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Multicenter Comparison of Levels of Antibody to the *Neisseria meningitidis* Group A Capsular Polysaccharide Measured by Using an Enzyme-Linked Immunosorbent Assay

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There is no standard immunoassay for evaluating immune responses to meningococcal vaccines. We developed an enzyme-linked immunosorbent assay to measure total levels of antibody to *Neisseria meningitidis* group A capsular polysaccharide. Five laboratories measured the antibody levels in six paired pre- and postvaccination serum samples by using the enzyme-linked immunosorbent assay. Methylated human serum albumin was used to bind native group A polysaccharide to microtiter plate surfaces. The between-laboratory coefficients of variation for pre- and postvaccination sera had ranges of 31 to 91 and 17 to 31, respectively. The mean laboratory coefficients of variation for pre- and postvaccination sera, respectively, were 17 and 11 (Molecular Biology Laboratory, Centers for Disease Control), 12 and 15 (Immunodiagnostic Methods Laboratory, Centers for Disease Control), 22 and 19 (Dana-Farber Cancer Institute), 38 and 38 (Bacterial Polysaccharide Laboratory, U.S. Food and Drug Administration), and 11 and 10 (Praxis Biologics, Inc.). Standardization of this enzyme-linked immunosorbent assay should allow interlaboratory comparison of meningococcal vaccine immunogenicity, thus providing a laboratory-based assessment tool for evaluating meningococcal vaccines.

No standard immunoassay for the quantitative measurement of total levels of antibody to *Neisseria meningitidis* capsular polysaccharide in serum exists. The development of polysaccharide-protein conjugate meningococcal vaccines has emphasized the need for a standardized and reproducible assay to evaluate the immunogenicity of new vaccines and to correlate antibody levels with functional assays and minimum protective antibody levels.

The necessity for having a standard immunoassay before beginning vaccine immunogenicity studies was exemplified by a between-laboratory comparison of *Haemophilus influenzae* type b pre- and postvaccination antibody levels measured by a radioactive antigen-binding assay (24). Before any agreement on a standard assay, as much as a 6-fold difference in geometric mean antibody levels occurred between laboratories, and levels in some individual serum samples varied as much as 64-fold (24). Lack of a standard assay for measuring the immune response to *H. influenzae* type b vaccine made it impossible to compare results of different vaccines evaluated in different laboratories during the first 3 years of vaccine evaluation.

Antibody responses to the capsular polysaccharides of *N. meningitidis* have been measured by various serologic methods: capillary precipitation (10), bactericidal antibody (9), opsonization (19), passive hemagglutination (13), immunofluorescence (9), latex agglutination (20, 23), solution-phase radioimmunoassay (7, 11, 13, 14), solid-phase radioimmunoassay (25), and enzyme-linked immunosorbent assay (ELISA) (4, 5, 15, 22). Of the quantitative procedures reported, only radioimmunoassays and the ELISA accurately measured total capsular polysaccharide antibody levels with the serum volumes typically obtained from young children. Because it is simpler and safer to perform, ELISA is preferred for routine measurement of immunoglobulin class and subclass levels (21) and is the method of choice for a standardized meningococcal assay.

This study was undertaken to compare results of total levels of antibody to meningococcal group A polysaccharide obtained by an ELISA. The between-laboratory comparison showed the ELISA to be sensitive and reproducible, indicating that the ELISA is an acceptable method for the routine measurement of these antibody levels.

(Preliminary results of this study were presented at the International Workshop on Meningococcal Immunology, 24 August 1990, in Atlanta, Ga.)

MATERIALS AND METHODS

Study design. Sera for evaluation were obtained from six healthy white adults (designated serum sample numbers 2, 5, 14, 16, 20, and 24) who were immunized subcutaneously with a single injection (50 μg of each polysaccharide per 0.5 ml) of *N. meningitidis* vaccine (MENOMUNE; lot 9H11153; Connaught Laboratories, Inc., Swiftwater, Penn.) containing group A, C, Y, and W-135 polysaccharides. None of the subjects had been previously vaccinated with a meningococcal vaccine or had a known history of invasive meningococcal disease. After informed consent was obtained, blood specimens were collected from each subject at the Centers

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for Disease Control (CDC) Serum Bank just prior to vaccine administration and then 14 to 33 days postimmunization. Prevaccination sera for each sample were designated "a" and postvaccination sera were designated "b." The following postvaccination serum samples were collected on the indicated days after immunization: 2b, 14 days; 5b, 22 days; 14b, 26 days; 16b, 33 days; 20b, 26 days; and 24b, 29 days. All sera were stored at −70°C in coded vials. The six pre- and six postimmunization serum samples were divided into aliquots and distributed to the following laboratories: Molecular Biology Laboratory, CDC (CDC1); Immunodiagnostic Methods Laboratory, CDC (CDC2); Bacterial Polysaccharide Laboratory, U.S. Food and Drug Administration, Bethesda, Md. (FDA); Dana-Farber Cancer Institute, Boston, Mass. (D-F); and Praxis Biologics, Inc., Rochester, N.Y. (PRAX). All laboratories were blinded as to the source of the specimen and to the results from other laboratories.

ELISA. The ELISA procedure developed at FDA was used for this study (2). Meningococcal group A polysaccharide, meningococcal polysaccharide reference serum (PB-2 from a single donor was assigned the value of 4,800 U of total antibody to meningococcal group A polysaccharide per ml by one of the authors [C.E.F.] by using calibration procedures described previously [8]), and methylated human serum albumin were provided by one of the authors (C.E.F., Office of Biologics Research and Review, FDA). Equal volumes of methylated human serum albumin (16) and *N. meningitidis* group A polysaccharide, in coating buffer (10 mM phosphate-buffered saline [pH 7.4]), were mixed dropwise to give a final concentration of 5 μg/ml each and were used to coat (100 μl per well) Immulon 1 microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.). The plates were sealed and incubated for 6 to 7 h at 28°C. Plates were then washed five times with wash buffer (10 mM Tris [Bio-Rad Laboratories, Rockville Center, N.Y.] and 0.1% Brij 35 [Sigma Chemical Company, St. Louis, Mo.] in 0.85% saline), and 100 μl of serum-conjugate (S-C) buffer (5% newborn bovine serum, 0.1% Brij 35, and 0.05% sodium azide in phosphate-buffered saline) was added to each well.

Serum diluted in S-C buffer (100 μl) was added to the first well of the dilution series, after which seven triplicate or duplicate (PRAX) twofold dilutions of PB-2 and eight duplicate twofold dilutions of test sera were made by well-to-well transfer in the microtiter plates by using a multichannel pipette (100 μl was transferred and mixed five times in each well). Two laboratories prepared serum dilutions in separate tubes (FDA) or microtiter dilution plates (PRAX) rather than in the microtiter plate wells and then transferred 100 μl to the appropriate wells. The plates were sealed and incubated overnight at 4°C. Plates were washed five times as described above; 100 μl of alkaline phosphatase-labeled affinity-purified goat antibody to human immunoglobulins G, A, and M (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1:500 in S-C buffer was added to each well; and the plates were incubated for 2 h at 28°C (the same lot of conjugate was used by each laboratory). The plates were washed five times as described above, 100 μl of substrate-buffer (1 mg of *p*-nitrophenyl phosphate [Sigma] per ml in 1 M Tris [pH 9.8] containing 0.3 mM $MgCl_2 \cdot 6H_2O$) was added to each well, and the plates were incubated for 30 to 50 min at ambient temperature. The enzyme reaction was allowed to proceed to an absorbance value of approximately 1.5 to 2.0, at which time the plates were read (FDA only), or the reaction was stopped by adding 100 μl of 0.4 M NaOH to each well and the absorbance values were read at 410 nm; the minimum detectable limit of the assay was about 10 antibody units per ml.

Inhibition assays. Inhibition assays were done with a single dilution of the standard reference serum (PB-2) and the six postvaccination serum samples. The final dilution of each serum sample was chosen to give an absorbance value of approximately 1.0 after 30 min of color development. The sera were diluted in S-C buffer, and 150 μl of diluted serum was then divided into aliquots and placed into seven 1.5-ml centrifuge tubes. Six 10-fold dilutions of meningococcal group A polysaccharide (diluted in S-C buffer) were prepared starting at the highest concentration (200 μg/ml). An equal volume (150 μl) of meningococcal group A polysaccharide was added to six of the diluted-serum tubes; 150 μl of S-C buffer alone was added to one tube as the 0% inhibition control. The serum and polysaccharide dilutions were allowed to react while they were rotated for 1 h at ambient temperature (approximately 21°C). The mixture was then added in duplicate wells to a microtiter plate (100 μl per well). The plates were incubated overnight at 4°C and treated as described above in the ELISA procedure.

Assay variables. Three laboratories (CDC1, CDC2, and D-F) calculated antibody values by extrapolation from a PB-2 standard dilution curve plotted by using a four-parameter logistic model (6), FDA used a logit-log plot (8), and PRAX used a log-log plot. Three laboratories (CDC1, CDC2, and D-F) used sterile, pyrogen-free distilled water in all buffers and for washing; PRAX used sterile, pyrogen-free water only for coating of antigen; and FDA did not use sterile pyrogen-free water in the ELISA. Two laboratories (CDC1 and CDC2) washed plates with a manual plate washer (Skatron, Inc., Sterling, Va.), and the other three laboratories used automated plate washers. PRAX used 5% fetal bovine serum instead of 5% newborn bovine serum. Three laboratories (CDC1, CDC2, and D-F) made twofold serum dilutions in the antigen-coated microtiter plate wells; the other laboratories first made dilutions in plastic tubes or polystyrene microtiter plates and then transferred the diluted sera to the microtiter assay plate wells. Four laboratories (CDC1, CDC2, D-F, and PRAX) read A_{410} values and corrected for plate imperfection at A_{630}; the FDA laboratory did not correct for plate imperfection.

Statistical methods. Each serum dilution was assayed in duplicate, and the total meningococcal group A antibody (in antibody units per milliliter) was determined by averaging all values from each dilution that fell within the working range of the PB-2 standard dilution curve (absorbance values between approximately 0.05 and 2). The between-laboratory mean antibody level (in antibody units per milliliter), standard deviation, and coefficient of variation (CV) were calculated by averaging the mean antibody value reported by each laboratory for each serum sample. Mean pre- and postvaccination antibody levels among laboratories were compared for each serum sample by using the Kruskal-Wallis test (3).

RESULTS

Antibody level comparison. Increases in antibody levels (ratio of pre- and postvaccination serum means) ranged between 5- and 101-fold and are shown in Fig. 1. Serum sample 2b, collected 14 days postvaccination, had the lowest mean postvaccination antibody level and the lowest increase in antibody level. An increase in antibody level did not correlate with the time of collection of the postvaccination serum sample for the other five serum samples. Samples 14 (prevaccination, 111 U/ml; postvaccination, 2,408 U/ml) and

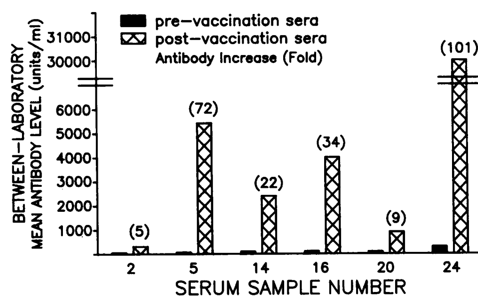

FIG. 1. Between-laboratory mean antibody levels (units per milliliter) in pre- and postvaccination sera from individuals vaccinated with meningococcal group A polysaccharide. The fold increase (ratio of post- to prevaccination antibody level) in antibody level is given in parentheses.

20 (prevaccination, 98 U/ml; postvaccination, 910 U/ml) were collected 26 days postvaccination; sample 14 showed a 22-fold increase in antibody level, while sample 2 showed only a fivefold increase.

The mean antibody level, standard deviation, and CV from the three independent assays for six prevaccination and six postvaccination serum samples reported by the five laboratories are given in Table 1. The between-laboratory antibody levels for each of the pre- and postvaccination serum samples were significantly different ($P < 0.001$, Kruskal-Wallis test [3]).

The percent difference between the antibody level for each serum sample reported by each laboratory and the between-laboratory mean antibody level for each of the six pre- and postvaccination serum samples are shown in Fig. 2A and B, respectively. The ±50% bounds in Fig. 2A and B represent 50% (0.5-fold) above and 50% (0.5-fold) below the mean

TABLE 1. Between-laboratory comparison of total antibody levels to *N. meningitidis* group A polysaccharide in pre- and postvaccination serum samples

Sample no.	Laboratory	Prevaccination			Postvaccination		
		Mean (U/ml)[a]	SD (U/ml)[b]	CV[c]	Mean (U/ml)	SD (U/ml)	CV
2	CDC1	59	13	22	289	54	19
	CDC2	38	6	17	341	77	23
	D-F	28	5	16	237	39	16
	FDA	164	133	81	405	152	38
	PRAX	28	5	18	303	43	14
	Between-laboratory	63	58	91	315	63	20
5	CDC1	67	8	13	4,842	459	10
	CDC2	57	6	11	5,248	494	9
	D-F	53	6	12	4,427	472	11
	FDA	143	60	42	7,984	2,435	31
	PRAX	56	6	10	4,642	431	9
	Between-laboratory	75	38	51	5,428	1,460	27
14	CDC1	159	33	21	2,609	307	12
	CDC2	79	15	18	2,907	397	14
	D-F	84	23	28	2,167	576	27
	FDA	145	41	28	2,523	1,114	44
	PRAX	87	11	12	1,832	199	11
	Between-laboratory	111	38	34	2,408	416	17
16	CDC1	170	38	22	4,370	376	9
	CDC2	70	10	15	5,172	1,039	20
	D-F	110	36	33	3,274	663	20
	FDA	146	26	8	3,336	1,335	40
	PRAX	87	3	3	3,878	279	7
	Between-laboratory	117	41	35	4,006	789	20
20	CDC1	95	11	11	796	79	10
	CDC2	63	3	5	934	157	17
	D-F	63	10	16	851	70	8
	FDA	193	83	43	1,206	306	25
	PRAX	77	3	4	763	45	6
	Between-laboratory	98	55	56	910	178	20
24	CDC1	439	67	15	36,373	2,349	7
	CDC2	260	22	8	38,641	2,163	6
	D-F	204	59	29	17,078	5,247	31
	FDA	368	89	24	24,460	12,497	51
	PRAX	253	50	20	37,300	4,644	13
	Between-laboratory	305	96	31	30,770	9,530	31

[a] Mean total meningococcal group A polysaccharide antibody level per milliliter.
[b] SD, one standard deviation calculated by using values from three independent assays from each laboratory.
[c] The CV was the standard deviation divided by mean units per milliliter × 100.

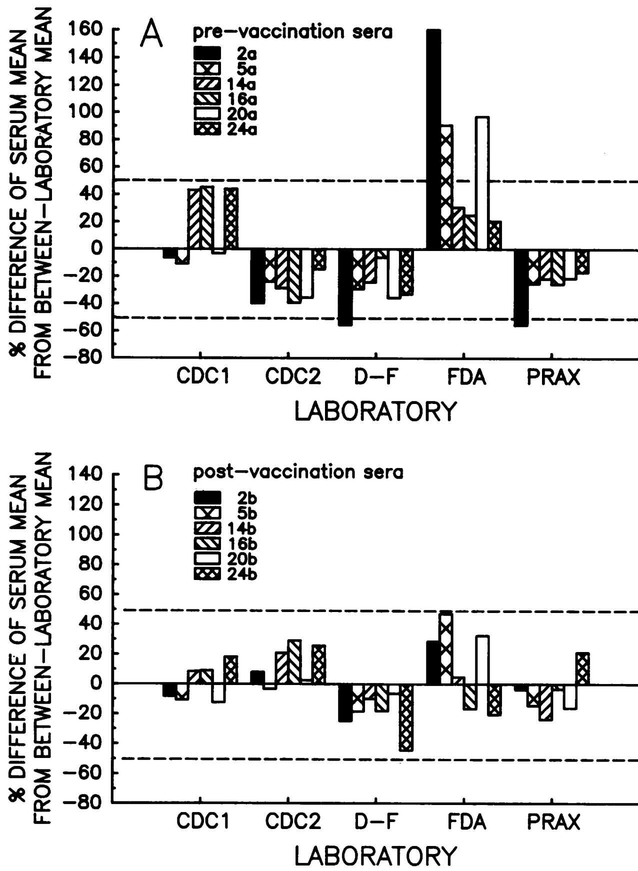

FIG. 2. Percent difference of the sample mean from the between-laboratory mean antibody level for each prevaccination (A) and postvaccination (B) serum sample. The dotted lines indicate the bounds defined by the ±50% difference (±0.5-fold) from the between-laboratory means.

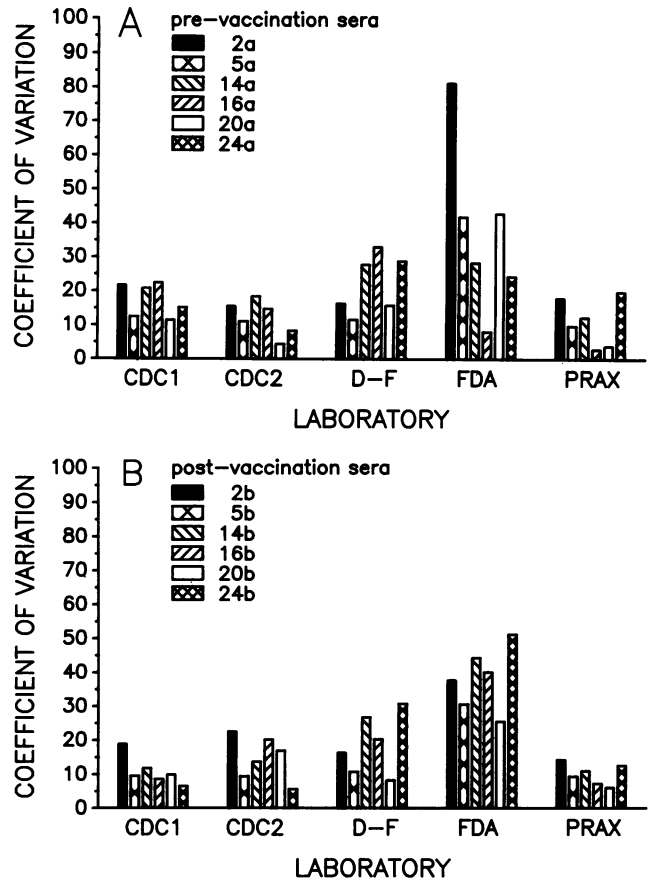

FIG. 3. Between-laboratory comparison of the CV for prevaccination (A) and postvaccination (B) sera.

antibody level for each serum sample. The average percent difference (absolute value) from the between-laboratory means for all prevaccination serum samples measured by each laboratory, as shown in Fig. 2A, was 37%; it was 18% for all postvaccination serum samples (Fig. 2B). Serum sample 2a showed the highest amount of between-laboratory variability. Antibody levels reported for serum sample 2a by D-F, FDA, and PRAX differed from the between-laboratory mean antibody level (63 U/ml) by 56% (28 U/ml), 160% (164 U/ml), and 56% (28 U/ml), respectively (Fig. 2A). Only two other prevaccination serum samples (5a and 20a reported by FDA) had antibody levels that differed from their respective between-laboratory mean antibody values by more than 45%. Three laboratories (CDC2, D-F, and PRAX) reported antibody levels for all six prevaccination serum samples below the between-laboratory mean antibody level, all FDA values were above the mean, and CDC1 reported levels above and below the mean. Antibody levels for serum sample 2a reported by FDA (164 U/ml) and by D-F and PRAX (both 28 U/ml) differed by 600%; this was the largest difference in antibody level between any given pre- or postvaccination serum sample.

Postvaccination antibody levels differed by no more than 47% (serum sample 5b reported by FDA) from their respective between-laboratory mean antibody levels (Fig. 2B). Only at D-F were all postvaccination antibody levels in sera below the between-laboratory mean antibody level; the other four laboratories reported levels above and below the mean level. A greater percent difference in antibody level was observed when mean antibody levels of selected serum samples determined in different laboratories were compared. Postvaccination antibody levels reported for serum sample 24b by D-F (17,078 U/ml) differed by 126% from antibody levels reported by CDC2 (38,641 U/ml) and differed by 118% from those reported by PRAX (37,300 U/ml). None of the other antibody levels for a given postvaccination serum sample differed from each other by more than 80%.

The CVs for pre- and postvaccination sera for all laboratories are shown in Fig. 3A and B. The overall mean prevaccination CV (average of all prevaccination serum CVs) was 20, and the overall postvaccination CV (average of all CVs for postvaccination serum) was 19. The within-laboratory mean CVs for pre- and postvaccination sera (average of the CVs for all pre- and postvaccination sera reported from a laboratory), respectively, were 17 and 11 for CDC1, 12 and 15 for CDC2, 22 and 19 for D-F, 38 and 38 for FDA, and 11 and 10 for PRAX. The overall between-laboratory CVs for prevaccination sera ranged between 31 (serum sample 24a) and 91 (serum sample 2a), and those for postvaccination sera ranged between 17 (serum sample 14b) and 31 (serum sample 24b) (Table 1).

Antibody binding inhibition. A competitive inhibition assay was used to inhibit binding of specific serum antibody to the solid-phase polysaccharide antigen. The inhibition curves for the PB-2 reference serum sample and six post-

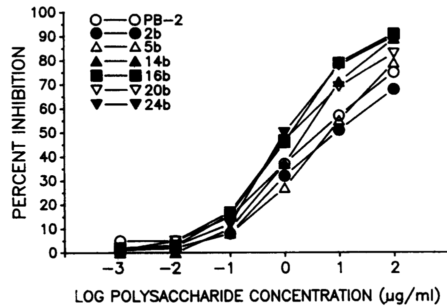

FIG. 4. Inhibition of antibody binding for the standard reference serum (PB-2) and six postvaccination serum samples. Native polysaccharide was tested with diluted serum and was assayed as described for the standardized ELISA. The reciprocal of the final dilution of each serum sample was as follows: PB-2, 1,800; 2b, 80; 5b, 2,000; 14b, 900; 16b, 1,200; 20b, 200; and 24b, 10,000.

vaccination serum samples are shown in Fig. 4. The percent inhibition of each serum sample when 100 and 10 μg of group A polysaccharide per ml, respectively, were added was as follows: PB-2, 75 and 57%; 2b, 68 and 51%; 5b, 79 and 55%; 14b, 89 and 71%; 16b, 90 and 79%; 20b, 83 and 69%; and 24b, 91 and 78%. The percent inhibition varied only by ±2% when polysaccharide (10 μg/ml) and diluted sera were allowed to react in the ELISA for 4 h at 4°C instead of overnight at 4°C (data not shown). The concentrations (in micrograms per milliliter) of meningococcal group A polysaccharide needed for 50% inhibition of each serum sample were as follows: PB-2, 7.7; 2b, 9.4; 5b, 8.6; 14b, 5.8; 16b, 4.3; 20b, 5.1; and 24b, 4.3. Serum sample 2b had the lowest percent inhibition as well as the lowest antibody level. This serum sample had a dilution curve slope that was shallower than that of the reference standard, suggesting a lower overall antibody affinity. Serum sample 24b had the highest percent inhibition as well as the highest antibody level. This serum had a dilution curve slope that was steeper than that of the reference standard, suggesting a higher overall antibody affinity.

Variations of ELISA procedure. Laboratories CDC1 and CDC2 compared plates that were stored for as long as 5 days at 4°C (0.5% sodium azide was added to antigen-coating buffer for storage). When freshly coated plates and stored plates were compared in parallel assays, antibody levels and assay variability were not significantly affected (data not shown). However, significantly reduced absorbance values were observed when microtiter plates were coated with antigen, dried, and stored. CDC1 obtained essentially equivalent antibody levels when dilutions were made independently with an automatic dilutor, in separate plastic tubes before they were transferred to microtiter plates, or by serial twofold dilutions with a multichannel pipette in the antigen-coated microtiter plate wells (data not shown). Twofold dilutions in the microtiter plate wells were the easiest and most rapid way to prepare dilutions.

The use of fetal bovine serum instead of newborn bovine serum by PRAX did not appear to affect the total antibody levels obtained by ELISA. An advantage of newborn bovine serum is that it is generally less expensive than fetal bovine serum.

DISCUSSION

A laboratory-based assessment of new and developing vaccines should include the development and within- and between-laboratory evaluation of standardized immunoassays prior to the initiation of immunogenicity and efficacy studies. There is no standard immunoassay for evaluating immune responses to *N. meningitidis*. Since new meningococcal conjugate vaccines are being actively developed, it is important that a standardized assay(s) be available to enable between-laboratory comparisons of antibody levels and the subsequent determination of functional antibody activity and minimum protective antibody level.

The purpose of this study was to evaluate a quantitative ELISA and to compare the between-laboratory variations in determination of the level of antibody to *N. meningitidis* group A polysaccharide. We found that the between-laboratory pre- and postvaccination antibody levels were significantly different ($P < 0.001$). However, when antibody levels for each serum sample reported by each laboratory were compared with their respective between-laboratory mean antibody levels, prevaccination sera differed from the mean antibody level (percent difference) by an average of 37%, and postvaccination sera differed by only 18%. There is no consensus on an upper limit for an acceptable between-assay CV; however, a CV of 30 has been used (12), and in our experience this is a reasonable limit. Jeffcoate and Das (12) determined that about one-third of the between-laboratory CV is caused by differences in calculation procedures. Given this consideration, a CV of 30% could be judged at the 20% level if a CV of 10% was assumed to be due to calculation differences.

The between-laboratory variation was greatest in the prevaccination sera, in which all six serum samples had CVs of greater than 31; this is not surprising, given the low levels of antibody. All postvaccination serum samples, however, had between-laboratory CVs of less than or equal to 31, and none of the postvaccination serum samples differed from their respective between-laboratory mean antibody levels by more than 47%. Since a minimum protective antibody level is not known for *N. meningitidis*, the lower antibody detection limit necessary for the ELISA is also not known. A higher value of the lower assay limit should decrease the variability observed with the prevaccination sera, since the highest variability was observed with the sera with the lowest antibody levels.

In this assay, we used methylated human serum albumin to bind negatively charged native polysaccharide to the plate surface. This eliminated the need for chemical modification of the polysaccharide and the possible loss of some native epitopes. It also reduces the lot-to-lot variability sometimes observed with chemical modification of a polysaccharide (21).

Three methods of data transformation were used in this study (four-parameter logistic-log, logit-log, and log-log models). There is no consensus on which curve-fitting technique should routinely be used for immunoassays (17); some methods describe the data with greater precision than others. It has been shown that the four-parameter logistic-log and logit-log models are superior to the log-log model (17). However, the log-log model works well when, as in this study, patient sera are serially diluted over a range of dilutions and the concentrations are averaged over all points. The logistic-log model should be the model of choice for a standard assay.

Nonspecific antibody binding can be influenced by the quality of water used in the ELISA (18). CDC2 initially had high nonspecific antibody binding because of contamination of automatic plate washer reservoir lines, even with the presence of a bacteriostatic agent. The nonspecific binding

was reduced when sterile, pyrogen-free distilled water was used in all buffers. Sterile, pyrogen-free distilled water (type 1 water) is recommended for the preparation of all buffers used in the ELISA.

In this study, we measured antibody levels in healthy white adults from a single geographic location. To ensure that the assay is generally applicable, antibody levels need to be measured in young children and adults from other geographic locations, such as persons from the countries in sub-Saharan Africa that form the "meningitis belt." A high level of unwanted or nonspecific background binding in an ELISA has been observed in sera from children in this area of Africa (1). However, because of the high incidence of human immunodeficiency virus-positive sera in this area, serum samples are routinely heat treated. CDC1 and CDC2 have observed increased nonspecific binding with heat-treated sera (data not shown).

The ELISA was shown to be sensitive and reproducible and allows comparisons (within-laboratory and between-laboratory) of levels of antibody to group A polysaccharide. This assay has also been successfully used with meningococcal group C polysaccharide (2). We suggest that this assay be used as a basis for comparison with other immunoassay procedures for the quantitation of antibody levels to *N. meningitidis* group A and C polysaccharides. The recommended standard procedure(s) for quantitation of meningococcal polysaccharide antibody levels is likely to evolve from such comparisons.

To aid in the development of a standardized assay, a meningococcal standard reference serum is being distributed by FDA (C.E.F.), and quality control sera, methylated human serum albumin, and meningococcal group A and C polysaccharides are available from CDC (G.M.C.). CDC will make available a data-handling and analysis program (BASIC program for IBM-compatible computers) for evaluation.

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