenile rhesus monkeys. Infect. Immun. **65:**1045–1052.
10. **Ey, P. L., G. J. Russell-Jones, and C. R. Jenkin.** 1980. Isotypes of mouse IgG. I. Evidence for non-complement fixing IgG1 antibodies and characterization of their interference with IgG2 sensitization of target red blood cells for lysis by complement. Mol. Immunol. **17:**699–710.
11. **Frasch, C. E.** 1995. Vaccines: past, present and future prospects, p. 245–283. *In* K. A. V. Cartwright (ed.), Meningococcal disease. Wiley & Sons, New York, N.Y.
12. **Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein.** 1969. Human immunity to the meningococcus. I. The role of humoral antibodies. J. Exp. Med. **129:**1307–1326.
13. **Gotschlich, E. C., T. Y. Liu, and M. S. Artenstein.** 1969. Human immunity to the meningococcus. III. Preparation and immunochemical properties of the group A, group B and group C meningococcal polysaccharides. J. Exp. Med. **129:**1347–1366.
14. **Griffis, J. M., and D. K. Goroff.** 1982. IgA blocks IgM and IgG-initiated immune lysis by separate molecular mechanisms. J. Immunol. **130:**2882–2885.
15. **Jennings, H. J., C. Lugowski, and F. E. Ashton.** 1984. Conjugation of meningococcal lipopolysaccharide R-type oligosaccharides to tetanus toxoid as a route to a potential vaccine against group B *Neisseria meningitidis*. Infect. Immun. **43:**407–412.
16. **Jennings, H. J., R. Roy, and A. Gamian.** 1986. Induction of meningococcal group B polysaccharide-specific IgG antibodies in mice using an *N*-propionylated B polysaccharide-tetanus toxoid conjugate vaccine. J. Immunol. **137:** 1708–1713.
17. **Jennings, H. J., A. Gamian, F. Michon, and F. E. Ashton.** 1989. Unique intermolecular bactericidal epitope involving the homosialopolysaccharide capsule on the cell surface of group B *Neisseria meningitidis* and *Escherichia coli* K1. J. Immunol. **142:**3585–3591.
18. **Kasper, D. L., J. L. Winkelhake, W. D. Zollinger, B. L. Brandt, and M. S. Artenstein.** 1973. Immunochemical similarity between polysaccharide antigens of *Escherichia coli* O7:K1(L):NM and group B *Neisseria meningitidis*. J. Immunol. **110:**262–268.
19. **Lifely, M. R., C. Moreno, and J. C. Lindon.** 1987. An integrated molecular and immunological approach towards a meningococcal group B vaccine. Vaccine **5:**11–26.
20. **Lifely, M. R., S. C. Roberts, W. M. Shephard, J. Esdaile, Z. Wang, A. Cleverly, A. A. Aulaqi, and C. Moreno.** 1991. Immunogenicity in adult males of a *Neisseria meningitidis* group B vaccine composed of polysaccharide complexed with outer membrane proteins. Vaccine **9:**60–66.
21. **Lifely, M. R., and J. Esdaile.** 1991. Specificity of the immune response to the group B *Neisseria meningitidis*. Immunology **74:**490–496.
22. **Mandrell, R. E., F. H. Azmi, and D. M. Granoff.** 1995. Complement-mediated bactericidal activity of human antibodies to poly α2→8 *N*-acetylneuraminic acid, the capsular polysaccharide of *Neisseria meningitidis* serogroup B. J. Infect. Dis. **172:**1279–1289.
23. **Moran, E. E., B. L. Brandt, and W. D. Zollinger.** 1994. Expression of the L8 lipopolysaccharide determinant increases the sensitivity of *Neisseria meningitidis* to serum bactericidal activity. Infect. Immun. **62:**5290–5295.
24. **Raff, H. V., D. Devereux, W. Shuford, D. Abbott-Brown, and G. Maloney.** 1988. Human monoclonal antibody with protective activity for *Escherichia coli* K1 and *Neisseria meningitidis* group B infections. J. Infect. Dis. **157:**118–126.
25. **Rosenquist, E., E. A. Hoiby, G. Bjune, P. Brandtzaeg, K. Bryn, O. Closs, and L. O. Froholm.** 1990. Antibodies against meningococcal lipopolysaccharides after vaccination and meningococcal disease, p. 105. Seventh International Pathogenic Neisseria Conference. Walter de Gruyter and Co., Berlin, West Germany.
26. **Rosenquist, E., E. A. Hoiby, E. Wedege, K. Bryn, J. Kolberg, A. Klem, E. Ronnild, G. Bjune, and H. Nokleby.** 1995. Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine. Infect. Immun. **63:**4642–4652.
27. **Ross, S. C., P. J. Rosenthal, H. M. Berberich, and P. Densen.** 1987. Killing of *Neisseria meningitidis* by human neutrophils: implications for normal and complement deficient individuals. J. Infect. Dis. **155:**1266–1275.
28. **Schlesinger, Y., D. M. Granoff, and the Vaccine Study Group.** 1992. Avidity and bactericidal activity of antibody elicited by different *Haemophilus influenzae* type b conjugate vaccines. JAMA **267:**1489–1494.
29. **Tai, J. Y., F. Michon, and P. C. Fusco.** 1995. Antibody-dependent, complement-mediated bactericidal activity elicited by group B meningococcal conjugate vaccines in mice and nonhuman primates, abstr. G3, p. 159. *In* Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
30. **Verheul, A. F. M., J. A. M. Van Gaans, E. J. H. Wiertz, H. Snippe, J. Verhoef, and J. T. Poolman.** 1993. Meningococcal lipopolysaccharide (LPS)-derived oligosaccharide-protein conjugates evoke outer membrane protein- but not LPS-specific bactericidal antibodies in mice: influence of adjuvants. Infect. Immun. **61:**187–196.
31. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides, p. 83–91. *In* R. I. Whistler (ed.), Methods in carbohydrate chemistry, vol. V. Academic Press, Ltd., New York, N.Y.
32. **World Health Organization Expert Committee on Biological Standardization.** 1976. Requirements for meningococcal polysaccharide vaccine, p. 51–75. World Health Organization Report Series 594. World Health Organization, Albany, N.Y.
33. **Zollinger, W. D.** Unpublished data.
34. **Zollinger, W. D., R. E. Mandrell, J. M. Griffiss, P. Altieri, and S. Berman.** 1979. Complex of meningococcal group B polysaccharide and type 2 outer membrane protein immunogenic in man. J. Clin. Invest. **63:**836–848.
35. **Zollinger, W. D., J. E. Boslego, C. E. Frasch, and L. O. Froholm.** 1984. Safety of vaccines containing meningococcal group B polysaccharide. Lancet **ii:**166.
36. **Zollinger, W. D., and R. E. Mandrell.** 1983. Importance of complement source in bactericidal activity of human antibody and murine monoclonal

antibody to meningococcal group B polysaccharide. Infect. Immun. **40:**257–264.
37. **Zollinger, W. D., B. L. Brandt, and E. C. Tramont.** 1986. Immune response to *Neisseria meningitidis*, p. 346. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 3rd ed. American Society for Microbiology, Washington, D.C.
38. **Zollinger, W. D., and E. E. Moran.** 1991. Meningococcal vaccines—present and future. Trans. R. Soc. Trop. Med. Hyg. **85:**37–43.
39. **Zollinger, W. D., C. E. Frasch, S. J. N. Devi, E. E. Moran, and P. J. Snoy.** 1994. Bactericidal antibody responses of juvenile rhesus monkeys to conjugate B polysaccharide vaccines, p. 441–442. *In* J. S. Evans, S. E. Yost, M. C. J. Maiden, and I. M. Feavers (ed.), Proceedings of the Ninth International Pathogenic Neisseria Conference. SSC, Reading, England.

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