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Protection Against Group B *Neisseria meningitidis* Disease: Preparation of Soluble Protein and Protein-Polysaccharide Immunogens

CARL E. FRASCH* AND MARK S. PEPPLER†

Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20205

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Although effective polysaccharide vaccines have been developed for meningococcal groups A, C, Y, and W135, the purified group B polysaccharide has proven to be nonimmunogenic. Earlier studies indicated that serotype 2 outer membrane protein vaccines induced bactericidal antibodies in animals and protected them from meningococcal challenge. However, a similar vaccine induced only low levels of antiprotein antibodies in both adults and children (C. E. Frasch et al., *in* J. B. Robbins et al., ed., *Seminars in Infectious Disease* vol. 4, p. 263–267, 1982). Methods were therefore developed to produce more immunogenic serotype 2 protein vaccines. We found that, by growing the organism for 65 to 72 h at 32°C, three to four times more outer membrane protein was released into the culture medium than could be extracted from overnight-grown cells. The outer membranes were therefore purified directly from the broth by ultrafiltration followed by ammonium sulfate precipitation. Most of the lipopolysaccharide was selectively removed from the membranes by treatment with the nonionic detergent Brij-96. The Brij-96 was then removed and the resulting vaccine was filter sterilized. Some vaccines were prepared by combining equal parts of detergent-treated membrane protein and high-molecular-weight group B polysaccharide producing highly soluble vaccines. These new vaccines were compared by using an enzyme-linked immunosorbent inhibition assay to an insoluble vaccine (E-06) found to be poorly immunogenic in humans. A human serum with serotype 2 specificity was used in the inhibition assay, and 5 μg of E-06 was required for 50% inhibition, whereas <1 μg of the soluble vaccines was required. Addition of group B polysaccharide slightly increased the inhibitory capacity of the protein component.

The major cell surface antigens of *Neisseria meningitidis* causing invasive disease in humans are the capsular polysaccharide, outer membrane proteins, and lipopolysaccharide (LPS). Bactericidal antibodies against these surface antigens play a major role in protection against meningococcal disease (13, 16). A high frequency of meningococcal disease has been observed among individuals lacking in one of the higher complement components and, therefore, unable to mount serum bactericidal killing (19).

Currently in the United States and many European countries, group B organisms are responsible for much of the meningococcal disease (1, 5, 8), but unfortunately the purified high-molecular-weight group B meningococcal polysaccharide has proven to be nonimmunogenic in humans (24). This lack of immunogenicity is as yet unexplained since high levels of group B polysaccharide-specific bactericidal antibodies are found in the sera of most children and adults (L. F. Mocca and C. E. Frasch, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 20th, abstr. no. 193, 1980). The B polysaccharide in association with outer membrane proteins was used by Zollinger et al. (27) to immunize adults and was found to stimulate bactericidal antibodies. Thus, immunogenicity of the group B polysaccharide may be improved by use of noncovalent complexes of group B polysaccharide and protein, although covalent protein B polysaccharide complexes have proven to be nonimmunogenic (14).

Our approach to development of an effective group B vaccine has been to evaluate the immunogenicity and possible protective effects of outer membrane protein vaccines. Group B and C *N. meningitidis* have been subdivided into 15 to 18 different serotypes based upon immunologically distinct major outer membrane proteins (8, 25). Of these serotypes, only a few are associated with significant levels of meningococcal dis-

† Present address: Laboratory of Microbial Structure and Function, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, MT 59840.

ease (1, 5, 8). In recent studies in Canada and Belgium serotype 2 accounted for 65% of group B meningococcal disease and 78% of group C disease (1, 5), yet serotype 2 was isolated from only 3% of non-case contact group B carriers (2). Serotype 2 was therefore chosen for developmental vaccine studies.

Particulate serotype 2 protein vaccines were evaluated in a guinea pig chamber implant model (11) and a mouse bacteremia model (3) and found to protect against challenge with group B serotype 2 organisms. Although these vaccines were immunogenic in animals, a similar vaccine was poorly immunogenic in humans (10). The poor immunogenicity could have been due in part to protein denaturation during extraction or purification or both.

In this communication, the conditions for preparation of soluble protein vaccines are presented. We found that high yields of outer membrane vesicles (OMV) could be obtained from the supernatants of 72-h cultures. A nonionic detergent, Brij-96, was used to selectively solubilize approximately 90% of the LPS yet less than 5% of the protein from purified OMV. The resulting serotype 2 vaccines were soluble and severalfold more antigenic than particulate serotype 2 vaccines. By "soluble" we mean that the vaccines appeared to be soluble by visual inspection. In contrast, insoluble or particulate vaccines had obvious precipitates.

MATERIALS AND METHODS

Strains and growth conditions. The group B meningococcal serotype 2 strain M986-NCV-1, used for vaccine preparation, is a naturally occurring nonencapsulated mutant selected with antiserum agar (4). For vaccine preparation the strain was grown in Fernbach flasks containing a filter-sterilized dialysate of tryptic soy broth (Difco Laboratories, Detroit, Mich.) with shaking at 125 rpm for 24 to 72 h at 32 to 33°C. Typically, 17 liters of sterile dialysate was prepared and distributed to 12 2,800-ml baffled Fernbach flasks (Bellco Glass, Inc., Vineland, N.J.).

Preparation of soluble vaccine. The vaccine preparation method was based upon comparative studies presented in Results. The 65-h broth cultures were centrifuged twice at 13,000 × *g* for 15 min to remove cells. The wet-weight cell yield was approximately 5 to 8 g per liter of broth. The broth supernatant was recentrifuged at 20,000 × *g* for 20 min to remove residual cellular debris. Approximately 2 mg each of DNase (Sigma Chemical Co., St. Louis, Mo.) and RNase (Sigma) was added to 15 liters of cell-free supernatant. After 30 min at room temperature, the material was placed in an ice bath and concentrated to 0.5 liter, using 100,000-molecular-weight PTHK Pellicon membranes (Millipore Corp., Bedford, Mass.) in a Pellicon cassette system (Millipore). Two separate 2-liter volumes of distilled water were added to the concentrate, and ultrafiltration was continued to achieve a final volume of 0.3 to 0.5 liter. The filtration apparatus was washed with an additional 400 ml of distilled water which was added to the concentrate. The outer membrane protein was precipitated by addition of solid ultrapure ammonium sulfate (Schwarz/Mann, Orangeburg, N.Y.) to 50% saturation. After standing overnight at 4°C, the precipitate was recovered by centrifugation at 10,000 × *g*, dissolved in distilled water, and then reprecipitated with 50% saturated (final concentration) ammonium sulfate. The precipitate was collected by centrifugation at 10,000 × *g*, dissolved in distilled water, centrifuged at 5,000 × *g* to remove insoluble debris, and then dialyzed overnight at 4°C against 0.05 M NaCl to remove residual ammonium sulfate. The OMV preparation was adjusted to 1 to 2 mg per ml of protein.

The nonionic detergent Brij-96 (polyoxyethylene [10] oleyl ether; Sigma) was used to remove LPS. The OMV preparation was adjusted to contain 30 mM Tris (pH 8.5)–2 mM EDTA. Brij-96 and sodium deoxycholate were added to give final concentrations of 5% (wt/vol) and 0.2% (wt/vol), respectively. The material was centrifuged at 150,000 × *g* for 4 h. The LPS-containing supernatant was discarded, and the pellet was redissolved in the same detergent mixture and recentrifuged. The resultant pellet was dissolved in 5% (wt/vol) sodium deoxycholate in Tris-EDTA buffer and sterile filtered through a 0.45-μm cellulose acetate membrane filter into a sterile collection flask. Aliquots, 10 ml, were aseptically placed into 50-ml glass screw-cap centrifuge tubes. A 3.0-ml test sample was placed into a separate centrifuge tube. Four volumes of sterile-filtered 95% ethanol (0.2-μm polycarbonate filter; Nuclepore Corp., Pleasanton, Calif.) were added to each tube to precipitate the protein. The vaccine was pelleted by low-speed centrifugation and washed twice with filtered ethanol.

The test sample was used to estimate the amount of ethanol-precipitated vaccine protein. An appropriate amount of 3% (wt/vol) lactose was then prepared in distilled water, and to a portion, high-molecular-weight group B polysaccharide (provided for our studies by Connaught Laboratories, Swiftwater, Pa.) was added to a concentration of 500 μg/ml. The vaccine protein was then dissolved to 500 μg/ml in each of the lactose solutions. Vaccine lots containing the group B polysaccharide were designated with a VB suffix; those without, with a V suffix.

These vaccines were compared with the previously investigated particulate vaccines (3, 11), of which lot E-06 is representative. Vaccine E-06 was prepared as described for lot E-05 (11).

Chemical and physical analysis. The OMV and vaccine preparations were analyzed for their protein content by the Lowry method (17), using bovine serum albumin (fraction V) as a standard. LPS content was measured by determination of 2-ketodeoxyoctonate (KDO) (18), using KDO (Sigma) as a standard, and by determination of glucosamine and phosphatidylethanolamine (as ethanolamine), using an amino acid analyzer (23). The protein composition of the OMV and vaccines was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a modified Laemmli system (23). Electron microscopy was performed on vesicle and vaccine preparations on Parlodion (Ladd Research Industries, Burlington, Vt.)-coated grids and negatively stained with 1% sodium phosphotungstic acid, pH 6.1.

To estimate the amount of protein-bound group B

polysaccharide, the protein-polysaccharide vaccines were applied to 20 to 50% potassium tartrate linear gradients and centrifuged in a swinging-bucket rotor at 200,000 × *g* for 18 h. The single protein band was removed and analyzed for protein and for group B polysaccharide as sialic acid, using the resorcinol-HCl method (21).

The endotoxin content of the vaccines was estimated by the *Limulus* amoebocyte lysate gelatin test of Hochstein et al. (13). The minimum pyrogenic dose of vaccine was determined in rabbits as detailed in the *Code of Federal Regulations*, Title 21, §610.13.

ELISA inhibition. Soluble and particulate vaccines were compared for their ability to inhibit human serotype 2 antibodies. The enzyme-linked immunosorbent assay (ELISA) procedure was performed as described previously (7), using a conjugate prepared from the immunoglobulin G fraction of a goat anti-human immunoglobulin and alkaline phosphatase (type VIII; Sigma).

The tubes were sensitized with 2 μg of M986-NCV-1 OMV per ml in 0.1 M Tris buffer, pH 8.5. The test vaccines were diluted in twofold dilutions from 400 to 50 μg/ml in phosphate-buffered saline, pH 7.4, containing 0.1% (wt/vol) Brij-35. Volumes of 25 μl were added to the antigen-containing tubes, followed immediately by a standard human anti-type 2 serum diluted to yield an absorbance value of approximately 2.0 calculated at 100 min. The micrograms of vaccine required for 50% inhibition of the standard serum was determined from the slope of an inhibition curve for each vaccine.

The standard human serum used in the ELISA inhibition test was selected after comparison of several sera. Whereas most of the sera were inhibited only slightly by LPS-depleted OMV preparations, the serum selected was inhibited over 80%. By comparison, post-vaccination serum from an adult immunized with vaccine lot 790626VB was also inhibited over 80%, whereas his preserum was inhibited approximately 15%.

RESULTS

Growth conditions for recovery of OMV. Electron micrographs show numerous outer membrane evaginations or blebs on the surface of meningococcal cells (6). Since these blebs (OMV) are released into the culture medium during growth of the organism, recovery of OMV from the medium should be less denaturing than lithium chloride extraction from the cells as done previously (8). The group B type 2 vaccine strain was grown at 32 to 33°C for up to 72 h (Fig. 1). No significant decrease in viability occurred over the 72-h growth period. The OMV were recovered from the broth by ultracentrifugation and from the cells by lithium chloride extraction. Maximum yields of cell-extracted protein occurred by 24 h of growth, whereas three to four times more protein was obtained from the broth at 72 h.

The protein composition of the naturally released OMV was compared with outer membrane material extracted from whole cells with

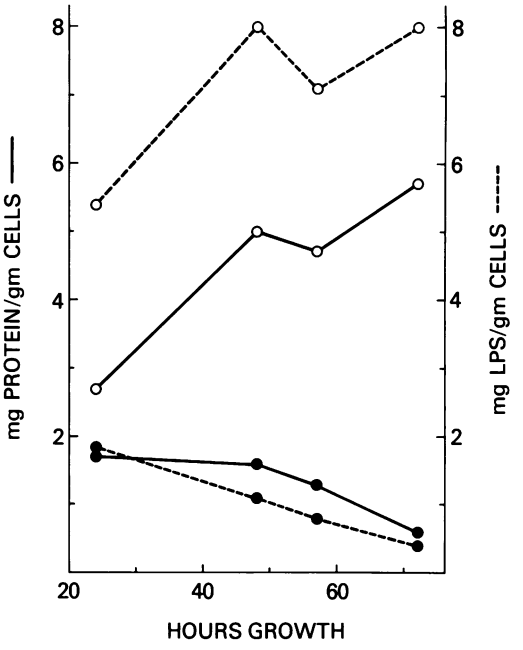

FIG. 1. Release of OMV material during growth of strain M986-NCV-1 in tryptic soy broth. Ultracentrifuge-pelletable material was recovered from the broth (○) and compared for protein and LPS content to outer membrane material extractable from the cells (●) by lithium chloride. Data presented represent the average of two experiments.

LiCl by SDS-PAGE (Fig. 2). The increased yield of protein from the broth at 48 to 72 h was not due to cell lysis, but rather to continued release of OMV. The only major difference in proteins recovered from the cells and broth was increased amounts of the 28,000-molecular-weight protein in the OMV recovered from the culture supernatant.

Preparation of soluble vaccines with low endotoxin content. Nonionic detergents have been used to reduce the LPS content (9, 11), but detergent treatment generally renders the OMV insoluble, probably due to delipidation of the membrane. A major problem in preparation of OMV vaccines is therefore to remove the LPS, yet retain solubility of the protein component upon removal of detergent.

Precipitation with 50% saturated ammonium sulfate at 4°C was found to be superior to ultracentrifugation for recovery of OMV from concentrated cell-free broth (Table 1). The three lots prepared by ammonium sulfate precipitation contained less LPS than the other three lots. Half of a concentrated cell-free broth was ultracentrifuged and the other half was precipitated with ammonium sulfate (see OMV lot 790510, Table 1). Both methods yielded equivalent

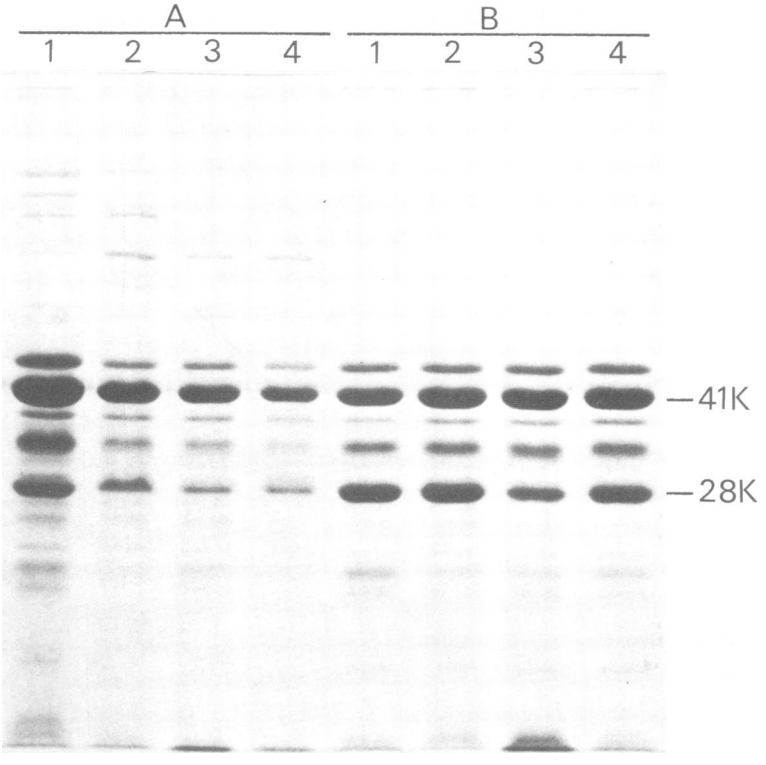

FIG. 2. Comparison by SDS-PAGE of OMV material obtained from the culture broth with that extracted from the cells. (A) Ultracentrifuge-pelleted OMV extracted from cells; (B) ultracentrifuge-pelleted OMV from cell-free broth. The OMV were obtained from the cultures after 24 (lane 1), 48 (lane 2), 57 (lane 3), and 72 (lane 4) h.

TABLE 1. Comparison of ultracentrifugation and ammonium sulfate precipitation for recovery of OMV from broth

OMV lot	Culture age (h)	OMV recovery method	mg of protein/g of wet cells	mg of LPS/g of wet cells	μg of LPS/mg of protein
790619	57	Centrifuge	1.77	1.65	930
790716	72	Centrifuge	1.46	1.76	1,200
790510C	65	Centrifuge	2.32	1.96	840
790510P	65	SAS[a]	2.44	1.37	560
790522	65	SAS	8.27	2.85	350
790626	65	SAS	5.86	4.74	810

[a] SAS, Saturated ammonium sulfate.

amounts of protein, but the ammonium sulfate-precipitated material contained approximately 30% less LPS.

Several nonionic detergents including the Brij series, Nonidet P-40, and Emulphogene BC-720, as well as deoxycholate, were compared in Tris-EDTA buffer (pH 8.5) for their ability to remove LPS from the OMV (data not shown). Two detergents, Brij-96 and Nonidet P-40, removed 80% or more of the LPS, as measured by reduction in glucosamine content, and removed over 90% of the phosphatidylethanolamine, the predominant phospholipid of the outer membrane. These detergents were then examined for selective removal of LPS (Table 2). They were compared with Emulphogene BC-720 and sodium deoxycholate which have been used for previous vaccines (3). Brij-96 was the most effective of the detergents used in solubilizing primarily the LPS; about 90% of the protein remained within

TABLE 2. Detergent treatment of OMV for removal of LPS

OMV prepn	Detergent[a]	μg of protein in sample	% Protein remaining	μg of LPS in sample	μg of LPS/mg of protein
1	None	2,160	100	820	380
	EBC-720	1,700	78	160	94
	NP-40	1,030	53	120	116
	Brij-96	2,120	98	236	111
2	None	890	100	1,250	1,404
	EBC-720	600	68	80	133
	NP-40	280	32	40	143
	Brij-96	805	89	100	124
3	None	1,060	100	1,125	1,061
	DOC	520	49	45	87

[a] Abbreviations: EBC-720, Emulphogene BC-720; NP-40, Nonidet P-40; DOC, sodium deoxycholate.

TABLE 3. Analysis of meningococcal protein and protein group B meningococcal polysaccharide vaccines

Vaccine	Detergent used to prepare	Protein (μg/ml)	B polysaccharide (μg/ml)	LPS (μg/ml)[a]		Vaccine solubility[b]
				KDO	GlcN	
E-06	Emulphogene BC-720	100	0	7.5	12.1	0
790228V	Deoxycholate	100	0	17.1	ND[c]	0
790228VB	Deoxycholate	100	100	17.1	ND	+++
790309V	Deoxycholate	100	0	24.3	ND	+++
790309VB	Deoxycholate	100	100	24.3	ND	++++
790626V	Brij-96	100	0	16.2	22.0	++++
790626VB	Brij-96	100	100	16.2	22.0	++++

[a] The LPS content of the vaccines was estimated by KDO and glucosamine (GlcN) content. Purified LPS from the vaccine strain contains 5% KDO and 12.5% glucosamine by weight.

[b] Solubility was judged by gross visual appearance of vaccine in water after removal of detergent by ethanol. Solubility was graded: ++++, water clear; +++, slightly opalescent; ++, opalescent; + fine precipitate present; 0, large particulate precipitate.

[c] ND, Not done.

the ultracentrifuge-pelletable fraction.

Detergent-treated OMV often became water-insoluble white floccular precipitates when the detergent was removed by ethanol precipitation (Table 3). Solubility was judged by visual appearance of the vaccine in water after removal of detergent. An insoluble vaccine had a visible precipitate, whereas a soluble vaccine could be opalescent to water clear. Brij-96-treated OMV remained water soluble after the detergent was removed. The solubility of these preparations was further enhanced by addition of group B meningococcal polysaccharide. Addition of B polysaccharide to protein vaccine preparations that would otherwise be insoluble (vaccine 790228) rendered them soluble.

Chemical composition of vaccines. The amount of protein and group B meningococcal polysaccharide in the vaccines was adjusted in the final product to 100 μg/ml. The amount of protein-bound group B polysaccharide estimated as sialic acid in three different VB vaccines was found to be 17.8 ± 0.9 μg of sialic acid per 100 μg of protein. The LPS content was estimated by measuring the KDO and glucosamine contents of the vaccine (Table 3). Analysis of purified LPS prepared by phenol-water treatment of lysozyme-treated cells (15) from the vaccine strain indicated measurable KDO and glucosamine contents of 5.0 and 12.5%, respectively. However, glucosamine was not found in the major outer membrane proteins of the vaccine strain (22), indicating that the measured glucosamine was from the LPS. Measurement of KDO and glucosamine gave comparable results, both showing that the amount of LPS in different vaccines was variable.

The mean cell wet weight and protein yields for seven different vaccine lots were determined (data not shown). The mean cell yield from 15 liters of medium was 108 ± 16 g and the OMV protein was 7.2 ± 2.3 mg per g of cells before detergent treatment. The mean vaccine yield was 3.1 ± 0.8 mg per g of cells. In contrast, the

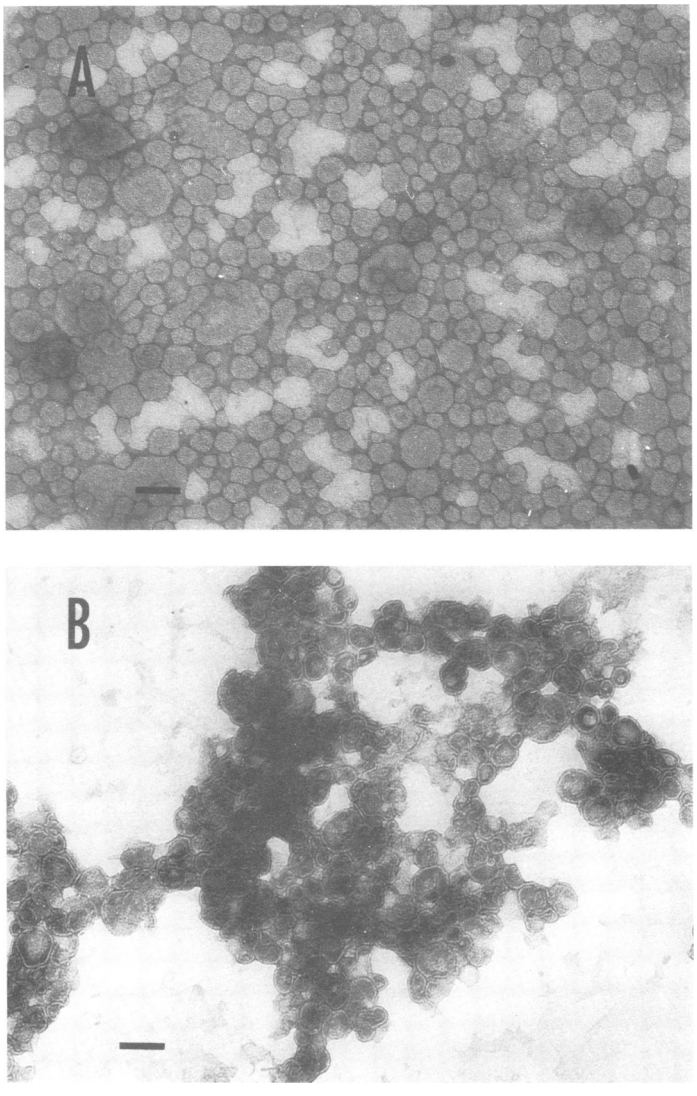

FIG. 3. Electron microscopic appearance of OMV before and after detergent treatment. (A) Untreated purified OMV; (B) Emulphogene-treated vesicles which became insoluble upon removal of detergent; (C) Brij-96-treated OMV in presence of group B meningococcal polysaccharide. Bar, 100 nm.

OMV protein yield by hot LiCl extraction was approximately 2 mg per g of cells (see Fig. 1), whereas vaccine protein was about 1 mg per g of cells.

Physical-chemical characteristics of vaccines. The physical appearance of insoluble Emulphogene BC-720-treated protein vaccines was compared by electron microscopy with soluble Brij-96-treated vaccines with and without added group B meningococcal polysaccharide (Fig. 3). Whereas untreated vesicles excluded the negative stain (Fig. 3A), detergent treatment rendered them permeable to sodium phosphotungstate. Selective removal of lipid by treatment with *n*-butanol also rendered OMV permeable to the negative stain (Frasch, unpublished data). We wanted to know whether large particulate vaccines became insoluble through vesicle aggregation or coalescence of many small vesicles. Emulphogene-treated OMV aggregated sufficiently to produce insoluble precipitates (Fig. 3B). The soluble protein-B polysaccharide complex vaccine showed no evidence of vesicle aggregation (Fig. 3C).

The proteins present in a representative Brij-96-treated vaccine were compared by SDS-PAGE with those in the untreated ammonium sulfate-purified OMV (Fig. 4). The ammonium

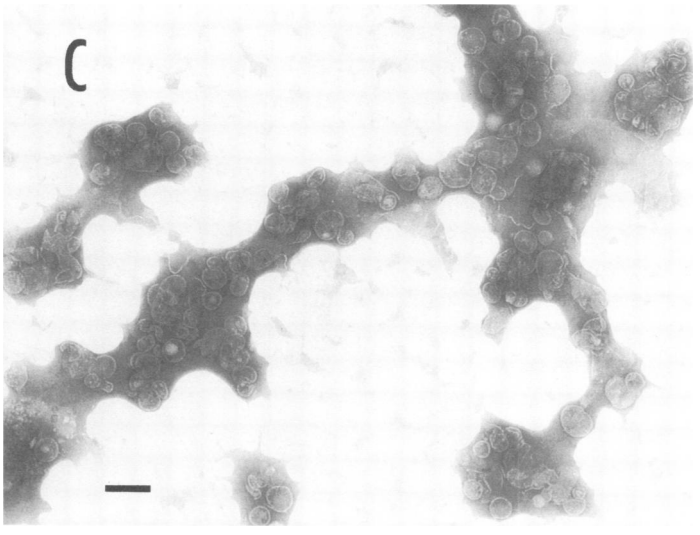

FIG. 3—*Continued*

sulfate-purified OMV contained several higher-molecular-weight proteins not found in ultracentrifuge-purified OMV (see Fig. 2). Detergent treatment removed most of these high-molecular-weight proteins.

ELISA inhibition. An ELISA inhibition assay was used to test potential vaccines for their capacity to inhibit human serotype 2-reactive antibodies (Fig. 5). Particulate vaccines (D-05 and E-06) prepared by earlier methods and previously evaluated in a mouse bacteremia model (3) were compared with soluble vaccines prepared as described in this paper. Vaccine E-06 required 5.0 μg to inhibit 50% of the anti-type 2 antibody, whereas the best soluble vaccines showed greater than 70% inhibition at 1.0 μg. Vaccine 790228, prepared with deoxycholate, was particulate without added B polysaccharide, but soluble when noncovalently complexed with B polysaccharide. Much less of the soluble form of this vaccine was required to achieve comparable levels of inhibition. The inhibition experiments suggested that vaccine solubility was a major factor in ability to bind human serotype 2-reactive antibodies, although the polysaccharide could have also induced favorable protein conformational changes.

DISCUSSION

Earlier reports from our laboratory have described the development and testing of serotype 2 protein vaccines (3, 11). These first vaccines were made by extracting the bacteria with LiCl at 50°C and treating the resultant OMV either with the nonionic detergent Emulphogene BC-720 or with deoxycholate to remove the LPS. Upon removal of detergent with ethanol and reconstitution in 0.15 M NaCl, these vaccines became insoluble due to vesicle aggregation. Such vaccines were first tested in a guinea pig chamber implant model (11) and later in a mouse bacteremia model (3). In both of these models, serotype 2 proteins prepared by the above method were immunogenic and induced protection against challenge with group B and C serotype 2 meningococci. One Emulphogene-treated vaccine (lot E-06) has recently been shown to be a poor immunogen in both adults and children (10). Zollinger et al. reported that labile antigenic determinants were present in the outer membrane protein fraction (W. D. Zollinger, H. Hansel, and R. E. Mandrell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, E98, p. 58). These observations suggest that the use of heat in our extraction procedure or the particulate nature of the final vaccine or both may have contributed to the vaccine's poor immunogenicity.

Zollinger et al. have successfully combined heat-extracted deoxycholate-treated outer membrane complex with group B polysaccharide to produce a soluble vaccine which induces both serotype 2 protein and group B polysaccharide bactericidal antibodies in adult volunteers (27). We initiated the present studies to develop protein vaccines that were soluble with or without added group B polysaccharide. Actively growing meningococci produce abundant outer membrane evaginations or blebs which are released into the medium. DeVoe and Gilchrist observed that released OMV represented a substantial proportion of the total cell material released during growth (6). The large number of OMV may be related to absence of a peptidoglycan-

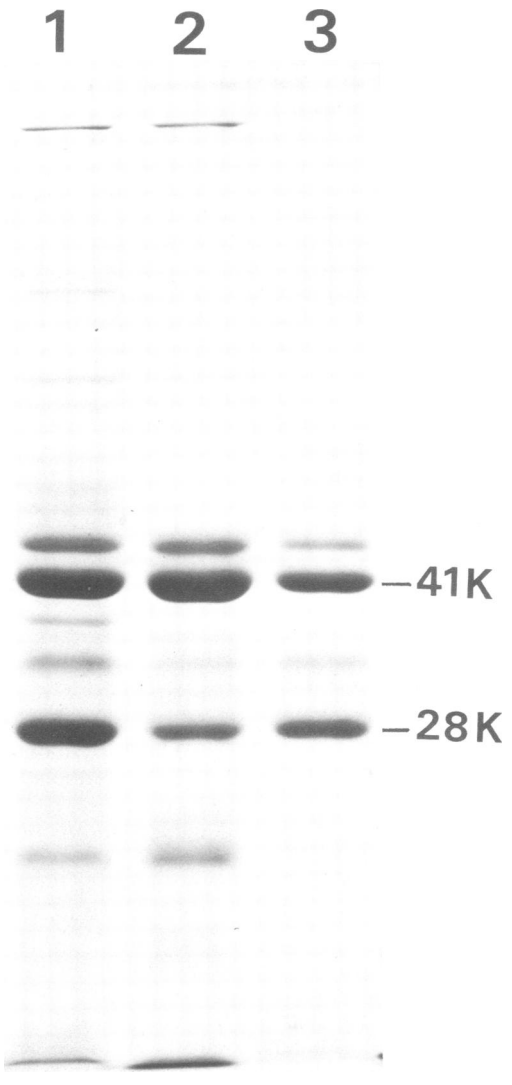

FIG. 4. Comparison of protein vaccine lot 790626V with known serotype 2 OMV by SDS-PAGE. Lane 1, Purified 790626 OMV before detergent treatment; lane 2, vaccine 790626V; lane 3, known LiCl-extracted M986-NCV-1 OMV.

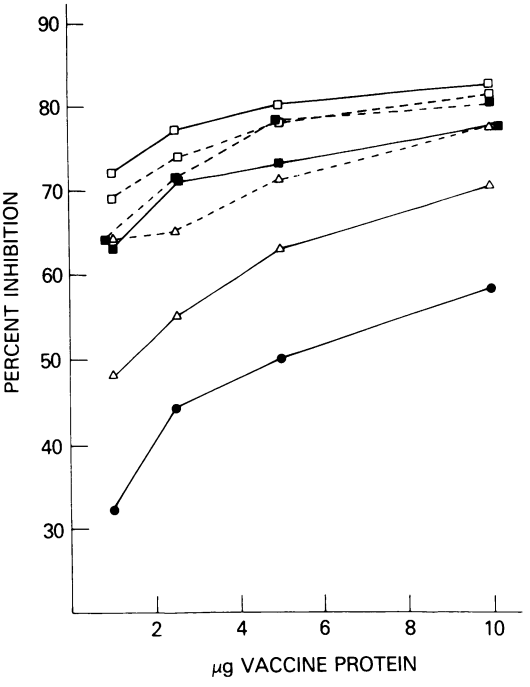

FIG. 5. Inhibition of anti-OMV antibodies by soluble and particulate serotype 2 protein vaccines in an ELISA inhibition assay. Particulate vaccine E-06 (●) was compared with soluble vaccines 790228 (△), 790309 (■), and 790626 (□). Solid lines denote protein vaccines; broken lines denote combined protein-group B polysaccharide vaccines. Vaccine 790228 was insoluble in the absence of polysaccharide.

bound lipoprotein in *N. meningitidis* (Frasch, unpublished data). Studies on *Escherichia coli* support this conclusion (20). *E. coli* normally has an outer membrane lipoprotein covalently bound to the peptidoglycan and produce few outer membrane blebs, whereas mutants lacking the lipoprotein produce many blebs.

The relative proportions of proteins present within the naturally released OMV were somewhat different from that seen in lithium chloride-extracted vesicles (see Fig. 2). The increased amount of 28,000-dalton protein in the naturally released vesicles probably reflects its relative concentration in the native outer membrane. This protein is on the exterior surface of the outer membrane and is easily removed by low concentrations of nonionic detergents (Frasch, unpublished data). The 50°C lithium chloride extraction procedure therefore probably causes release of both vesicle-bound and free 28,000-dalton protein.

The ammonium sulfate OMV purification procedure was chosen over ultra-centrifugation, because we found that a lower amount of LPS in the final vaccine could be achieved when the starting OMV preparation contained lower amounts of LPS. In addition, we found ultracentrifugation of large volumes, even after concentration by ultrafiltration, to be more time-consuming than purification of the OMV by selective precipitation by ammonium sulfate.

Our goal to produce a soluble protein vaccine was achieved through use of a gentle OMV isolation procedure, choice of a suitable detergent for removal of LPS, and control of the ionic strength. Detergent solubilization of membrane fractions often renders them insoluble upon re-

moval of detergent. In contrast, the Brij-96-treated membrane vesicles remained quite water soluble upon removal of the detergent by ethanol precipitation of the protein. The observed solubility could be due to residual bound detergent, since high-affinity detergent binding sites have been described for membrane proteins (2). Brij-96-treated membranes could, however, be rendered insoluble by increasing the ionic strength of the menstruum used to dissolve the vaccine. These characteristics are those of a colloidal suspension. In our distinction between particulate and soluble vaccines we know that both vaccines consist of approximately 60-nm membrane vesicles (see Fig. 3), but only the vesicles behave as colloids in the soluble vaccines. Addition of group B polysaccharide imparts a strong negative charge on the vesicles, thus improving the solubility of the vaccine. The protein vaccines were lyophilized in lactose, an agent shown to stabilize meningococcal polysaccharide vaccines (22). We found that lyophilization of the protein in the presence of lactose allowed for rapid solubilization upon reconstitution with normal saline.

Particulate serotype 2 protein vaccines elicited good immune responses in animals, yet were poor immunogens in humans (11, 26). A correlate was therefore needed to indicate the level of immune response in animals that could predict good immunogenicity in humans. The particulate vaccine E-06 appeared to provide good protection in the mouse bacteremia model (3), yet when this vaccine was compared with the soluble protein and protein-polysaccharide vaccines by ELISA inhibition, it was severalfold less effective in inhibiting human serotype antibodies. The soluble protein vaccines were subsequently examined in the mouse bacteremia model and found to be at least 10 times more effective than E-06 (18a). The soluble protein and protein polysaccharide vaccines are therefore being evaluated in animal models for their potential as human immunogens. A good animal or in vitro correlate of immunogenicity in humans may be achieved by comparison of human immune response data with protective levels in the mouse bacteremia model and with ELISA inhibition.

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