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Comparison and Correlation of *Neisseria meningitidis* Serogroup B Immunologic Assay Results and Human Antibody Responses following Three Doses of the Norwegian Meningococcal Outer Membrane Vesicle Vaccine MenBvac

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The prediction of efficacy of *Neisseria meningitidis* serogroup B (MenB) vaccines is currently hindered due to the lack of an appropriate correlate of protection. For outer membrane vesicle (OMV) vaccines, immunogenicity has primarily been determined by the serum bactericidal antibody (SBA) assay and OMV enzyme-linked immunosorbent assay (ELISA). However, the opsonophagocytic assay (OPA), surface labeling assay, whole blood assay (WBA), and salivary antibody ELISA have been developed although correlation with protection is presently undetermined. Therefore, the aim of the study was to investigate further the usefulness of, and relationships between, MenB immunologic assays. A phase II trial of the OMV vaccine, MenBvac, with proven efficacy was initiated to compare immunologic assays incorporating the vaccine and six heterologous strains. Correlations were achieved between the SBA assay, OMV ELISA, and OPA using human polymorphonuclear leukocytes and human complement but not between an OPA using HL60 phagocytic cells and baby rabbit complement. Correlations between the surface labeling assay, the SBA assay, and the OMV ELISA were promising, although target strain dependent. Correlations between the salivary antibody ELISA and other assays were poor. Correlations to the WBA were prevented since many samples had results greater than the range of the assay. The study confirmed the immunogenicity and benefit of a third dose of MenBvac against the homologous vaccine strain using a variety of immunologic assays. These results emphasize the need for standardized methodologies that would allow a more robust comparison of assays between laboratories and promote their further evaluation as correlates of protection against MenB disease.

Meningococcal serogroup B (MenB) disease remains a significant international health problem with high mortality and morbidity. Development of an effective MenB capsular polysaccharide vaccine is hindered by the poor immunogenicity of the polysaccharide (48) and concerns over the possible induction of autoimmune antibodies (14). Therefore, the development of MenB vaccines has focused on subcapsular antigens either as outer membrane vesicles (OMVs) or individual antigens.

Several candidate OMV vaccines have been developed and tested in large-scale efficacy studies in Norway, Cuba, Brazil, and Chile (4, 7, 10, 42). Efficacy estimates varied from 57 to 83% in those over 4 years, but no protection was demonstrated in children less than 2 years with a two-dose schedule. Analysis of the immunologic responses to OMV vaccines have been complicated by the wide range of responses observed in vaccine recipients, although a relationship between PorA OMP-specific antibodies and serum bactericidal antibody (SBA) has been noted (4, 40, 45). Therefore, since these vaccines are based on a single meningococcal disease causing isolate, concerns about the ability of these vaccines to offer cross-protection against heterologous virulent meningococci have arisen (29, 30, 43, 46). These findings may be of particular importance in countries where MenB disease is of a multiclonal nature such as in The Netherlands and the United Kingdom. How-

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ever, OMV vaccination has successfully contributed to the control of a clonal MenB epidemic in Cuba (39, 42) and subsequent outbreaks in Brazil (10). Furthermore, the introduction of a "tailor-made" OMV vaccine is hoped to curtail the continuing clonal MenB epidemic in New Zealand (35).

The prediction of efficacy of meningococcal MenB vaccines is currently hindered by the lack of an appropriate correlate of protection. Immunogenicity of polysaccharide vaccines for serogroups A, C, Y, and W135 has been evaluated in the SBA assay and the enzyme-linked immunosorbent assay (ELISA) to determine the specific anticapsular antibody. For OMV vaccines, immunogenicity has primarily been determined by the SBA assay, which has recently been evaluated as the appropriate correlate of protection resulting in the proposal of a tentative protective SBA titer of ≥4 (20). Previously, SBA cutoffs were not utilized in MenB vaccine studies, with reliance on ≥4-fold increases from before to 1 month after the last scheduled vaccination, which may underestimate protection (35, 47). Specific immunoglobulin G (IgG) antibodies to meningococcal OMVs and ≥4-fold increases from before to after vaccination have also been determined by ELISA, although correlation with protection is presently undetermined.

Since OMV vaccines contain subcapsular epitopes that may induce a range of immunologic responses, other mechanisms of protection involved in immunity to MenB disease may be appropriate correlates of protection. Demonstration of bactericidal killing of meningococci by opsonophagocytosis (8, 11, 41), coupled with the opsonophagocytic assay (OPA) being established as a correlate of protection for *Streptococcus pneumoniae* (27), has led to the development of OPA against MenB (1, 2, 16). In addition, a surface labeling assay, which detects antibody binding to meningococci, has also been developed (1, 2, 16), but further data are required to determine whether these assays may add to our knowledge of correlates of protection. The whole-blood assay (WBA), which measures the bactericidal activity of blood, including phagocytosis, in conjunction with complement-mediated lysis has been postulated as being more sensitive than the SBA assay (22, 23, 32). However, the WBA requires large volumes of fresh blood, is difficult to standardize and control, and may therefore not be suitable for vaccine trials.

The induction of herd immunity was of paramount importance in the control of meningococcal serogroup C (MenC) disease after the introduction of MenC conjugate vaccines (38). This was brought about by the reduction in carriage (25), presumably mediated by responses at mucosal surfaces. Salivary antibody ELISAs have been used previously in meningococcal vaccine studies (5, 18) and warrant further evaluation due to the importance of the possible effects of mucosal antibody on carriage and due to the assay's simplicity and noninvasive sample collection.

Despite comparisons between some MenB immunologic assays (1, 2, 33), the lack of an understanding of relationships between assays has hindered the assessment of the immunogenicity of MenB vaccines. The availability of an OMV vaccine with proven efficacy enabled the further investigation of the usefulness and relationships between MenB immunologic assays, including the SBA assay, OMV ELISA, OPA, WBA, salivary antibody ELISA, and surface labeling assay with diverse MenB target strains. This was completed with sera and saliva collected from a Phase II study using the Norwegian OMV vaccine, MenBvac, enabling relationships between assays to be determined to further our understanding of correlates of protection for MenB.

Previous studies of single-strain OMV vaccines have determined the cross-reactive response to limited numbers of isolates (36, 40, 43, 45), but to our knowledge none have utilized a panel of diverse isolates, such as those prevalent in the United Kingdom. Therefore, the secondary aim of the present study was to investigate the ability of MenBvac to induce antibody responses to the homologous strain and heterologous strains, currently representative of those causing disease in the United Kingdom.

TABLE 1. Serogroup, serotype, serosubtype, sequence type, and clonal cluster of meningococcal target strains used in immunologic assays

Designation	Serogroup	Serotype	Serosubtype		Sequence type	Clonal cluster
			VR1	VR2		
44/76-SL	B	15	7	16	32	32
NZ 98/254	B	15	7-2	4	44	41/44
M01-240101	B	NT	19-1	15-11	1049	269
M01-240013	B	NT	22	9	275	269
M01-240149	B	4	7-2	4	41	41/44
M01-240185	B	2a	5-1	10-8	11	11
M01-240355	B	1	22	14	213	213

NT, nontypeable.

MATERIALS AND METHODS

Vaccination and specimen collection. The vaccine, MenBvac, has been described previously (40). Briefly, the vaccine contained deoxycholate-extracted OMVs from the meningococcal strain 44/76 (B:15:P1.7,16) which were adsorbed to aluminum hydroxide.

Vaccination was administered by intramuscular injection into the nondominant deltoid muscle using a three-dose "0-6-12-week" schedule. Blood and salivary samples were obtained prior to the first vaccination, 6 weeks after each dose, and again 1 year after dose 3.

Study group. A single group, consisting of 31 healthy adult (>18 years old) laboratory workers (16 male and 15 female) from Manchester Public Health Laboratory, Manchester, United Kingdom (now the Health Protection Agency [HPA] North West, Manchester Laboratory), and The Institute of Child Health (ICH), London, United Kingdom, were enrolled. Ethical approval was gained from Public Health Laboratory Service and ICH committees.

Immunologic assays. The immunogenicity of the vaccine was determined by the SBA assay, WBA, and OMV ELISA completed at the Manchester HPA; OPA and surface labeling assays completed at the HPA, Centre for Emergency Preparedness and Response (HPA Porton), and salivary ELISA completed at the Department of Cellular and Molecular Medicine, University of Bristol. A subset of samples were also analyzed in the OPA at Norwegian Institute of Public Health (NIPH) to compare the two OPA assays.

Target strains. The target strains used in the present study are detailed in Table 1. These included the homologous MenBvac vaccine strain (44/76-SL) and the New Zealand OMV vaccine (MeNZB) strain (NZ 98/254). MenB strains with currently the most prevalent United Kingdom serosubtypes P1.7-2,4, P1.19,15, P1.22,14, P1.22,9, and P1.5-1,10-8, which were accountable for 31.8, 13.0, 8.8, 5.7, and 1.18%, respectively, of the MenB disease in 2000 and 2001 (24) were obtained from the HPA Meningococcal Reference Unit (MRU) and incorporated into the assays.

SBA assay. SBA assays were performed against all target strains (Table 1) as previously described (12) using a starting dilution of 1/2. Briefly, human serum, at 25%, was used as an exogenous source of human complement, with titers expressed as the reciprocal of the final dilution giving ≥50% SBA killing at 60 min from the control column (inactive complement/no test sera).

TABLE 2. SBA GMTs at baseline and after each dose of MenBvac

Target strain	SBA GMT (95% CI)[a]				
	Baseline	6 wk after dose 1	6 wk after dose 2	6 wk after dose 3	1 yr after dose 3
44/76-SL	2.1 (1.4–3.2)	7.0** (4.2–11.6)	7.1 (4.4–11.4)	9.2 (5.5–15.5)	4.8*## (2.8–8.3)
NZ 98/254	3.4 (1.8–6.7)	9.8** (4.8–19.8)	8.7 (4.1–18.5)	8.2 (4.0–17.1)	4.8*# (2.2–10.4)
M01 240013	7.6 (4.3–13.4)	10.7* (6.1–18.7)	11.5 (6.4–20.6)	13.5 (8.1–22.7)	9.0 (5.0–16.2)
M01 240101	10.5 (5.4–20.4)	14.6* (8.2–26.2)	10.4 (5.2–20.6)	16.4 (8.7–30.9)	9.2 (4.8–17.6)
M01 240149	3.4 (2.0–5.6)	4.7 (2.8–7.9)	4.2 (2.3–7.9)	5.3 (2.7–10.2)	4.4* (2.4–7.8)
M01 240185	3.0 (1.7–5.1)	3.4 (2.1–5.5)	3.4 (1.9–5.9)	4.3 (2.3–8.1)	2.8* (1.5–5.2)
M01 240355	6.2 (3.6–10.8)	12.5** (7.2–21.6)	11.8 (6.1–22.7)	17.4 (9.3–32.7)	8.7* (5.0–15.1)

[a] *, $P < 0.05$ for change from previous bleed; **, $P < 0.001$ for change from previous bleed; #, $P < 0.05$ for change from baseline; ##, $P < 0.001$ from baseline. *P* values were calculated using the nonparametric sign test and not using GMTs.

OMV ELISA. Specific IgG OMV ELISA endpoint titers (EPT) against OMV antigen preparations from all target strains (Table 1) were determined as previously described (9).

Opsonophagocytic assay (HPA Porton). The OPA assay was performed against all target strains (Table 1). To U-bottom 96-well microtiter plates (Sterilin, United Kingdom), 20 μl of test sera diluted 1:10 in OP buffer (Hanks balanced salts solution [Sigma-Aldrich, United Kingdom] containing 2% Marvel skimmed milk powder [Premier International Foods, United Kingdom], 1.2 mM $CaCl_2$ [Sigma-Aldrich, United Kingdom], and 1 mM $MgSO_4$ [Sigma-Aldrich, United Kingdom]) was added to 10 μl of bacteria at 6.25×10^8 ml^{-1} in OP buffer [bacterial stocks had previously been stained internally with 10 μg of 2′,7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)] ml^{-1}, acetoxymethyl ester (Calbiochem, United Kingdom), and then the mixture was fixed with 0.2% [wt/vol] sodium azide for 48 h); 10 μl of baby rabbit complement (Pel-Freez) was added, followed by incubation for 7.5 min with shaking (900 rpm) at 37°C. HL60 phagocytic cells (European Collection of Cell Cultures, United Kingdom) at 2.5×10^7 ml^{-1} in OP buffer (50 μl) were added, followed by incubation with shaking at 37°C for 7.5 min. The HL60 cell line was differentiated with 0.8% *N*,*N*-dimethylformamide (DMF) (Sigma-Aldrich, United Kingdom) for 5 days prior to use. The reaction was stopped by addition of 80 μl of ice-cold Dulbecco phosphate-buffered saline (PBS; Sigma-Aldrich, United Kingdom) containing 0.02% EDTA (Sigma-Aldrich, United Kingdom) (DPBS-EDTA). Horizontal gates in the BCECF channel (fluorescein equivalent) were set against a complement-only, no-antibody control to include ca. 10% of the population. For each sample, 7,500 live HL60 cells were measured, and the percentage of cells showing BCECF fluorescence in the appropriate gate (% gated) was multiplied by the mean fluorescence of the gated population (X-mean) to give a fluorescence index (FI). The FI of each test was divided by the FI of the complement-only no-antibody control to give a FI ratio (FIR).

Opsonophagocytic assay (NIPH). Opsonophagocytic activity against the live homologous strain 44/76-SL (Table 1) was measured as the respiratory burst using human complement as previously described (2).

Surface labeling assay. The surface labeling assay was completed against all target strains (Table 1). To V-bottom 96-well microtiter plates (Sterilin, United Kingdom) 2 μl of test sera and 198 μl of azide-fixed bacteria at an A_{600} of 0.1 in a blocking buffer (BB) of PBS plus 1% bovine serum albumin (BSA; Sigma-Aldrich, United Kingdom) were added. After incubation at 4°C for 2 h, the plates were washed twice by centrifugation at 3,060 × *g* for 5 min, followed by the addition of 220 μl of BB. The resultant pellets were resuspended in 200 μl of BB containing a 1:500 dilution of goat anti-human IgG FITC (Jackson Immunoresearch) and incubated in the dark for 1 h at room temperature with shaking. Plates were washed once more with 200 μl of BB, and the resuspended contents were added to 300 μl of BB in tubes for flow cytometry analysis. Horizontal gates in the fluorescein isothiocyanate (FITC) channel were set against a conjugate-only control to include ca. 10% of the population. For each sample, the fluorescence of 25,000 cells was measured, and the percentage of cells showing FITC fluorescence (percent gated) was multiplied by the mean fluorescence of the gated population (X-mean) to yield an FI value. The FI of the conjugate-only control was subtracted from the FI of each test to give a final reading (FI-C).

WBA. The WBA was performed as previously described (22, 23) with the minor modification of collecting whole blood in lithium heparin vacuettes. The results were expressed as the percentage killing of meningococci at 90 min as calculated from control counts at the incubation start. The WBA was performed with target strains 44/76-SL, NZ 98/254, and M01 240013 (Table 1).

Salivary antibody ELISA. Both specific IgA and IgG antibody levels against OMV antigen preparations from 44/76-SL (Table 1) were determined by using an ELISA as previously described (5, 21).

Data analysis. The SBA assay, the OMV ELISA, the surface labeling assay, the salivary antibody ELISA, and the Porton and NIPH OPA results from each time point were log transformed, and geometric means calculated with 95% confidence intervals (95% CI). For the SBA assay, OMV ELISA and NIPH OPA titers were geometric mean titers (GMTs); for the surface labeling assay, geometric mean FI minus conjugate background (GMFI); for the salivary antibody ELISAs, geometric mean arbitrary units (GMAUs); and for the Porton OPA, geometric mean FI ratios over the complement-only background (GMFIRs). In the SBA assay, titers of <2 were assigned a value of 1 for data analysis. Average fold changes (with 95% CI) from baseline to 6 weeks after the third dose were also calculated. The SBA and OMV ELISA responses were further considered as a ≥4-fold increase from baseline to 6 weeks after the third dose. Proportions with ≥4-fold increases in the SBA were calculated incorporating all ≥4-fold rises (including subjects with a baseline titer of <2 and a titer of 4 at 6 weeks after the third dose) and then only including individuals with a minimum titer of 8 at 6 weeks after the third dose. The WBA response was considered as a binary variable, with a response of ≥50% killing or equal to 100% killing at each time point.

Paired *t* tests were used to test for significant changes in results between successive time points and from baseline to 6 weeks and 1 year after the third dose. The nonparametric sign test was used to compare responses between time points for SBA assay and NIPH OPA due to the non-normal distribution of log responses and for salivary ELISAs due to small numbers. The sign test simply compares the numbers of individuals with an increase in response to those with a decrease.

TABLE 3. Numbers and percentages of subjects showing SBA titers of ≥4 at baseline and after each dose of MenBvac

Target strain	No. of subjects with SBA titers of ≥4/total no. of subjects tested (%)				
	Baseline	6 wk after dose 1	6 wk after dose 2	6 wk after dose 3	1 yr after dose 3
44/76-SL	9/30 (30)	23/31 (74.2)	24/28 (85.7)	22/25 (88)	13/23 (56.5)
NZ 98/254	10/28 (35.7)	20/31 (64.5)	16/25 (64)	17/25 (68)	12/24 (50)
M01 240013	20/28 (71.4)	23/31 (74.2)	21/25 (84)	21/25 (84)	18/24 (75)
M01 240101	21/28 (75)	26/31 (83.9)	17/24 (70.8)	23/25 (92)	17/24 (70.8)
M01 240149	13/28 (46.4)	17/31 (54.8)	14/25 (56)	14/25 (56)	13/24 (54.2)
M01 240185	11/28 (39.3)	15/31 (48.4)	11/25 (44)	13/25 (52)	8/24 (33.3)
M01 240355	17/28 (60.7)	25/31 (80.7)	20/25 (80)	22/25 (88)	18/24 (75)

TABLE 4. Target strain-specific fold change from baseline to 6 weeks after the third dose of MenBvac as determined by each immunologic assay

Target strain	Fold change (95% CI)						
	SBA assay	OMV ELISA	HPA Porton OPA	NIPH OPA	Surface labeling	Salivary IgA ELISA	Salivary IgG ELISA
44/76-SL	5.0 (3.3–7.7)	10.4 (6.3–17.4)	1.5 (1.2–1.8)	21.1 (11.1–40.1)	2.4 (2.0–2.9)	1.18 (0.7–1.9)	1.54 (1.0–2.4)
NZ 98/254	3.1 (1.9–5.2)	6.3 (4.0–9.8)	3.0 (2.0–4.4)	ND	1.8 (1.4–2.2)	ND	ND
M01 240013	1.9 (1.2–2.8)	4.4 (3.2–6.1)	1.0 (0.8–1.1)	ND	1.4 (1.2–1.5)	ND	ND
M01 240101	1.5 (1.1–2.2)	2.7 (2.0–3.5)	1.1 (0.8–1.4)	ND	1.5 (1.4–1.7)	ND	ND
M01 240149	1.8 (1.1–2.9)	4.1 (3.0–5.8)	1.0 (0.9–1.1)	ND	1.3 (1.2–1.4)	ND	ND
M01 240185	1.7 (1.1–2.6)	4.5 (3.0–6.7)	1.0 (0.8–1.1)	ND	1.1 (1.0–1.3)	ND	ND
M01 240355	2.6 (1.5–4.2)	3.5 (2.5–5.0)	1.0 (0.9–1.2)	ND	1.5 (1.3–1.7)	ND	ND

ND, not done.

The relationship between the different immunologic assays (all on a log-10 scale) were explored by linear regression analysis, and the Pearson correlation coefficient was determined.

RESULTS

Compliance with sampling. Of 31 adult laboratory staff recruited (age range, 22.6 to 58.3), 27 of 31 (87.1%) received all three doses, with two subjects withdrawing after the first dose and two subjects withdrawing after the second dose. For sampling 31 of 31 subjects (100%) gave the first bleed, 29 of 31 (93.5%) gave the second bleed, 26 of 31 (83.9%) gave the third and fourth bleeds, and 25 of 31 (80.6%) gave the fifth bleed.

Antibody responses. SBA GMTs are given in Table 2, and the number and percentages of subjects with putative protective SBA titers of ≥4 given in Table 3. The proportion of subjects with baseline SBA titers of ≥4 ranged from 30 to 75%, dependent upon the target strain. From baseline to 6 weeks after the third dose, increases in SBA were achieved for all strains (P = 0.023 to <0.001) with the exception of M01 240101 and M01 240149 (with borderline nonsignificant increases [P = 0.057] for both). SBA fold changes from baseline to 6 weeks after the third dose ranged from 1.5 against M01 240101 to 5.0 against the homologous strain (Table 4). Between 6 weeks after the third dose and 1 year after the third dose there were decreases in SBA for all strains (P = 0.035 to 0.0013) with the exception of M01 240101 and M01 240013 (P = 0.064 and 0.14, respectively). However, SBA levels 1 year after the third dose were elevated in comparison to baseline levels for the homologous strain and NZ 98/254 (P = <0.001 and 0.022, respectively). The highest percentages of subjects with ≥4-fold rises in SBA (for both calculation methods) from baseline to 6 weeks after the third dose (Table 5) were against the homologous strain, with differing responses achieved against heterologous strains.

Specific anti-OMV IgG GMTs are given in Table 6 and percentage of subjects with ≥4-fold rises in EPT are given in Table 5. Baseline GMTs were similar for all target strains. Increases in the GMT from baseline to 6 weeks after the third dose were achieved against all strains (P = <0.001), ranging from 2.7-fold against M01 240101 to 10.4-fold against the homologous strain (Table 4). From 6 weeks after the third dose to 1 year after the third dose the GMTs decreased against all OMV antigen preparations (P = <0.001), although they remained elevated compared to the baseline levels (P = 0.022 to <0.001). The highest percentages of subjects with ≥4-fold rises in EPT from baseline to 6 weeks after the third dose (Table 5) were against the homologous strain (83.3%) with various responses against heterologous strains (29.2 to 62.5%).

OPA GMFIRs and surface labeling GMFIs from HPA Porton are given in Tables 7 and 8, respectively. Baseline GMFIRs and GMFIs differ between target strains. From baseline to 6 weeks after the third dose increases in OPA GMFIRs were achieved against the vaccine strain and one heterologous strain (NZ 98/254) (P = <0.001). The greatest fold change from baseline to 6 weeks after the third dose was achieved against NZ 98/254 (3-fold) (Table 4), with the homologous strain only showing half of this increase (1.5-fold). The GMFIR 1 year after the third dose decreased against the homologous strain, and the two heterologous strains NZ 98/254 and M01 240149 (P = 0.02 to 0.011).

Increases in surface labeling GMFIs from baseline to 6 weeks after the third dose were achieved against all strains (P = <0.001) with the exception of M01 240185 (P = 0.098). The greatest fold change from baseline to 6 weeks after the third dose was achieved against the homologous strain (2.4-fold) (Table 4), with lower increases for all heterologous strains (ranging from 1.1- to 1.8-fold). One year after the third dose GMFIs decreased for the homologous strain, and two

TABLE 5. Numbers and percentages of subjects showing ≥4-fold increases in SBA titer and an anti-OMV IgG endpoint titer from baseline to 6 weeks after the third dose of MenBvac

Target strain	No. of subjects with ≥4-fold increases/no. of subjects tested (%)		
	SBA		Anti-OMV IgG
	Calculation method 1[a]	Calculation method 2[b]	
44/76-SL	18/24 (75)	15/24 (62.5)	20/24 (83.3)
NZ 98/254	12/23 (52.2)	8/23 (34.8)	15/24 (62.5)
M01 240013	8/23 (34.8)	8/23 (34.8)	14/24 (58.3)
M01 240101	4/23 (17.4)	2/23 (8.7)	7/24 (29.2)
M01 240149	6/23 (26.1)	4/23 (17.4)	12/24 (50)
M01 240185	4/23 (17.4)	4/23 (17.4)	14/24 (58.3)
M01 240355	8/23 (34.8)	8/23 (34.8)	9/24 (37.5)

[a] Includes all subjects with ≥4-fold rises in SBA titer (including individuals with a baseline titer of <2 achieving a titer of 4 by 6 weeks after the third vaccination).

[b] Only includes subjects with a minimum titer of 8 at 6 weeks after the third vaccination (for individuals with a baseline titer of <2, a titer of ≥8 had to be attained 6 weeks after the third vaccination to constitute a ≥4-fold rise).

TABLE 6. Anti-OMV IgG GMTs at baseline and after each dose of MenBvac

Target strain	IgG GMT (95% CI)[a]				
	Baseline	6 wk after dose 1	6 wk after dose 2	6 wk after dose 3	1 yr after dose 3
44/76-SL	77.9 (52.3–116.0)	231.1** (152.2–351.0)	431.2** (321.8–577.6)	782.9** (557.8–1098.8)	214.5**## (141.9–324.4)
NZ 98/254	56.0 (34.0–92.3)	130.1** (83.5–202.7)	164.5 (99.5–272.2)	302.2** (201.2–453.9)	144.3**## (94.4–220.8)
M01 240013	31.5 (21.0–47.3)	61.8** (41.1–93.0)	70.5 (47.2–105.4)	143.3** (95.9–214.2)	52.9**## (34.8–80.5)
M01 240101	38.5 (26.9–55.2)	66.8** (45.7–97.8)	75.3 (50.6–112.0)	112.7* (76.5–166.1)	60.0**# (38.8–92.9)
M01 240149	47.7 (34.3–66.4)	87.6** (62.4–123.1)	100.7 (65.8–154.1)	182.9** (122.4–273.3)	91.6**## (64.4–130.1)
M01 240185	52.5 (33.9–81.2)	112.5** (70.7–179.0)	118.4 (73.1–191.9)	223.2** (142.5–349.6)	98.8**## (62.2–156.9)
M01 240355	38.4 (25.3–58.1)	61.6* (40.6–93.2)	76.8 (48.0–122.8)	131.6* (83.2–196.2)	65.7**# (42.6–101.5)

[a] *, $P < 0.05$ for change from previous bleed; **, $P < 0.001$ for change from previous bleed; #, $P < 0.05$ for change from baseline; ##, $P < 0.001$ from baseline.

heterologous strains, M01 240101 and M01 240149 ($P = 0.046$ to <0.001).

The OPA at NIPH was performed on a subset of samples ($n = 65$) against 44/76-SL with the GMTs presented in Table 9. Increases in the OPA GMT from baseline to 6 weeks after the first dose ($P = 0.001$), from 6 weeks after the 2nd dose to 6 weeks after the third dose ($P = 0.006$), and from baseline to 6 weeks after the third dose were achieved ($P = <0.001$) against the homologous strain.

The WBA was performed on subjects from the Manchester study site on the first four study bleeds with the results presented in Table 10. High baseline levels of percentage killing were demonstrated against all target strains assayed. The numbers of subjects assayed decreased at each time point, hindering interpretation. However, the percentage of subjects with ≥50 or equal to 100% killing increased after the first dose for all target strains. Further analysis of results was prevented by the high percentages of subjects (up to 71%) with killing equal to the highest measurable threshold of the assay (100% killing).

Salivary antibody levels were determined at the time of the first four bleeds against an OMV antigen preparation from 44/76-SL ($n = 9$ subjects), with the IgA and IgG GMAUs presented in Table 11. No significant differences were determined between successive time points or from baseline to 6 weeks after the third dose for specific anti-OMV IgA ($P = 0.77$). However, modest increases were demonstrated from 6 weeks after the second dose to 6 weeks after the third dose ($P = 0.05$) and from baseline or 6 weeks after the third dose for specific anti-OMV IgG ($P = 0.02$).

Comparison and correlation of results between immunologic assays. Correlations between immunologic assays were calculated between all available data. This included the results for baseline sera and for sera after each dose of vaccine, therefore covering the whole range of antibody levels. In addition, a subset of samples ($n = 65$) were analyzed in the OPA at the NIPH. The results from the WBA were not correlated with other assays due to sensitivity issues with the WBA. The Pearson linear correlation coefficients between assays for individual strains are given in Table 12. Overall, the correlation coefficients varied greatly between assay comparison and/or target strains with the strongest correlation between the NIPH OPA and the surface labeling assay with 44/76-SL. Table 4 shows the fold changes from baseline to 6 weeks after the third dose per target strain for all immunologic assays. The NIPH OPA, OMV ELISA, and SBA assay were the most sensitive assays, showing the greatest fold rises. The HPA Porton OPA, surface labeling, and IgA and IgG salivary ELISAs demonstrated the lowest fold increases.

DISCUSSION

To our knowledge this is the first comprehensive comparison and evaluation of MenB immunologic assays with a panel of varied wild-type target strains. Good correlations between SBA and anti-OMV IgG levels were achieved as in previous studies (1, 33, 40), although we have demonstrated this with a larger number of target strains. Reasonable correlations were also achieved between the NIPH OPA, the SBA assay, and the OMV ELISA, although these were not as strong as previously reported, where assays were all completed at the same laboratory (1, 33). Conversely, poor correlations were achieved between the Porton OPA and all other assays. Differences between correlations of the NIPH and Porton OPA to other assays are due to methodologic differences. The use of live or killed organisms in the assays is probably unlikely to account

TABLE 7. OPA GM FIRs from HPA Porton at baseline and after each dose of MenBvac

Target strain	Geometric mean FIR (95% CI)[a]				
	Baseline	6 wk after dose 1	6 wk after dose 2	6 wk after dose 3	1 yr after dose 3
44/76-SL	2.2 (1.8–2.9)	2.7 (2.2–3.4)	2.7 (2.1–3.3)	3.6* (2.7–4.8)	3.0* (1.7–5.4)
NZ 98/254	0.1 (0.1–0.2)	0.2* (0.2–0.3)	0.2 (0.2–0.3)	0.4* (0.3–0.4)	0.2* (0.1–0.5)
M01 240013	3.2 (2.7–3.9)	3.2 (2.8–3.8)	2.8* (2.4–3.3)	3.1 (2.6–3.7)	3.1 (2.0–4.6)
M01 240101	2.1 (1.6–2.7)	2.3 (1.9–2.9)	2.3 (1.7–3.0)	2.4 (1.9–2.9)	2.2 (1.2–3.8)
M01 240149	1.0 (0.8–1.2)	1.1 (0.9–1.3)	1.1 (0.9–1.3)	1.1 (0.9–1.3)	0.8* (0.5–1.3)
M01 240185	1.8 (1.6–2.0)	1.7 (1.5–1.9)	1.6 (1.4–1.9)	1.7 (1.5–2.0)	1.7 (1.2–2.4)
M01 240355	2.0 (1.7–2.3)	1.9 (1.6–2.3)	2.0 (1.6–2.4)	2.0 (1.7–2.4)	1.8 (1.2–2.6)

[a] *, $P < 0.05$ for change from a previous bleed.

TABLE 8. Surface labeling GM FIs at baseline and after each dose of MenBvac

Target strain	Geometric mean FI (range)[a]				
	Baseline	6 wk after dose 1	6 wk after dose 2	6 wk after dose 3	1 yr after dose 3
44/76-SL	1,038.4 (853.8–1,255.7)	1,404.1* (1,155.7–1,705.9)	1,616.9 (1,318.2–1,983.2)	2,388.2* (2,123.0–2,686.6)	1,315.4* (1,164.7–1,568.0)
NZ 98/254	1,321.0 (1,092.7–1,596.9)	1,799.6* (1,424.3–2,273.8)	2,038.9* (1,619.2–2,681.9)	2,263.6 (1,839.7–2,785.2)	1,528.8 (1,096.0–2,132.4)
M01 240013	1,047.8 (891.1–1,232.0)	1,336.0* (1,147.4–1,555.6)	1,278.9 (1,036.7–1,577.7)	1,381.8 (1,152.3–1,657.0)	1,189.5 (938.9–1,506.9)
M01 240101	1,428.7 (1,217.4–1,676.8)	1,760.6* (1,490.0–2,080.3)	1,886.2* (1,538.0–2,312.2)	2,032.2 (1,745.3–2,366.1)	1,665.4* (1,232.8–2,249.8)
M01 240149	2,217.2 (1,904.6–2,581.0)	2,600.5* (2,142.4–3,156.5)	2,782.7 (2,216.3–3,493.9)	2,736.8 (2,338.6–3,202.8)	2,936.6* (2,357.0–3,658.7)
M01 240185	1,702.7 (1,389.7–2,086.0)	2,150.3 (1,840.2–2,512.6)	2,105.6 (1,768.5–2,506.9)	2,109.8 (1,793.8–2,481.5)	2,104.8 (1,847.0–2,398.6)
M01 240355	2,290.1 (1,907.2–2,749.8)	2,690.5* (2,303.8–3,142.2)	3,026.4* (2,491.3–3,676.6)	3,252.8 (2,833.4–3,734.4)	2,256.9 (1,444.8–3,525.5)

[a] *, $P < 0.05$ for change from previous bleed.

for the observed differences despite greater quantities of PorB (class 3) OMP accessible on killed meningococci (28), but different effector cells, complement sources, and incubation conditions (3) may be responsible. In addition, methods used to calculate the opsonophagocytic response differ greatly between the two assays, with the Porton OPA measuring immunologic activity at a single dilution compared to the measurement by titer at the NIPH. These differences in methodologies highlight the requirement for interlaboratory standardization.

Correlations between the surface labeling assay and all other assays with the exception of the Porton OPA and salivary IgA ELISA are promising, although they vary depending on the target strain. Correlations using WBA results were not completed due to sensitivity issues. These results emphasize the need for standardized methodologies such as that recently published for the MenB SBA assay (6). Such standardization would allow more robust comparisons of results between laboratories and promote their further evaluation as correlates of protection against MenB disease. Another point of consideration is that some of the observed differences could be due to differences in the maintenance and culture of the target strains. This has been previously demonstrated in the SBA assay with 44/76-SL (6) and may also have an effect on the other immunologic assays/target strains used in the present study.

Previous studies of OMV vaccines have relied upon ≥4-fold rises in both SBA titer and anti-OMV IgG from baseline to after the final vaccination. An attempt was made to correlate fold increases between assays but was unsuccessful, with no relationships determinable (data not presented). In the present study, a greater proportion of subjects achieved ≥4-fold increases in anti-OMV IgG than in SBA titer, and greater fold increases were shown in the OMV ELISA, suggesting the presence of nonbactericidal antibody. Interestingly, it was not always the same subjects that achieved ≥4-fold rises in SBA titer and anti-OMV IgG (data not presented). For the heterologous strain M01 240101, low proportions (<30%) of subjects achieved ≥4-fold rises in SBA titer and anti-OMV IgG. However, 6 weeks after the third dose, 92% of subjects had a putative protective SBA titer of ≥4 against M01 240101, indicating that the use of ≥4-fold increases may underestimate protection, as previously suggested (35, 47). This is of particular importance when subjects have high baseline levels of SBA/anti-OMV IgG as in the present study.

The present study incorporated a three-dose "0-6-12-week" schedule, which differs from previous studies that have either used a 0-8-16-week schedule or administered the third dose as a booster. Increases in antibody against the majority of target strains after the third dose were determined by all assays with the exception of the WBA in comparison to after the second dose. This confirmed the benefit of a third dose of MenBvac in increasing the immunologic response to homologous and heterologous strains as previously reported (15, 33, 36, 40, 43).

In the SBA and surface labeling assays, greater baseline levels of antibody were directed against strains representative of those prevalent in the United Kingdom than against the homologous strain. This is perhaps not unexpected and is probably attributable to prior natural exposure to meningococci. The percentage of subjects achieving a putative protective SBA titer of ≥4 against the homologous strain at 6 weeks after the third dose was 88% compared to 100 and 96% in previous three-dose studies in Atlanta (15) and Iceland (20), respectively. One year after the third dose, the percentage decreased to 56.5% compared to 85% in the Icelandic study, although in

TABLE 9. OPA GMTs from NIPH at baseline and after each dose of MenBvac

Time point	GM titer (95% CI) for target strain 44/76-SL[a]
Baseline	2.38 (1.15–4.93)
6 wk after dose 1	12.7* (6.21–25.96)
6 wk after dose 2	13.83 (7.32–26.11)
6 wk after dose 3	39.74* (28.02–56.37)

[a] *, $P < 0.05$ for change from previous bleed. P values were calculated using the nonparametric sign test and not using GMTs.

TABLE 10. Numbers and percentages of subjects showing ≥50% and equal to 100% killing in the WBA at baseline and after each dose of MenBvac

Target strain	Time point	No./total no. tested (%) showing:	
		≥50% killing	100% killing
44/76-SL	Baseline	15/18 (83.3)	3/18 (16.7)
	6 wk after dose 1	16/17 (94.1)	9/17 (52.9)
	6 wk after dose 2	15/16 (93.8)	6/16 (37.5)
	6 wk after dose 3	8/9 (88.9)	3/9 (33.3)
NZ 98/254	Baseline	9/19 (47.4)	3/19 (15.8)
	6 wk after dose 1	12/17 (70.6)	12/17 (70.6)
	6 wk after dose 2	15/16 (93.8)	6/16 (37.5)
	6 wk after dose 3	8/9 (88.9)	2/9 (22.2)
M01 240013	Baseline	11/18 (61.1)	3/18 (16.7)
	6 wk after dose 1	13/17 (76.5)	6/17 (35.3)
	6 wk after dose 2	14/15 (93.3)	7/15 (46.6)
	6 wk after dose 3	5/9 (55.6)	2/9 (22.2)

TABLE 11. Salivary IgG and IgA GMAUs at baseline and after each dose of MenBvac against the OMV antigen preparation from 44/76-SL

Immunoglobulin	GMAUs (95% CI)[a]			
	Baseline	6 wk after dose 1	6 wk after dose 2	6 wk after dose 3
IgA	144.0 (103.0 to 205.5)	164.5 (127.0 to 205.1)	117.0 (93.8 to 227.7)	194.0 (117.0 to 245.1)
IgG	17.0 (11.9 to 22.6)	19.0* (15.2 to 22.3)	16.0 (11.5 to 26.4)	22.6 (18.1 to 32.0)

[a] *, $P < 0.05$ for change from previous bleed. *P* values were calculated using the nonparametric sign test and not using GMAUs.

that study the third dose was administered 10 months after the second dose as a booster (36).

Significant rises in anti-OMV IgG against the homologous strain were detected after each dose of vaccine and is in agreement with previous studies (33, 36, 40). Comparison of the proportions of subjects with ≥4-fold rises in SBA titer to previous studies is complicated due to differences in calculation methods. For baseline samples with a titer of <2, a ≥4-fold increase has been defined both as a minimum titer of 4 after vaccination (31, 40) and the more stringent minimum titer of 8 after vaccination (26, 37), with other studies not detailing the method of calculation (7, 15, 36, 43). The more stringent titer of ≥8 has been applied due to the reported uncertainty in titers of less than 4 (26). However, in our SBA assay the reproducibly of samples with titers of 2 have been reliably confirmed (data not presented), ensuring the validity of a minimum titer of 4 after vaccination to determine ≥4-fold rises in SBA. In the present study the proportion of subjects achieving ≥4-fold rises in SBA titer varied when the two calculation methods were used, highlighting the importance of declaration of the method of calculation in studies.

The WBA has been used in a previous study of an OMV vaccine in infants (32) but, to our knowledge, this is the first report of its use in a study with adults. As previously reported, the WBA is more sensitive than the SBA assay (22, 23, 32), with killing being detected in subjects with no SBA activity. However, it is labor-intensive, it is difficult to standardize, and it cannot be repeated using stored samples. The WBA was only completed on a subset of subjects due to the requirement of the assay to be commenced within 2 h of the collection of the whole blood. High levels of percent killing were measured before and after vaccination against the homologous strain, with many subjects achieving 100% killing, preventing the quantification of responses to the vaccine. We therefore suggest that whereas the WBA may be of value in investigating meningococcal pathogenesis (19, 23) or as a research tool in small-scale infant studies where subjects may not reach the maximum measurable threshold of 100% killing, this approach is not readily applicable to large trials or studies of vaccine efficacy in adults.

We have previously demonstrated an increase in specific antimeningococcal salivary IgA and IgG after serogroup A and C conjugate vaccination (5). In contrast, no increases in salivary anti-OMV IgA against the homologous strain were detected in the present study as in agreement with a previous MenBvac study (18). We did find a modest increase in salivary IgG after intramuscular OMV vaccination, but the persistence and biological significance remains uncertain. Due to the possibility of mucosal anti-OMV antibody contributing to herd immunity, further evaluation of immunological markers of mucosal immunity are required and may be of particular importance in any future intranasal vaccination studies.

TABLE 12. Target strain-specific Pearson's linear correlation coefficients of assay results (contains all available data)

Assay	Pearson's linear correlation coefficient for target strain:						
	44/76-SL	NZ 98/254	M01 240013	M01 240101	M01 240149	M01 240185	M01 240355
SBA assay vs OMV ELISA	0.60	0.67	0.62	0.61	0.68	0.57	0.76
SBA assay vs Porton OPA	0.34	0.32	0.13	0.13	0.29	0.0007	0.08
SBA assay vs NIPH OPA	0.72	ND[a]	ND	ND	ND	ND	ND
SBA assay vs surface labeling	0.61	0.72	0.39	0.43	0.38	0.24	0.63
SBA assay vs salivary IgA ELISA	0.12	ND	ND	ND	ND	ND	ND
SBA assay vs salivary IgG ELISA	0.06	ND	ND	ND	ND	ND	ND
OMV ELISA vs Porton OPA	0.22	0.45	0.14	0.23	0.34	0.11	0.22
OMV ELISA vs NIPH OPA	0.78	ND	ND	ND	ND	ND	ND
OMV ELISA vs surface labeling	0.68	0.73	0.64	0.71	0.53	0.37	0.61
OMV ELISA vs salivary IgA ELISA	0.03	ND	ND	ND	ND	ND	ND
OMV ELISA vs salivary IgG ELISA	0.39	ND	ND	ND	ND	ND	ND
Porton OPA vs NIPH OPA	0.32	ND	ND	ND	ND	ND	ND
Porton OPA vs surface labeling	0.29	0.40	0.19	0.22	0.16	0.03	0.04
Porton OPA vs salivary IgA ELISA	0.03	ND	ND	ND	ND	ND	ND
Porton OPA vs salivary IgG ELISA	0.04	ND	ND	ND	ND	ND	ND
NIPH OPA vs surface labeling	0.81	ND	ND	ND	ND	ND	ND
NIPH OPA vs salivary IgA ELISA	0.09	ND	ND	ND	ND	ND	ND
NIPH OPA vs salivary IgG ELISA	0.46	ND	ND	ND	ND	ND	ND
Surface labeling vs salivary IgA ELISA	0.11	ND	ND	ND	ND	ND	ND
Surface labeling vs salivary IgG ELISA	0.50	ND	ND	ND	ND	ND	ND
Salivary IgA ELISA vs salivary IgG ELISA	0.22	ND	ND	ND	ND	ND	ND

[a] ND, not done.

Although previous studies have determined the cross-reactive response to the MenBvac against homologous, isogenic, and other vaccine strains, none have investigated a diverse panel of wild-type isolates such as in the present study. Various levels of cross-reactive SBA and anti-OMV IgG were induced against heterologous strains, although comparisons are complicated by the high prevaccination levels of SBA against some strains. The heterologous strain with the greatest number of subjects achieving ≥4-fold rises in SBA and anti-OMV IgG was the MeNZB vaccine strain NZ 98/254. Interestingly, in the SBA assay, the phenotypically identical strain M01 240149 only had half the number of subjects attaining ≥4-fold increases, although the percentage of subjects with an SBA titer of ≥4 was similar. These findings are similar to previous reports wherein different P1.7-2,4 wild-type isolates have demonstrated significantly different SBA activity (13, 44), suggesting that the standardization of strains between laboratories is crucial for the compatibility of the data. For the United Kingdom representative isolates, the SBA responses were generally poor, with low percentages of subjects achieving ≥4-fold increases. However, heterologous vaccine responses were complicated by the high proportions of subjects with a baseline SBA titer ≥4. One year after the third dose, the number of subjects with an SBA titer of ≥4 was similar to baseline levels, although anti-OMV IgG remained elevated compared to baseline levels.

Although SBA activity is crucial in the protection against meningococcal disease (7, 17, 31), opsonophagocytosis may be involved in immunity to MenB disease and may be an additional correlate of protection. The OPA has been used to investigate the immunogenicity of MenBvac previously (1, 2, 33), but to our knowledge this is the first study to incorporate an OPA and the surface labeling assay with a number of phenotypically diverse target strains. The surface labeling assay detected rises in GMT from baseline to 6 weeks after the third dose for all but one heterologous strain, whereas the Porton OPA only detected similar rises for the homologous vaccine strain and one heterologous strain. Significant rises in opsonophagocytic activity against the homologous vaccine strain were detected 6 weeks after the first and third dose (NIPH OPA) and from baseline to 6 weeks after the third dose (Porton and NIPH OPA). This is in contrast to previous studies that have reported significant increases after each dose of vaccine (1, 2, 33). Such discrepancies are likely to be due to differences in vaccination schedule, the number of samples, and subject ages. The WBA was only completed for a subset of subjects for two heterologous strains due to its labor-intensity. Increases in percent killing were noted after vaccination with all target strains, although sensitivity issues prevented further quantification of the responses for many subjects.

In the present study we have confirmed the immunogenicity of MenBvac against the homologous vaccine strain by using a variety of immunologic assays. The benefit of a third dose was demonstrated, and the responses to heterologous strains representative of those causing United Kingdom disease were apparent, although generally limited. Single-strain OMV vaccines will probably not provide sufficient protection in countries with diverse MenB disease such as the United Kingdom. However, it is hoped that the introduction of an MeNZB into New Zealand will curtail the current MenB epidemic (34), and OMV-based vaccines still currently offer the only means of protection against MenB disease. These results highlight the requirement for a MenB vaccine that will induce a cross-reactive response against all MenB organisms and the necessity for standardized and validated MenB immunologic assays.

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