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Development and use of a serum bactericidal assay using pooled human complement to assess responses to a meningococcal group A conjugate vaccine in African toddlers

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Abstract

Background: A meningococcal group A polysaccharide conjugate vaccine (PsA-TT) has been developed for African countries affected by epidemic meningitis caused by *Neisseria meningitidis*. Complement-mediated serum bactericidal antibody (SBA) assays are used to assess protective immune responses to meningococcal vaccination. Human complement (hC') was used in early studies demonstrating antibody mediated protection against disease, but it is difficult to obtain and standardize. We developed and evaluated a method for sourcing hC', then used the SBA assay with hC' (hSBA) to measure bactericidal responses to PsA-TT vaccination in 12 to 23 month-old African children. Methods: Sera with active complement from 100 unvaccinated blood donors were tested for intrinsic bactericidal activity, SBA titer using rabbit complement (rSBA), and anti-group A PS antibody concentration. Performance criteria and pooling strategies were examined then verified by comparisons of three independently prepared hC' lots in two laboratories. hSBA titers of clinical trial sera were then determined using this complement sourcing method. Results: Two different functional antibody tests were necessary for screening hC'. hSBA titers determined using three independent lots of pooled hC' were within expected assay variation among lots and between laboratories. In African toddlers, PsA-TT elicited higher hSBA titers than meningococcal polysaccharide or Hib vaccines. PsA-TT immunization or PS challenge of PsA-TT primed subjects resulted in vigorous hSBA memory responses, and titers persisted in boosted groups for over a year. Conclusions: Quantifying SBA using pooled hC' is feasible and showed that PsA-TT was highly immunogenic in African toddlers.

BACKGROUND

Group A *Neisseria meningitidis* has caused devastating recurrent epidemics in countries of the African Meningitis Belt. A vaccine has been developed with the goal of eliminating epidemic meningitis from the region (1, 2). Human responses to new meningococcal vaccines are evaluated serologically by several methods. Clinical efficacy for a group A polysaccharide (PS) vaccine was originally correlated with anti-polysaccharide Ig antibody ≥2 µg/mL as quantitated by radioimmunoassay (RIA) (3). Since specific antibody-dependent, complement-mediated bactericidal killing is the primary mechanism of human immunity to *N. meningitidis* (4), functional antibody responses, as measured in serum bactericidal antibody (SBA) assays, have been and are currently used to evaluate new meningococcal vaccines.

Several types of SBA assays have been used to assess natural or vaccine induced immunity to *N. meningitidis*. Intrinsic SBA assays do not require the addition of externally sourced complement but instead rely on the active complement in the individual test serum. These assays are performed with limited dilution of the serum, usually 1:4, and require serum collection and storage methods that preserve complement activity. Quantitative SBA assays use serially diluted heat-inactivated sera with an exogenous complement source. Exogenous sources include active human (hSBA) or animal sera; the most common animal source being baby rabbit complement (rSBA). The development of novel meningococcal PS-protein conjugate vaccines for use in infants and young children, and recognition that some complement and complement regulatory components, such as human factor H, are species-specific, have made complement source an important consideration for SBA assays (5). The original studies of human immunity were based on bactericidal activity in the presence of human complement (hC'), both intrinsic SBA and

hSBA (4, 6), whereas for the licensure of monovalent group C conjugate vaccines in Europe, rSBA was utilized (7). The correlation and agreement between rSBA and hSBA titers are not strong, especially for sera from pediatric populations (8, 9). Unfortunately, hSBA assays are challenging because of the difficulty of obtaining suitable hC' and the observation that assay results can vary depending on the individual complement source.

To identify hC' sources and improve the consistency and reliability of hSBA assays, we assessed the prevalence of suitable complement sources among sera collected from a group of healthy adult blood donors, and we evaluated pooling as an approach to provide consistency between lots of hC' when used in hSBA assays. Using the screening criteria and pooling methods we identified, we then assessed the hSBA responses of randomized subsets of subjects in a study of PsA-TT vaccine in African toddlers 12-23 months of age (2).

METHODS

Human complement source preparation and qualification. Whole blood (3 x 10 mL) from healthy adult blood donors who met routine blood donation screening at the National Institutes of Health, was placed on ice for transportation, allowed to clot at 37^{o}C for 15 minutes, centrifuged at 5^{o}C to separate serum, aliquoted, immediately frozen on dry ice, and stored at -80^{o}C. These individual hC' sources were tested for intrinsic bactericidal activity, rSBA titer, total anti-A PS IgG IgA and IgM (IgGAM) antibody concentration, and total hemolytic complement activity (RC001, The Binding Site Group Ltd, Birmingham, UK) as described below. Each of the first 100 blood donations was assigned sequentially by enrollment order to one of three groups (A, B or C) and individual sera within each group were selected if they exhibited lack of intrinsic

killing, rSBA titer ≤8, IgGAM concentration <30 µg/mL (the upper 95% confidence interval of the geometric mean concentration for the first 100 donor sera), and normal complement activity. Equal volumes of individual hC' sources that met screening criteria were pooled on ice, aliquoted and stored at -80°C until use (hC' lots C11, C12 and C13 from groups A, B and C respectively). Sera that met the intrinsic and rSBA criteria but had high total anti-A PS antibody concentrations of >30 µg/ml were evaluated as a separate hC' pool (hC' lot C14). Pooled hC' was qualified by applying the same screening criteria as the individual sera. Subsequent hC' pools created for testing clinical study samples were further qualified by using the hC' pool as a complement source in hSBA assays testing negative, low-titer positive and high-titer positive control sera with the requirement that the assigned hSBA titers (+/- one 2-fold dilution) were obtained.

Intrinsic SBA growth curve assay. The *N. meningitidis* group A target strain CDC lab no. F8238 (phenotype A:4,21:P1.20,9, L11) as previously described by Maslanka et al. (10) was sub-cultured onto brain-heart infusion with horse serum (BHI-HS) agar and incubated overnight with 5% CO_2 at 37ºC, was streaked for confluence onto BHI-HS agar, and incubated with 5% CO_2 at 37ºC for four hours. Cells were scraped from plates and suspended in Dulbecco's phosphate buffered saline with calcium and magnesium (BioWhittaker, Walkersville, MD), 0.1% glucose (Sigma, St. Louis, MO), and 0.1% gelatin (Bio-Rad, Hercules, CA) (DPBSGG); and adjusted to ~80% transmittance at 530 nm, then diluted 1:5x$10^4$to yield ~2.4-4 x 10^4colony forming units (CFU)/ml. A 1:4 final dilution of the complement source, either active or heat inactivated, and a 1:4 final dilution of the bacterial suspension, in DPBSGG was incubated at 37ºC with shaking. Ten microliters was plated on BHI-HS agar in duplicate at time 0, 15, 30, 60 and 90 minutes.

Plates were incubated overnight and colonies counted. Anti-meningococcal group A/C/Y/W reference serum pool, CDC 1992 (code no. 99/706, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK) (11), 1:20 dilution was added to the hC' source as a bactericidal positive control. Active hC' sources that supported growth of strain F8238 equivalently when compared to a known negative serum or the heat inactivated sample were considered intrinsically negative. Sera that did not support growth were qualitatively labeled as bacteriostatic when the growth curve was flat or bactericidal when the growth curve showed distinct killing.

Exogenous complement SBA assay. The rSBA and hSBA assays performed in the CBER laboratory were conducted using the microtiter plate with agar overlay assay described by Maslanka et al. (10) adapted to a 40 µL reaction volume with the following modifications: 10 µL of strain F8238 diluted to 1:2x10^5cfu/mL in DPBSGG (50-80 colonies per 10 µL) was added to 20µL/well of serial 2-fold dilutions (1:4 to 1:1024) of heat inactivated serum in DPBSGG. Ten microliters of baby rabbit complement (Pel Freez Biologicals, Milwaukee, Wisconsin) or pooled hC' was added for rSBA or hSBA respectively. Sealed plates were incubated at 37ºC with shaking at 110 rpm for 90 minutes. Fifty microliters/well warm (48ºC) tryptic soy broth (Becton, Dickinson and Co., Sparks, MD) with 1% noble agar (Becton, Dickinson and Co., Sparks, MD) (TSB/noble agar) was added, cooled for five minutes, and an additional 25 µL/well TSB/noble agar cap added. CFU per well after overnight incubation at 37ºC with 5% CO_2 were counted using a dissecting microscope. The SBA titer was the reciprocal of the highest dilution resulting in ≥50% killing compared to the average colony count for active complement only control wells. Sera were run in duplicate, and the assigned titer was the geometric mean of the duplicates

136 rounded down to the nearest step titer if the duplicate titers were within 4-fold of each other. Sera
137 with discrepant duplicates or with curves that crossed the 50% killing threshold more than once
138 were repeated a maximum of two times, and if unresolved were called indeterminate.
139
140 hSBA assays performed at the Public Health England laboratory, Manchester, UK, used the
141 internationally standardized rSBA drip plate method (10) with pooled human complement lots
142 prepared at CBER and shipped overnight on dry ice.
143
144 **Anti-A PS ELISA.** Anti-group A PS IgG and total IgGAM antibody concentrations were
145 determined using a standardized ELISA as previously described (12), except antigen coating was
146 at 4ºC, the wash buffer was PBS/0.05% Tween-20 (Sigma, St. Louis, MO), serum/conjugate
147 buffer was PBS/0.05% Tween-20/5% normal calf serum (Hyclone, Logan, Utah), and alkaline
148 phosphatase labeled goat anti-human IgG (Sigma, St. Louis, MO) or alkaline phosphatase
149 labeled goat anti-human IgG, IgA, IgM (Sigma, St. Louis, MO) were used. The optical density
150 was read at 405nm using uQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments,
151 Inc., Winooski, VT) and antibody concentration determined by 4-parameter logistical regression
152 using serum CDC 1992 (11) as the reference serum.
153
154 **Test sera.** Centers for Disease Control and Prevention (CDC) meningococcal quality control
155 sera (collected after meningococcal ACWY polysaccharide vaccination) (11), standard reference
156 serum (CDC1992) (11), de-identified sera from vaccinated laboratory personnel obtained as part
157 of routine laboratory safety surveillance, and sera obtained 28 days after vaccination with PS or
158 PsA-TT during the Phase 1 study conducted in young adults in India (13) were used in the

evaluation of complement screening and pooling methods. To assess the feasibility of the methods in supporting clinical trials, sera from a randomized subset of subjects (n=180) from the Phase 2 study of PsA-TT conducted in African toddlers 12-23 months of age (2) were tested. The clinical study subjects received a primary immunization with PsA-TT, quadrivalent polysaccharide vaccine (PsACWY), or a *Haemophilus influenzae* type b conjugate vaccine (Hib). Each primary immunization group was randomized to receive PsA-TT, 1/5 dose of PsACWY or Hib at 10 months following the primary immunization (Supplemental Figure 2). Aliquots of sera drawn 28 days following the primary immunization, 10 months following the primary immunization prior to the second immunization, 28 days following the second immunization, and at 24 months after the primary vaccination, coded and blinded to vaccine group, were heat inactivated at 56^{o}C for 30 min prior to testing then stored at -20^{o}C.

Statistical analysis. For the methods development assays using different lots of pooled hC', fold differences between the titers of an individual serum for each complement lot tested within an individual laboratory were compared. For inter-laboratory comparisons, the median titer of values reported for each sample within each laboratory were compared. Negative samples were assigned a value of 2 (1/2 of the reciprocal of the limit of detection). Titers ≥1024 were defaulted to 1024 for analysis.

hSBA results from the Phase 2 study subsets were compared by vaccine group for sera obtained at 28 days after primary vaccination and 10 months later, prior to the second immunization, using a t-test for the GMTs and Fisher's Exact Test for the proportion of subjects with hSBA titer ≥8. The threshold titer of 8 was used to define a positive sample to allow for assay

variability around the starting dilution of 1:4. GMT and proportion with hSBA titers ≥8 for the time points of 28 days after the second immunization and two years after primary immunizations are shown with 95% confidence intervals (CI) but are observational only due to the small sample size following subdivision of the initial study subset groups each into three different secondary vaccination groups (n = 13 to 22 per group). Negative values were assigned a value of 2 as above for GMT calculations.

Ethics. These studies were reviewed and exempted by the FDA Research Involving Human Subjects Committee. Blood donor sera were de-identified except for age, sex, and race of the donor, and were assigned a unique number that was used by the National Institutes of Health blood bank to exclude receipt of samples from the same donor. PsA-TT trial sera were encoded by an accession number with no identifiers. Appropriate ethics approvals were obtained for the clinical trials from which the test sera originated (2, 13).

RESULTS

Demographics of blood donors. One hundred sequential blood donors were used to develop our complement source screening and pooling methods. These donors were 18 to 77 years of age, (average 41.3 years), 32% female, 68% male, 74% Caucasian, 21% African American, 4% Hispanic, and 1% Native American. All donors met standard screening tests for blood donations and were excluded if they had previously received a meningococcal vaccine. Each blood donation was assigned sequentially by enrollment order to one of three groups (A, B, or C) for use in one of three independent complement lots. Demographic characteristics were similar between the three groups (Supplemental Table 1).

205

206 **Serologic evaluation of blood donor complement sources.** Overall, 51 of 100 individual
207 samples lacked intrinsic killing of F8238, 48 samples had an rSBA titer ≤8, and 31 sera met both
208 functional screening criteria. Concentrations of anti-group A PS IgG (Supplemental Figure 1)
209 and IgGAM (data not shown) did not predict rSBA titer or intrinsic killing. Within each of the
210 three donor groups, samples that were intrinsically negative, had an rSBA titer ≤8, and had an
211 IgGAM anti-A PS antibody concentration below the upper 95% CI of the geometric mean
212 concentration (GMC) for all 100 complement source samples were pooled (lots C11, C12, and
213 C13). Lot C14 was comprised of samples from the three donor groups that met the intrinsic and
214 rSBA criteria but had anti-A PS IgGAM concentrations of >30 µg/ml.

215

216 **Pooled hC' performance.** In the CBER laboratory, performance of the hC' lots was initially
217 tested in hSBA assays of 21 sera comprising seven CDC QC sera (11), CDC1992, and 13 de-
218 identified sera from laboratory personnel obtained before or after meningococcal quadrivalent
219 polysaccharide vaccination. These assays indicated that low (<4 to 32) but similar titers (+/- one
220 2-fold dilution) were obtained for the majority of sera using each complement pool lot (data not
221 shown). Based on these data, performance of the complement lots across the range of expected
222 post-vaccination titers was tested using post-immunization sera from the PsA-TT Phase 1 clinical
223 study (13) in both the CBER and PHE laboratories.

224

225 In the CBER laboratory (Figure 1A), 15 sera were tested; three sera were tested with each of four
226 complement lots, and 12 sera were tested using lots C12, C13, and C14. Valid titers were
227 obtained in 44 of the 48 tests, while two were indeterminate (sample 22015 with C12 and C13),

and two were ≥64 but had insufficient sample remaining to test at higher dilutions (samples 11013 and 22006 with C12). Overall, results between different lots for 43/44 tests and 14/15 sera were within one 2-fold dilution of the median titer which is the expected assay variability.

In the PHE laboratory, twenty PsA-TT Phase 1 clinical study sera (the 15 sera tested in the CBER laboratory and five additional) were tested using lots C11, C12, and C13, and then each serum was repeated a fourth time with one of the three complement lots (four assays for each serum). Valid titers were determined in 71 of the 80 assays. Of the 16 sera with two valid titers from the same complement lot, two differed by more than 4-fold, one had titers that were 4-fold different, and 13 were within a single 2-fold dilution of each other (data not shown). The repeated complement lot titer was used for lot comparison only if the first assay with the same lot was not valid. Data for 18 samples with valid titers from more than one complement lot are shown in Figure 1B. Overall, of 52 valid tests among 18 sera, 45 (87%) were within expected assay variability regardless of lot. Due to limited complement volume, indeterminate or inconsistent results were not repeated.

The results obtained in the CBER and the PHE laboratories were compared by using the median value for the 15 PsA-TT Phase 1 clinical study sera tested in both laboratories. Median titers were similar: of 14 samples, two samples were discrepant between laboratories, being <4 and 4 in one laboratory and mid-range positive (16 and 64) in the other (Figure 1C). One sample was excluded because the two valid titers within one laboratory were too discrepant (<4 and 128) to assign a meaningful median titer.

hSBA immune responses in clinical study sera. We utilized the same screening and pooling methods to prepare hC' for testing hSBA responses in the PsA-TT vaccine study conducted in African children 12 to 23 months of age (2). The clinical study design and hSBA sample subsets are shown in Supplemental Figure 2. Samples collected at four time points from 180 randomly selected study participants were tested. hSBA GMTs at 28 days after primary vaccination, and 10 months later are shown in Figure 2, and the proportions of subjects with hSBA titers ≥8 are shown in Table 1. Similar to the reported results of rSBA and anti-A PS IgG concentration (2), PsA-TT was immunogenic in toddlers following a single dose: the hSBA GMT at 28 days after the primary immunization was higher for the PsA-TT group ($p<0.0001$) and remained higher than the PsACWY group 10 months later ($p < 0.005$). Also, a higher proportion of subjects who received the PsA-TT vaccine had titers ≥8 ($p<0.0001$).

All subjects had been randomized to one of three secondary immunization groups and samples drawn for immunogenicity testing at 28 days after secondary immunization and two years after study enrollment. The analysis of hSBA responses following the second immunization was limited by the small number of hSBA subset subjects in each secondary group. However, the hSBA GMTs for the groups that received PsA-TT or $1/5^{th}$ dose PsACWY following a primary dose of PsA-TT (PP, PM; Figure 2,) were consistent with a memory response in primed subjects. At two years following study enrollment, the majority of PsA-TT primed subjects who received either a PsA-TT or $1/5^{th}$ PsACWY as their second immunization had hSBA titers ≥8 (28/37, 76%; Table 1). hSBA GMTs at 28 days following PsA-TT or $1/5^{th}$ PsACWY administered 10 months after PsACWY (MP, MM; Figure 2) were less than those of the PsA-TT primed groups. The 4 groups that received a single meningococcal vaccine (MH, PH, HM, HP) all had GMT

point estimates at 28 days post the second vaccination that were lower than the MP group and all were similar to HH at 2 years following study enrollment (data not shown). The reverse cumulative distribution curves for the PsA-TT primary immunization group at each time point are consistent with a strong booster response in PsA-TT primed subjects and persistence of hSBA at the 2-year time point for the majority of PsA-TT primed and boosted subjects (Figure 3).

DISCUSSION

In this study we successfully developed a strategy for screening and pooling human complement sources and produced three independent lots of complement that gave reproducible results in group A hSBA assays of adult sera. The strategy was based on excluding donor sera with functional antibody activity combined with pooling sera from six to nine individuals as the final complement source. Both of the functional antibody screening tests appeared to provide data on which possible complement sources to exclude. Less than half of the excluded sera failed both the intrinsic and rSBA criteria, so a single functional screening test would not have identified all excluded sources. Those that failed one functional screening test also could not be distinguished from the suitable complement sources by anti-A PS IgG or IgGAM concentration. Although it is not clear why some complement source sera with high rSBA titers were intrinsically negative and some intrinsically bactericidal sera were not positive in the rSBA assay, our approach of using both functional assays as screening criteria likely contributed to the successful identification of sera that could be used to create pools of complement that remained negative in terms of direct bacterial killing and performed consistently among lots.

297 The presence of measurable anti-A PS antibody was common (92%) among our population of
298 adult blood donors. The results of the fourth complement lot (C14), comprised of functionally
299 negative sera with high anti-group A PS antibody concentrations, suggest that anti-PS IgGAM,
300 when not functional in either killing assay, is not a necessary exclusion criterion; however
301 further study is needed. Anti-A PS antibody in this population is most likely through exposure to
302 cross-reacting polysaccharides found in various enteric bacteria (14, 15).
303
304 We found that hSBA titers obtained using different pooled complement lots were within the
305 expected 4-fold assay variability (a single 2-fold dilution above or below a given titer). These
306 data suggest that using suitable pooled human complement sources is a feasible approach for
307 development of a reproducible group A hSBA assay. Since serum is a highly complex biologic
308 reagent, establishing the similarity of individual single sources could be difficult. Pooling likely
309 averages many unmeasured factors, reducing complement lot to lot variability, simplifying
310 complement qualification, and thereby increasing the ability of the assay to generate data that are
311 reproducible and consistent among studies.
312
313 The hSBA responses observed in African toddlers following one or two doses of PsA-TT
314 indicate that a strong functional immune response followed immunization with the conjugate
315 vaccine. Based on a threshold titer of 8, the results from 28 days after the primary vaccination for
316 subjects who did not receive a meningococcal vaccine identify approximately 87% of that
317 population below the threshold. Vaccination with the conjugate vaccine decreased the proportion
318 of the population with hSBA titers <8 to below 26%, while a much smaller vaccine effect was
319 observed in this young population with the PS vaccine. These serologic observations are

consistent with what would be expected immunologically. In PsA-TT primed subjects, the hSBA results suggest a vigorous response to a second PsA-TT immunization or to PS challenge, and hSBA in these two groups at two years after primary immunization was clearly distinct from the persistence of hSBA in the remaining groups. Although sample sizes were small in each group, these data suggest persistence of functional antibody responses in this young population following a second dose.

The rapid decline in hSBA titers following a single dose has been observed with other group A meningococcal conjugate vaccines (16-20), and is consistent with observations reported in this population for rSBA GMT and IgG GMC. However, the magnitude of hSBA response is much less, and by threshold analyses, hSBA persists in a smaller proportion of the immunized group than indicated by rSBA or IgG ELISA immunoassays (2). Differences among the assays were also observed in our previous report of hSBA responses to PS or conjugate vaccination in the MVP PsA-TT Phase 1 study. hSBA determined using pooled human complement as described here was compared with rSBA, anti-PS antibody, and opsonophagocytosis (21). In that study, the greatest correlation between assays was for the hSBA and IgG ELISA concentration in the conjugate vaccinated group. Without efficacy data for group A conjugate vaccines in this age group, the clinical significance of differences between serologic assays is currently not known. Vaccine efficacy of polysaccharide vaccines in young children has been examined (22), but a Cochrane study of meningococcal polysaccharide vaccine efficacy concluded that the existing data are inadequate to evaluate age differences or duration of efficacy following PS vaccination (23). Therefore, historical clinical efficacy information does not help in interpretation of differences between the serologic results for the PS arm of this clinical study. Expanded

343 immunologic studies and careful epidemiologic surveillance after vaccine implementation in
344 African nations may contribute to the interpretation of differences between these immunoassays.
345
346 In this study, methods were successfully developed for processing, screening, and pooling
347 human sera for use as a complement source in *N. Meningitidis* group A hSBA assays. The PsA-
348 TT monovalent conjugate vaccine was highly immunogenic in African toddlers 12-23 months of
349 age, and persistence of hSBA activity was seen for over a year following a second dose. The
350 hSBA assay using a pooled human complement source is a practical, reliable method for
351 assessing immune responses to meningococcal vaccines and can generate data that may have
352 substantive relevance in evaluating vaccine use.
353
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458 **Table 1.** Proportion of subjects with hSBA titers ≥8 - intention to treat population
459

		n ≥8/N;% (95% CI)			
Primary vaccine	Secondary vaccine	28 days after primary	10 months after primary	28 days after 2nd vaccine[#]	14 months after 2nd vaccine [#]
PsA-TT		42/57; 73.7*** (60.3, 84.5)	11/55; 20.0** (10.4, 33.0)		
	PsA-TT			11/13; 84.6 (54.6, 98.1)	12/15; 80.0 (51.9, 95.7)
	PsACWY			22/22; 100.0 (84.6, 100.0)	16/22; 72.7 (49.8, 89.3)
	Hib			3/18; 16.7 (3.6, 41.4)	4/19; 21.1 (6.1, 45.6)
PsACWY		20/61; 32.8* (21.3, 46.0)	1/61; 1.6 (0.0, 8.8)		
	PsA-TT			13/19; 68.4 (43.4, 87.4)	7/20; 35.0 (15.4, 59.2)
	PsACWY			9/17; 52.9 (27.8, 77.0)	5/18; 27.8 (9.7, 53.5)
	Hib			2/21; 9.5 (1.2, 30.4)	8/22; 36.4 (17.2, 59.3)
Hib		8/62; 13.1 (5.8, 24.2)	3/59; 5.1 (1.1, 14.1)		
	PsA-TT			6/16; 37.5 (15.2, 64.6)	5/16; 31.3 (11.0, 58.7)
	PsACWY			4/22; 18.2 (5.2, 40.3)	3/20; 15.0 (3.2, 37.9)
	Hib			1/18; 5.6 (0.1, 27.3)	4/19; 26.3 (9.1, 51.2)

460 *** Fisher's exact test of % ≥8: p<0.0001 for PsA-TT vs. PsACWY and vs. Hib at 28 days after
461 primary immunization.
462 ** Fisher's exact test of % ≥8: p=0.0014 for PsA-TT vs. PsACWY and p=0.0212 for PsA-TT vs.
463 Hib at 10 months after primary immunization.
464 * Fisher's exact test of % ≥8: p=0.0169 for PsACWY vs. Hib at 28 days after primary
465 immunization
466 [#] No significance testing performed
467

468 **FIGURE LEGENDS**

469 **Figure 1.** hSBA titers of serum samples determined using independent pooled human
470 complement (hC') lots. Two-fold serum dilutions from 1:4 to 1:1024 were tested. Titers <4 are
471 shown as values of 2. A. Comparison of hSBA titers for 15 samples using hC' lots C12, C13,
472 and C14 analyzed in the CBER laboratory; data for 3 samples tested with lot C11 are included.
473 Indeterminate titers (n=2) and titers ≥64 with insufficient sample to test at higher dilutions (n=2)
474 are not shown. B. Comparison of reported titers for 18 samples tested using hC' lots C11, C12,
475 and C13 in the PHE laboratory. Two samples with data available for only one hC' lot are not
476 shown. C. Comparison of the median values of 14 samples between the CBER and PHE
477 laboratories. Sample 33005 is not shown as a valid median titer (panel B) was not determined.
478
479 **Figure 2.** The hSBA geometric mean titers (GMT) are shown by vaccination group at 28 days
480 and 10 months after primary immunization (H= Hib, M= PsACWY, P=PsA-TT). For groups
481 that received a second meningococcal immunization, GMTs are shown at 28 days after the
482 second immunization and at 2 years post enrollment. The first letter designates the first
483 immunization and the second letter designates the second immunization. Error bars show +/-
484 one standard deviation.
485
486 **Figure 3.** Reverse cumulative distribution curves for hSBA responses to PsA-TT vaccination.
487 Shown is the proportion of subjects with hSBA titers at or above the x-axis value for: the PsA-
488 TT primary immunization group at 28 days (P 1 mo) and 10 months (P 10 mo) after primary
489 immunization, PsA-TT primed subjects who received a second PsA-TT dose (PP) or a $1/5^{th}$ dose
490 PsACWY challenge (PM) at 28 days (PP 11 mo and PM 11 mo, respectively) and 14 months (PP

24 mo and PM 24 mo, respectively) after the second immunization. The distribution of hSBA titers among the reference PsACWY vaccine group is shown at 28 days after primary immunization (M 1 mo).

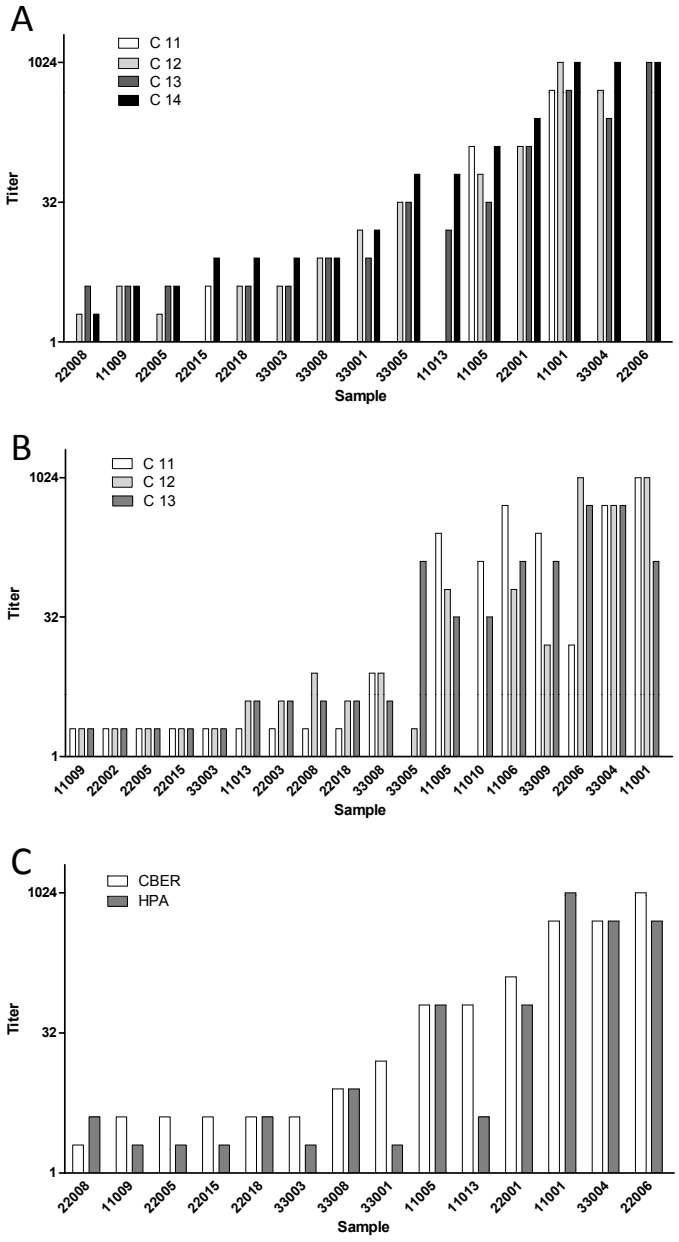

Figure 1

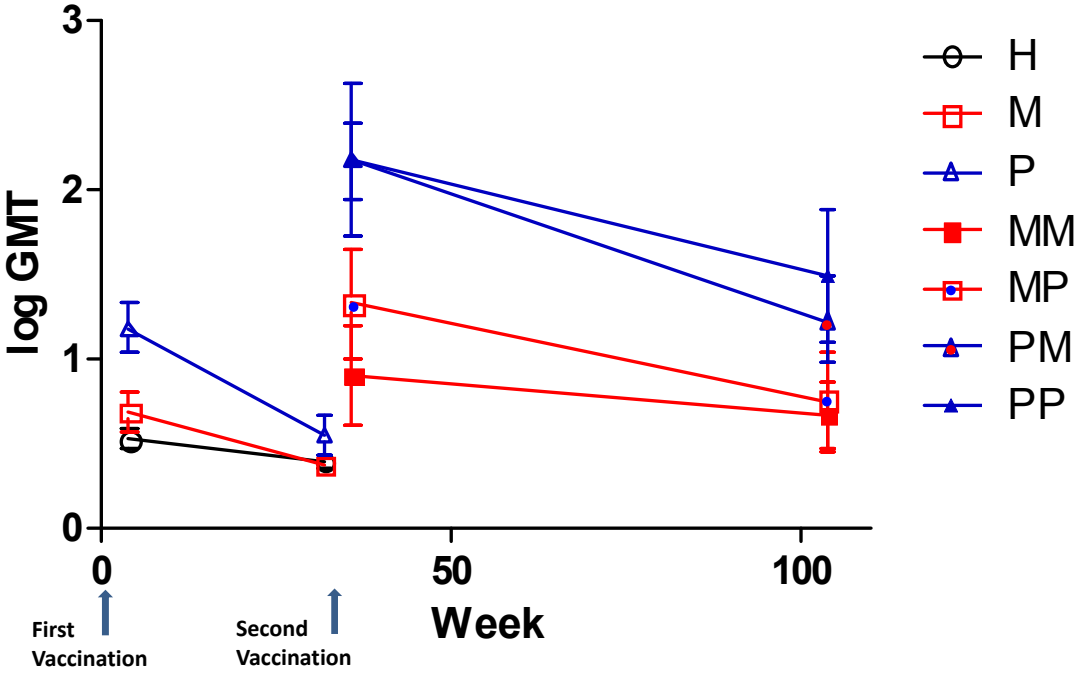

Figure 2

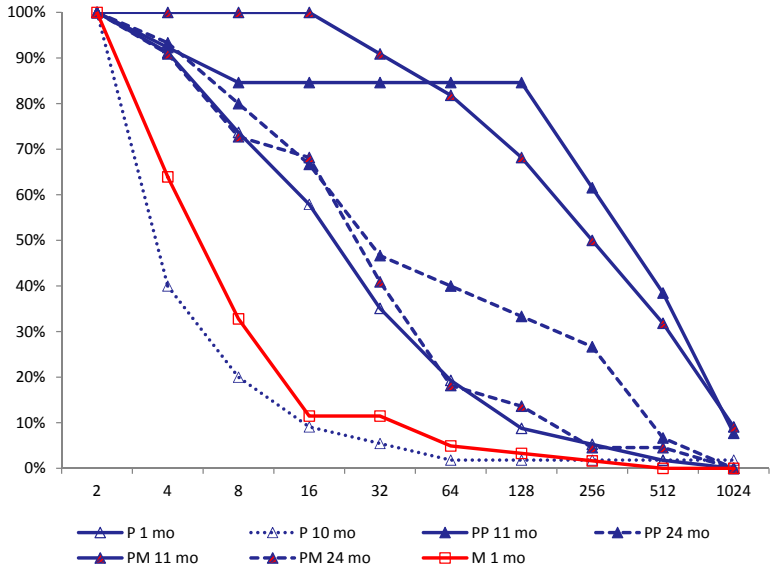

Figure 3